Working group “Insect Pathogens and Entomoparasitic Nematodes”

Preceedings of the Meeting

“Biological Control – its unique role in organic and integrated production”

at

Zagreb (Croatia)

16 – 20 June, 2013

Edited by:
Johannes A. Jehle, Renata Bazok, Neil Crickmore, Miguel López-Ferber, Itamar Glazer, Enrique Quesada-Moraga, Michael Traugott

IOBC-WPRS Bulletin
The content of the contributions is in the responsibility of the authors.
We would like to thank our sponsors for support:
Local Organizing Committee:

University of Zagreb, Faculty of Agriculture
Renata Bažok
Maja Čačija
Darija Lemić
Antonela Kozina
Tomislav Kos
Ivana Pajač
Ivan Juran
Irena Dušak
Damir Bertić

Scientific Committee:

Johannes Jehle, Federal Research Centre for Cultivated Plants, Julius Kühn Institute, Germany, Convenor of Working Group
Neil Crickmore, University of Sussex, United Kingdom, Subconvenor Bacteria
Itamar Glazer, The Volcani Center, Israel, Subconvenor Entomoparasitic Nematodes
Miguel Lopéz-Ferber, Ecole des Mines d’Alès, France, Subconvenor Viruses
Enrique Quesada-Moraga, University of Cordoba, Spain, Subconvenor Fungi
Michael Traugott, University of Innsbruck, Austria, Subconvenor Soil Insect Pests
Renata Bažok, University of Zagreb, Faculty of Agriculture, Croatia
Božena Barić, University of Zagreb, Faculty of Agriculture, Croatia
Tanja Gotlin Čuljak, University of Zagreb, Faculty of Agriculture, Croatia
Dinka Grubišić, University of Zagreb, Faculty of Agriculture, Croatia
Preface

Biological control of insect and slug pest using insect pathogenic micro-organisms and viruses as well as parasitic nematodes plays an important role in pest control and sustainable food production. Therefore the subject of the 14th Meeting of the IOBC-WPRS Working Group “Insect Pathogens and Entomoparasitic Nematodes”, held on June 16 to 20, 2013 in Zagreb, is: “Biological control – its unique role in organic and integrated production”.

Since the last meeting of the WG in Innsbruck 2011, significant changes can be observed in the society, in politics, and in biocontrol industry. More and more consumers request organically produced fruits and crops or products grown with low input of chemical pesticides. The Directive 2009/128/EC on the sustainable use of pesticides came into effect in the European Union and aims to reduce the risks of the use of chemical pesticides; and a consequence, non-chemical pest control measures is given priority. Biological Control can do this. The market of biocontrol industry grows and multi-national companies (re)discover their interest in biocontrol products as promising cornerstones in their portfolio.

In 49 oral contributions, 41 poster presentations, a round table discussion, and two workshops, the most recent progress and new challenges in the use of insect pathogens and entomoparasitic nematodes will be presented and discussed. More than 110 delegates from 25 countries have registered and will exchange news and views from different regions in this world. Therefore, we hope that this meeting will stimulate further international collaboration and exchange of scientists and students.

We are proud that more than 20 students attend this meeting. For some of them it will be their first international congress and the first opportunity to present their data to an international audience. We are grateful to the Ministry of Science, Education and Sport of the Republic of Croatia, to our sponsors, and to the IOBC-WPRS for their generous financial support of the meeting. Your contributions made it possible to support students’ attendance to this meeting.

Organizing such an international meeting is a lot of work. We cordially thank all members of the local organization team for their cordial hospitality, their everlasting dedication and hard work to make this meeting a success. We also thank to Faculty of Agriculture of the University of Zagreb for providing us space for workshops and all other services we needed for a successful organization.

Because this Bulletin was prepared to be handed out at the meeting, the contributions were reviewed and edited in a very short time. We wish to thank all the editorial board members, in particular the subgroup convenors and the technical editor Ute Koch, who spent many hours of working time, evenings and weekends to prepare this Bulletin in time.

Johannes Jehle
Working Group Convenor

Renata Bazok
Local organizer
List of participants

ABDEL-RAHMAN, Mohamed
Plant Protection Research Institute
Giza, Egypt
alaaa4@hotmail.com

AIUCHI, Daigo
Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan
aigo@obihiro.ac.jp

ALAWI, Zakiya
Diwan of Royal Court - Oman Botanic Garden
Muscat, Sultanate of Oman
zakiya.alalawi@omanbotanicgarden.com

ALRIJEIBY, Shadia
Diwan of Royal Court - Oman Botanic Garden
Muscat, Sultanate of Oman
shadia.alrijeiby@omanbotanicgarden.com

ASANO, Shin-Ichiro
Hokkaido University
Sapporo, Japan
sangaku@abs.agr.hokudai.ac.jp

BALOG, Emese
SZIE Plant Protection Institute
Gödöllő, Hungary
Balog.Emese@mkk.szie.hu

BARIĆ, Božena
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
baric@agr.hr

BAŽOK, Renata
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
rbazok@agr.hr

BENJAMIN, Emmanuel
Technische Universität München
Freising, Germany
emmanuel.benjamin@tum.de

BERLING, Marie
CREA
Bonneville, France
mberling@crea.fr

BESSE, Samantha
Natural Plant Protection
Pau, France
samantha.besse@arysta.com

BLACKSHAW, Rod
Plymouth University
Drake Circus, Plymouth, United Kingdom
rblackshaw@plymouth.ac.uk

BOARIA, Andrea
University of Padua
Marostica, Italy
andrea.boaria@studenti.unipd.it

BRANDL, Michael A.
Georg-August-University
Göttingen, Germany
michael.brandl@agr.uni-goettingen.de

BÜCHS, Wolfgang
Federal Research Centre for Cultivated Plants
Julius Kühn Institute, Braunschweig, Germany
wolfgang.buechs@jki.bund.de

BURJANADZE, Medea
Agricultural University of Georgia
Tbilisi, Georgia
medeabu@yahoo.com
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BENUZZI, Massimo</td>
<td>Biogard Division of CBC (Europe) Cesena (FC), Italy <a href="mailto:mbenuzzi@cbceurope.it">mbenuzzi@cbceurope.it</a></td>
</tr>
<tr>
<td>BUTT, Tariq</td>
<td>Swansea University, College of Science Swansea, Wales, United Kingdom <a href="mailto:t.butt@swan.ac.uk">t.butt@swan.ac.uk</a></td>
</tr>
<tr>
<td>CABALLERO, Primitivo</td>
<td>Universidad Publica de Navarra Pamplona, Spain <a href="mailto:pcm92@unavarra.es">pcm92@unavarra.es</a></td>
</tr>
<tr>
<td>DOBEŠ, Pavel</td>
<td>Masaryk University, Faculty of Science Brno, Czech Republic <a href="mailto:pavel.dobes@mail.muni.cz">pavel.dobes@mail.muni.cz</a></td>
</tr>
<tr>
<td>CHUBINISHVILI, Mariam</td>
<td>Agricultural University of Georgia Tbilisi, Georgia <a href="mailto:m.chubinishvili@agruni.edu.ge">m.chubinishvili@agruni.edu.ge</a></td>
</tr>
<tr>
<td>DOMÍNGUEZ, Tania</td>
<td>University of Granada, Faculty of Sciences Granada, Spain <a href="mailto:tandf@correo.ugr.es">tandf@correo.ugr.es</a></td>
</tr>
<tr>
<td>CIORNEI, Constantin</td>
<td>Forest Research and Management Institute Băcău Station, Romania <a href="mailto:ciorneitinel@yahoo.com">ciorneitinel@yahoo.com</a></td>
</tr>
<tr>
<td>ECKARD, Sonja</td>
<td>Agroscope, Swiss Federal Research Station Zürich, Switzerland <a href="mailto:sonja.eckard@art.admin.ch">sonja.eckard@art.admin.ch</a></td>
</tr>
<tr>
<td>CRUZ BARRERA, Fredy M.</td>
<td>Corpoica Mosquera, Cundinamarca, Colombia <a href="mailto:fcruz@corpoica.org.co">fcruz@corpoica.org.co</a></td>
</tr>
<tr>
<td>EHLERS, Ralf-Udo</td>
<td>e-nema GmbH Schwentinental, Germany <a href="mailto:ehlers@e-nema.de">ehlers@e-nema.de</a></td>
</tr>
<tr>
<td>CUTHBERTSON, Andrew</td>
<td>The Food and Environment Research Agency Sand Hutton, York, United Kingdom <a href="mailto:andrew.cuthbertson@fera.gsi.gov.uk">andrew.cuthbertson@fera.gsi.gov.uk</a></td>
</tr>
<tr>
<td>EIBEN, Ute</td>
<td>Prophyta GmbH Malchow, Germany <a href="mailto:ueiben@prophyta.com">ueiben@prophyta.com</a></td>
</tr>
<tr>
<td>ČAČIJA, Maja</td>
<td>University of Zagreb, Faculty of Agriculture Zagreb, Croatia <a href="mailto:mcacija@agr.hr">mcacija@agr.hr</a></td>
</tr>
<tr>
<td>EILENBERG, Jørgen</td>
<td>University of Copenhagen, Faculty of Life Sciences, Frederiksberg C, Denmark <a href="mailto:jei@life.ku.dk">jei@life.ku.dk</a></td>
</tr>
<tr>
<td>DEMIR, Ismail</td>
<td>Karadeniz Technical University Trabzon, Turkey <a href="mailto:idemir@ktu.edu.tr">idemir@ktu.edu.tr</a></td>
</tr>
<tr>
<td>ENKERLI, Jürg</td>
<td>Agroscope Reckenholz-Tänikon (ART) Zürich, Switzerland <a href="mailto:juerg.enkerli@art.admin.ch">juerg.enkerli@art.admin.ch</a></td>
</tr>
<tr>
<td>DEMIRBAĞ, Zihni</td>
<td>Karadeniz Technical University Trabzon, Turkey <a href="mailto:zihni@ktu.edu.tr">zihni@ktu.edu.tr</a></td>
</tr>
<tr>
<td>ESTER, Albert</td>
<td>Ester Research &amp; Consultancy Lelystad, The Netherlands <a href="mailto:a.ester@tele2.nl">a.ester@tele2.nl</a></td>
</tr>
</tbody>
</table>
Dimitrova, Desislava Vasileva
University of Copenhagen, Faculty of Life Sciences, Frederiksberg C, Denmark
desivd@dsr.life.ku.dk

Fernandez-Bravo, Maria
University of Cordoba
Cordoba, Spain
002febrm@uco.es

Fischer, Esther
University of Applied Science Zürich
Wädenswil, Switzerland
esther.fischer@zhaw.ch

Gayán-Bolado, María
University of Córdoba
Córdoba, Spain
o02febrm@uco.es

Fischler, Esther
University of Applied Science Zürich
Wädenswil, Switzerland
esther.fischer@zhaw.ch

Graillot, Benoît
Natural Plant Protection
Pau, France
benoit.graillot@mines-ales.fr

Giraldo-Jurado, Inmaculada
University of Córdoba
Córdoba, Spain
g72gajui@uco.es

Hantzsche, Miriam
Applied University Bielefeld
Bielefeld, Germany
miriam.hantzsche@fh-bielefeld.de

Gebhardt, Manuela
Federal Research Centre for Cultivated Plants,
Julius Kühn Institute, Darmstadt, Germany
manuela.gebhardt@jki.bund.de

Houghton, Gemma
ADAS
Cambridge, United Kingdom
Gemma.houghton@adas.co.uk

Gkounti, Vasiliki
Benaki Phytopathological Institute
Kifissia, Attiki, Greece
vgkounti@agro.auth.gr

Huber, Jürg
IBMA/DA
Darmstadt, Germany
juerg.huber@jki.bund.de

Glazer, Itamar
ARO Volcani Center
Rishon Le Zion, Israel
glazerit@agri.gov.il

Gotlin, Tanja
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
tgotlin@agr.hr

Juraj, Ivan
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
ijuran@agr.hr

Kleespies, Regina G.
Federal Research Centre for Cultivated Plants,
Julius Kühn Institute, Darmstadt, Germany
regina.kleespies@jki.bund.de
GRABENWEGER, Giselher
Agroscope Reckenholz-Tänikon (ART)
Zürich, Switzerland
giselher.grabenweger@art.admin.ch

KONRAD, Roger
Andermatt Biocontrol AG
Grossdietwil, Switzerland
konrad@biocontrol.ch

KOS, Tomislav
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
tkos@agr.hr

LÓPEZ-FERBER, Miguel
Ecole Nationale Supérieur des Mines d'Alès
Alès, France
miguel.lopez-ferber@mines-ales.fr

KÖZINA, Antonela
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
akozina@agr.hr

KÜHNE, Stefan
Federal Research Centre for Cultivated Plants,
Julius Kühn Institute, Kleinmachnow,
Germany
stefan.kuehne@jki.bund.de

KYEI-POKU, George
Natural Resources Canada, Canadian Forest Service,
Sault Ste. Marie, Ontario, Canada
gkyeipok@NRCAN.gc.ca

KÜHNE, Stefan
Federal Research Centre for Cultivated Plants,
Julius Kühn Institute, Kleinmachnow,
Germany
stefan.kuehne@jki.bund.de

LÜTH, Peter
Prophyta GmbH
Malchow, Germany
peterlueth@prophyta.com

KOZINA, Antonela
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
akozina@agr.hr

LÓPEZ-FERBER, Miguel
Ecole Nationale Supérieur des Mines d'Alès
Alès, France
miguel.lopez-ferber@mines-ales.fr

LAZNIK, Žiga
Biotechnical Faculty
Ljubljana, Slovenia
ziga.laznik@bf.uni-lj.si

MAURHOFER, Monika
Swiss Federal Institute of Technology Zürich
Zürich, Switzerland
monika.maurhofer@usys.ethz.ch

LEMİĆ, Darija
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
dlemic@agr.hr

MIKAIA, Nona
Sokhumi State University
Tbilisi, Georgia
nonamikaia@gmail.com
LINDE, Andreas
University of Applied Sciences Eberswalde
Eberswalde, Germany
alinde@hnee.de

MITTEREGGER, Maria
University of Innsbruck
Innsbruck, Austria
maria.mitteregger@student.uibk.ac.at

MOORE, Sean
Citrus Research International
Port, South Africa
seanmoore@cri.co.za

POPE, Tom
Harper Adams University
Newport, United Kingdom
tpope@harper-adams.ac.uk

MURATOĞLU, Hacer
Karadeniz Technical University
Trabzon, Turkey
hacermuratoglu@yahoo.com

PRZY KLÉN, Michael
University of Applied Sciences Bielefeld
Bielefeld, Germany
michael.przyklenk@fh-bielefeld.de

MURILLO, Rosa
Universidad Publica de Navarra
Pamplona, Spain
rosa.murillo@unavarra.es

PÜŽA, Vladimir
Biology Centre ASCR v.v.i.
České Budějovice, Czech Republic
vpuz@seznam.cz

NALÇACIOĞLU, Remziye
Karadeniz Technical University
Trabzon, Turkey
remziye@ktu.edu.tr

QUESA DA-MORAGA, Enrique
University of Córdoba
Córdoba, Spain
equesada@uco.es

NERMUT, Jiří
Biology Centre ASCR v.v.i.
České Budějovice, Czech Republic
Jirka.Nermut@seznam.cz

RAUCH, Hannes
University of Innsbruck
Innsbruck, Austria
rauchhannes@hotmail.com

ORESTE, Monica
DISSPA, University of Bari
Bari, Italy
monica.oreste@uniba.it

RONDOT, Yvonne
Hochschule Geisenheim University
Geisenheim, Germany
yvonne.rondot@hs-gm.de

PAJAČŽIVKOVIĆ, Ivana
University of Zagreb, Faculty of Agriculture
Zagreb, Croatia
ipajac@agr.hr

RUIU, Luca
Dipartimento di Agraria
Sassari, Italy
lucaruiu@uniss.it

PADEL, Anant
University of Applied Sciences Bielefeld
Bielefeld, Germany
anant.patel@fh-bielefeld.de

SAUER, Annette J.
Federal Research Centre for Cultivated Plants,
Julius Kühn Institute, Darmstadt, Germany
annette.sauer@jki.bund.de

PELZER, Bianca
University of Applied Sciences Bielefeld
Bielefeld, Germany
bianca.pelzer@fh-bielefeld.de

SCHUMANN, Mario
Georg-August-University
Göttingen, Germany
Mario.Schumann@agr.uni-goettingen.de
SEZEN, Kazim
Karadeniz Technical University
Trabzon, Turkey
sezen@ktu.edu.tr

TÓTH, Timea
Research and Extension Centre for Fruit Growing, Ujfehértó, Hungary
timi42@gmail.com

SIERPINSKA, Alicja
Forest Research Institute
Raszyn, Poland
A.Sierpinska@ibles.waw.pl

TRAUGOTT, Michael
University of Innsbruck
Innsbruck, Austria
Michael.Traugott@uibk.ac.at

SIMÓN, Oihane
Consejo Superior de Investigaciones Científicas, Mutilva, Spain
oihane.simon@unavarra.es

TURÓCZI, György
SZIE Plant Protection Institute
Gödöllő, Hungary
Turoczi.Gyorgy@mkk.szie.hu

SKRZECZ, Iwona
Forest Research Institute
Raszyn, Poland
I.Skrzecz@ibles.waw.pl

VIDAL, Stefan
Georg-August-University Göttingen, Germany
svidal@gwdg.de

STRASSER, Hermann
University of Innsbruck
Innsbruck, Austria
hermann.strasser@uibk.ac.at

VÍLCHEZ, Susana
University of Granada
Granada, Spain
svt@ugr.es

TAIBON, Judith
University of Innsbruck
Innsbruck, Austria
Judith.Taibon@uibk.ac.at

VIRTO, Cristina
UPNA
Pamplona, Spain
cristina.virto@unavarra.es

TOEPFER, Stefan
CABI Europe-Switzerland
Hódmezővásárhely, Hungary
s.toepfer@cabi.org

VLÁK, Just M.
Wageningen University
Wageningen, The Netherlands
just.vlak@wur.nl

TOOTH, Miklós
Plant Protection Institute ATK MTA
Budapest, Hungary
toth.miklos@agrar.mta.hu

VOJTEK, Libor
Masaryk University, Faculty of Science
Brno, Czech Republic
libor.vojtek@mail.muni.cz

TOY, Dönüş
Karadeniz Technical University
Trabzon, Turkey
donustoy@hotmail.com

WANDELER, Heiri
Andermatt Biocontrol AG
Grossdietwil, Switzerland
wandeler@biocontrol.ch
WENNMANN, Jörg
Federal Research Centre for Cultivated
Plants,
Julius Kühn Institute, Darmstadt, Germany
joerg.wennmann@jki.bund.de

WILLIAMS, Trevor
Instituto de Ecologia A C
Xalapa, Mexico
trevor.inecol@gmail.com
Contents

Sponsors ........................................................................................................................................... I

Organizing committee ..................................................................................................................... III

Preface ........................................................................................................................................... V

List of participants ......................................................................................................................... VII

Contents ......................................................................................................................................... XV

Plenary Session: State-of-the-art in microbial control

Authorization of biological control agents - theory and practice
Ralf-Udo Ehlers ................................................................................................................................. 3-7

Dynamics of baculovirus as insect biocontrol agent
Just M. Vlak, Monique M. van Oers ............................................................................................ 9-10

Insect pathogenic fungi: what was obtained and where to go?
Jørgen Eilenberg ............................................................................................................................ 11-13

Fungi

Session 1: Entomopathogenic fungi in the control of soil-dwelling pests

Biological control of wireworms with entomopathogenic fungi
S. Eckard, M. A. Ansari, T. M. Butt, J. Enkerli, G. Grabenweger .............................................. 17-20

Monitoring of the entomopathogenic fungus Beauveria brongniartii in cockchafer infested areas of the Euroregion Tyrol
Johanna Mayerhofer, Jürg Enkerli, Roland Zelger, Hermann Strasser ................................. 21-25

Susceptibility of Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae) to entomopathogenic fungi: Laboratory assays and field trials
Hannes Rauch, Roland Zelger, Stefan Hutwimmer, Hermann Strasser .................................. 27-31

Efficacy of biological control agents for the control of western corn rootworm
Emese Balog, Bui Xuan Hung, György Turóczi, József Kiss ..................................................... 33-36

Exploring synergistic effects of semiochemicals, entomopathogenic fungi and nematodes against root-herbivores
Michael A. Brandl, Mario Schumann, Stefan Vidal ................................................................. 37
Session 2: Above-ground use of entomopathogenic fungi in protected and open field crops

Entomopathogenic fungi ecology and diversity from different Mediterranean ecosystems
Maria Fernández-Bravo, Enrique Quesada-Moraga, Inmaculada Garrido-Jurado ................................................................. 41

Efficacy of two strains of Beauveria bassiana entomopathogenic fungus on the red palm weevil in France and in Spain
Samantha Besse, Ludovic Crabos, Karine Panchaud ......................................................... 42

Beauveria bassiana strain ATCC 74040 interferes with oviposition behavior of Mediterranean fruit fly
Luca Ruiu, Giovanni Falchi, Edith Ladurner ............................................................. 43-46

Pathogenicity of an indigenous strain of the entomopathogenic fungus Beauveria bassiana on larvae and adults of the sisal weevil, Scyphophorus acupunctatus Gyllenhall (Coleoptera: Curculionidae)
V. T. Gkounti, D. Markoyiannaki, D. C. Kontodimas ..................................................... 47-49

Microbial control of European red spider mite (Panonychus ulmi) with Beauveria bassiana strain ATCC 74040
Edith Ladurner, Massimo Benuzzi, Andrea Braggio, Sergio Franceschini, Veselin Zivkovic ................................................................. 51-55

Mycopathogens of the corn leaf aphid, Rhopalosiphum maidis (Fitch.) infesting wheat plants at Assiut, Egypt
Ahmed Y. Abdel-Mallek, Mohamed A. A. Abdel-Rahman, Gamal H. A. Hamam ............. 56

Session 3: New strategies for delivering and monitoring of entomopathogenic fungi

Exploiting vine weevil behaviour to disperse an entomopathogenic fungus
Tom Pope, Charlotte Arbona, Harriet Roberts, Jude Bennison, John Buxton, Gill Prince and Dave Chandler ................................................................. 59-62

Field persistence of Metarhizium spp. strains applied as biocontrol agents against ticks (Ixodes ricinus)
Maria Mitteregger, Sarah Sonderegger, Hermann Strasser ......................................................... 63-67

Vertical transmission of an endophytic strain of Beauveria bassiana (Ascomycota; Hypocreales) colonizing opium poppy Papaver somniferum
Enrique Quesada-Moraga, Blanca B. Landa del Castillo, Cristina López-Díaz ............. 68

Development of a novel fermentation and formulation process for an endophytic Beauveria bassiana strain
Rieke Lohse, Desiree Jakobs-Schönwandt, Anant Patel ................................................................. 69-73

Development of analytical tools to monitor the fate of Metarhizium anisopliae metabolites in the environment
Judith Taibon, Sonja Sturm, Christoph Seger, Hermann Strasser, Hermann Stuppner ................................................................. 75-78

Cross-species transferability of 41 microsatellite markers for Metarhizium spp.
Andy Lutz, Franco Widmer, Adrian Leuchtmann, Jürg Enkerli ................................................................. 79-83
Posters

A review of the use of entomopathogenic fungi for the control of *Bemisia tabaci* (Hemiptera: Aleyrodidae) in the UK
Andrew G. S. Cuthbertson ................................................................. 87-90

Effect of entomopathogenic fungi against *Trialeurodes vaporariorum* and its parasitoid *Encarsia formosa*: preliminary laboratory assays
Monica Oreste, Michele Poliseno, Eustachio Tarasco .............................. 91

Laboratory *Beauveria bassiana* (Bals.) Vuill. bioassays on spruce bark beetle (*Ips typographus* L.)
Ana-Maria Andrei, Daniela Lupâştean, Constantin Ciornei,
Ana-Cristina Fătu, Mihaela Monica Dinu ........................................ 93-96

Effect of local strains of *Beauveria bassiana* (BB024) and *Metarhizium anisopliae* (M 7/2) against the fallweb worm *Hyphantria cunea* (Lepidoptera: Arctiidae) in Georgia
Medea Burjanadze, Elena Nakaidze, Mariam Arjevanidze, Tea Abramishvili ........ 97-101

Highly effective *Beauveria pseudobassiana* strain (Dm-5) against the great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Scolytidae)
Ismail Demir, Seda Kocacevik, Ali Sevim, Mahmut Eroglu, Zihni Demirbag ........ 102

Laboratory testing of insect associated fungi for the control of wireworms (*Agriotes* sp. L.)
Jaka Razinger, Matthias Lutz, Hans-Josef Schroers,
Gregor Urek, Jürg Grunder .................................................................. 103-107

Laboratory and semi-field trials on the effects of *Beauveria bassiana* (JW-1, ATCC 74040) against soil-dwelling stages of *Frankliniella occidentalis* (Thysanoptera: Thripidae)
Andrea Boaria, Alberto Pozzebon, Mauro Pesce, Mauro Lorenzon,
Carlo Duso .......................................................................................... 109-112

Prevalence of the species *Beauveria pseudobassiana* among tick-associated fungal isolates from the Republic of Moldova
Polina V. Mitkovets, Natalia V. Munteanu, Galina V. Mitina, Yuri S. Tokarev,
Alexandr A. Movila, Ion Toderas, Regina G. Kleespies, Andreas Leclerque ........ 113-117

Evaluation of indigenous *Beauveria* isolates as potential agents for emerald ash borer management and the development of a diagnostic marker to monitor a post-release isolate
George Kyei-Poku, Shajahan Johny ..................................................... 119-124

Isolation and identification of endophytic entomopathogenic fungi from dent corn
Daigo Aiuchi, Tatsumi Takanami, Sayaka Toba, Minehiro Ishii,
Shin-ichiro Asano, Masanori Kitae ................................................ 125-128

Endophytic establishment of the entomopathogen *Beauveria bassiana* in *Vitis vinifera* plants
Yvonne Rondot, Annette Reineke ...................................................... 129

Effect of temperature, water activity and UV-B radiation on conidia germination and colony growth of *Beauveria bassiana* isolates from soil and phylloplane
Maria Fernández-Bravo, Inmaculada Garrido-Jurado, Monica Oreste,
Enrique Quesada-Moraga ................................................................. 130
Viruses

Session 1

Deletion genotypes influence occlusion body potency and production in insects infected by a *Spodoptera frugiperda* nucleopolyhedrovirus isolate from Colombia

_Gloria Barrera, Trevor Williams, Laura Villamizar, Primitivo Caballero, Oihane Simón_ .......................................................... 133-136

On the role of baculovirus photolyases in DNA repair upon UV damage of occlusion bodies

_Magdalena A. Biernat, Primitivo Caballero, Just M. Vlak, Monique M. van Oers_ ........................................................................ 137-142

Effect of top spray drying and freeze drying on the photostability and insecticidal activity of a *Spodoptera frugiperda* nucleopolyhedrovirus (SM NPV 003) formulation

_Mauricio Cruz, Martha Liliana Chaparro, Laura Fernanda Villamizar, Martha Isabel Gomez_ ............................................................. 143-147

Variations in the susceptibility to CpGV in populations of the codling moth, *Cydia pomonella*

_Benoît Graillot, Christine Blachere, Samantha Besse, Myriam Siegwart, Miguel López-Ferber_ ........................................................... 149-153

Characterisation of novel CrleGV isolates for false codling moth control - lessons learnt from codling moth resistance to CpGV

_John Opoku-Debrah, Sean Moore, Martin Hill, Caroline Knox_ ................................................................. 155-159

Elucidation of a novel mode of resistance of codling moth against *Cydia pomonella* granulovirus by homogenization experiments

_Annette J. Sauer, Eva Fritsch, Karin Undorf-Spahn, Johannes A. Jehle_ .......................................................... 161-165

Session 2

Biological control of the box tree moth *Cydalima perspectalis* with *Anagrapha falcifera* nucleopolyhedrovirus (AfNaNPV)

_Jana Rose, Johannes A. Jehle, Regina G. Kleespies_ .......................................................... 169-172

Interactions between structural proteins of *Chilo iridescent virus*

_Emine Özşahin, Remziye Nalcacioglu, Just M. Vlak, Monique M. van Oers, Zihni Demirbağ_ ................................................................. 173

Natural populations of *Spodoptera exigua* are infected by multiple viruses: implications for the production and use of virus insecticides

_Cristina Virto, David Navarro, M. Mar Tellez, Salvador Herrero, Trevor Williams, Rosa Murillo, Primitivo Caballero_ .......................... 175-177

Estimating the importance of maternal and paternal contributions to the vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeM NPV)

_Cristina Virto, Carlos A. Zárate, Rosa Murillo, Primitivo Caballero, Trevor Williams_ .......................................................... 179-181

Baculoviruses for the biological control of cutworms (*Agrotis* spp.)

_Jörg T. Wennmann, Gianpiero Gueli Alletti, Johannes A. Jehle_ .................................................. 183-186
Posters

Insecticidal activity of a spray dried formulation based on a Colombian Spodoptera frugiperda nucleopolyhedrovirus
Judith Elena Camacho, Martha Isabel Gómez, Mauricio Cruz, Laura Fernanda Villamizar ................................................................. 189-193

Cydia pomonella granulovirus knockout mutants: The potential role of pe38 in overcoming codling moth resistance
Manuela Gebhardt, Karolin E. Eberle, Johannes A. Jehle ................................. 194

Sequence analysis of CpGV-R5 isolate, able to efficiently control CpGV-M resistant insects: relation between biological activity and genome
Benoît Graillot, Samantha Besse, Christine Blachère-Lopez, Jérôme Olivares, Myriam Siegwart, Miguel López-Ferber ............................. 195-199

Functional characterization of serine/threonine protein kinase gene (AMV197) of Amsacta moorei entomopoxvirus
Hacer Muratoglu, Remziye Nalcacioglu, Srini Perera, Basil Arif, Zihni Demirbag ................................................................. 201

Transcriptional analysis of CpGV isolates in Cydia molesta
Dönüs Toy, Diana Schneider, Zihni Demirbag, Johannes A. Jehle ................................. 202

An examination of stress-related activation of SeMNPV in covertly infected Spodoptera exigua
Cristina Virto, David Navarro, María Mar Tellez, Rosa Murillo, Trevor Williams, Primitivo Caballero ................................................ 203-205

Functional analysis of Chilo iridescent virus zinc-binding matrix metalloproteinase gene
Aydın Yesilyurt, Hacer Muratoglu, Zihni Demirbag, Remziye Nalcacioglu ................. 206

Soil pests

Latest field results on the biological control of Diabrotica virgifera virgifera with nematodes
Ralf-Udo Ehlers ................................................................. 209

Development of new formulations for soil pest control
Miriam Hanitzsch, Michael Przyklenk, Bianca Pelzer, Anant Patel .................. 211-215

Click beetles disperse widely across farmland: what else do we need to know?
Rod Blackshaw, Robert S. Vernon ................................................................. 217-220

Distribution and abundance of Agriotes ustulatus L. adults on pheromone traps in four regions in Croatia
Antonela Kozina, Maja Čačija, Renata Bažok ................................................................. 221-225

Efforts to develop female-targeted attractants for click beetles – a summary
Miklós Tóth, Lorenzo Furlan, József Vats, Éva Bálintné Csonka, István Szarukán, Teodora B. Toshova, Mítko Subchev, Dimitar I. Velchev, Christine M. Woodcock, John C. Caulfield, Patrick Mayon, John A. Pickett, Michael A. Birkett ................................................................. 221-225

New perspectives for wireworm control based on an improved understanding of their feeding ecology
Michael Traugott, Karin Staudacher, Nikolaus Schallhart, Corinna Wallinger .......... 231
Posters

Exploratory use of geometric morphometrics in the identification of wireworm species
Darija Lemić, Katarina Mikac, Hugo A. Benítez, Maja Čačija, Antonela Kožina, Renata Bažok ............................................................ 235

Development of novel biocontrol encapsulation techniques for garlic extracts: first results
Bianca Pelzer, Miriam Hanitzsch, Anant Patel ..................................................... 237-240

The project ATTRACT: Protection of crops from soil-borne insect pests with a novel attract and kill strategy

IPM (Fungi/Bacteria)

IPM microbial control based strategies

Combined use of entomopathogenic fungi and their extracts to improve the control of the cotton leafworm Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae)
Inmaculada Garrido-Jurado, Gloria Resquín-Romero, Enrique Quesada-Moraga .... 245

Insecticidal activity of a semi-purified extract from Metarhizium brunneum (Ascomycota: Clavicipitaceae) against the red palm weevil Rhynchophorus ferrugineus (Coleoptera: Curculionidae)
Inmaculada Garrido-Jurado, Óscar Dembilio, Josep Anton Jacas, Lola Ortega, Carlos Campos, Enrique Quesada-Moraga ................................................................. 246

Subterranean control of an arboreal pest: EPNs and EPFs for FCM
Sean Moore, Candice Coombes, Aruna Manrakhan, Wayne Kirkman, Martin Hill, Ralf-Udo Ehlers, John-Henry Daneel, Jeanne de Waal, Jo Dames, Antoinette Malan ............................................................... 247-250

Do plant-associated insect toxin producing pseudomonads have the potential for the biocontrol of insect pests?
M. Maurhofer, B. Ruffner, P. Flury, M. Péchy-Tarr, E. Fischer, P. Kupferschmied, C. Keel ............................................................................................. 251

Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays
P. Flury, B. Ruffner, M. Péchy-Tarr, P. Kupferschmied, C. Keel, M. Maurhofer .......... 252

Colorado potato beetle (Leptinotarsa decemlineata Say) – control strategies in organic farming using biological insecticides (azadirachtin, Bacillus thuringiensis var. tenebrionis, pyrethrum and spinosad)
Stefan Kühne, Uta Priegnitz, Benjamin Hummel, Frank Ellmer ............................. 253-256
Nematodes

Update on life cycle of entomopathogenic nematodes
Ralf-Udo Ehlers ................................................................. 259-260

Aiming to eradicate small hive beetle Aethina tumida
using entomopathogenic nematodes
Andrew G. S. Cuthbertson, James J. Mathers, Lisa F. Blackburn, Gay Marris,
Mike A. Brown, Giles E. Budge ........................................ 261-265

The development of mollusc-parasitic nematode Phasmarhabditis hermaphrodita
(Nematoda: Rhabditidae) in different substrates
Jiří Nermut, Vladimír Půža ................................................ 267-270

New nematodes associated to Rhynchophorus ferrugineus
(Coleoptera: Curculionidae): preliminary description
Monica Oreste, Francesca De Luca, Elena Fanelli,
Alberto Troccoli, Eustachio Tarasco .................................. 271

The role of bacterial symbionts in the competition
of entomopathogenic nematode species
Vladimír Půža, Jiří Nermut, Zdeněk Mráček ........................ 273-276

Research and development for a nematode-based biological control solution
for western corn rootworm in maize
Stefan Toepfer, Ulrich Kuhlman .......................................... 277-282

Posters

Development of a method to establish entomopathogenic nematodes (EPN)
in arable soils by using farm-suitable field equipment
Wolfgang Büchs .................................................................. 285

Biosafety analysis of the Bacillus pumilus 15.1 strain
through a Caenorhabditis elegans pathogenicity assay
Juan F. Caña Roca, Diana C. García, Juan I. Vílchez-Morillas,
Maximino Manzanera, Tania Domínguez, Antonio Osuna, Susana Vílchez ...... 287-290

The indigenous entomopathogenic nematode searching results
at different agrocenosis of Georgia
M. Chubinishvili, Ts. Chkhubianishvili, M. Kakhadze, I. Malania, I. Rijamadze ....... 291

Field evaluation of entomopathogenic nematodes for controlling
fall webworm Hyphantria cunea (Lepidoptera: Arctiidae) in West Georgia
Oleg Gorgadze, Manana Lortkipanidze, Patrick Tailliez, Medea Burjanadze,
Madona Kuchava ................................................................ 293-296

Feeding activity and survival of slug Arion lusitanicus (Gastropoda: A rionidae)
exposed to the rhabditid nematode, Phasmarhabditis hermaphrodita
(Nematoda: Rhabditidae)
Dinka Grubišić, Tina Hamel, Tanja Gotlin Čuljak, Ana Loparić,
Mirjana Brmež ........................................................................ 297-300

New insights to insect response to the infection by nematobacterial complex
Pavel Hyršl, Pavel Dobeš, Badrul Arefin, Lucie Kučerová,
Robert Markus, Zhi Wang, Michal Žurovec, Ulrich Theopold ......................... 301
Compatibility of five different entomopathogenic nematode (Nematoda: Rhabditida) species with registered insecticides and fungicides under laboratory conditions
Žiga Laznik, Stanislav Trdan ................................................................. 303-308

Susceptibility of Phytodecta fornicata (Coleoptera: Chrysomelidae) to Heterorhabditis bacteriophora
Ivana Majić, Emilija Raspudić, Marija Ivezić, Mirjana Brmež, Ankica Sarađlić, Andrea Mirković ................................................................. 309-312

The susceptibility of mulberry moth to infection by entomopathogenic nematodes, Heterorhabditis bacteriophora and Steinernema carpocapsae
Nona Mikaić ................................................................. 313-316

Attract and kill against western corn rootworm larvae with entomopathogenic nematodes
Mario Schumann, Felicitas Kaemena, Anant Patel, Stefan Vidal ..................... 317-318

Bacteria

Poster

Cloning strategy for recovering phage-displayed Cry1Aa13 mutants from phages with affinity towards proteins present in the gut of Ceratitis capitata
Tania Domínguez, Juan F. Caña Roca, Diana C. García, Antonio Osuna, Susana Vílchez ................................................................. 321-325

Efficacy evaluation of different Bacillus thuringiensis sv kurstaki strain EG2348 formulations against Malacosoma neustria (Lepidoptera: Lasiocampidae)
Luca Ruiu, Achille Loi, Giovanni Falchi, Edith Ladurner, Andrea Braggio, Pietro Luciano ................................................................. 327-330

Development of a new bio-insecticide for controlling lepidopteran pests
Kazım Sezen, Remziye Nalçacığoğlu, Ismail Demir, Hüseyin Tepe, Islam Yıldız, Zihni Demirbağ ................................................................. 331

Bioluminescence determination of antibacterial activity of Bombyx mori and Galleria mellonella haemolymph
Libor Vojtek, Pavel Dobes, Ender Buyukguzel, Pavel Hyrsl ................................................................. 333-338

Intramolecular cleavage at the loop between α3-helix and α4-helix is critical for cytotoxic activity of Cry8Da
Takuya Yamauchi, Hisanori Bando, Shin-ichiro Asano ................................................................. 339-342

Electron microscope and genetic analysis of an intracellular bacterium associated with the common rough woodlouse, Porcellio scaber (Isopoda, Porcellionidae)
Regina G. Kleespies, Andreas Leclerque ................................................................. 343-347
Miscellaneous

Poster

Impact of various oilseed rape productions on biological potential of endogaecic active ground beetles (Coleoptera: Carabidae)
Ivan Juran, Tanja Gotlin Čuljak, Wolfgang Büchs, Dinka Grubišić, Ivan Sivčev ........ 351

Ground beetles (Coleoptera: Carabidae) in sugar beet fields as the base for conservation biological control
Tomislav Kos, Renata Bažok, Zrinka Drmić, Željka Graša ........................................... 353-357

Impact of Entomophaga maimaiga on gypsy moth populations in Bulgaria
Plamen Mirchev, Andreas Linde, Daniela Pilarska, Plamen Pilarski, Margarita Georgieva, Georgi Georgiev ................................................................. 359-363
Plenary session:
State-of-the-art in microbial control
Authorisation of biological control agents - theory and practice

Ralf-Udo Ehlers
e-nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany
e-mail: Ehlers@e-nema.de

Abstract: The legislation of the European Union regarding the placement of plant protection products on the market (Regulation (EC) No 1107/2009) and also the Directive 2009/128/EC on the sustainable use of pesticides pave the ground for increasing use of biological control agents in Europe. Both clearly give priority to the use of alternative, non-chemical control measures. However, the general practice in member states is different. Whereas member states largely seem to ignore the potential of biological control, chemical control companies have increasing interest in biological control and have acquired several biocontrol companies in order to get access to biocontrol biodiversity and know-how.

Introduction

The history of pesticide regulation has been a process of replacement of one chemical group by another, which often exhibited another set of problems. This process was accompanied by the development of more and more stringent rules taking into account scientific reports of damage caused by synthetic compounds and anticipated risks of new compounds. Governments responded to reports of damage with the development of new rules to ensure that similar impacts will not occur with new compounds. Currently the ban of neonicotinoide compounds is discussed because of their potential influence on bee health. Since the introduction of regulation in Europe, registration requirements and guidance documents had always been developed in consultation with multinational agrochemical companies. Other than regulation of synthetic compounds, regulations for biological plant protection products have not evolved within such a process:

- Regulation of biological control agents (BCAs) was not a gradual evolution involving industry
- Regulation was not based on scientific reports of damages, as there are hardly any reports on damage of BCAs
- BCAs have no evolution of regulatory rules; rules for synthetic compound were implemented on biocontrol without consultation of biocontrol industry

In order to discuss more adapted approaches to regulation of biologicals and avoid exaggerating regulation requirements, the EU supported the policy support action REBECA (Regulation of Biological Control Agents). REBECA made several valuable proposals how the system could be improved in order to accelerate the placement of BCAs on the market (Ehlers, 2011). These proposals have been discussed but, apart from minor improvements, major changes have not been made since. New regulations are still implemented without a scientific analysis of potential risks related to the use of BCAs (currently for instance the Biocide Regulation 528/2012).

Waivers for data requirements are still limited. One example: The agent Bacillus thuringiensis israelensis (Bti) is used to control mosquitoes. Bti is considered the safest insecticide by the WHO (WHO, 1999). It may possibly be the number one product in sales in
the world-wide biocontrol market and negative impacts on the environment are scarce or completely absent. A registration for the used against tipulids, a new indication, would require data on toxicity, chemical properties, analytical methods for quality control and evaluation of toxicity and non-target effects. Costs have been estimated to surpass 0.5 million € for a Reg. (EC) No 1107/2009 Annex1 listing. Such expenses are not acceptable for a product, which has been used for decades without causing any damage. Many other products are not coming to the market because the costs for registration are too high.

One of the future tasks of the European Community (EC) common agriculture policy (CAP) is the greening of agricultural practice. When the Directive 91/414/EEC, regulating the placement of plant protection products on the market, was replaced by Regulation (EC) No 1107/2009, several good steps towards greening of CAP were taken: The new regulation, together with the Directive 2009/128/EC on the sustainable use of pesticides (SUD), now gives priority to non-chemical plant protection methods. However, the attempts by the EC to support introduction of biocontrol are most often foiled by member states (MS), which do not follow these rules when handling authorisation of plant protection products. As a consequence, chemical products are given priority.

**Articles 53 of Regulation (EC) No 1107/2009**

Article 53 of the Regulation (EC) No 1107/2009 provides information on the authorisation of plant protection products (PPP) in emergency situations: “By way of derogation from Article 28, in special circumstances a Member State may authorise, for a period not exceeding 120 days, the placing on the market of plant protection products, for limited and controlled use, where such a measure appears necessary because of a danger which cannot be contained by any other reasonable means.” Several cases of authorisation of chemical products exist although BCAs would have been available.

**The SUD Directive**

Regulation (EC) No 1107/2009 references the Directive 2009/128/EC on the sustainable use of pesticides (SUD) for seven times. In this directive Article 14 indicates that “the MS shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods, so that professional users of pesticides switch to practices and products with the lowest risk to human health and the environment.” EU regulations are implemented in each MS in the moment they are issued. Directives have to be transferred into MS laws. The deadline for directive 2009/128/EC is January 2014. Some MS currently negotiate with the Commission to prolong this period.

It is interesting to analyse how MS want to reach the goal of pesticide reduction and whether they consider BCAs to substitute for chemical compounds. Biocontrol community could certainly help MS to put this directive into practice.

MS were asked to develop National Action Plans (NAPs) to provide strategies for reduction of pesticide use. Some MS have produced these plans others are still working on their plans. The German Action Plan was published 2005 and is a colourful document of 46 pages. Within this document the word “Biological Control” occurs a single time and documents the use of Trichogramma on 3,000 ha of corn seed production. Biological control is of much larger importance in Germany but this is not recognised by the Ministry of Agriculture and related organisations, which develop strategies to reduce the use of pesticides.
EU supported projects like ENDURE or PURE also give little attention to biological control. Considerable amounts of funds are spent on re-defining IPM instead of supporting the introduction of biological control strategies. Are the EU plans to prioritise biocontrol lip services with little political consequences and even less practical influence for safety of food and the environment?

**Example: Control of the invasive maize pest corn rootworm**

The corn rootworm *Diabrotica virgifera virgifera* is an invasive pest in Europe. It causes major damage to corn and MS and the EU try to limit spreading of the pest in Europe. Eradication programmes have failed and it has been spreading from the Balkan region into Austria, Poland, Germany, Italy and France. The pest overwinters in the egg stage, thus rotation is a possibility to control the pest. However, costs for rotation are much higher than control measures, even biological control.

No chemical compound has an official authorisation according to Reg. (EC) No 1107/2009 for control of this pest. However, MS have given emergency authorisations for 120 days (according to Art. 53 of Reg. (EC) No 1107/2009) for neonicotinoids (imidacloprid, thiametoxam and clothianidin) for seed treatment of corn and/or the pyrethroid tefluthrin in a granular formulation. Since 10,000 bee hives in Germany had suffered from major mortality due to seed treatment with clothianidin in 2008, German authorities did not permit the use of neonicotinoids again. Plant protection products containing neonicotinoids (thiametoxam, imidaclorpid, clothianidin) are under suspect to contribute to the Colony Collapse Disorder of bees (e.g., Henry et al., 2012).

As *Diabrotica* is a quarantine pest, the Article 53 of Regulation (EC) No 1107/2009 provides appropriate justification for authorisation because of emergency. However, in Article 53 the words “any other reasonable means” are important to be considered. “Reasonable” means that the alternatives should be as effective as chemical measures and should be economically viable. Current authorisation practice in member states for control of the invasive pest *Diabrotica virgifera virgifera* never took into consideration non-chemical alternatives.

Since 2011 a biological control product based on the entomopathogenic nematode *Heterorhabditis bacteriophora* is on the market. The nematodes have been field tested in Hungary for 7 years, in Austria for 5 years and in Italy for 3 years in numerous trials and the results indicate that the nematode *H. bacteriophora* achieved equally high control results compared to the chemical seed treatment with a neonicotinoid or application of the granular pyrethroid (e.g. Toepfer et al., 2010). Nematodes are approximately 60 €/ha more expensive than chemical control with tefluthrin in Germany. Hence, “other reasonable means” exist.

The general practice of MS authorities is to renew article 53 authorisations every year, even for a product which has not even an authorisation for other indications. Tefluthrin had been rejected by the EC because of negative side-effects on soil biota and it was re-submitted in 2008. It was then authorised only for use as insecticide for pelleting of sugar beet seeds. But it has no Annex 1 listing to be used as granular formulation with additional compounds and in high concentration in the soil.

In 2011 and 2012, only tefluthrin was authorised in Germany for control of larvae. Because of the negative effects on soil biota, German authorities allow the use only every three years. However, many growers who could not rotate after growing maize were obliged to control the larvae because of its quarantine status (see also EU recommendation 2006/565/EC and Decision 2003/766/EC). So, these growers either missed to control the
quarantine pest breaking the EU laws or the growers did not comply with requirements on the use of tefluthrin allowing the use only every three year. It would have been possible to avoid this illegal situation by use of biological control. However, resistance against using a biological control method were so severe from all different organisations that biological control was not used. Only this year 2013, for the first time, it will be used on 150 ha with financial support by the State Ministry in Baden-Wuerttemberg, Germany. There is light at the end of the tunnel.

**Example: Authorisation of antibiotic in fire blight control**

Many cases of ignorance towards available biological alternatives can be reported. Another case is the authorisation of the antibiotic compound streptomycin-sulphate for control of fire blight (*Erwinia amylovora*). Over several years this antibiotic was authorised in Germany. Again, no registration on Annex 1 exists for control of fire blight and not even for any other indication. In general, antibiotics are excluded from use in plant protection because of concerns regarding their use in human chemotherapy. Resistance against antibiotics in rapidly achieved after continuous use and can occur also under field situations. However, grower organisations urgently requested authorisation to get the emergence registration years over years, although biological products were available, like the biological control agent *Aureobasidium pullulans*. In Germany for the 2013 season progress is now made and biological compounds were also given an emergency authorisation.

**The new EU Biocid Regulation (EU) No 528/2012**

In Article 3 of the Regulation (EU) No 528/2012 definitions are provided for the purposes of this Regulation. Under point 1 microorganisms are defined: (b) "micro-organism" means any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including lower fungi, viruses, bacteria, yeasts, moulds, algae, protozoa and microscopic parasitic helminths. Without consultation of biological control academia or industry, helminths have been included under “microorganisms”. Similar attempts were prevented before Regulation (EC) No 1107/2009 was implemented. After the draft version was made available by the Commission, COST Action 850 “Biocontrol Symbiosis” together with several stakeholders from industry and the Environmental Protection Agency (EPA), in charge of pesticide regulation in the USA, could persuade the EU officials to remove nematodes from the definition of microorganisms. But someone has had the idea of changing the practice for the biocide directive without a risk-damage analysis or a cost-benefit analysis of this regulatory measure. Is our safety regulation managed by incompetent and autistic personal?

**Member states disregard biocontrol, chemical companies discover their potential**

Whereas MS authorities and often also governmental R&D organisations ignore the potential of biological control agents, chemical industry has discovered the benefits. In 2011 and 2012 a series of takeovers of companies specialising in biocontrol were reported. In a press release (28 November 2012) it was announced that BASF has completed the acquisition of Becker Underwood from Norwest Equity Partners, a US-based private equity investment company,
for a purchase price of US$ 1.02 billion (785 million Euro). With the acquisition, BASF is now a leading global provider of technologies for biological seed treatment as well as producer of entomopathogenic nematodes Heterorhabditis and Steinernema. On 19 September 2012, Syngenta announced that it acquired Pasteuria Bioscience Inc., a US-based biotechnology company developing and commercialising biological products to control plant-parasitic nematodes, using the naturally occurring soil bacterium Pasteuria penetrans. Syngenta acquired the company for US$ 86 million, with additional deferred payments of up to US$ 27 million. On 21 January 2013, Bayer CropScience announced the completion of its purchase of Prophyta GmbH, Germany, a leading supplier of microbial crop protection products. Prophyta, founded in 1992, provides the product Contans for control of Sclerotinia spp. and Bioact, Paecilomyces lilacinus, for control of Meloidogyne spp. in greenhouse vegetables. A part from these acquisitions, chemical industry is heavily investing into R&D of biological control and registration of products based on microbial control agents can soon be expected. Of course, we have experienced engagement into biological control before with unsuccessful outcome, why these activities are very critically followed. But the situation has changed: Political directions, like greening of CAP, problems with residues of synthetic compounds in food and changing consumer behaviour set other priorities. We will experience more input into biological control, despite the fact that some stakeholder are still stuck in the paradigms of the last century.

References

Dynamics of baculovirus as insect biocontrol agent

Just M. Vlak, Monique M. van Oers
Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

Abstract: Baculoviruses are pathogens causing disease in lepidopteran, dipteran and hymenopteran insects. They are largely specific and form a viable biological alternative to chemical insecticides or to genetically-modified crops in the control of insect pests. There are over seven hundred reported baculoviruses, but only a fraction of these has been developed and registered as biocontrol agent. Baculoviruses are considered safe for humans and non-target animals, even when used in food crops. Drawbacks are their slow speed of action and UV-sensitivity, but these can be mitigated to some extent by ‘smart-spraying’ techniques and by using proper formulations.

Baculoviruses were strongly promoted in the 1970s in the wake of ‘Silent Spring’ and many small and big size companies started to develop baculoviruses commercially. Many research centers emerged and studied different aspects of baculovirus genetics and ecology. In the 1980s both in vivo and promising in vitro production technologies were set up and novel insight in the molecular genetics of baculoviruses allowed engineering of baculoviruses with improved insecticidal properties. However, in vitro production technology never matured to the extent that could compete with in vivo production and the societal discussion on genetically modified baculoviruses halted further development in that direction.

In the 1990s major industries reduced their baculovirus activities or withdrew from the scene altogether and only a few baculovirus products and companies survived until the present day. Also many of the research centers on baculoviruses have disappeared for a variety of reasons and one wonders whether there is enough vitality, or novel developments and opportunities to foresee another wave of interest in baculoviruses as biocontrol agent. This is the topic of this contribution and an effort to highlight recent and exciting developments and novel opportunities in this area. The interest in baculoviruses as vector for the expression of foreign genes and for gene delivery in a human or veterinary setting (vaccins, gene therapy) never ceased to exist and is even enhanced with the recently registered human vaccines (human papilloma virus, influenza), therapeutics (prostate cancer) and gene therapy applications (lipoprotein lipase deficiency) products based on this vector system. Research in this area also has a positive feedback for the further understanding of baculoviruses as infectious agent.

There a number of emerging areas in baculovirus research that are relevant for insect biocontrol and worth discussing. Although there is substantial information on the molecular genetics and functional genomics of baculoviruses, much less is known on the ecology and behavior of these viruses and the genes associated with these processes. Baculoviruses have evolved from a common ancestor, but have diverged and adapted to their respective hosts to optimize their own survival, not necessarily by killing their host fast but optimizing virus dispersal. Understanding these processes better and identifying the virus and host genes driving these processes, calls for a detailed understanding of the hosts’ behaviour and its biological and behavioral response to virus infection. This could mean that not only baculoviruses show species specificity but also the host response may be species specific. This aspect will be highlighted with a few examples. A second aspect to discuss is the notion
that baculoviruses species are in fact a cloud of related genotypes (baculovirus isolates or strains) but also mixture of genotypes within each isolate, the relative proportion of which may determine the outcome of infection. The recently observed cases of resistance against baculoviruses can be overcome by using other strains of the virus, highlighting the importance of strain selection, identification and characterization. The inference of this information for the use of baculoviruses as biocontrol agent will be discussed.

**Key words:** baculoviruses, biocontrol, biodiversity, behavior

**References**


Insect pathogenic fungi: what was obtained and where to go?

Jørgen Eilenberg
Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frb. C., Denmark
e-mail: jei@life.ku.dk

Abstract: Since its start in 1991 the IOBC working group ‘Insect Pathogens and Insect Parasitic Nematodes’ has held 13 meeting, plus some subgroup meetings. Papers on fungal entomopathogens have been presented at all meetings, and likewise, fungi have been part of presentations at all Annual Meetings in Society for Invertebrate Pathology (SIP). By looking into especially the most recent presentations and literature I will discuss the status of insect pathogenic fungi in biological control: what was obtained and where to go?

Key words Insect pathogenic fungi, Hypocreales, Entomophthorales

The fungal species: we have more species than we thought

The species concept of insect pathogenic fungi has moved significantly towards a splitting of several well-known Hypocreales into clades, which can be considered as species, eventually cryptic species. The species Metarhizium anisopliae is not any more ‘just’ M. anisopliae, but should be split into several species, including M. brunneum and M. robertsii. It is a challenge for approval authorities and for scientists to learn if a study on ‘M. anisopliae’ do really refer to that species or merely refers one of the other species. The same development has appeared for Beauveria bassiana. Also, the application of new nomenclature rules has significantly influenced our perception of the identity of certain hypocrealean fungi (Humber, 2012), for example if the name of the teleomorph should be used rather than the name of the anamorph (Vega et al., 2012).

The Entomophthorales have been less subjected to significant changes in naming, probably due to the fact that is has for long been known, that correct identification of these specialist fungi besides morphological data also need the inclusion of pathobiological and molecular data. For example, the genus Entomophthora consists of a number of species with differences both with respect to morphology, molecular biology and pathobiology (host range), and by that can be documented as being an old lineage (Jensen et al., 2009).

Field ecology: it is complicated

The field ecology of entomopathogenic fungi was outlined by Hesketh et al. (2010). A major element is, how transmission of the disease takes place in nature, where the spore concentration would normally be far below the levels used for biocontrol experiments. On one hand, there will surely be hot-spots with higher spore concentrations and also, there might be several types of interactions with hosts allowing host and fungus to meet and allow spores to stick to cuticle and readily infect. On the other hand it appears that for example different clades/species of Metarhizium have different natural ecology (Steinwender et al., 2012) and...
by that the concentration of each of these clades are even lower than the concentration of the genus in total. A recent project IMBICONT (2012-2015), a bi-lateral collaboration between University of Sao Paulo and University of Copenhagen, has as one aim to study more in detail the field ecology and the interaction of insect pathogenic fungi and their hosts at molecular level.

**Bio-assays: methods are well established**

Performing bio-assays is an indispensable way, first to screen several isolates against a specific target insects, then to detail more the conditions governing successful infections and finally to test performance of a selected isolate in the laboratory before semi-field and field application. The IOBC proceedings and the SIP abstracts and proceedings include many such studies, especially with Hypocreales. The present knowledge is summarized by Inglis et al. (2012) for Hypocreales and by Hajek et al. (2012) for Entomophthorales and these chapters contain some main guidelines. Some papers and presentations on bio-assays from the latest years (especially on Hypocreales) appear be of high technical value for the selection and further work on specific fungal isolates against specific insect target species, while contributions to new general approaches and methodologies are more rare.

**Production and formulation: new approaches on their way**

Recently the knowledge on mass production of Hypocreales was compiled (Jaronski & Jackson, 2012). Their book chapter contains the full information package needed for small scale to medium scale laboratory production as well as significant considerations about real mass production. Further development of insect pathogenic fungi needs emphasis on production and formulation and new approaches are needed. A recent EU supported project INBIOSOIL (2012-2015) has as one aim to study effects on target and non-target of novel formulations of Hypocreales.

**Biocontrol strategies: Inundation, inoculation or conservation?**

A basic question concerning insect pathogenic fungi: do they fit in all the mentioned three strategies for biocontrol. Obviously yes concerning inundation and inoculation, since the existence of hypocrealean fungi on the market in Europe and elsewhere over decades documents that these fungi can act in both strategies. The perspectives for conservation are also really high, although the potential cannot be explored more fully before the recent knowledge of species complexes with different ecology and pathology are taken more into consideration.

**Acknowledgements**

Support to our research is currently due to the European Union (INBIOSOIL), The Danish Strategic Research Council (IMBICONT and FUNSECPROT) and The National Foundation for Research (Centre for Social Evolution).
References


Fungi

Session 1: Entomopathogenic fungi in the control of soil-dwelling pests
Biological control of wireworms with entomopathogenic fungi

S. Eckard¹, M. A. Ansari², T. M. Butt², J. Enkerli¹, G. Grabenweger¹
¹Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstr. 191, 8046 Zürich, Switzerland; ²Department of Biosciences, College of Science, Swansea University, Singleton Park, Swansea, SA2 8PP, UK

Abstract: Three species of wireworms, Agriotes lineatus, A. obscurus and A. sputator, are economically important soil pests in arable and vegetable crops in Europe. Fungi of the genus Metarhizium are natural pathogens of wireworms. We tested the virulence of three European Metarhizium strains in laboratory experiments and found a maximum mortality of up to 80% four weeks post inoculation. We further investigated stability of the virulence of the most effective strain ART2825 after ten times of subcultivation on artificial media. There was no difference in virulence compared to a treatment of larvae that were infected with freshly host-passed conidia. We conclude that Metarhizium strain ART2825 is a potential candidate for the control of wireworms and we will continue to validate its efficacy under field conditions.

Key words: Agriotes lineatus, A. obscurus, A. sputator, entomopathogenic fungi, potato, Metarhizium, virulence, host passage

Introduction

Wireworms, the soil-dwelling larvae of click beetles, cause economically significant damage particularly on potato tubers. The species responsible for this damage are Agriotes lineatus, A. obscurus and A. sputator in many European regions (e.g. Burghause & Schmitt, 2011, Parker & Howard, 2001). Currently, wireworms are controlled with chemical insecticides (Kuhar et al., 2003). Alternative control methods of wireworms for use in organic or integrated farming systems are not yet available, although highly anticipated by the EU “sustainable use” directive 2009/128/EC.

Species of the fungal genus Metarhizium are natural pathogens of a broad range of insects including wireworms (Kabaluk et al., 2005). However, virulence of Metarhizium strains differs significantly against certain species of wireworms (Ansari et al., 2009). Additionally, virulence may attenuate after successive subcultivation on artificial media (reviewed in Butt et al., 2006). The aim of our investigations was to identify virulent strains of entomopathogenic fungi (EPF) against different species of wireworms and to determine their stability after repeated subcultivation on artificial media.

Material and methods

Wireworm larvae and fungal strains
Larvae of A. obscurus, A. lineatus and A. sputator used in these experiments originated from a greenhouse rearing, established with field collected adult click beetles (Kölliker et al., 2009). Three Metarhizium strains were tested: (1) BIPESCO 5 (= F52), isolated from codling moth, Cydia pomonella, Austria, (2) ART2825 isolated from A. obscurus, Switzerland and (3) V1002 isolated from A. lineatus, UK. Each fungus strain was passaged through Greater Wax...
moth larvae (Galleria mellonella), and then re-isolated from single conidia colonies and maintained on Sabouraud dextrose agar (modified after Strasser et al., 1996).

Bioassay with different Metarhizium strains
Ten larvae of each wireworm species were dipped into a suspension of $10^8$ conidia ml$^{-1}$ water with 0.03% Tween® 80 for 20 s. Each larva was incubated separately in a small cup with about 30 g non-sterile wet field soil. A slice of carrot was placed in each cup as food source and was replaced weekly. Cups of each treatment were kept in a plastic box and incubated under controlled conditions (at 23 °C and 65% relative humidity). The number of dead larvae was assessed weekly for eight weeks. Cadavers were incubated until mycosis by Metarhizium spp. was clearly visible on the insect’s cuticle. A water/Tween®80 treatment and an insecticide treatment with Ethoprophos (9.6 mg per cup according to 60 kg ha$^{-1}$) served as controls. The whole experiment was repeated three times.

Bioassay with freshly host-passed and in vitro subcultured conidia
The strain ART2825 was selected for further investigations because of its virulence in the previous experiment. Conidia from in vivo and in vitro cultivation were tested against wireworms. Conidia for the first treatment originated from the tenth subsequent cultivation of the fungus on SDA plates, while conidia for the second treatment were directly harvested from fresh Agriotes cadavers. A treatment with a water/0.05% Tween®80 solution served as control. Eight larvae of A. lineatus and A. obscurus and 12 larvae of A. sputator were infected per treatment and each treatment had four replicates. Larvae were dipped into suspensions of $10^6$ conidia ml$^{-1}$ for 5 s and incubated in 10 g of moist peat substrate. Incubation and mortality was assessed as described above.

Statistical analyses
We analyzed the data for effects on the mortality caused by the treatment with a linear mixed effect model based on maximum likelihood. The statistical software R (version 2.14.1) with the function “lmer” was used. The status of the larvae (alive/killed by treatment) was considered as dependent variable with a binomial distribution. In the bioassay comparing the virulence of strains, the treatment was used as the independent variable. In the host passage bioassay the presence of host passaged conidia (yes/no) was used as the independent variable. The block represented by a box containing a treatment of wireworms was considered as random factor.

Results and discussion
Virulence of different Metarhizium strains
ART2825 was the most effective strain against A. obscurus. An average of 80% of the larvae died of mycosis (Figure 1). This strain killed significantly more A. obscurus larvae than BIPESCO 5 ($z = 0.00014$) and V1002 ($z = 0.0063$). Additionally, ART2825 was the most efficient strain by killing more than half of the A. obscurus larvae within two to three weeks. Results were similar for A. lineatus, with more than half of the larvae killed by ART2825 within four weeks and an average mortality of 70% after eight weeks. A. sputator was less susceptible to ART2825 (50% mortality) than BIPESCO 5 (60%) and V1002 (70%).
Effect of a host passage on the virulence of *Metarhizium* strain ART2825

The cultivation background of the EPF inoculum had no effect on the virulence of ART2825 in the host passage test. An average of 75% of the *A. obscurus* larvae died of mycosis when treated with conidia directly harvested from *A. obscurus* cadavers. A similar mortality rate of 70% was achieved with inoculum derived from the tenth subsequent in vitro cultivation of the *Metarhizium* strain. Results were similar for *A. lineatus* and *A. sputator* with generally lower mortality rates (data not shown).

Bioc

ontrol agents and strategies against wireworms have been research topic for several years (e.g. Ansari et al., 2009; Kabaluk, 2007). ART2825 is a European *Metarhizium* strain which showed high virulence against two important wireworm species, *A. obscurus* and *A. lineatus*. Our results are in accordance with those from Kölliker et al., 2011, who demonstrated significantly higher mortality of wireworms caused by ART2825 in comparison with a commercially available biocontrol product based on *Beauveria bassiana*.

Stability of virulence is an important criterion of biocontrol agents in commercial use. The attenuation of virulence due to successive subcultivation on artificial media is a common phenomenon and may be a problem for mass production of fungal biocontrol agents (reviewed in Butt et al., 2006; Ansari & Butt, 2011). Virulence may, however, be restored with host passages, and it has been demonstrated that a single host passage may be sufficient (e.g. Fargues & Robert, 1983). We could not find signs of attenuation in ART2825 after ten subcultivations on artificial medium and therefore conclude that the virulence of this strain may be retained in commercial mass production systems.
In conclusion, ART2825 is a promising candidate for the development of a biological wireworm control product. Additional studies under simulated and actual field conditions are currently being conducted to confirm the potential of this strain as a biocontrol agent.

Acknowledgements

We would like to thank Sven Bacher from the University of Fribourg for support with the statistical analyses. We are also thankful for the technical assistance of Deborah Kaiser and Christian Schweizer. Our research is funded by means of the 7th framework programme of the EU, as part of the project INBIOSOIL “innovative biological products for soil pest control”, http://inbiosoil.uni-goettingen.de.

References


Monitoring of the entomopathogenic fungus Beauveria brongniartii in cockchafer infested areas of the Euroregion Tyrol

Johanna Mayerhofer¹, Jürg Enkerli², Roland Zelger³, Hermann Strasser¹
¹University of Innsbruck, Institute for Microbiology, Technikerstrasse 25, 6020 Innsbruck, Austria; ²Swiss Federal Research Station for Agroecology and Agriculture, Reckenholzstrasse 191, 8046 Zürich, Switzerland; ³Research Centre for Agriculture and Forestry Laimburg, Laimburg 6, 39040 Ora/Auer (BZ), Italy

Abstract: The persistence of the entomopathogenic fungus Beauveria brongniartii in soils in the Euroregion Tyrol has been evaluated over a period of two decades. The fungal product Melocont® Pilzgerste was successfully applied on the fields in different concentrations and intervals between 1989 and 2012. In July and August 2012 the soil samples were drawn and analyzed on selective media to determine the occurrence of Beauveria spp. Preliminary results from microsatellite analysis showed that the re-isolated Beauveria strains from the test sites, which had been treated during the last four years, were identified as the production strain. New insights into colonisation, mobility and persistence of B. brongniartii in soils will be discussed in the presentation.

Key words: Beauveria brongniartii, biological control agent, persistence, monitoring, microsatellites

Introduction

The entomopathogenic fungus Beauveria brongniartii [(Sacc.) Petch anamorphic Hypocreales: Cordycipitaceae] is a commercially available biological control agent (BCA). It occurs naturally in many habitats around the world. In Europe it infects mainly the cockchafers Melolontha melolontha and M. hippocastani (Coleoptera: Scarabaeidae). The scarabs Melolontha spp. are major pest in agro/forst systems and are commonly found in all provinces of the alpine region. To control Melolontha spp. in the province of Tyrol B. brongniartii has been successfully applied and combined with mechanical treatment for more than two decades. Monitoring the persistence of an entomopathogenic fungus in soils is an integral part of the risk assessment of microbial pesticides. In 2012 the Pesticides Unit and the Panel on Plant Protection Products and their Residues (PPR) of the EFSA issued a tender to generate a guidance document on how to conduct risk assessment of microbial pesticides. This guidance document should support the EU Regulation (EC) No 1107/2009. To facilitate the assessment of risks posed on the environment by biological control agents Laengle and Strasser (2010) proposed a risk index (RI). The RI is composed of five components including persistence. Data on the survival of B. brongniartii and other entomopathogenic fungi are not only essential for risk assessment of biological control agents but are required for registration of new microbial pesticides and help to improve biological control strategies.

Molecular methods enable the discrimination between different strains of fungi and therefore add an important tool to study the persistence of the applied biological control agent versus naturally occurring strains. Enkerli et al. (2001) developed a method to use simple sequence repeats, also called microsatellites, to discriminate between various strains of B. brongniartii. This study aimed to monitor B. brongniartii in Melolontha infested areas of
Tyrol over a period of two decades. Additionally, the occurrence of indigenous *Beauveria* strains was compared to the density of the applied production strain of Melocont® Pilzgerste.

**Material and methods**

**Selection of sampling sites**

For the soil sampling, 20 sites (i.e. meadows and orchards) with a history of cockchafer infestation in East, North, and South Tyrol were selected. The sites were categorized according to the treatments with various concentrations of the product Melocont® Pilzgerste at different time periods between 1989 and 2012 (Table 1). The control sites had never been treated with Melocont® Pilzgerste. The fungal pesticide was applied according to the manufacture’s guide lines (Agrifutur).

Table 1. Melocont® Pilzgerste treatments at sampling areas in the Euroregion Tyrol. With the exception of the control area (C) all sites were treated with different quantities of Melocont® Pilzgerste and time frames.

<table>
<thead>
<tr>
<th>Variations</th>
<th>Years of application</th>
<th>Number of treatments (T)</th>
<th>Rate of application (kg ha⁻¹ and T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1994-1997</td>
<td>1-2</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>2009-2012</td>
<td>1-2</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>1989-2012</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>

**Soil analysis**

Soil samples from the test and control plots were taken with a split tube sampler. 40 samples ha⁻¹ soil were drawn and combined in a plastic zip lock bag. Two horizons of each sample were analyzed: from 0 to 10 cm depth and from 10.5 to 20 cm depth. All soil plots were sampled in a Z-shape in July and August 2012. Soil samples were processed according to the standard protocol published by Laengle *et al.* (2005). To determine the number of fungal colony forming units (CFU) a selective medium was used (Strasser *et al*., 1996). Three colonies per plate were selected and isolated. These isolates were grown in semi-synthetic complete medium (CM; Enkerli *et al*., 2001) and fungal biomasses were filtered and washed with deionised water. Aliquots of 0.15 g of fresh biomass were frozen in liquid nitrogen and lyophilized.

**Genetic analysis**

After adding 0.15 g of glass beads (1 mm diameter) to the lyophilized fungal myzelia the samples were homogenized with a ball-mill (MM301, Retsch) at maximum speed for 15 to 45 seconds. For the DNA extraction the PL2 buffer of the DNA extraction kit Nucleo Spin Plant II (Machery & Nagel) was used. The following steps of the DNA extraction were performed according to the manufacturer’s manual. The PCR reaction and the analysis of the *Beauveria* specific microsatellite markers were performed according to Enkerli *et al.* (2001). Amplified gene fragments were visualized with an Applied Biosystems 3130 Genetic Analyzer (Hitachi) and the output data were displayed with the software GeneMarker® (SoftGenetics).
Evaluation of the infestation with cockchafers
Spade sampling techniques were used to evaluate the infestation rate of cockchafers. Eight to twelve square holes 50 cm long and up to 70 cm deep were dug and the number of larvae was assessed. Supplemental data on the infestation of the soil plots with larvae and the resulting damages of the crops were collected in terms of a questionnaire provided by farmers.

Results and discussion

Persistence of Beauveria brongniartii
The application of Melocont® Pilzgerste for four years in a Melolontha infested area lead to a continuous increase of the density of B. brongniartii in soil plots compared to the control field. After the end of the treatments with the BCA the density of B. brongniartii dropped from $1 \times 10^5$ to $2 \times 10^4$ spores g$^{-1}$ dry weight of soil which is the recommended fungal density to ensure epidemic levels in grasslands (Ferron, 1967; Figure 1). Fornallaz (1992) estimated a 10- to 50-fold reduction of B. brongniartii spores g$^{-1}$ dry weight of soil per year in the absence of the host. Fifteen years after the use of the microbial pesticide the fungus underneath the limit of detection which is defined as 200 cfu g$^{-1}$ dry weight of soil (Laengle et al., 2005). The successful treatments resulted in low numbers of larvae, followed by a reduction of the fungus which decreases rapidly without its host. These results are in accordance with the results provided by Kessler et al. (2004).

Figure 1. Presence of Beauveria spp. (median, upper and lower quartile) in test fields (T1, T2) and the control field (C). Arrows indicate applications with Melocont® Pilzgerste.
Soils which had been treated with the fungal pesticide for a period of four years prior to sampling (variation 2) contained approximately \(1 \times 10^5\) spores of \(B.\) brongniartii g\(^{-1}\) dry weight of soil (Table 2). In these sites the infestation rate with \(M.\) melolontha was estimated to range between 75 and 150 larvae m\(^{-2}\) on average before and below 5 larvae m\(^{-2}\) after the period of application. Furthermore, no relevant damages by \(M.\) melolontha have been reported by farmers and expert authorities since using the Melocont\textsuperscript{®} Pilzgerste. A continuous application of Melocont\textsuperscript{®} Pilzgerste resulted in a fungal density up to \(1 \times 10^4\) CFU g\(^{-1}\) dry weight of soil (Table 2). The lack of \(M.\) melolontha larvae testified the efficacy of the fungal pesticide in those fields. Twelve years after overcoming the plague by the European cockchafer the fungus was not present in the soil and no damages of the meadows were recorded. The determination of the number of larvae is in progress.

Table 2. Density of \(Beauveria\) spp. (median) and its host \(M.\) melolontha larvae (L2/3) in sites (a, b, c) which were treated with Melocont\textsuperscript{®} Pilzgerste 12 years ago (1) in the previous 4 years (2) and for two decades (3) and the control field (C). * Evaluation in progress.

<table>
<thead>
<tr>
<th></th>
<th>(Beauveria) spp. (CFU)</th>
<th>Number of larvae of (M.) melolontha</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7.8 E + 01</td>
<td>*</td>
</tr>
<tr>
<td>1.a</td>
<td>0.0 E + 00</td>
<td>(\leq 4^*)</td>
</tr>
<tr>
<td>1.b</td>
<td>0.0 E + 00</td>
<td>(\leq 4^*)</td>
</tr>
<tr>
<td>1.c</td>
<td>0.0 E + 00</td>
<td>(\leq 4^*)</td>
</tr>
<tr>
<td>2.a</td>
<td>9.0 E + 04</td>
<td>(\leq 5)</td>
</tr>
<tr>
<td>2.b</td>
<td>5.0 E + 05</td>
<td>(\leq 5)</td>
</tr>
<tr>
<td>2.c</td>
<td>1.5 E + 05</td>
<td>(\leq 5)</td>
</tr>
<tr>
<td>3.a</td>
<td>1.4 E + 04</td>
<td>(\leq 1)</td>
</tr>
<tr>
<td>3.b</td>
<td>3.0 E + 02</td>
<td>(\leq 1)</td>
</tr>
</tbody>
</table>

**Discrimination between the applied strain and indigenous strains**

The genetic analysis of the microsatellite markers is still in progress. Nevertheless, preliminary studies showed that the reisolated \(Beauveria\) strains from the test sites variation 2 were identified as the production strain.

**Acknowledgements**

We would like to thank DI Dr. Andreas Koutny and Ing. Mag. (FM) Peter Frank (Tiroler Landwirtschaftskammer) for supporting the soil sampling procedure, MSc Sarah Sonderegger for her assistance in processing the samples and DI Andreas Tschöll (Amt der Tiroler Landesregierung) and INBIOSOIL (Grant Agreement No. 282767) for financial support.

**References**


Fornallaz, C. 1992: Microbial control of the may beetle Melolontha melolontha L. with the fungus Beauveria brongniartii (Sacc.) Petch: Demecological aspects of various control methods. ETH Zürich.


Susceptibility of Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae) to entomopathogenic fungi: Laboratory assays and field trials

Hannes Rauch1,2, Roland Zelger1, Stefan Hutwimmer2, Hermann Strasser2
1Research Centre for Agriculture and Forestry Laimburg, Laimburg 6, 39040 Ora/Auer (BZ), Italy; 2University of Innsbruck, Institute for Microbiology, Technikerstrasse 25, 6020 Innsbruck, Austria

Abstract: Since the introduction of Diabrotica virgifera virgifera to Europe between the late 1980s and the early 2000s, the western corn rootworm subsequently has extended its presence across many parts of Europe and can currently be found in 20 European countries. Several different strategies aiming at the control of D. v. virgifera have at least partially limitations, making the biological control probably the most encouraging management method. Bioassays revealed two Metarhizium anisopliae strains and one Beauveria bassiana strain with the highest pathogenicity against 3rd instar larvae of D. v. virgifera. Although results obtained from fungal density measurements after fungal application in Hungarian croplands revealed quite displeased persistences, the potential of certain fungal strains for the control of the western corn rootworm can be considered indisputable. However, further lab assays as well as field trials are needed to confirm this high potential.

Key words: entomopathogenic fungi, Metarhizium anisopliae, western corn rootworm, biological control agents (BCA), EU funded project INBIOSOIL

Introduction

Since the reiterated accidental introduction of Diabrotica virgifera virgifera to Europe between the late 1980s and the early 2000s (Miller et al., 2005), the western corn rootworm (WCR) subsequently has extended its presence across many parts of Europe (Edwards and Kiss, 2011). Several different natural enemies of the western corn rootworm occur in the New World, e. g. fungi, bacteria, protista, viruses, nematodes, arthropod predators and parasitoids, all with different impact on WCR populations (Toepfer et al., 2009). Many of these, particularly specialized parasitoids, predators and pathogens, have been left behind in the area of origin, resulting in a lack of occurrence in most parts of Europe (Toepfer and Kuhlmann, 2004). Different management practices aiming at the control of the WCR, such as (i) crop rotation, (ii) the application of insecticides and (iii) the use of rootworm-resistant transgenic maize hybrids producing Bacillus thuringiensis (Bt) toxins have shown to exhibit several limitations (Gassmann et al., 2011). Thus, the use of biological control agents in order to protect plants from WCR feeding is currently probably the most encouraging one.

Entomopathogenic fungi, especially Beauveria spp. and Metarhizium spp., have already indicated to be efficient biological agents, suppressing WCR populations in several lab assays, semi-field- and field trials (Pilz et al., 2007; Pilz et al., 2009). Pre-investigations carried out in laboratory assays and field trials aimed at the efficacy verification of entomopathogenic fungal strains on the basis of bioassays. Furthermore, fungal density calculations of Beauveria sp. and Metarhizium sp. products in Diabrotica infested fields in Hungary were performed and damage rating on maize roots with the IOWA 1-6 scale (Hills and Peters, 1971) were evaluated.
Material and methods

Bioassays of biological control agents against Diabrotica v. virgifera larvae
The virulence/efficacy of 18 different fungal strains were compared by exposing 3rd instar larvae to defined spore suspensions of BCAs. Larvae were infected by exposing batches of up to 30 third-instar larvae (grown on Zea mays) to the spore suspensions: each batch was transferred to a filter paper in a 5-cm diameter funnel. Fifty ml of the particular spore suspension were gently poured over the larvae. After 5 s, the suspension was quickly drained by suction. After inoculation, five treated larvae, each, were placed on the roots of an individual 10-day old corn plant. Roots were placed on a wet filter paper and covered with a plastic foil before rolling. Plants were stored under high humidity conditions by covering with a plastic bag. Control larvae were exposed to 0.1% (vol/vol) Tween 80 and treated as the inoculated ones. Larval development at 25-28 °C was monitored for 10 days. Numbers of dead insects were recorded every day. The cause of death was determined by visually differentiating between larvae killed by the tested entomopathogens and death resulting from other causes. Dead larvae were transferred to selective S4G agar medium to determine the proportion of cadavers with resulting fungal emergence and sporulation.

Bioassay data was analyzed following the Probit method by Throne et al. (1995) using Mathematica software 4.2. LT<sub>50</sub> values were calculated using probit transformation of proportion of insects killed and logarithmic transformation of time.

Fungal densities in soil and evaluation of damages on maize plants
The fungal densities were assessed by determining the number of colony forming units (CFU) per gram of dry soil on a selective medium (Strasser et al., 1996). The natural occurrence of the entomopathogenic fungi Beauveria and Metarhizium was initially assessed at five trial sites in Hungary (Enying, Fono, Ozora, Gölle and Boyhad) in May 2005 and May/June 2006 in a soil depth of 0-10 cm and 10.5-30 cm. Totally twelve different treatments with Beauveria spp. and Metarhizium spp., e.g. as granules, wettable powder or in combination with the insecticide Carbofuran, were carried out during sowing in 2005 and 2006.

CFU measurements were also conducted in October/November 2005 as well as in June, August and November 2006. The trial sites at the three locations Enying, Fono and Ozora exhibited heavy feeding damages the year before treatments. Feeding damages were evaluated in treated trial sites in 2005 and 2006 depending on the time of harvest using the Iowa 1-6 scale (Hills and Peters, 1971) and compared to the control fields.

Results and discussion

Bioassays of biological control agents against Diabrotica v. virgifera larvae
From the 18 different fungal strains tested, two Metarhizium anisopliae (strain V38E and BIPESCO 5) and one Beauveria bassiana (strain KVL 0433) were identified as high pathogenic against 3rd instar larvae of Diabrotica v. virgifera. Probit transformation generated LT<sub>50</sub>-rates of 5.2 days and 4 days for spore suspension of 10<sup>6</sup> spores ml<sup>-1</sup> of M. anisopliae BIPESCO 5 and V38E, respectively.

Seven-day bioassays showed ED<sub>50</sub> of 1.76 x 10<sup>5</sup> and 7.4 x 10<sup>4</sup> spores ml<sup>-1</sup> for M. anisopliae V38E and BIPESCO 5, respectively. It is worth to notice that from Diabrotica larvae re-isolated reused spores of the strain V38E (V38E RI) exhibited a higher virulence compared to the parent strain (Figure 1).
Figure 1: Dose-response-curves of measured and with Probit calculated data of seven-day bioassays on 3rd instar larvae of *Diabrotica virgifera virgifera*. (Ex N2 = parental strain; RI = from larvae re-isolated spores).

**Fungal densities in soil and evaluation of damages on maize plants**

Evaluation of natural occurrence of *Beauveria* spp. revealed only a low abundance at all five locations. The maximal fungus density was measured at experimental sites in Fono and was 730 CFU per gram dry weight soil (CFU g\(^{-1}\) DW). *Metarhizium* spp. densities were found to be higher than 1000 CFU g\(^{-1}\) DW up to a soil depth of 30 cm at trial sites in Enying and Fono.

In spite of their fully vitality and absence of contamination (proved in quality assurance examinations), neither *Beauveria* nor *Metarhizium* was able to decisively establish itself at any of the experimental sites, regardless of the treatment and their application. Although *Metarhizium* spp. showed a slight increase of densities (maximal increase was >3 x 10\(^3\) CFU g\(^{-1}\) DW reached at location Enying), the minimal concentration of 5000 CFU g\(^{-1}\) DW for a sustainable control of *Diabrotica* larvae could, if any, only partially reached.

Only in one of three control fields the damage rate exceeded the economic threshold level of 3 on the IOWA scale (Journey & Ostlie, 2000). Thus, the larval feeding damages in control fields can be considered to be low to moderate, respectively, and did not significantly differ from the treated sites in 2005. Relative ample precipitation during summer in 2005 might have helped quickly to restore root losses and could be a plausible explanation for the general low larval feeding damages observed.
Prospects
Although the results obtained from the assessment of fungal densities in the Hungarian croplands revealed quite displeased fungal persistences, bioassays have shown the high pathogenicity of *M. anisopliae* (strain V38E and BIPESCO 5) and *B. bassiana* (strain KVL 0433) against 3rd instar larvae of *Diabrotica v. virgifera*. The formulated granular product GranMet-P®, based on BIPESCO 5 grown on barley kernels, is already registered in Austria for the control of the garden chafer *Phyllopertha horticola* (L.) and could thus potentially be applied in the field as safe BCA.

In Austria, *D. v. virgifera* was first detected in 2002 near the Slovakian border, from where it has continuously spread to western parts of the country. Well established WCR populations can currently be found in Styria, Lower Austria and Burgenland. In the province Styria, further investigations regarding the generation of efficacy data of entomopathogenic fungal strains (EPF) and the evaluation of possible synergies between EPF and efficacy enhancing agents (EEAs; i.e., semiochemicals, entomopathogenic nematodes) have already been initiated in the framework of the EU funded project INBIOSOIL (No. 282767) and are scheduled to last three years. The field trials will be complemented by controlled semi-field trials and laboratory assays aiming at the improvement of the applied products.

Acknowledgements
This research was partly supported by the European Community's Seventh Framework Programme grant (FP7 ENV.2011.3.1.9-1 ECO-INNOVATION, INBIOSOIL, Grant Agreement No. 282767), by The Austrian Research Promotion Agency – FFG 809806, by Agrifutur s.r.l. (Italy) and by Kwizda Agro Gmbh (Austria).

References


Efficacy of biological control agents for the control of western corn rootworm

Emese Balog1, Bui Xuan Hung2, György Turóczi1, József Kiss3
1Szent István University, Plant Protection Institute, H-2100 Gödöllő, Hungary; 2Can Tho University of Gent, Department of Plant Protection, Can Tho City, Vietnam

Abstract: The western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte, has been introduced to Europe more than 20 years ago, and it is a well-established maize pest in Hungary since 1995. The larvae of WCR cause damage on the maize roots. The efficacy of various biological control agents (BCAs), such as fermented cultures of various entomopathogenic toxin producing preparations of Bacillus thuringiensis, and some strains of the entomopathogenic conidial fungus Metarhizium anisopliae, was screened against the larvae of WCR but the practical application of them still needs additional research and development inputs. In in vitro tests, WCR larvae were treated with microbial products (fermented cell cultures or spore suspensions in various concentrations) at the second larval stage. Larvae were fed with freshly germinated maize roots and larval mortality was recorded until pupation. In greenhouse experiments maize plants were grown in pots placed in isolators. WCR eggs (20 for each plant) were put directly under the seeds. In greenhouse experiments the microbial preparations were applied at the time of sowing, in the same way as they were applied in the in vitro trials. One month after the planting, the root mass was measured, and the damage caused by larvae was determined based on the modified IOWA 1-6 scale. Most of the bacterial preparations and fungal strains proved to be effective both in killing WCR larvae and preventing root damage on maize plants. Some microbial treatments almost reached the efficacy of the control treatments (Tefluthrin (FORCE 1.5 G) and Bacillus thuringiensis var. tenebrionis (NOVODOR FC)) and can be considered as promising control agents of WCR.

Key words: western corn rootworm, Diabrotica, entomopathogenic fungi, Metarhizium, maize

Introduction

The western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte, (Coleoptera: Chrysomelidae) is a well-established maize pest in Hungary. It was first detected in Europe near Belgrade, Serbia, in 1992 (Báca, 1993). In 1995 it was first recorded in Hungary (Princzinger, 1996) but the modelling of the population dynamics of the WCR showed that it could have been present well before this time (Szalai et al., 2011).

The larvae of WCR feed on the maize roots causing the characteristic ‘gooseneck’ symptom and significant yield loss. The adults cause damage on above ground plant parts, frequently on the generative parts of maize, thus reducing the fertilization (Ball, 1957; Chiang, 1973).

Although various control tools (crop rotation, chemical insecticide treatments) can be applied by farmers to keep the population below economic threshold level, WCR is still considered as a major problem in maize production.

Entomopathogenic bacterial and fungal species thrive in the soils in Hungary (Pilz et al., 2007) and they are promising agents for the control of WCR. The entomopathogens studied until now proved to be sufficiently selective, they impose no risk for non-target organisms and they are considered harmless for human health. Among these entomopathogens, conidial
fungi (Deuteromycota) are the most promising because they have wide host range and inoculum production and formulation is comparatively easy (Turóczi, 2003).

Material and methods

In vitro test
WCR was bred in Petri dishes. Ten WCR eggs were put in the Petri dish on wet filter paper, covered by 2 g sterilized soil and incubated at 25 °C. The hatched larvae were fed with freshly germinated corn, presoaked in EDTA to prevent the growth of saprotrophic moulds.

At the second larval stage the larvae were treated with 2 ml of the microbial preparations. We applied fermented cell cultures of B. thuringiensis var. tenebrionis, (patent pending strains of Biofil Ltd.) in two different concentrations (10⁸ cfu ml⁻¹ and 10⁷ cfu ml⁻¹). Also, spore suspensions (also 10⁷ cfu ml⁻¹) of 5 strains of M. anisopliae (Met-4, -16, -34, -43, -51) were applied. Throughout the larval development the rate of mortality was recorded.

Greenhouse experiment
Two maize seeds were sown into pots of 15 cm diameter and the pots were grouped by six and placed in isolators. 20 WCR eggs were put directly under each seed. B. thuringiensis and M. anisopliae preparations were applied directly on the seeds in the same dosage as in in vitro trials. Altogether there were 21 treatments, each in 6 replications. Pherocon AM yellow sticky traps were put into the upper parts of the isolators to capture emerging adults.

One month following the planting, we recorded the plant height, the number of leaves, the root mass, the number of emerged adults and the root damage caused by larvae. Due to the limited term of experiment (maize in the isolators could reach a maximum height of about half a meter), the maximum root damage was measured on a modified IOWA 1-6 scale, (that allows to determine the root damage also on small maize plants, see in EPPO 1/212 (1) standard, EPPO Bulletin, 1999).

The efficacy of the microbial treatments was compared to untreated control and to seed treatments with insecticide Tefluthrin (Force 1.5 G) and Bacillus thuringiensis var. tenebrionis (Novodor FC).

Results and discussion

In in vitro test, all of the microbial preparations increased the mortality of WCR larvae. The recorded mortality was less dependent on the concentration of the applied preparations, but it varied significantly between the individual microbial strains.

In greenhouse experiments there was no damage in control treatment without WCR eggs (Control 0) and the most serious damage was recorded in control treatment with WCR eggs (Control WCR). Some of the microbial preparations proved to be effective in the reduction of root damage (Figure 1).

The efficacy of the bacterial preparations and the M. anisopliae strains were highly variable, some of them were significantly different from control treatments. Several microbial treatments almost reached the efficacy of NOVODOR treatment either in larval mortality or in the reduction of root damage.

The examined B. thuringiensis preparations and the M. anisopliae fungal strains are promising control agents of WCR. Their efficacy under field conditions will be tested in further experiments.
Figure 1. Larval damage on modified IOWA 1-6 scale. Bacterial strains (M and K) were applied at concentrations of $10^8$ cfu ml$^{-1}$ and $10^7$ cfu ml$^{-1}$ (marked with 8 and 7, respectively), respectively. Metarhizium (Met) strains were applied at a concentration of $10^7$ cfu ml$^{-1}$.

Acknowledgements

Research was supported by the grant GOP-1.1.1-11-2012-0059 „Development of environment friendly product with the use of entomopathogenic organisms”.

References


Exploring synergistic effects of semiochemicals, entomopathogenic fungi and nematodes against root-herbivores

Michael A. Brandl, Mario Schumann, Stefan Vidal
Department of Crop Sciences, Agricultural Entomology, Georg August University Goettingen, Grisebachstr.6, 37077 Göttingen, Germany

Abstract: Root-herbivores may cause important economic yield losses; furthermore secondary stress factors such as increased water deficiencies occur. So far, little is known about their ecology, especially with regard to host finding strategies. Several studies have shown that semiochemicals may serve as host location cues. Recent studies have shown the use of host specific signals by western corn rootworm larvae (Diabrotica virgifera virgifera LeConte) for locating its host maize (Zea mays). Taking into account these studies and the recently discovered synergistic effects of entomopathogenic fungi and nematodes offer potential for refined biological control strategies. Furthermore, political presets by the EU request innovative techniques and the implementation of sustainable strategies for suppressing root-herbivores, respectively.

We aim at controlling larvae of western corn rootworms and wireworms (Agriotes spp.) in maize by combining semiochemicals, known as components in “attract” or “confuse” strategies, with Metarhizium anisopliae and Heterorhabditis bacteriophora known as kill components. The concept uses biological control agents in co-formulated capsules for preservation. Furthermore, either attractant or repellent semiochemicals, with regard to the targeted strategy, are added. We focus on CO₂ as the attractive component in the “attract & kill” strategy and botanicals as the repellent component in the “confuse & kill” strategy. To date, three different strains of M. anisopliae (Bipesco5, ART2825 and EAMa 01/58-Su) have been tested in bioassays and in the greenhouse. Bipesco5 and ART2825 showed most promising potential, although all strains were not as efficient as a standard insecticide. Different conidial and nematode concentrations will be tested in the future to identify the most virulent concentrations for synergistic effects and for capsule formulations.

Key words: Agriotes, wireworm, Diabrotica virgifera virgifera, control strategies
Session 2: Above-ground use of entomopathogenic fungi in protected and open field crops
Entomopathogenic fungi ecology and diversity from different Mediterranean ecosystems

María Fernández-Bravo, Enrique Quesada-Moraga, Inmaculada Garrido-Jurado
Universidad de Córdoba, Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, 14071 Córdoba, Spain
e-mail: o02febrem@uco.es

Abstract: The objective of the present study is to provide new insights on the presence, diversity and ecology of entomopathogenic fungi (EF) in the soil and the phylloplane in ephemeral (sunflower) and permanent (olive and holm oak) Mediterranean agroforestry systems with different management strategies, organic or conventional, dehesa or reforestation. For that, soil and phylloplane samples from the same geographical sampling point were gathered in the four seasons and in the four cardinal directions using a high sampling effort. From 272 soil samples and 840 phylloplane samples, 693 EF isolates were obtained. Their genetic diversity were characterised by the molecular marker based on elongation factor 1-alpha (EF1-α). Nine species were found, including the genera Beauveria, Metarhizium, Paecilomyces and Purpureocillium. B. bassiana was detected more frequently in all ecosystems and even in the phylloplane (21.65% of isolates), whereas B. amorpha, P. lilacinus and P. marquandii were rarely detected (0-0.29%, 0-0.43%, and 0.14-0.43%, respectively). All ecosystems showed no diversity of EF according to Shannon-Weaver index (H’), which was lower than 1. Likewise, the five ecosystems presented a highest fungal dominance of one species (B. bassiana) as indicated by Simpson dominance index (D) and Pielou’s evenness ratio (J’) values lower than 1. For each agroforestry system, differences between habitats in diversity of EF were also detected with the lower Jaccard’s index values (J), and therefore higher differences, observed for fungal communities from phylloplane and soil.

In general, the phylloplane habitat showed more isolates and diversity than soil for all ecosystems and species. Ecosystems ranking according to number of fungal isolates were: holm oak dehesa > holm oak reforestation > organic olive orchard > traditional olive orchard > sunflower plantation, which could indicate that the higher the ecosystem modification the lower the presence and diversity of EF.

Key words: phylloplane, soil, diversity index, Beauveria, Metarhizium, Paecilomyces, Purpureocillium
Efficacy of two strains of *Beauveria bassiana* entomopathogenic fungus on the red palm weevil in France and in Spain

Samantha Besse\(^1\), Ludovic Crabos\(^1\), Karine Panchaud\(^2\)

\(^1\)Natural Plant Protection (N.P.P.), Arysta LifeScience Group, 35 avenue Léon Blum, 64000 Pau, France; \(^2\)Vegetech, 33 Chemin de la Source, 83260 La Crau, France

**Abstract:** NPP/Arysta LifeScience, helped by Vegetech company, works on the implementation of an alternative biological control method against the red palm weevil, *Rhynchophorus ferrugineus*. Such a tool would limit the environmental impact of treatments. A first trial in outdoor cages, set up at the end of 2010 in France, has shown the interest of two strains of the entomopathogenic fungus *Beauveria bassiana*. During autumn 2011 and spring 2012, two new trials were carried out, in semi-natural conditions, in France and in Spain, in order to validate previously obtained results. They demonstrate that *Beauveria bassiana* strain 147 (active ingredient of Ostrinil\(^\circ\), already registered in France for the treatment of palm trees against *Paysandisia archon*, the palm borer), is at least as efficient as imidacloprid, the chemical reference, and that strain NPP111B005 shows an increased efficacy.

**Key words:** *Rhynchophorus ferrugineus*, palm tree, *Beauveria bassiana* strain 147, *Beauveria bassiana* souche NPP111B005, biological control
Beauveria bassiana strain ATCC 74040 interferes with oviposition behavior of Mediterranean fruit fly

Luca Ruiu¹, Giovanni Falchi¹, Edith Ladurner²
¹Dipartimento di Agraria, University of Sassari, Via E. de Nicola, 07100 Sassari, Italy; ²CBC (Europe) S.r.l., BIOGARD Division, Via XXV Aprile, 44, 24050 Grassobbio (BG), Italy

Abstract: The entomopathogenic fungus Beauveria bassiana is known to interact with insects in several ways. The present work reports the results of observations on the potential of Beauveria bassiana strain ATCC 74040 against the Mediterranean fruit fly, Ceratitis capitata, with special regard to disturbance effects on oviposition behaviour. A commercial formulation (Naturalis) and different fungal preparations (pure conidia, hyphae, culture supernatants) were applied to orange fruits offered to ovipositing medflies. A significantly lower number of fly visits and oviposition punctures were recorded on fruits treated with Naturalis and with pure conidia than on control fruits. The observed effects are examined on the basis of additional proteomic and genomic observations, and the potential molecular implications of the rodlet layer of aerial conidia are discussed.

Key words: Ceratitis capitata, oviposition, Beauveria bassiana, microbial control

Introduction

Studies with the entomopathogenic fungus Beauveria bassiana can be listed among the first significant experiences with microbial control. Since then, the increasing knowledge on this entomopathogenic species, including its biology, the mechanism of action against different target insects, and the improvement in formulation and application techniques led to the commercialization of different products (Wraight et al., 2001). In general, the insecticidal action related to conidia germination and hyphae penetration inside the insect’s body is associated with various molecules including different protein families like chitinases, proteases and other extracellular enzymes (Ortiz-Urquiza et al., 2010). Furthermore, recent investigations based on the whole genome shotgun sequencing of B. bassiana strain ARSEF 2860, evidenced the presence of genes encoding for bacterial-like toxins, some of which showing similarities to Bacillus thuringiensis Cry toxins (Xiao et al., 2012). However, differences among strains in terms of insecticidal potential have been demonstrated, and different strains may therefore express enhanced action against specific targets (Castrillo et al., 2008). Ceratitis capitata Wiedemann (Diptera: Tephritidae), also known as the Mediterranean fruit fly, is a multivoltine and polyphagous pest species affecting numerous host fruits. The management of this pest is still mainly based on repeated applications of synthetic chemicals. In order to implement sustainable and integrated crop protection, the integration of these conventional control methods with biological control tools would be highly desirable. Previous studies (Ortu et al., 2009) showed that applications of Naturalis on fruits compared to a blank (inert co-formulants of this bioinsecticide) result in a significant reduction of oviposition punctures of C. capitata.

In the studies herein reported we thus decided to further investigate the mechanisms involved in the disturbance effects of B. bassiana strain ATCC 74040 on the oviposition behaviour of C. capitata.
**Material and methods**

**Fungal fractions**
Experiments were conducted with *B. bassiana* strain ATCC 74040 which was isolated and purified from the commercial formulation Naturalis® (CBC (Europe) S.r.l., Nova Milanese, Italy). To collect pure conidia, the microorganism was cultured on Sabouraud dextrose agar (SDA) plates at 28 °C. Conidia were then collected by scraping from plates into 0.1% Triton solution followed by filtration, when necessary. The conidia suspension purity was checked under a phase microscope and quantification was based on Thoma chamber counting. To collect hyphae and culture supernatants 24-48 h after conidia germination, liquid cultures of the microorganism were grown on Sabouraud broth. The effects of the formulated product Naturalis, of pure conidia, hyphae and of culture supernatants on the oviposition behaviour of *C. capitata* in comparison to an untreated or blank (containing all components of the formulation except conidia) control was then tested in no-choice tests.

**Insects and No-choice tests**
*C. capitata* females were provided by the insect rearing facility of the University of Sassari (Sassari, Italy). After emergence, females were kept for 5 days in mating cages (40x40x40 cm) with males, and allowed to mate and to grow up gonads. Then, groups of 5 mated females each were transferred inside Plexiglas cages (30x30x30 cm) with two lateral faces covered with gauze to allow ventilation. In different experiments, either a treated fruit or a blank or an untreated (control) fruit was offered to females in each cage for oviposition (5 replicates per treatment). To estimate the number of female visits/fruit, fruits were observed for 1 min every hour, and the number of females landing on fruits was recorded. After 48 h, fruits were removed from the cages, and the number of oviposition punctures per fruit was counted. The different fungal fractions were applied using a spray volume of 10 ml per fruit, which was sufficient to ensure thorough coverage of fruits. In the case of Naturalis and of its blank, the product was applied at a concentration of 1.5 µl ml⁻¹ following label recommendations. The culture supernatants were applied tal quale, while pure conidia were applied at a concentration of 10⁵ conidia ml⁻¹. Hyphae were quantified with optical measures and assayed at a concentration comparable to conidia biomass. The numbers of female visits/fruit and oviposition punctures/fruit were compared across treatments using 1-way ANOVA, followed by LSD test for post-hoc comparisons of means.

**Molecular studies**
Genomic and proteomic approaches were followed to investigate the protein profile of the different fungal fractions tested in the no choice tests. The whole protein profile of the different fractions was resolved by mono- and bi-dimensional electrophoresis (SDS-PAGE), followed by peptide mass fingerprinting using trypsin digestion and MALDI mass spectrometry for main components. Fungal DNA was extracted and used for the detection and sequencing of genes possibly connected with the insecticidal mode of action (i.e. chitinases, proteinases) and the interaction with insects (i.e. hydrophobins) (Kumar et al., 2011).

**Results and discussion**

Both the number of female visits/fruit and the number of oviposition punctures/fruit were significantly lower on fruits treated with Naturalis or with pure conidia than on blank or untreated control fruits, respectively. On fruits treated with hyphae and culture supernatants,
instead, no significant reduction in the number of visits/fruit and in the number of oviposition punctures/fruit in comparison to the control was recorded (Table 1).

In the proteomic and genomic analysis of B. bassiana strain ATCC 74040, different molecules involved in the interaction between insects and the fungus were identified, and among these a chitinase (chit1 homologous), a cuticle-degrading proteinase (CDEP1 homologous) (Kumar et al., 2011) and two hydrophobins (hyd1 and hyd2 homologous) forming a rodlet layer, conferring aerial conidia hydrophobic features (Bidochka et al., 1995).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of visits/fruit</th>
<th>Oviposition punctures/fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Blank)</td>
<td>17.4 ± 1.4 a</td>
<td>8.4 ± 0.5 a</td>
</tr>
<tr>
<td>Naturalis®</td>
<td>5.0 ± 1.1 b</td>
<td>1.2 ± 0.4 b</td>
</tr>
<tr>
<td>Experiment group 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>18.6 ± 1.4 a</td>
<td>10.4 ± 1.1 a</td>
</tr>
<tr>
<td>Pure conidia (10^5 conidia ml^-1)</td>
<td>5.6 ± 0.6 b</td>
<td>3.4 ± 0.8 b</td>
</tr>
<tr>
<td>Hyphae</td>
<td>18.4 ± 1.5 a</td>
<td>8.6 ± 1.0 a</td>
</tr>
<tr>
<td>Culture supernatants</td>
<td>20.6 ± 1.8 a</td>
<td>10.6 ±1.5 a</td>
</tr>
</tbody>
</table>

*For each experiment group, means in the same column followed by different letters are significantly different (LSD test, P < 0.05).

Table 1. Mean number (m ± SE) of female visits/fruit and of oviposition punctures/fruit recorded in the different treatments.*

Oviposition deterrent effects were observed for the commercial product (Naturalis) and for pure conidia suspensions, while no such effects emerged for the other fungal fractions tested (hyphae and culture supernatants). Conidia were thus identified as the main fraction responsible for the observed effects. However, these inhibitory effects were detected only when conidia were applied at a concentration equal to or exceeding 10^5 conidia ml^-1 (data not reported). Furthermore, on fruits treated with preparations obtained by centrifugation of 2-day-old cultures in Sabauraud broth, thus in the immediate post-germination phase of conidia, no significant inhibiting effects were detected (data not reported). Therefore, since the effect of the conidia on medfly oviposition behaviour was observed immediately after treatment application and for the following 48 h, and considering that conidia take at least 2 days to germinate, all further investigations were focused on the main components and structure of intact conidia.

Given the results of our studies, we assumed that the physical and biochemical properties of conidia, in particular the hydrophobic layer of conidia on the fruit surface, may impair the ability of medflies to detect orange-derived stimuli, such as orange odours and fruit humidity content (Levinson et al., 2003), known to affect oviposition. According to this assumption, the hydrophobins of the external conidia rodlet layer may be of primary importance in inhibiting medfly oviposition (Bidochka et al., 1995). The implication of hydrophobins in the fungal biocontrol potential has already been suggested by Wang et al. (2013). It has also been shown that insects can be repelled by hydrophobic particle film barriers (i.e kaolin), because coated plants become visually or tactually unrecognizable as a host and insect movement and behavior can be affected by the attachment of particles to their body (Glenn et al., 1999). In
line with this, B. bassiana conidia might work in a similar way. However, in addition to a barrier-effect determined by conidia on fruits, we cannot exclude that part of the observed effects could be due to volatile organic compounds released by the fungus and with inhibitory effect on C. capitata (Crespo et al., 2008).

At present, further investigations are being conducted to clarify the specific role of conidia surface compounds. These studies would further support the potential of B. bassiana strain ATCC 74040 in protecting fruits in integrated medfly management programs.

Acknowledgements

This study was financially supported by Sardegna Ricerche (Project Bioinnovation: Incentivo Ricerca Polaris 2010).

References


Pathogenicity of an indigenous strain of the entomopathogenic fungus Beauveria bassiana on larvae and adults of the sisal weevil, Scyphophorus acupunctatus Gyllenhal (Coleoptera: Curculionidae)

V. T. Gkonti1,2, D. Markoyiannaki2, D. C. Kontodimas2
1Aristotle University Thessaloniki (AUTH), Laboratory of Agricultural Zoology and Parasitology, Greece; 2Benaki Phytopathological Institute (BPI), Department of Entomology and Agricultural Zoology, Greece

Abstract: The sisal weevil is a severe pest of both ornamental and cultivated agave species. As the use of synthetic insecticides causes undesirable effects, the evaluation of potential biological control agents is necessary. Field collected adults and larvae of Scyphophorus acupunctatus were used to evaluate the pathogenicity of an indigenous strain of the entomopathogenic fungus Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales). Different concentrations of spore suspensions were tested. As in some cases 100% mortality was achieved it is indicating that this strain could serve as a potential biological control agent of the sisal weevil.

Key words: Beauveria bassiana, Scyphophorus acupunctatus, indigenous strain, biological control agent, entomopathogenic fungi

Introduction

The sisal weevil Scyphophorus acupunctatus Gyllenhal is one of the most important pests of agave species, which attacks both cultivated and ornamental plants (Gonzalez-Castillo et al., 2011). In addition, other ornamental plants (Beaucarnea recurvata, Dasylirion longissimum, Dracaena draco, Furcraea foetida, Yucca spp. Polianthes tuberosa) have been reported as hosts of the sisal weevil (Kontodimas & Kallinikou, 2010). Suppression of the weevil by the use of synthetic insecticides on ornamental plants as well as in cultivated agave plants for spirit (tequila) production pose a threat both to human health and the environment. Thus, effective alternative control methods such as the use of bioinsecticides or the implementation of classical or conservation biological control are desirable for replacing conventional methods of pest management.

The weevil lives and develops in a protected habitat, on the button of the leaves or inside the plant’s head (Lock, 1962), where it is protected by its limited number of predators and parasitoids (Velázquez et al., 2006). Therefore, the use of entomopathogenic fungi that have the ability to infest individuals into their protected habitat could serve as potential biological control agents against the sisal weevil. It has been shown that different species or even different strains within the same species may exhibit different behaviour in terms of host range, pathogenicity and temperature optimum levels for development (Shah & Pell, 2003).

In the present study an indigenous strain of Beauveria bassiana (Balsamo) Vuillemin from Greece obtained from a naturally infected cadaver of Rhynchophorus ferrugineus Olivier (Coleoptera: Curculionidae) was evaluated for its pathogenicity, as R. ferrugineus and S. acupunctatus both belong to the same tribe (Rhynchophorini). The fungus was applied in three different concentrations and mortality caused to different developmental stages of the insect was recorded.
Material and methods

Insects
Insects were collected from infested ornamental plants of agave, Agave americana, located in Arditto's hill in Athens (37° 58' 06", 23° 44' 18"). Adults were collected by hand while whole infested plants were removed and taken to the laboratory for the isolation of large larvae. Both adults and larvae were placed in polyester cages and kept under laboratory conditions at 25 ± 1 °C, 50-65% relative humidity (R.H.) and 12 h light:12 h dark photoperiod. Weevils were provided with apple slices until used in trials.

Fungal isolates
The B. bassiana strain used was obtained from the entomopathogenic fungi collection of Benaki Phytopathological Institute (Attica, Greece). The initial strain was isolated from a naturally infected R. ferrugineus cadaver found in Ellinikon region (Attica, 37°53'15"N 23°43'42"E). Aqueous conidial suspensions were prepared by scraping the surface of 2 weeks old cultures, grown on Sabouraud Dextrose Agar at 25 °C in dark, into aqueous solution of 0.2% Tween 80. Conidia concentrations were counted with the use of an haemocytometer.

Bioassays
Infection of adults and larvae was achieved by contact with the insects’ cuticle. Each treatment was replicated three times, with each replicate consisting of a group of 5 individuals. Application was accomplished by immersing individuals in groups of 5 in aqueous conidial suspensions for 60 s. All treatments contained a control group, were aqueous solutions containing 0.2% Tween 80 were used. Concentrations of 4 x 10^6, 2 x 10^7 and 4 x 10^6 conidia ml^-1 were tested. Mortality was recorded daily for up to 11 and 21 d for larvae and adults respectively. Additionally, cadavers were kept individually in sterile Petri dishes lined with moistened filter paper, in a dark environment at 25 °C and checked for external signs of fungal infection.

Statistical analysis
Data on percentage mortality were arcsin transformed to homogenise variances and to meet ANOVA requirements. Transformed data were analyzed with one way ANOVA (SPSS 8.0, SPSS Inc., Chicago, IL).

Results
Mortality of adults
Adult weevils exhibited 100% mortality in the high concentration treatment. Mortality differed significantly (F = 32.49; df = 3.8; P = 0.001) between concentrations of 4 x 10^6 and 4 x 10^7 conidia ml^-1, while control group exhibited zero mortality (Table 1).

Mortality of larvae
All treatments in all concentration levels caused high mortality to larvae of S. acupunctatus, reaching up to 100%. Low levels of mortality were observed in control treatments, which did not exceed 6.7 ± 0.06%, differing significantly from the treatment groups (F = 41.41; df = 3.8; P = 0.001) (Table 1).
**Mycelium development**

All isolated cadavers previously treated with the fungus developed visible mycelium on their surface within a week. The examination of the conidia under microscope ascertained the involvement of the entomopathogenic fungus *B. bassiana* in their death. Cadavers of larvae and adults deriving from control groups did not develop mycelium of *B. bassiana* or of any other entomopathogenic fungus.

Table 1. Average mortality (%) (± standard error)\(^1\) of sisal weevil adults and larvae after 21 and 11 days, respectively.

<table>
<thead>
<tr>
<th>Developmental stage of <em>S. acupunctatus</em></th>
<th>conidia concentration</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(4 \times 10^7)</td>
<td>(2 \times 10^7)</td>
</tr>
<tr>
<td>Adults</td>
<td>100% a</td>
<td>86.6 ± 0.21% ab</td>
</tr>
<tr>
<td>Larvae</td>
<td>100% a</td>
<td>100% a</td>
</tr>
</tbody>
</table>

\(^1\) Means within rows followed by the same letter are not significantly different (Tukey-b, \(a = 0.05\))

**Discussion**

Results indicate that this indigenous strain of *B. bassiana* has a high virulence on both adults and larvae of *S. acupunctatus*. Hence, it could serve as a potential biological control agent. It is of high importance that the pest attacks the plant from its base and that populations develop mainly under the ground surface having direct contact with the soil. *B. bassiana* is a soil borne fungus, so enhancement of the ground with its conidia could constitute a part of an IPM approach against the sisal weevil. Further research is required for the field evaluation of the pathogenic capacity of the certain strain.

**References**


Microbial control of European red spider mite (Panonychus ulmi) with Beauveria bassiana strain ATCC 74040

Edith Ladurner¹, Massimo Benuzzi¹, Andrea Braggio¹, Sergio Franceschini¹, Veselin Zivkovic²

¹CBC (Europe) S.r.l., BIOGARD Division, Via XXV Aprile 44, 24050 Grassobbio (BG), Italy; ²VINS 2000 d.o.o., Ustanicka 64/XV, 11000 Belgrade, Serbia

Abstract: The European red spider mite, Panonychus ulmi, can cause severe damage on many fruit crops, especially on apple. Outbreaks of mite populations usually occur in summer on warm and humid days. Natural occurring predator populations may not always be able to keep the pest under control, especially because of the likely occurrence of a lag in time in build-up of prey and predator populations and due to the use of non-selective chemical pesticides. The efficacy of the microbial control agent Beauveria bassiana strain ATCC 74040 (Naturalis®) against P. ulmi was tested in open trials on apple. In one of the trials, also observations on the potential side effects of the formulated product on natural occurring predator populations (Phytoseiulus spp. and Stethorus punctillum) were made. The microbial control agent showed high efficacy against P. ulmi in all field trials, and did not adversely affect predator populations. Beauveria bassiana strain ATCC 74040 can be considered a valuable tool to be integrated into P. ulmi control strategies.

Key words: Panonychus ulmi, Beauveria bassiana strain ATCC 74040, microbial control, selectivity

Introduction

The European red spider mite, Panonychus ulmi (Koch), is considered a secondary pest in fruit crops. Conditions favouring P. ulmi outbreaks are warm and humid summer days and the excessive use of fertilizers (Cuthbertson & Murchie, 2005). The mite is usually not a problem in orchards, where predator populations are well established, but injury may reach economic importance in commercial orchards due to the effects of non-selective chemical sprays killing beneficials (Costa-Comelles et al., 1990). However, when no chemical sprays are applied, natural occurring predator populations alone may not be able to keep the pest under control, because of the likely occurrence of a lag in time in build-up of prey and predator populations (Cuthbertson & Murchie, 2005). An integrated control programme based on the use of acaricides which are safe to predators is therefore desirable.

Microbial control agents, such as strains of the entomopathogenic fungus Beauveria bassiana (Balsamo) Vuillemin, can be considered interesting candidates to be included into P. ulmi control strategies. This microbial control agent acts primarily by contact. Once attached to the host’s cuticle, the conidiospores germinate producing penetration hyphae, which enter and proliferate inside its body. The proliferation of the fungus inside the host leads to its death. However, pathogenicity towards arthropods and thus also selectivity towards beneficials is variable among different strains of the fungus (Monio et al., 1998). In our studies we investigated the efficacy of B. bassiana strain ATCC 74040, known to effectively control the Tetranychid mite Tetranychus urticae (Chandler et al., 2005; Duso et al., 2008), and to show little or no adverse effects on several beneficials (Duso et al., 2008; Simoni et al., 2010; Ladurner et al., 2012). The strain has been included into the EU list of approved active substances (Regulation EU 540/2011) in 2009. The formulated product used
in our studies was Naturalis\textsuperscript{®} (CBC (Europe) Srl - BIOGARD Division, Italy), an oil
dispersion (OD) containing at least $2.3 \times 10^7$ viable spores ml\textsuperscript{-1} of B. bassiana strain ATCC
74040.

The results of three field trials evaluating the efficacy of Naturalis\textsuperscript{®} against the European
red spider mite on apple are reported. In addition, in one of the trials, observations on side
effects of the product on natural occurring predator populations of P. ulmi were conducted.

Material and methods

In 2011-2012, three efficacy trials were carried out on apple (Malus domestica Borkh.) in
compliance with EPPO guidelines and Principles of Good Experimental Practice (GEP). One
trial (Trial n. 1) was conducted in Serbia in 2011 (45°09’N, 20°11’E), and two other trials
(Trial n. 2 in 2011 and Trial n. 3 in 2012) were conducted in Italy (44°52’N, 11°40’E). In all
trials, the efficacy of Naturalis\textsuperscript{®} against P. ulmi was compared to that of a chemical reference
treatment and an untreated control (Table 1). To compare the different treatments, a
randomized complete block design with 3 (Trial n. 1) or 4 (Trials n. 2 and n. 3) replicates per
treatment was used (plot size: 5 trees), respectively. In all trials, treatments were applied twice
using a spray volume of 1000 l ha\textsuperscript{-1}. The 1\textsuperscript{st} treatment application was conducted when the
target mite was already present on the crop.

Table 1. Tested products, application rates and timing of applications in the three trials.

<table>
<thead>
<tr>
<th>N.</th>
<th>Active substance</th>
<th>Formulated product (conc. a.s.)</th>
<th>Applied rate</th>
<th>Timing (dd/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trial n. 1 (Serbia 2011)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>Naturalis (2.3x10\textsuperscript{7} spores/ml)</td>
<td>1.5 l/ha</td>
<td>28/06, 04/07</td>
</tr>
<tr>
<td>2</td>
<td>Abamectin Kraft</td>
<td>1.8 EW (18 g/l)</td>
<td>1.0 l/ha</td>
<td>28/06, 04/07</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trial n. 2 (Italy 2011)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>Naturalis (2.3x10\textsuperscript{7} spores/ml)</td>
<td>1.25 l/ha</td>
<td>03/08, 08/08</td>
</tr>
<tr>
<td>2</td>
<td>Fenazaquin Pride</td>
<td>200 SC (13.8%)</td>
<td>0.75 l/ha</td>
<td>03/08</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trial n. 3 (Italy 2012)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>Naturalis (2.3x10\textsuperscript{7} spores/ml)</td>
<td>1.5 l/ha</td>
<td>17/07, 20/07</td>
</tr>
<tr>
<td>2</td>
<td>Tebufenpyrad Masai</td>
<td>20 WP (20.0%)</td>
<td>0.6 kg/ha</td>
<td>17/07</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In all trials, the number of live mobile stages of P. ulmi was counted on 25 randomly
selected leaves per plot. The 1\textsuperscript{st} (preliminary) assessment was conducted just before the 1\textsuperscript{st}
application, while the final assessment was conducted 7-10 d after the last application (10 d in
Trial n. 1, 9 in Trial n. 2, and 7 in Trial n. 3), when the target mite population had reached its
peak in the untreated control. In addition, an intermediate assessment was carried out 3 d after
the 2\textsuperscript{nd} application in Trial n. 1 and just before the 2\textsuperscript{nd} application in Trial n. 2 and 3.
Furthermore, in Trial n. 3, also the number of live mobile stages of the spider mite predators Phytoseiulus spp. and Stethorus punctillum per 25 leaves was assessed during the study period. The final efficacy in reducing the number of live mobile stages of P. ulmi per 25 leaves of the different treatments was calculated according to Henderson-Tilton’s formula.

At each assessment, the number of live mobile stages of P. ulmi per 25 leaves (Trial n. 1-3) and the number of live mobile stages of Phytoseiulus spp. and S. punctillum per 25 leaves (Trial n. 3) were compared across treatments using one-way ANOVAs, followed by the Student-Newman-Keuls test for post hoc comparisons of means.

**Results and discussion**

Significant differences among treatments in the number of live mobile stages of P. ulmi per 25 leaves were not observed at the preliminary assessment (Table 2). Pest distribution at the beginning of the trial was thus homogeneous among treatments. In all trials, mite infestation increased considerably over time in the untreated control. At the final assessment, the mite infestation was always significantly lower in plots treated with B. bassiana strain ATCC 74040 than in untreated control plots, with mean efficacy values of the microbial control agent always exceeding 70% (Table 2).

Table 2. Number of live mobile stages of P. ulmi per 25 leaves (mean ± standard deviation) at the 3 assessments in the different treatments and trials, and mean efficacy (%) in reducing the number of live mobile stages per 25 leaves at the final assessment (Bb = B. bassiana strain)*.

<table>
<thead>
<tr>
<th>N.</th>
<th>Treatment</th>
<th>Preliminary assessment</th>
<th>Intermediate assessment</th>
<th>Final assessment</th>
<th>Efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>34.3 ± 13.5 a</td>
<td>75.0 ± 49.3 a</td>
<td>121.5 ± 130.3 a</td>
<td>74.6</td>
</tr>
<tr>
<td>2</td>
<td>Abamectin</td>
<td>29.8 ± 12.0 a</td>
<td>44.0 ± 36.5 a</td>
<td>67.8 ± 55.5 a</td>
<td>83.1</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td>32.8 ± 11.0 a</td>
<td>362.3 ± 154 b</td>
<td>440.3 ± 181 b</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>75.5 ± 42.8 a</td>
<td>52.5 ± 21.0 b</td>
<td>11.5 ± 9.0 a</td>
<td>93.4</td>
</tr>
<tr>
<td>2</td>
<td>Fenazaquin</td>
<td>54.0 ± 33.4 a</td>
<td>8.0 ± 1.6 a</td>
<td>1.5 ± 1.0 a</td>
<td>98.9</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td>61.0 ± 20.6 a</td>
<td>72.5 ± 17.5 b</td>
<td>140.8 ± 74.4 b</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>169.3 ± 27.6 a</td>
<td>67.3 ± 11.7 b</td>
<td>154.0 ± 28.3 b</td>
<td>71.9</td>
</tr>
<tr>
<td>2</td>
<td>Tebufenpyrad</td>
<td>158.5 ± 38.7 a</td>
<td>12.0 ± 10.5 a</td>
<td>41.0 ± 20.9 a</td>
<td>92.0</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td>146.0 ± 39.2 a</td>
<td>163.5 ± 49.5 c</td>
<td>473.0 ± 79.9 c</td>
<td>-</td>
</tr>
</tbody>
</table>

* Different letters within the same column and for the same trial indicate significant differences (SNK test: $P < 0.05$).

In two trials the final efficacy of Naturalis® was statistically comparable to that of the chemical standard, while in Trial n. 3 the latter showed significantly higher efficacy than the tested product. However, in this trial, the initial infestation level was considerably higher than
in the other two trials (approx. 150 versus less than 100 mites per 25 leaves). Entomopathogenic fungi have a slow mode of action compared to chemical pesticides. In fact, infection by B. bassiana strain ATCC 74040 can take between 24 and 48 h depending on the temperature (BCPC, 2004). Furthermore, based on the results of Duso et al. (2008) and Simoni et al. (2010), it can be assumed that against mites the strain acts primarily as an ovicide. Starting with applications of Naturalis® at the very first appearance of P. ulmi would therefore be advisable.

In our trial, the microbial control agent did not affect the natural occurring predator populations present in the field. Our field observations confirm the results of previous studies, in which little or no side effects of Naturalis® on Phytoseiid mite species were observed (Duso et al., 2008; Simoni et al., 2010). The microbial control agent can thus be considered a valuable tool to be integrated into sustainable P. ulmi control programmes.

Table 3. Number of live mobile stages of Phytoseiulus spp. and S. punctillum per 25 leaves (m ± s.d.) at the 3 assessments in the different treatments (Bb = Beauveria bassiana strain)*.

<table>
<thead>
<tr>
<th>N.</th>
<th>Treatment</th>
<th>Preliminary assessment</th>
<th>Intermediate assessment</th>
<th>Final assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N. live mobile stages of Phytoseiulus spp. per 25 leaves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>0.5 ± 1.0 a</td>
<td>0.5 ± 1.0 a</td>
<td>3.5 ± 1.9 b</td>
</tr>
<tr>
<td>2</td>
<td>Tebufenpyrad</td>
<td>0.5 ± 1.0 a</td>
<td>0.5 ± 1.0 a</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td>1.0 ± 1.2 a</td>
<td>1.5 ± 1.9 a</td>
<td>5.5 ± 1.9 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. live mobile stages of S. punctillum per 25 leaves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bb ATCC 74040</td>
<td>n.a</td>
<td>6.5 ± 1.7 b</td>
<td>6.5 ± 1.3 b</td>
</tr>
<tr>
<td>2</td>
<td>Tebufenpyrad</td>
<td>n.a</td>
<td>2.8 ± 0.5 a</td>
<td>3.3 ± 0.5 a</td>
</tr>
<tr>
<td>3</td>
<td>Untreated control</td>
<td>n.a.</td>
<td>6.3 ± 2.1 b</td>
<td>6.8 ± 1.0 b</td>
</tr>
</tbody>
</table>

* Different letters within the same column and for the same trial indicate significant differences (SNK test: P < 0.05).

n.a. = data not assessed.

References


**Mycopathogens of the corn leaf aphid, Rhopalosiphum maidis (Fitch.) infesting wheat plants at Assiut, Egypt**

Ahmed Y. Abdel-Mallek¹, Mohamed A. A. Abdel-Rahman², Gamal H. A. Hamam¹

¹Assiut University, Botany Department, Assiut, Egypt; ²Plant Protection Research Institute, A.R.C., Egypt
e-mail: alaaa4@hotmail.com

**Abstract:** The present study was carried out during 2008 and 2010 wheat growing seasons. Mycopathogens of the corn leaf aphid, *Rhopalosiphum maidis* were investigated under natural conditions. Seven species of entomopathogenic fungi, including five Entomophthorales and two Hyphomycetes were surveyed and identified infecting the corn leaf aphid. Entomophthorales were represented by five species belonging to three families. Ancylistaceae was represented by one genus, *Conidiobolus* including three species, namely *C. coronatus*, *C. obscurus*, and *C. thromboides*. Entomophthoraceae was represented by two genera, *Pandora* and *Zoophthora* including two species, *Pandora (= Erina) neoaphidis* and *Zoophthora radicans*. The identified species of Hyphomycetes fungi belonging to order Moniliales were represented by two Moniliaceae species, namely *Beauveria bassiana* and *B. alba*. The species *Beauvaria bassiana*, *B. alba* and *Zoophthora radicans* represented the predominant fungi species followed by *Pandora* and *Conidiobolus obscurus*.

**Key words:** Mycopathogens, corn leaf aphids
Session 3:
New strategies for delivering and monitoring of entomopathogenic fungi
Exploiting vine weevil behaviour to disseminate an entomopathogenic fungus

Tom Pope1*, Charlotte Arbona1, Harriet Roberts1, Jude Bennison1, John Buxton1, Gill Prince2 and Dave Chandler2
1ADAS Boxworth, Battlegate Road, Cambridge, CB23 4NN, UK; 2University of Warwick, Warwick Life Sciences, Wellesbourne, Warwick, CV35 9EF, UK; *Current address: Harper Adams University, Edgmond, Newport, Shropshire, TF10 8NB, UK

Abstract: Control of adult vine weevil (Otiorhynchus sulcatus) is currently reliant on the use of insecticides. However, using insecticide applications targeted against this pest is difficult, as they need to be applied at dusk, and are often incompatible with integrated pest management programmes. This study investigated the potential of a novel control strategy that uses artificial refuges containing spores of an entomopathogenic fungus and exploits vine weevil behaviour to disseminate the pathogen throughout weevil populations.

Preliminary experiments identified a simple plastic crawling insect trap as a suitable artificial vine weevil refuge. Subsequent semi-field experiments using fluorescent powders in place of an entomopathogenic fungus spore formulation showed that vine weevil aggregation behaviour and movement between refuges effectively disseminated the powders throughout weevil populations.

Key words: vine weevil, Otiorhynchus sulcatus, entomopathogenic fungus, refuge, aggregation

Introduction

Vine weevil (Otiorhynchus sulcatus) remains one of the most serious pests of soft fruit and nursery stock crops. Despite non-chemical options for the control of vine weevil larvae, such as use of entomopathogenic nematodes and the entomopathogenic fungus (EPF), Metarhizium anisopliae, control of adult weevils is currently reliant on insecticide applications.

This study investigated the potential of exploiting vine weevil behaviour to achieve control of this pest through the use of an EPF. The approach is based on the fact that adult weevils are nocturnal and seek refuge during the day, show aggregation behaviour and can be infected by EPF (Moorhouse et al., 1992). Given these features of vine weevil biology it may be possible to use artificial refuges to deliberately infect weevils with spores of an EPF. Vine weevil aggregation behaviour and movement between refuges may then allow dissemination of these spores throughout the weevil population.

Material and methods

Insect rearing

Adult vine weevils were collected from commercial strawberry and raspberry crops. Weevils were kept in small groups in ventilated plastic containers. Each container had a source of moisture (damp tissue paper), refuge (corrugated cardboard) and food source (Taxus baccata leaves). The weevils were kept in a controlled temperature laboratory at 21 °C.
Artificial refuge testing

Three simple artificial refuge designs were tested in these experiments: (1) Roguard™ (BASF plc, UK) – plastic crawling insect trap (80 mm diameter x 15 mm) with four small entrances (20 mm x 5 mm); (2) Roachmaster™ (Russell IPM, UK) – plastic crawling insect trap (110 mm x 80 mm x 15 mm), hinged along one side. Each Roachmaster was modified for use as a vine weevil refuge by inserting a piece of corrugated cardboard inside the trap; (3) ‘WeeVille’ refuge was a novel design based on a block of yew wood (80 mm diameter x 30 mm). A refuge was created by cutting a series of grooves (5 mm x 5 mm) in the wood.

Artificial refuges were tested in gauze cages (50 cm x 50 cm x 50 cm) placed in a glasshouse compartment at ADAS Boxworth (Cambridge, UK) maintained at 20 °C. Each cage contained a damp cotton wool pad, yew leaves and one or more artificial refuges. Twenty vine weevil adults were released into each cage during daylight hours. The position of each weevil was recorded 24 h and 48 h after release. Data were subjected to χ² analysis. The experimental design was as follows:

a) Single refuge (no choice) experiment – a Roguard, Roachmaster or ‘WeeVille’ refuge was placed into each cage. Each refuge design was tested on the same day. The experiment was replicated six times.

b) Two refuge (choice) experiments – two refuges of different designs were placed into each cage. Based on the results of the single refuge experiment, two comparisons were completed: Roguard + Roachmaster and Roguard + ‘WeeVille’. Each comparison was replicated six times.

Determining potential efficacy of artificial refuges in spreading spores of an EPF

Roguard refuges were tested in large gauze cages (145 cm x 145 cm x 152 cm) placed in a ventilated polytunnel at ADAS Boxworth. Temperature data loggers were placed in these cages throughout the experimental period. Cages were prepared in one of two ways:

a) Two strawberry grow-bags were placed into each cage. Each bag was one metre-long and was planted with five strawberry plants (cv. Elsanta).

b) Sixteen Euonymus fortunei (cv. Emerald Gaiety) plants grown in 1.5 l pots.

Forty adult weevils were released into each cage and then 24 h later 12 Roguard refuges were spread evenly throughout the cage (on the floor and close to plants). Each refuge contained 0.2 g of a hydrophobic fluorescent powder (Swada, Stalybridge, UK). The fluorescent powder served to mark weevils entering the refuge and was used to simulate an EPF spore formulation. The powders used were brightly coloured and fluoresced under ultraviolet light, allowing easy identification of weevils that had entered a refuge or had come into contact with a weevil that had. Weevils were collected seven days after placing the refuges in the cages and scored for the presence of fluorescent powder. The strawberry grow-bag and Euonymus experimental designs were replicated seven and eight times, respectively.

Determining potential efficacy of weevil to weevil contact in spreading spores of an EPF

Large gauze cages were prepared with Euonymus plants as previously described. Thirty five weevils were released into each cage and 24 h later 12 of the Roguard refuges were placed into each cage and arranged as previously described. However, in this experiment each refuge was clean and contained no fluorescent powder. Finally, five adult vine weevils that had been coated in fluorescent powder were released into each cage. These weevils were also marked with water based paint. All weevils were collected seven days after releasing the fluorescent
powder coated weevils into the cages. Weevils were scored for the presence of fluorescent powder, excluding those that were coated with powder at the start of the experiment. The experiment was replicated eight times.

**Results and discussion**

**Artificial refuge testing**

a) Single refuge (no choice) experiment - refuge design significantly affected the proportion of adult vine weevils within a refuge during daylight hours both 24 h ($\chi^2 = 17.80$, $P < 0.001$) and 48 h ($\chi^2 = 21.06$, $P < 0.001$) after the weevils were released into cages (Figure 1). Individual comparisons between the treatments showed that a significantly higher proportion of weevils were found within the Roguard or Roachmaster refuges compared to the ‘WeeVille’ refuges after 24 h and 48 h. In addition, after 48 h the proportion of weevils within Roguard refuges was significantly higher than in Roachmaster refuges.

![Figure 1](image_url)

**Figure 1.** Mean percent adult vine weevils within each refuge design presented in a no-choice environment 24 h and 48 h after weevils were released into cages. Different letters indicate a significant difference ($P < 0.05$).

b) Two refuge (choice) experiments - a significantly higher proportion of weevils were found within Roguard refuges than in Roachmaster refuges when placed together within a cage (Figure 2a). This difference was seen both after 24 h ($\chi^2 = 7.48$, $P = 0.006$) and 48 h ($\chi^2 = 15.38$, $P < 0.001$). Similarly, significantly more weevils were found within Roguard refuges when placed together with ‘WeeVille’ refuges within a cage (Figure 2b) after 24 h ($\chi^2 = 55.02$, $P < 0.001$) and 48 h ($\chi^2 = 58.01$, $P < 0.001$).

**Determining potential efficacy of artificial refuges in spreading spores of an EPF fungus**

a) Strawberry grow-bags – 92% of adult vine weevils were recovered from the cages seven days after Roguard refuges containing fluorescent powder were placed into the cages. Of those recovered 94% had come into contact with fluorescent powder. Mean temperatures in cages during this experiment were 22.8-24.1 °C (daytime) and 11.0-13.3 °C (night time).
b) Potted Euonymus – 94% of adult vine weevils were recovered from the cages seven days after Roguard refuges containing fluorescent powder were placed into the cages. Of those recovered 88% had come into contact with fluorescent powder. Mean temperatures in cages during this experiment were 24-26 °C (daytime) and 12-13 °C (night time).

Figure 2. Mean percent adult vine weevils within Roguard + 'WeeVille' refuges presented in a choice environment 24 h and 48 h after weevils were released into cages. Different letters indicate a significant difference (P < 0.05).

Determining potential efficacy of weevil to weevil contact in spreading spores of an EPF

Seven days after introducing the adult vine weevils coated in fluorescent powder 75% of the adult vine weevils recovered had come into contact with fluorescent powder. Mean temperatures during the experiment were 26-28 °C (daytime) and 14-15 °C (night-time).

Conclusions

These results indicate that simple plastic crawling insect traps can be used as artificial vine weevil refuges. Furthermore, vine weevil aggregation behaviour and movement between refuges effectively disseminated fluorescent powders put in these refuges or onto a small number of weevils. Work is currently underway to test the efficacy of a suitable EPF spore formulation in the refuges.

Acknowledgements

We thank the Chemicals Regulations Directorate (CRD) on behalf of Defra for funding the research (project PS2134) and Karen Jones for designing and making the ‘WeeVille’ refuges.

References

Field persistence of *Metarhizium* spp. strains applied as biocontrol agents against ticks (*Ixodes ricinus*)

Maria Mitteregger, Sarah Sonderegger, Hermann Strasser  
Leopold-Franzens University Innsbruck, Institute of Microbiology, Technikerstrasse 25, 6020 Innsbruck, Austria  

Abstract: In two semi-field trials the persistence of three *Metarhizium* strains (BIPESCO 5, ARSEF 3297, ARSEF 4556) after foliar spray application was monitored and bioassays with *Tenebrio molitor* larvae were made to preclude a negative effect on germination and vitality of conidia by using the adhesive agent Neo-wett™ and the antifoaming agent Antischiuma Schaumstop™. Three different outdoor conditions (i.e. unprotected, rainfall protected, rainfall protected and fully covered) were tested and all strains showed an adequate persistence after 25 days (20-50%). BIPESCO 5 conidia were re-isolated from foliage even after heavy rain showers in open sites after 44 days. Conidia showed high virulence in bioassay with and without Neo-wett™ and Antischiuma Schaumstop™: 90% of all larvae were killed after 5 to 20 days. No negative effects of the adhesive- and antifoaming agent on the germination ability and vitality of *Metarhizium* conidia were observed.

Key words: *Metarhizium anisopliae*, *M. brunneum*, additives, above ground application

Introduction

*Metarhizium* is one of the most important entomopathogenic fungus currently used as a bioinsecticide and bioacaricide, respectively, for the control of a variety of arthropod pests. The above ground application of the fungus is a promising new approach for the control of ticks reported by Stafford & Allan (2010). However, little is known about the persistence of entomopathogenic fungal propagules on plant surfaces (Inglis et al., 2001). Inglis et al. (2001) and Jaronski (2010) published general information regarding the influence of abiotic and biotic factors on the efficacy of mycopesticides in foliar applications. The authors emphasized that the main environmental factors are solar radiation, temperature, humidity, rain, wind, leaf surface chemistry and phylloplane microbiota. The use of stickers in conidial suspensions for spray application is announced to enhance high conidia concentrations on leaf surfaces and consequently a good persistence on foliage (Inglis et al., 2001). Preliminary studies have shown that *Metarhizium* conidia with a conidia density of $2 \times 10^5$ conidia cm$^{-2}$ leaf do not persist longer than three weeks. To ensure a successful reduction of the ticks the aim of this study was to improve the persistence of *Metarhizium* conidia on foliage by using the adhesive agent Neo-wett™ (sticker) and the antifoaming agent Antischiuma Schaumstop™.

Material and methods

Fungal isolates

Three different strains of *Metarhizium* spp. were used in this study: *Metarhizium* anisopliae var. anisopliae strain BIPESCO 5 (mycological collection, University of Innsbruck, Austria), isolated from *Cydia pomonella*; *M. anisopliae* (ARSEF 4556) and *M. brunneum* (ARSEF 3297), isolated from *Boophilus* sp. (Acarina: *Ixodidae*) in Florida/USA and Mexico (Ansari &
Butt, 2011). Both strains were provided by Dr. Tariq M. Butt, from Swansea University, as technical spore powder products produced on rice.

**Quality control tests**

All technical spore powder products were characterised based on the Standard protocols published by Laengle et al. (2005): (i) purity of the products, (ii) viability of conidia and (iii) virulence of the technical spore powder propagules.

**Influence of adhesive- and antifoaming agent**

Testing influence of the adhesive agent Neo-wett™ (Kwizda, 10640.001) and the antifoaming agent Antischiuma Schaumstop™ (BASF Italia Srl) on conidia germination, bioassays with Tenebrio molitor larvae were conducted with several variations. Suspensions were tested as follows: (a) conidia with 0.1% (v/v) sterile Tween® 80 solution (positive control), (b) conidia with 450 mg l\(^{-1}\) Dodine (1-dodecylguanidium acetate) and 70 mg 100 ml\(^{-1}\) Cycloheximid (negative control), (c) conidia with tap H\(_2\)O, (d) conidia with tap H\(_2\)O and 0.05% (v/v) Neo-wett™, (e) conidia with tap H\(_2\)O and 0.0015% (v/v) Antischiuma Schaumstop™, (f) conidia with tap H\(_2\)O, 0.05% (v/v) Neo-wett™ and 0.0015% (v/v) Antischiuma Schaumstop™.

**Spray application**

Phaseolus vulgaris (bean plants) and Malus domestica (apple plants) were used in this study. Before spray application the plants were separated in four groups due to the use of three different Metarhizium products and one untreated control. All bean plants per treatment and variation were sprayed once with a conidial suspension of 1 x 10\(^8\) conidia ml\(^{-1}\) and all apple plants with a conidial suspension of 1.5 x 10\(^7\) ml\(^{-1}\) (BIPESCO 5) and 5 x 10\(^7\) conidia ml\(^{-1}\) (ARSEF 3297), respectively (total spray volume 400 ml). The H\(_2\)O conidial suspension was supplemented with a Neo-wett™-solution [0.05% (v/v); Kwizda, 10640.001]. The suspensions were shaken for 2 min to avoid clumping of the conidia. A variation in preparing the spore suspensions were made in the semi field trial with apple trees: The conidial suspensions were first incubated in the sonication bath for 10 min, then filtrated through a cotton cloth and finally supplemented with Neo-wett™ [0.05% (v/v)]. All suspensions were applied with a Spray-Matic 1.25 P aerosol can (Birchmeier) with a volume capacity of 5 l for 2 to 3 s per plant. After a short air drying period this procedure was repeated until the total dose of 400 ml was applied to the leaves. This application technique assured that the upper and bottom side of the bean and apple leaves were covered with a fine conidia film, with a recommended concentration of more than 2 x 10\(^5\) conidia cm\(^{-2}\) leaf.

**Monitoring persistence on foliage**

Standard protocol by Hutwimmer et al. (2007) was used for monitoring the persistence of conidia on leaf surfaces over time of 25 and 44 days, respectively. The treated bean plants (n = 7; per product and variation) and apple plants (n = 6) were grown in four different environments: one set of plants was kept in the greenhouse and the other three series were preserved outdoor under three different conditions: open field (unprotected), roofed (rainfall protected) and roofed, fully covered with canvas cover. At the sampling date, one leaf per treatment and station was cut off the bean shrubs (n = 7) and the apple trees (n = 6) with a sterilised scissor and put into plastic zip lock bags to process the samples in the laboratory. The leaves were put into sterilised 100 ml Erlenmeyer flasks containing 50 ml sterilised 0.1% (v/v) Tween® 80 solution. The flasks were shaken for 15 s to wash down all conidia from the leaves. The harvested and dried leaves were used to determine the surface area of the processed leaves. The former conidia suspension was diluted to obtain approximately 6 conidia µl\(^{-1}\). The suspension (50 µl) was plated on Metarhizium selective S4G agar plates (n = 3). The plates were incubated at 25 °C and 60% relative humidity for up to two weeks. The colony forming units (CFUs) were counted to calculate the vital spore density cm\(^{-2}\) and leaf.
Results and discussion

Sonderegger (2012) reported that all three strains (BIPESCO 5, ARSEF 3297, ARSEF 4556) showed persistence on leaves but the number of detectable conidia per cm² leaf significantly decreased between 4 and 8 days after spray application, respectively. Although the same conidia concentration was tested, in our study all three strains showed an adequate persistence even after 25 days in all outdoor environments (Figure 1). BIPESCO 5 strain was persistent over an observation period of 44 days. More than 9% of vital BIPESCO 5 conidia were determined. A more rapidly decrease of the conidia viability of strain ARSEF 4556 and BIPESCO 5 was assessed in the unprotected open field system. Monitored meteorological data lead to the conclusion that heavy rainfall periods had a significant impact on the persistence of conidia. During the semi-field trial with Phaseolus vulgaris three and with Malus domestica six heavy rain showers occurred (> 8 mm rainfall per day) and affected the plants in the unprotected environment. In the greenhouse the number of conidia decreased faster and only BIPESCO 5 showed an adequate persistence till the end of the experiment (Figure 1). The declined persistence can be traced back to the fact, that the greenhouse conditions were suboptimal, because the bean- and apple plants were exposed to extreme heat (> 45 °C) for more than 8 h a day because of the missing air condition in the building.

Figure 1: Persistence of Metarhizium spp. conidia on leaves of Phaseolus vulgaris and of Malus domestica during a time interval of 25 and 44 days, respectively, in four different environments: Greenhouse, open field (unprotected), open field and roofed (rainfall protected), open field and fully covered with a canvas cover. Comparison of three different Metarhizium technical spore powder products: BIPESCO 5 from June 2012 (…), BIPESCO 5 (—), ARSEF 4556 (– –) and ARSEF 3297 (– –) from July 2012.
An essential part of a successful spray application is to maintain the virulence of the biological control agent. No negative effects on the vitality of Metarhizium conidia were estimated by adding the adhesive agent Neo-wett™ and the antifoaming agent Antischiuma Schaumstop™. With the exception of the positive control substances Dodine and Cycloheximid (two potent fungicides) all three production strains were highly virulent despite the addition of the additives to the conidia suspensions. A fifty percent mortality of the Tenebrio larvae was estimated for all BIPESCO 5 and ARSEF 4556 products within 5 to 10 days (Figure 2). ARSEF 3297 conidia showed a decreased virulence, especially in the water-, Neo-wett™- and Antischiuma Schaumstop™ suspensions. Nevertheless, ninety percent of the larvae were still killed by all tested agents after an incubation time of 5 to 20 days (Figure 2).

Figure 2: Influence of the adhesive agent Neo-wett™, the antifoaming agent Antischiuma Schaumstop™ and Tween® 80 and the two fungicides Dodine and Cycloheximid on the virulence of Metarhizium spp. conidia (LT50 and LT90). Following variations were tested: Neo-wett™; Antischiuma Schaumstop™; Neo-wett™ and Antischiuma Schaumstop™; Tween® 80 [0.1% (v/v); positive control], 450 mg l⁻¹ Dodine (1-dodecylguanidium acetate) and 0.7 mg ml⁻¹ Cycloheximid (negative control) and tap water. BIPESCO 5 ( ■), ARSEF 4556 ( ▲) and ARSEF 3297 (○).

Summarizing, the proposed conidia density for foliage treatment of 2 x 10⁵ conidia cm⁻² leaf (Sonderegger, 2012) ensures that the conidia persist on leaf surface for more than three weeks. Even after heavy rainfalls conidia were re-isolated in the open field site after 44 days. The use of the adhesive agent Neo-wett™ and the antifoaming agent Antischiuma Schaumstop™ is recommended to enhance the persistence of Metarhizium on leaf surfaces.
Acknowledgements

This research was partly supported by the European Community's Seventh Framework Programme grant (FP7_ENV.2011.3.1.9-1 ECO-INNOVATION, INBIOSOIL, Grant Agreement No. 282767). We would like to thank Dr. Roland Zelger, Research Centre Laimburg (Italy) for providing the potted plant cultures.

References


Vertical transmission of an endophytic strain of *Beauveria bassiana* (Ascomycota; Hypocreales) colonizing opium poppy *Papaver somniferum*

Enrique Quesada-Moraga¹, Blanca B. Landa del Castillo², Cristina López-Díaz³

¹Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus de Rabanales, Edificio C4 Celestino Mutis, 14071 Cordoba, España (Spain); ²Department of Crop Protection, Institute of Sustainable Agriculture, Spanish Council for Scientific Research (CSIC), P.O. Box 4084, 14080 Cordoba, Spain

e-mail: equesada@uco.es

**Abstract:** *Beauveria bassiana* (Balsamo) Vuill. strain EABb 04/01-Tip obtained from larvae of the opium poppy borer *Iraella luteipes* (Hymenoptera; Cynipidae) endophytically colonizes opium poppy (*Papaver somniferum* L.) plants. The goal of this study has been to use a species-specific two-step nested PCR for identifying and monitoring this strain in plant tissues and to ascertain whether the fungus is transmitted vertically via seeds. Surface-sterilized seeds were treated (dressed) with a conidial suspension and endophytic colonization of the plant tissues by the fungus was monitored and ascertained throughout different plant growth stages including seedling, rosetta, principle of notching, end of notching, capsule formation and in new seeds. Use of the nested PCR protocol showed that all plants obtained from seeds dressed with the fungus were endophytically colonized at the different growth stages, and more importantly the endophyte was detected in 50% of the seed samples formed in the new capsules. Three seed lots obtained from three independent capsules showing *B. bassiana* colonized seeds were selected, and their seeds were surface disinfested and sown in order to monitor the possible presence of the fungus in the tissues of the new plants at the same phenological stages. In total, 24 plants were obtained from the above mentioned seeds, and the fungus was detected in plant tissues and even seeds of 19 plants, therefore demonstrating that the fungus was transmitted vertically from maternal plants via seeds, which to the best of our knowledge is reported for the first time for an entomopathogenic fungus.

**Key words:** entomopathogenic fungi, endophytic colonization, systemic protection, species-specific two-step nested PCR
Development of a novel fermentation and formulation process for an endophytic Beauveria bassiana strain

Rieke Lohse, Desiree Jakobs-Schönwandt, Anant Patel
University of Applied Sciences Bielefeld, Department of Engineering and Mathematics, Wilhelm-Bertelsmann-Str.10, 33602 Bielefeld, Germany

Abstract: There is an increasing demand for alternative or complementary crop protection strategies. A novel approach could be the use of the entomopathogenic and endophytic fungus Beauveria bassiana isolate ATP-04. To use the endophyte as a commercial biocontrol agent, the fungus has to be mass-produced. B. bassiana was raised in shake flask cultures to produce submerged conidiospores (SCS) which are reported to show a higher shelf life than mycelium and blastospores (BS). It was found that in mineral media with 5% sugar beet molasses B. bassiana produced 0.1 x 10^10 SCS g^{-1} sucrose in 192 h. By adding 50 g l^{-1} NaCl 48 h after inoculation the SCS yield increased to 1.4 x 10^10 SCS g^{-1} sucrose. The scale-up to a 2 l stirred tank reactor was carried out at 25°C, 200-600 rpm and 1 vvm at pH 5.5. A total spore yield of 5.2 x 10^10 spores g^{-1} sucrose corresponding to a SCS yield of 0.2 x 10^10 SCS g^{-1} sucrose was obtained after 216 h. Also the yield of SCS increased to 1.1 x 10^10 SCS g^{-1} sucrose by the addition of NaCl. After fermentation the B. bassiana was formulated in a novel spray formulation that delivers the fungus on oilseed rape leaves, increases persistence, germination and growth on leaves as well as penetration, colonization and efficacy in bioassays with Plutella xylostella.

Key words: endophyte, Beauveria bassiana, submerged conidiospores, fermentation, spray formulation

Introduction

B. bassiana is an entomopathogenic fungus that can colonize a wide array of plant species (Ownley, 2010) many of them of economic interest. This endophytic B. bassiana shows efficacy against a wide range of insect pests from within the plants and has the potential of becoming a cost-effective biocontrol agent (Khachatourians, 1986). For application as a commercial biocontrol agent the endophytic B. bassiana has to be mass-produced and formulated in such a fashion that it colonizes plants and protects them from insect pests from within, just as transgenic plants do.

Most publications on cultivation of B. bassiana deal with solid-state fermentation of epiphytic B. bassiana isolates and therefore with the mass-production of aerial conidia and mycelium (e.g. Kang et al., 2005). However the preferred method for large-scale production of microorganisms is submerged cultivation. The obvious advantages of a submerged cultivation are that the fungus produces spores in a relatively short time with high yields under controlled sterile conditions as well as a simpler scale-up in contrast to solid-state fermentation (Feng et al., 1994, Patel et al., 2010). In a submerged cultivation B. bassiana forms BS, SCS and mycelium. BS are relatively large, thin-walled and single-celled hyphal bodies (Bidochka et al., 1987). SCS, on the other hand, are small, spherical, more uniform in size and show a higher shelf-life than BS. They arise from fungal mycelia or directly from BS in a process known as microcycle conidiation (Thomas et al., 1987).
With regard to formulation, there are no systematic investigations on the development of novel spray formulations for endophytic entomopathogenic fungi which leads to an increased colonisation of oilseed rape plants (Burges, 1998).

The objectives of the present work were to produce SCS in a cost-effective culture medium on lab scale, to increase SCS yield by addition of NaCl and scale-up the process to a 2 l stirred-tank reactor. Furthermore, we will show data on delivery of formulations on oilseed rape leaves, persistence of fungus, germination and growth on leaves, penetration, colonization and efficacy in bioassays with *Plutella xylostella*.

**Material and methods**

**Strain**
Beauveria bassiana isolate ATP-04 was provided by the Georg-August-University, Department of Crop Sciences/Agricultural Entomology, Goettingen. The strain was raised at 25 °C on SDA agar containing 1% casein peptone, 2% glucose and 1.5% agar-agar at pH 5.5. Temperature optimum was determined at 25 °C and pH optimum at 5-6 (data not shown).

**Cultivation**
B. bassiana was grown in different liquid media in shake flasks with three baffles. Aerial conidia from agar plates (see above) were used as a starter inoculum. The shake flask cultures were inoculated with the spore suspension to give an initial spore density of 5.0 x 10⁴ spores ml⁻¹. The flasks were incubated at 25 °C, 150 rpm and pH 5.5. At seven different times after inoculation different sterile NaCl stock solutions were added to the “osmotic stress” cultures, varying the final NaCl concentration in the media. Batch fermentation was carried out in a 2 l stirred tank reactor (Sartorius Stedin System GmbH, Germany). Fermentation was started by inoculating 300 ml of a carbon source stock solution with 7.5 x 10⁸ aerial conidia (5.0 x 10⁴ spores ml⁻¹). The fermentation was carried out at 25 °C, 1 vvm and 200-600 rpm.

**Analytics**
For the determination of fungal dry biomass 15 ml samples were centrifuged for 10 min at 20000 xg, washed two times with dd H₂O and centrifuged again. The cell suspensions were dried at 115 °C with a moisture analyzer (Sartorius, Germany). The colony forming units (CFU) of BS and SCS were determined by spreading 100 µl of diluted samples on agar plates (see above) and incubating at 25 °C for 4-6 days.

**Results and discussion**
In total, 23 technical culture media based on different carbon sources, minerals and technical yeast extracts were screened. The most promising culture medium was a mineral medium with 5% sugar beet molasses, which consists of 50% sucrose. In this culture medium B. bassiana produced 5.32 ± 0.24 x 10¹⁰ total spores g⁻¹ sucrose at 192 h after inoculation. But the yield of SCS was only 0.12 ± 0.04 x 10¹⁰ SCS g⁻¹ sucrose.

Sugar beet molasses is a residue of the agricultural industry and consequently it is a low-cost source. Therefore, the cost of 1 l culture medium amounts to only 0.33 €. However the problem of this cultivation is the low concentration of SCS. One potential solution for this problem is the selective production of SCS by osmotic stress. To this end the influence of
different times of addition and final concentrations of NaCl on the production of SCS was investigated.

In the control, where no salt was added but the same amount of water after the same time span, a concentration of $0.02 \pm 0.00 \times 10^9$ SCS ml$^{-1}$ was obtained. In contrast, 48 h after inoculation the addition of $50 \text{ g l}^{-1}$ NaCl led to a concentration of $0.35 \pm 0.03 \times 10^9$ SCS ml$^{-1}$ corresponding to a yield of $1.40 \pm 0.12 \times 10^{10}$ SCS g$^{-1}$ sucrose at the end of the cultivation (Figure 1).

![Graph showing influence of different times of addition of NaCl on the production of SCS.](image)

Figure 1. Influence of different times of addition of $50 \text{ g l}^{-1}$ NaCl on the production of SCS.

Thus, the amount of SCS was increased 17.5-fold by the addition of NaCl at the appropriate time. It was observed that the highest yield of total spores was obtained without any addition of NaCl. When NaCl was added to the cultivation broth, the yield of total spores (TS) decreased and a shift from BS to SCS was observed. The earlier the addition of NaCl the higher was the concentration of SCS and the lower the concentration of total spores.

Figure 2A shows a fermentation of $B. \text{ bassiana}$ in a 2 l stirred-tank reactor without addition of NaCl. In this fermentation $B. \text{ bassiana}$ produced $1.29 \pm 0.04 \times 10^9$ TS ml$^{-1}$ corresponding to a yield of $5.16 \pm 0.16 \times 10^{10}$ TS g$^{-1}$ sucrose at the end of the fermentation. Therefore, the cost of $10^{12}$ total spores can be estimated at 0.26 €. But concentration of SCS was only $0.06 \pm 0.00 \times 10^9$ SCS ml$^{-1}$. The amount of dry biomass increased at the beginning of the fermentation because the fungus produced mycelium. After 72 h, 21 g biomass l$^{-1}$ were obtained. Then the amount of mycelium decreased to the end of the fermentation. This could be due to the limitation of substrates. 96 h after inoculation the concentration of viable spores started to decrease to a yield of $3.12 \times 10^{10}$ TS g$^{-1}$ sucrose at the end of the fermentation. For the selective production of SCS $50 \text{ g l}^{-1}$ NaCl was added to the culture broth after 48 h (Figure
In contrast to a cultivation without NaCl the concentration of SCS could be increased to $0.28 \pm 0.01 \times 10^9$ SCS ml$^{-1}$ at the end of the fermentation. This represents a 5-fold increase of the SCS yield. Furthermore, B. bassiana did not produce mycelium during the fermentation in contrast to the fermentation without NaCl addition.

It could be shown that wetters based on non-ionic surfactants could decrease the contact angle on the leaf from 110° to < 25° resulting in an increase of the wetted leaf area compared to the control based just on water. However, some wetters decreased viability of spores by > 90%. Besides, we will show data on delivery of formulations on oilseed rape leaves, persistence of fungus, germination and growth on leaves, penetration, colonization and efficacy in bioassays with $P$. xylostella.

![Figure 2. Cultivation of B. bassiana without NaCl (A) and in presence of NaCl (B) in a 2 l stirred tank reactor. The concentrations of TS, BS, SCS and correlation of spore counts with biomass and CFU are shown.](image)

**Acknowledgements**

This research was funded by the BMBF, Germany (FKZ 17N1510).

**References**


Development of analytical tools to monitor the fate of Metarhizium anisopliae metabolites in the environment

Judith Taibon¹,², Sonja Sturm¹, Christoph Seger¹,³, Hermann Strasser², Hermann Stuppner¹
¹Leopold-Franzens University Innsbruck, Department of Pharmacognosy, Innrain 80/82, 6020 Innsbruck, Austria; ²Leopold-Franzens University Innsbruck, Institute of Microbiology, Technikerstrasse 25, 6020 Innsbruck, Austria; ³Institute of Medical and Chemical Laboratory Diagnostics (ZIMCL), University Hospital Innsbruck, Anichstraße 35, 6020 Innsbruck, Austria

Abstract: Destruxins (dtxs) are structurally closely related cyclic hexadepsipeptides secreted as relevant metabolites by the entomopathogenic fungus Metarhizium anisopliae. To monitor dtxs in fungal culture broth, plant derived matrices and cell cultures, a fast and selective off-line SPE UHPLC-DAD/MS method was established. Sample preparation was carried out by a solid phase extraction (SPE) on a reversed phase material. Optimal purification was achieved by a washing step with 40% (v/v) methanol, removing most of the polar components. The highest amounts of dtxs were obtained by using 85% (v/v) methanol for elution. An UHPLC-DAD system hyphenated to a Q-TOF mass spectrometer was utilized to separate and detect the dtx congeners. A sub-2µm particle size column was used as stationary phase, with a water/acetonitrile solvent gradient at a flow rate of 0.3 ml min⁻¹ serving as mobile phase. A total analysis time of 12 min was achieved with the UHPLC-DAD assay with the dtx congeners eluting from 1 min to 8 min with a higher resolution of the peaks compared to previous HPLC-DAD assays. Besides the available reference compounds dtxA, dtxB, dtxE, dtxE-diol 18 dtx derivatives were tentatively identified by analyzing TOF-MS data.

Key words: Metarhizium anisopliae, entomopathogen, metabolites, destruxin, HPLC-DAD/MS

Introduction

The entomopathogenic fungus Metarhizium anisopliae plays an important role as a biological control agent (BCA), and has been used for about 130 years (Zimmermann, 2007). It is known that this fungus has an effect against many pest insects including the larvae of wireworms, western corn rootworm, black vine weevil and scarids. Fungi produce a wide variety of biological active compounds, such as secondary metabolites or toxins, which can act as pathogenicity determinants by improving the infection and colonisation of the host insect (Strasser et al., 2011).

The main metabolites produced by the fungus M. anisopliae are destruxins (dtxs), cyclic hexadepsipeptides composed of an α-hydroxy acid and five amino acid residues. They exhibit a wide variety of biological activities, for example important cytotoxic effects. Overall they are best known for their insecticidal and phytotoxic activities (Pedras et al., 2002).

However, there are concerns regarding whether these fungi and their produced secondary metabolites entail risks to humans and environment, the EU funded project INBIOSOIL (“Innovative biological products for soil pest control, No. 282767”) will implement the RAFBCA-REBECA decision scheme, which has been tested in case studies on fungal metabolites and crude extracts (Strauch et al., 2011). Concerning the question whether destruxins pose a risk to human health the aim of this study is to assess if theses metabolites enter the food chain. Consequently, protocols for isolating, qualitative verification and
quantitative determination of selected metabolites from *M. anisopliae* out of model crops have to be developed.

As prerequisite a HPLC–DAD/MS assay to monitor destruxins in fungal culture broth based on a previously reported assay (Seger et al., 2004) was established: The novel method shall serve as basis for further assay development in food matrices.

**Material and methods**

**Cultivation of Metarhizium anisopliae**

Metarhizium anisopliae var. anisopliae was cultivated in Sabouraud dextrose (SD) liquid medium (S2G, Merck 1.08339, Vienna) supplemented with 2% (w/v) glucose (NeoLab 4445.5000 Heidelberg, Germany), at 25 °C and 65% relative humidity for 2 weeks. Inoculation was done by pipetting 500 µl of the stock inoculum per flask. The submerged culture was stirred at 250 rpm to ensure a biomass of up to 10 g l⁻¹ dry weight. Before harvesting the liquid lost due to evaporation was replaced with deionized water. All batches were unified to one pooled sample of culture broth. Non-inoculated medium was used as a control for analytics. The pooled culture broth was centrifuged and the supernatant was filtrated over a tared filtration gauze.

**Sample preparation from culture filtrate**

Sample preparation was carried out by a solid phase extraction (SPE) on a Strata C18-E reversed phase material (Strata C18-E, Phenomenex, Aschaffenburg, Germany). Optimal purification was achieved by a washing-step with 40% (v/v) methanol. For elution of the destruxins a 85% (v/v) methanolic solution was used.

**HPLC-DAD/MS conditions**

An Agilent 1200 UHPLC-DAD system (Agilent) was utilized to separate and detect dtx congeners. A Zorbax Eclipse XDB-C18 column (50 x 2.1 mm, 1.8 µm particle size, Agilent) was used as stationary phase, with a water (A) / acetonitrile (B), each containing 0.02% (v/v) acetic acid, gradient at a flow rate of 0.3 ml min⁻¹ serving as mobile phase. A Bruker microTOF-QII mass spectrometer (TOF-MS; Bruker Daltonics, Bremen, Germany) was used to detect and identify dtx congeners. Experiments were performed in positive ESI-mode.

**Assay validation**

For calibration functions methanolic dilution series of dtxA, dtxB, dtxE reference standards were prepared. The method was fully validated including the limit of detection (LOD) and limit of quantification (LOQ) values, repeatability and reproducibility.

**Results and discussion**

**Sample preparation**

A solid phase extraction (SPE) was developed to isolate, concentrate, and purify dtx congeners from *M. anisopliae* culture broth prior to HPLC analysis. The developed sample preparation protocol makes it possible to extract dtxs and to clean up samples within three steps.

To determine the optimum ratio of methanol and water for the wash step and the elution step six different methanolic solutions were prepared increasing in 5% steps. Experiment was conducted in 5 replicates for each concentration and sample preparation done as described above: first using the different wash solvents at the same elution conditions and then using the
different elution solvents at the same wash conditions. For evaluation which ratio of water and methanol can be used as wash solvent, so that polar components were removed and analytes were retained from the sorbent, the eluate was measured. From 25% (v/v) until 40% (v/v) methanol the analyte yields were rather constant. Up to 40% (v/v) methanol they decrease which indicates that analytes started to elute already in the wash step (Figure 1a). Using the 40% (v/v) methanolic solution for the wash-step all undesired polar analytes can be removed so that dtxs can be eluted with the optimized 85% (v/v) methanolic solution (Figure 1b) without disturbing compounds.

Figure 1: (a) Comparison of analyte yields for dtx E-diol. Mean results (n = 5) achieved by using different methanolic wash solvents. (b) Comparison of analyte yields for dtx E-diol. Mean results (n = 5) achieved by using different methanolic elution solvents. Vertical bars indicate the standard deviation (SD).

**HPLC-DAD method development**

The method development was carried out using *M. anisopliae* culture broth samples. Best results could be achieved using the Zorbax Eclipse XDB-C18 rapid resolution column. The addition of acidic additives showed differences in the resolution of peaks regarding the more polar compounds eluting in the first minutes. As final solvents water (A) and acetonitrile (B), each containing 0.02% (v/v) acetic acid, were used. With this composition of the solvents and the optimized gradient peaks were better separated, a higher resolution can be achieved and baseline can be stabilized.

**HPLC-DAD method validation**

Assay validation was performed using *M. anisopliae* culture broth samples and dilution series of reference material in methanol. For all dilution series of dtxA, dtxB and dtxE linear calibration functions could be reached. The calibration range of dtxA was between 3.1 mg l⁻¹ to 600 mg l⁻¹, of dtx B between 0.5 mg l⁻¹ to 200 mg l⁻¹ and for dtxE between 0.5 mg l⁻¹ to 400 mg l⁻¹. The LODs of dtx A, B and E ranged between 0.05 mg l⁻¹ and 0.4 mg l⁻¹ and the LOQs between 0.14 mg l⁻¹ and 1.2 mg l⁻¹.

**Identification of destruxins from culture filtrate**

With the optimized method it was possible to separate 22 analytes (Figure 2) within eight minutes. Compared with the method of Seger et al. (2004) more peaks can be baseline-separated, the method showed a higher resolution than the previous assay. Tentative peak identification was facilitated by analyzing and comparing TOF-M S data using exact masses and specific fragmentation pattern with data from literature (Seger et al., 2004; Jegorov et al., 1998).
Figure 2: Separation of destruxin congeners (e.g. dtx A, B, E, E-diol): Representative HPLC-DAD chromatogram of a Metarhizium anisopliae culture broth, sample recorded at 210 nm.

Acknowledgements

This research has been supported by the European Community’s Seventh Framework Programme grant (FP7_ENV.2011.3.1.9 - ECO-INNOVATION, INBIOSOIL, Grant Agreement No. 282767).

References


Cross-species transferability of 41 microsatellite markers for *Metarhizium* spp.

**Andy Lutz**, **Franco Widmer**, **Adrian Leuchtmann**, **Jürg Enkerli**

1Molecular Ecology, Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstrasse 191, 8046 Zürich, Switzerland; 2Plant Ecological Genetics, Institute of Integrative Biology, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland

**Abstract:** The genus *Metarhizium* includes insect pathogenic fungal species, which are used as biological control agents (BCAs). Genetic tools for identification and monitoring of such BCAs are important. A genotyping tool based on 41 simple sequence repeat (SSR) markers has been developed for *M. anisopliae* s.l. However, detailed phylogenetic analyses based on a multifocus approach revealed that *M. anisopliae* s.l. is a cryptic species complex of nine different *Metarhizium* species. According to this new taxonomy, the 41 SSR markers were isolated from *M. brunneum*, *M. robertsii* or *M. anisopliae* s.s. The goal of this study was to assess the transferability of the 41 SSR markers to individual species of the former *M. anisopliae* species complex. Successful PCR-amplification of SSR markers was observed in all species but the number of loci yielding PCR products varied among species. Amplification of individual SSR loci did not always yield products for all strains of a particular species and not all were polymorphic. The study revealed that SSR markers can be transferred to different species of the former *M. anisopliae* species complex. However, the number of available SSR markers strongly depends on the species to be analyzed. The markers will provide a valuable tool for identification and monitoring of *Metarhizium* BCAs and they will allow investigation of genetic diversity and population structure of seven species of the former *M. anisopliae* species complex.

**Key words:** simple sequence repeats, SSR markers, *Metarhizium* spp., genotyping

**Introduction**

Entomopathogenic fungi of the genus *Metarhizium* constitute an important biotic component in the natural regulation of arthropod populations including agronomically important pests. *Metarhizium* spp. have a history in use as biocontrol agents (Meyling & Eilenberg, 2007) and various products are commercially available (Srivastava et al., 2009). In a recent study the taxonomy of *Metarhizium* spp. and in particular the *Metarhizium anisopliae* species complex (*M. anisopliae* s.l.) has been revised based on a multifocus phylogenetic analysis (Bischoff et al., 2009). Within *M. anisopliae* s.l. nine terminal taxa are now recognized, i.e. *M. acridum*, *M. anisopliae* s.s., *M. brunneum*, *M. globosum*, *M. guizhouense*, *M. lepidiotae*, *M. majus*, *M. pingshaense* and *M. robertsii* (Figure 1). Currently, species affiliation of *Metarhizium* isolates is performed by sequencing the 5′ end of elongation factor 1-alpha, and subsequent alignment of obtained sequences to reference sequences as described by Bischoff et al. (2009).

A availability of efficient tools that allow genotyping, and detection, of a fungal BCA is crucial to allow monitoring of an applied BCA or assessment of its host and habitat type dependent occurrence, population structure, or its possible effects on non-target organisms. A genotyping tool, which is based on 41 single sequence repeat (SSR) markers (microsatellites) has been developed for *M. anisopliae* s.l. (Enkerli et al., 2005, Oulevey et al., 2009).
Microsatellites are short DNA sequence motives (1 to 6 bases) that occur as tandem repeats. The number of repeats in each particular SSR locus can be highly variable (length polymorphism) between individuals, which makes them effective for identification purposes and population genetic analysis (Queller et al., 1993). Microsatellite markers are amplified by PCR and the size of the resulting products (allele size) are determined by gel or capillary electrophoresis. According to the new taxonomy, the 41 SSR markers have been isolated from M. brunneum (27 SSR markers), M. robertsii (6 SSR markers) or M. anisopliae s.s. (8 SSR markers). The goal of this study was to assess the transferability of the 41 SSR markers to individual species of the former M. anisopliae species complex.

Figure 1. UPGMA phylogenetic tree based on an alignment of 21 sequences of the 5' end of elongation factor 1-alpha (~ 630 bp). 18 sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and 3 sequences were sequenced in the frame of this study (ART 500, ART 714 and ART 2062).

Material and methods

Fungal strains and DNA extraction
A collection of 50 fungal strains including the Metarhizium species M. acridum, M. anisopliae s.s., M. brunneum, M. guizhouense, M. lepidiotae, M. majus, M. pingshaense and M. robertsii was used. Fungal strains were obtained from three different collections: USDA-
ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY, USA), CBS collection (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) and Agroscope Reckenholz-Tänikon (ART) collection. M. globosum was not included in this study as only one strain is available from above culture collections. Strains were grown and maintained on Sabouraud-Dextrose-Agar (SDA, Difco BD, Franklin Lakes, NJ, USA), mycelium was grown and harvested as described by Schneider et al., 2011. DNA of Metarhizium strains was extracted from 20 mg lyophilized mycelium using the NucleoSpin® Plant II Kit (Macherey-Nagel, Easton, PA) according to manufacturer’s instructions. Genomic DNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA).

SSR marker amplification and data analysis
The 41 SSR markers previously developed (Enkerli et al., 2005; Oulevey et al., 2009) were tested on all strains of the isolate collection. PCR amplifications were performed as described by Oulevey et al. (2009). Amplification products were analysed on an ABI 3130xl sequencer equipped with 36 cm capillaries filled with POP-7 polymer (Applied Biosystems, Foster City, CA, USA) and using GENESCAN® 400 HD [ROX] Size Standard (Applied Biosystems, Foster City, CA, USA) as internal size standard. Allele sizes were scored with the program GenMarker v1.91 (SoftGenetics LLC, State College, PA, USA).

Results and discussion
The number of SSR loci yielding scorable PCR products varied strongly among the different Metarhizium spp. (Table 1.). For M. brunneum PCR amplification of 40 SSR loci, including the 27 SSR loci isolated from this species (Oulevey et al. 2009), revealed scorable products. For the species M. anisopliae s.s., M. pingshaense, M. robertsii, and M. guizhouense 36, 34, 36, and 33 SSR loci, respectively, revealed scorable PCR products, whereas for the species M. majus, M. lepidiotae and M. acridum amplification products were obtained from 27, 24 and 10 SSR loci, respectively. However, SSR loci yielding scorable products did not always yield products for all strains of a particular species. For example, for M. brunneum 33 of the 40 SSR loci, including 20 of the loci isolated from this species, yielded products for all the 11 M. brunneum strains analyzed. Seven SSR loci yielded PCR products from 6 to 10 of the 11 M. brunneum strains only. For M. pingshaense, M. anisopliae s.s M. robertsii, M. majus, M. guizhouense and M. lepidiotae, 30, 30, 28, 22, 21, and 14 SSR markers revealed PCR products from all the strains, respectively. Sequence differences in the primer annealing sites among different species or strains are most likely the reason for the differences in successful PCR amplification. Polymorphic loci (i.e. different alleles are detected at a locus within a species) were observed in all species. The largest number of loci displaying within species polymorphism was detected for M. brunneum (37 polymorphic loci) followed by M. pingshaense and M. anisopliae s.s. (27 polymorphic loci). Loci displaying species specific alleles across different species were also observed.

The present study showed that SSR markers isolated from M. brunneum, M. robertsii, M. anisopliae s.s. can be transferred to different species of the former M. anisopliae species complex. The number of SSR markers that can be applied strongly depends on the species to be analyzed and available markers for a certain Metarhizium species do not necessarily correspond to the markers available for another Metarhizium species. This fact explains previous observations, where SSR markers were not consistently amplified from all strains of the M. anisopliae species complex (Enkerli, unpublished). However, there are markers that
can be used to analyze more than one *Metarhizium* spp. at the same time. For example, 26 SSR markers are available that can be applied to *M. brunneum* and *M. robertsii* and 14 of these 26 markers are polymorphic in both species. The study revealed that for seven of the nine species of the former *M. anisopliae* species complex 15 to 37 polymorphic SSR markers are available. These markers will provide a valuable tool for identification and monitoring of *Metarhizium* BCAs and they will allow investigation of genetic diversity and population structure of seven species of the former *M. anisopliae* species complex.

Table 1. Cross-species transferability of the 41 *Metarhizium* spp. SSR markers. The number of strains analyzed per species, the number of SSR loci yielding PCR products, the number of SSR loci successfully amplified from all strains of a single species and the number of polymorphic loci are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Nr. of strains analyzed</th>
<th>SSR markers yielding PCR products</th>
<th>Amplification from all strains</th>
<th>Polymorphic loci</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. acridum</em></td>
<td>9</td>
<td>10</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td><em>M. anisopliae s.s.</em></td>
<td>4</td>
<td>36</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td><em>M. brunneum</em></td>
<td>11</td>
<td>40</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td><em>M. guizhouense</em></td>
<td>5</td>
<td>33</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td><em>M. lepidiotae</em></td>
<td>4</td>
<td>24</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td><em>M. majus</em></td>
<td>6</td>
<td>27</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td><em>M. pingshaense</em></td>
<td>4</td>
<td>34</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td><em>M. robertsii</em></td>
<td>5</td>
<td>36</td>
<td>28</td>
<td>23</td>
</tr>
</tbody>
</table>

**Acknowledgements**

The project was supported by funding from the EU-project INBIOSOIL.

**References**


Posters
A review of the use of entomopathogenic fungi for the control of Bemisia tabaci (Homoptera: Aleyrodidae) in the UK

Andrew G. S. Cuthbertson
The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK

Review

The sweetpotato whitefly, Bemisia tabaci Gennadius (Homoptera: Aleyrodidae) is a major pest of economically important crops worldwide (Xu et al., 2012). Bemisia tabaci damages crops by feeding on phloem sap and the large amounts of sticky honeydew produced can lower the rate of leaf photosynthesis. This species of whitefly is also a vector of many plant viruses (Powell et al., 2012). Within the United Kingdom (UK), B. tabaci remains a notifiable pest subject to a policy of eradication if found on propagators premises, plants moving in trade, and containment/eradication if outbreaks occur at nurseries (Cuthbertson et al., 2011). Entomopathogenic fungi can penetrate and cause the death of many economically important pests. They can form effective biological alternatives to chemical pesticides. Studies by Cuthbertson et al. (2005a, 2010, 2012) have demonstrated their potential and have determined that the second and third instars of B. tabaci are the most susceptible life-stages to both Lecanicillium muscarium and Beauveria bassiana (Figure 1).

![Figure 1. The susceptibility of the immature stages of Bemisia tabaci to the entomopathogenic fungi Lecanicillium muscarium on verbena plants. Columns with the same letter are not significantly different. Water control: □; Lecanicillium muscarium + 0.02% Agra1: □ (Cuthbertson et al., 2005a).](image)

Due to the differences in sensitivity of fungal species to different formulations of the same insecticide, information is required on the compatibility of each entomopathogenic fungi and chemical product to be used within a given IPM strategy. Formulations of different insecticides may differ in toxicity to fungi due to the use of different surfactants. Further, fungi species may also differ in sensitivity to different formulations of the same insecticide.
Therefore, information regarding compatibility between entomopathogenic fungi and each chemical product for an IPM system needs to be tested individually within the ecosystem in which it will be applied. The optimum use of an IPM system for pest control may also require sequential rather than simultaneous applications of insecticides and entomopathogenic fungi (Cuthbertson & Walters, 2005).

Within the UK, only Cuthbertson et al. (2005b, 2010, 2012) have investigated the combination of chemicals routinely used for the control of whitefly with fungi. Here promising results have been obtained. In regards to mixing chemicals with L. muscarium, direct exposure for 24 h to imidacloprid, nicotine and teflubenzuron resulted in very low spore germination, unsuitable for commercial use. Only the active ingredient buprofezin provided an acceptable level of spore germination.

The implementation of an IPM scheme may require sequential rather than simultaneous applications of insecticides and entomopathogenic fungi but few previous studies have tested the effect of dry insecticide residues on fungal activity. Recent work (Cuthbertson et al., 2005b) has shown that when L. muscarium was applied to plants sprayed 24 h earlier with a standard commercial application of one of three contact insecticides or with treatment using a systemic insecticide, no significant reduction in infectivity (mycelial growth) was detected in any cases. Therefore, L. muscarium could be applied sequentially with imidacloprid, buprofezin, nicotine and teflubenzuron in a commercial IPM strategy. In similar trials, B. bassiana proved suitable for tank mixing with a range of products including petroleum oils (Cuthbertson et al., 2012). Following sequential applications of L. muscarium and chemicals, mortalities of up to 90% of B. tabaci second instars were recorded (Table 1) (Cuthbertson et al., 2005b). Sequential treatments offer a greater flexibility in timing applications against various life stages of the pest.

Table 1. The results of experiments investigating the effect of chemical residues on tomato plants on the infectivity of Lecanicillium muscarium (ca 1.5 x 10^5 spores cm^{-2} of leaf area) against Bemisia tabaci second instar larvae. The second treatment application was applied 24 hours following the first treatment. Data represent the model derived percentage mortality (± 95% confidence intervals) of B. tabaci larvae 3 days after final treatment application. Within both rows and columns means with the same letter exhibit overlapping confidence intervals (Cuthbertson et al., 2005b).

<table>
<thead>
<tr>
<th>Insecticide tested</th>
<th>No. of B. tabaci</th>
<th>1st appl.</th>
<th>2nd appl.</th>
<th>A Water</th>
<th>B Insecticide</th>
<th>C Insecticide</th>
<th>D Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprofezin (30ml/100l)</td>
<td>673</td>
<td></td>
<td></td>
<td>5.6 ± 5.2</td>
<td>67.0 ± 10.6</td>
<td>68.5 ± 10.4</td>
<td>77.4 ± 9.2</td>
</tr>
<tr>
<td>Nicotine (500ml/5l)</td>
<td>521</td>
<td></td>
<td></td>
<td>7.7 ± 3.1</td>
<td>69.7 ± 4.9</td>
<td>68.9 ± 4.9</td>
<td>63.6 ± 5.3</td>
</tr>
<tr>
<td>Imidacloprid (0.2g/l)</td>
<td>480</td>
<td></td>
<td></td>
<td>7.8 ± 11.3</td>
<td>89.1 ± 14.7</td>
<td>89.2 ± 16.9</td>
<td>91.4 ± 13.3</td>
</tr>
<tr>
<td>Teflubenzuron (500ml/1000l)</td>
<td>674</td>
<td>6.3 ± 10.0</td>
<td>52.1 ± 23.6</td>
<td>52.8 ± 21.6</td>
<td>75.6 ± 19.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The chemical groups most toxic to fungi are organophosphates and carbamates. Many commonly used insecticides, for example, buprofezin, have now been rendered ineffective in the UK against *Trialeurodes vaporariorum* (glasshouse whitefly) by the widespread appearance of resistance in populations. This product has now also just recently become unavailable for use in UK horticulture (Cuthbertson et al., 2011). *Bemisia tabaci* have also been shown to offer a degree of resistance to imidacloprid (Schuster et al., 2010) adding urgency to the development of alternative IPM approaches.

The ambient temperature and humidity are known to be important factors determining fungi efficacy. Trials have shown that for optimal use of *L. muscarium* favourable conditions for fungi survival and efficacy must be maintained for up to 6-8 h following application to plant foliage. As a result, no host plant effects are apparent (Figure 2) (Cuthbertson & Walters, 2005).

![Figure 2](image-url) **Figure 2.** Efficacy of *Lecanicillium muscarium* (10^7 conidia ml^-1 + 0.02% Agrafal) against second instar *Bemisia tabaci* on a range of host plants applied within a controlled environment cabinet, 20 °C, 85% relative humidity. Mortality recorded after 72 h. Bars are standard error of the means (± SEM) (Cuthbertson & Walters, 2005).

*L. muscarium* and *B. bassiana* have the potential to be important biological control agents of *B. tabaci*. Integrated approaches utilizing entomopathogens are showing much potential. Early instars of *B. tabaci* are proving most susceptible to infection, an important factor when wanting to target a quarantine species at as early a life-stage as possible in order to break the lifecycle. The levels of both direct and indirect compatibility of the fungi with chemical insecticides also increase their potential for incorporating them into strategies for the control of *B. tabaci*. Their use depends on further work in commercial-scale glasshouse crops and, if successful, they may contribute to the development of sustainable production systems through a reduction in the use of chemical insecticides and, consequently, a reduction of chemical residues on produce and insecticide resistance. Further research is now required to both fine tune the application techniques and optimum dose rates required for their use within the glasshouse environment.
Acknowledgements

The Bemisia tabaci research is funded by the Department for Environment, Food and Rural Affairs (UK).

References


Effect of entomopathogenic fungi against Trialeurodes vaporariorum and its parasitoid Encarsia formosa: preliminary laboratory assays

Monica Oreste, Michele Poliseno, Eustachio Tarasco
DISSPA – Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari “Aldo Moro”, Via Amendola 165/a, 70126 Bari, Italy

Abstract: Trialeurodes vaporariorum Westwood, the greenhouse whitefly, is one of the major pest affecting crops in greenhouse, particularly tomato and its biological control is possible releasing its parasitoid Encarsia formosa Gahan or using myco-insecticides based on the entomopathogenic fungus Beauveria bassiana Vuill. Balsam. Entomopathogenic fungi are generally considered not detrimental against insects natural enemies and useful arthropod fauna, despite of the lack of data, particularly about the effect of this fungi on parasitoids under laboratory and field condition.

Three isolates of B. bassiana and one of M. anisopliae were tested for their virulence against Trialeurodes vaporariorum Westwood (Hemiptera: Aleurodidae) nymphs (sub-pupae), performing $2 \times 10^6$ conidia ml$^{-1}$ suspensions and using the leaf disks method. The commercial myco-insecticide Naturalis (Intrachem Bio Italia, Italy), and the ATCC 74040 B. bassiana strain, contained into the commercial product, were included in the assays for comparison. The same isolates were also tested against whiteflies sub-pupae parasitized by Encarsia formosa Gahan (Hymenoptera: Aphelinidae) (Encarsia system, Biobest). A complete randomized block design with four replicates was used. The whiteflies mortality and the emergence of adult parasitoids was recorded daily for 7 days. For statistical analysis, cumulative mortality and cumulative number of surviving parasitoids (%) were calculated. Mean survival time and mean lethal time were determinated by the Kaplan-Meier procedure and the probit analysis respectively. Data were then analyzed performing the one way Analysis of Variance (ANOVA) and the HSD Tukey Test was used to compare means.

Results of our preliminary assay showed a good efficacy of tested entomopathogenic isolates against T. vaporariorum, with a final cumulative mortality (7 day after the inoculation) greater than the 80% for all the isolates and Mean Survival Times ranging from 3.4 to 4.5 days. The ATCC 74040 B. bassiana strain and the commercial product Naturalis, resulted not significantly different from our isolates. Not significative effects in decreasing the E. formosa adults emergence were detected among the fungal isolates. The emergence of parasitoids in the untreated control was satistically not different from that in the fungal treatments, except the case of Naturalis. The myco-insecticide Naturalis reduced the rate of E. formosa emergence (20.3% 7 days after inoculation while in the control was 57.65%), but not the ATCC 74040 strain isolated from this product. This effect is probably related to the improving effect of co-formulants, in term of adhesion, persistence and physical covering action.

Our results, except the case of commercial product, are not in contrast with other works which revealed that mature parasitoid larvae are able to complete their development when treated with entomopathogenic fungi. Several authors showed that the time interval between parasitization and fungal application is crucial for the parasitoid development. These aspects will be analyzed in future tests under laboratory and field conditions.

Key words: microbial pest control, greenhouse, non target insects
Laboratory Beauveria bassiana (Bals.) Vuill. bioassays on spruce bark beetle (Ips typographus L.)

Ana-Maria Andrei¹, Daniela Lupăștean², Constantin Ciornei³, Ana-Cristina Fătu¹, Mihaela Monica Dinu¹

¹Research - Development Institute for Plant Protection, Bd. Ion Ionescu de la Brad nr. 8, CP 013813, Sector 1, Bucharest, Romania; ²University “Ştefan cel Mare”, Universității Street, nr. 13, CP 720229, Suceava, Romania; ³Forestry Research Station, Ştefan cel Mare Street, nr. 23, CP 600359, Bacău, Romania

Abstract: The massive damages caused by Ips typographus in spruce forests in Romania, the severe restrictions on the use of chemical insecticides, the identification of natural Beauveria bassiana outbreaks in the Romanian forests infested with bark beetle and the isolation of a new B. bassiana strain, led to the development of some researches on the possibility to use this entomopathogenic fungi for reducing the damage caused by bark beetles. In laboratory conditions, the susceptibility of Ips typographus to infection by a naturally occurring B. bassiana strain was tested. Utilization of a B. bassiana conidial suspension (3.31 x 10^11 conidia ml^-1) induced beetle mortality, length of mother galleries reduction and larval galleries number reduction.

Key words: Ips typographus, Beauveria bassiana, biological control

Introduction

Every year, large areas of Romanian spruce forests are affected by the attack of European spruce bark beetle (Ips typographus). In Romania between 2006-2010, there were studies conducted concerning the amount of losses caused by I. typographus and the control measures that have been applied. Thus, in 2006 the I. typographus attacks had a moderate intensity as a result of control measures, which have been used to a relatively small number of trees (39,143 pieces); in 2007 the I. typographus infestation level increased due to the large number of windfall, especially in the North-Eastern Forest Districts (Iacobeni Broșteni, Dorna Cândrenilor and Moldovita); in 2008 the I. typographus infestation level continued to grow, creating favorable conditions of outbreaks. Between 2009-2010, there was a worsening of the forests health state, despite the control measures applied, mainly because of the large number of felled trees, which were not evacuated on time from the forest.

Considering the restrictions, which currently apply in certified forests according to Forest Stewardship Council standards, it is increasingly important to give biological control means, which has many advantages compared to chemical ones. Some studies regarding the bacterial flora associated with I. typographus concluded that Serratia liquefaciens may have potential as a biological control agent against the Eurasian spruce bark beetle (Muratoglu et al., 2011). The identification of natural B. bassiana outbreaks in the Romanian forests infested with Ips duplicatus (Sahlberg) (Dinu et al., 2012) and the isolation of a new B. bassiana strain infecting I. typographus (accession number given by the International Depositary: NCAIM (P) F 001,392), led to the development of research on the possibility to use this entomopathogenic microorganism to reduce damage caused by bark beetles.
Material and methods

B. bassiana experimental bioprodut was obtained using submerged cultivation procedure (Andrei, 2004). Logs required for the experiment were obtained from the forest, freshly felled and was supposed not to provide previously experienced infestations. I. typographus beetles were captured using pheromone traps. Beetles were examined under a microscope and they were branded and placed separately according to morphological characteristics. The laboratory tests were performed in special cages (100×34×32 cm) with wooden frame and side walls made of fine wire mesh, with mesh sizes smaller than I. typographus beetle size. The special cages were set out with a mobile sidewall for an easy biological material manipulation (Figure 1). Devices, which allowed controlled infestation of logs, were later made using Eppendorf tubes. Two individuals, one male and one female, were placed in each device.

Results and discussion

The holes made by males for penetrating the bark and the ventilation holes made by females throughout maternal gallery were used as penetration pathways for B. bassiana infectious inoculum in the cambial zone (between the bark and the wood). 300 ml fungal suspension m⁻² of bark was applied in the following three experimental variants: V1: 3.3 x 10¹¹ conidia ml⁻¹; V2: 9.9 x 10¹¹ conidia ml⁻¹; V3: 16.5 x 10¹¹ conidia ml⁻¹. A natural degree of hydration of the samples from laboratory was maintained by periodic spraying of water on the bark surface. 18 days after B. bassiana treatment, it was found that approx. 60% beetles from galleries were dead, covered with B. bassiana white mycelium (Figure 2).

By measuring the maternal galleries it was found that their length varied between 6.2 mm and 7.5 mm on logs treated with B. bassiana conidial suspension and between 7.6 mm and 9.9 mm on control logs (Figure 3). Averge number of larval galleries corresponding to each maternal gallery was also considered. The larval galleries number varied between 2.1 and 3.6 per cm maternal gallery on logs treated with B. bassiana conidial suspension and between 3.5 to 4.4 galleries per cm maternal gallery on control logs (untreated). For testing the significance of differences between the average length of maternal galleries in treated sections in different experimental variants and control sections, Student t-test was applied. After statistical processing of the experimental data, the results showed that B. bassiana infection resulted in a significant reduction in the maternal gallery length (Table 1 and Table 2).
treated trees, a reduction in average length of maternal galleries from 8 to 30% was observed, compared with control sections. The number of larval galleries corresponding to 1 cm of maternal gallery was 19 to 48% lower.

Table 1. Average length of maternal galleries on treated and control sections

<table>
<thead>
<tr>
<th>Variants</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>6.9</td>
<td>6.8</td>
<td>6.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Variance</td>
<td>0.263333</td>
<td>0.397037</td>
<td>0.169259</td>
<td>1.51</td>
</tr>
<tr>
<td>t-statistic</td>
<td>-2.82431</td>
<td>-4.33674</td>
<td>-3.86184</td>
<td>-</td>
</tr>
<tr>
<td>Significance of differences</td>
<td>insignificant</td>
<td>highly significant</td>
<td>significant</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Numerical evaluation of larval galleries corresponding to treated and control sections

<table>
<thead>
<tr>
<th>Variants</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>2.8</td>
<td>2.8</td>
<td>2.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Variance</td>
<td>0.453848</td>
<td>0.074444</td>
<td>0.671481</td>
<td>0.203333</td>
</tr>
<tr>
<td>t-statistic</td>
<td>-7.15335</td>
<td>-10.888</td>
<td>-4.35489</td>
<td>-</td>
</tr>
<tr>
<td>Significance of differences</td>
<td>highly significant</td>
<td>highly significant</td>
<td>highly significant</td>
<td>-</td>
</tr>
</tbody>
</table>

**Conclusions**

B. bassiana is a biological control agent effective in reducing the I. typographus populations because of the bark beetle susceptibility to fungal infection.

The main indicators of bark beetle population reduction following B. bassiana biological treatments were: maternal gallery length, average number of larval galleries for each maternal
gallery and high mortality rates recorded in the nuptial chambers of bark beetle from treated logs. There was a significant reduction of the number of eggs laid by insects in systems galleries of treated logs and a significant reduction of larval galleries number, too.

Acknowledgements

This work was carried out in the frame of the National Forest Administration (RNP-ROM.SILVA) research-development programme. We are grateful to technical staff of National Forest Administration in Suceava for helping in the field work.

References


**Effect of local strains of Beauveria bassiana (Bb024) and Metarhizium anisopliae (M 7/2) against the fallweb worm Hyphantria cunea (Lepidoptera: Arctiidae) in Georgia**

**Meedia Burjanadze, Elena Nakaidze, Mariam Arjevanidze, Tea Abramishvili**
Agricultural University of Georgia, 13 km David Agmashenebeli Alley, 0131 Tbilisi, Georgia

**Abstract:** The indigenous isolates of the entomopathogenic fungi Beauveria bassiana (Bb-024) and Metarhizium anisopliae (M7/2) against fifth to seventh instars larvae of Hyphantria cunea in two concentrations of $1 \times 10^7$ and $1 \times 10^8$ conidia ml$^{-1}$ were tested in the laboratory. Maximum mortality of larvae was observed 4-9 d after treatment. Both isolates were pathogenic to H. cunea larvae. The mean mortality caused by B. bassiana ranged from 59.8% to 84.3% and that of M. anisopliae ranged from 52% to 68%. The living larvae hidden under leaves and cordon made cocoons and transformed into pupae. The adult moths appeared from the overwintering pupae. They began to emerge massively and mated. The emergence of adults of B. bassiana was 69.6%, that of M. anisopliae was 60%, and that of the control was 55.7%. The larvae hatched 7 to 10 d later (the hatching rates were: B. bassiana – 76.3%, M. anisopliae – 70%, control – 89.5%). In case of B. bassiana treatment, dimorphic males emerged from pupae often showing undeveloped wings.

**Key words:** Hyphantria cunea, Beauveria bassiana, Metarhizium anisoplia, biocontrol

**Introduction**

The fall webworm (FWW), Hyphantria cunea Drury (Lepidoptera:Arctiidae), is a polyphagous pest having a very wide range of host plants. It has been established that FWW damage more than 400 plant species in Georgia (Edilashvili, 2002). H. cunea was introduced in 1970, the abundance of feeding plants and subtropical climate appeared favorable, the pests were well adapted and spread in western Georgia and the Black Sea coast within a short period of time. Nowadays, mechanical and chemical control methods are used to control H. cunea, but the insect mostly inhabits the populated and urban areas, where the application of chemical pesticides is prohibited. Therefore, it becomes necessary to use environmentally friendly tools such as biological control. Numerous experiments have been carried out using the microbial agents of this insect in Georgia (Albayrak Iskender et al., 2012; Chkhubianishvili et al., 2011; Gorgadze, 2000; Lortkipanidze et al., 2010; Supatashvili & Burjanadze, 2008). The strategy for biological control of H. cunea includes the use of entomopathogenic fungi (EPF) as well.

In this study, local isolates of Beauveria bassiana (Bb024) and Metarhizium anisopliae agg. (M 7/2) were tested against H. cunea larvae and the EPF’s pathogenicity was determined under laboratory conditions.
Material and methods

Fungal culture
The indigenous strains of B. bassiana Bb024 (IMI #501797) and M. anisopliae agg. M7/2 (IMI #501805) were isolated from soil samples using the ‘Galleria bait method’ (Zimmermann, 1986), then they were subjected to molecular identification and kept in CABI-UK Genetic Recourse Collection.

Inoculum preparation
Fungal suspensions of the isolates were prepared from 2 week-old cultures grown on PDA at 25 ± 2 °C, using distilled water containing 0.01% (w/v) Tween 80. The concentration of spores in the suspensions from each fungus was determined using a haemocytometer and adjusted to two concentrations of 1 x 10^7 and 1 x 10^8 conidia ml^-1 for bioassays.

Bioassays
The 5th and 7th instars (L5-L7) of larvae of H. cunea were collected manually from orchards and forest trees in West Georgia. Target insects used for the bioassay were treated with fresh cultural suspension of B. bassiana and M. anisopliae (1 x 10^7 and 1 x 10^8 conidia ml^-1), and placed in glass jar with mulberry tree leaves. They were kept at room temperature ~23 °C (day) and ~18 °C (night) and with 14 h (light)/10 h (dark) regime. Dead or infected larvae with fungal symptoms were removed and placed in moister environment for development of conidia. Mortality of larvae was recorded on 3-18 d after treatment.

Data analysis
All mortality data were corrected for control mortality using the formula of Abbott (1925). The percentage of larval mortality for each concentration was analyzed using one way ANOVA, means were separated by Tukey’s mean separation test. Mortality was considered significantly different at P < 0.01.

Results and discussion
Both fungal strains were pathogenic to larvae H. cunea. However, virulence considerably varied. Mycosis by B. bassiana Bb-024 was observed in L5-L6 larvae and in cocoons. With M. anisopliae M7/2 symptoms of mycosis were mostly observed in L6-L7 instars. Rapid development of mycosis was observed with Bb-024. Maximum mortality of larvae was marked 4-9 d after treatment, whereas with M7/2 the mortality was observed later on 5-13 d after treatment (Figure 1).

Both isolates were pathogenic to H. cunea larvae and the mean mortality ranged from 59.8% to 84.3% for B. bassiana and from 52% to 68% for M. anisopliae (Figure 2). Mortality caused by B. bassiana and M. anisopliae were significantly different depending on concentrations (p < 0.05). One-way ANOVA, single factor, α = 0.01: significant differences were found between the pairs of treatments: for B. bassiana 10^7 conidia ml^-1 and 10^8 conidia ml^-1: p = 0.0025, F = 18.7; for M. anisopliae 10^7 conidia ml^-1 and 10^8 conidia ml^-1: p = 0.000156, F = 44.6; and for B. bassiana 10^8 conidia ml^-1 and M. anisopliae 10^8 conidia ml^-1: p = 0.0001, F = 46. Hence, at the high concentration (10^8 conidia ml^-1) the promising isolate of B. bassiana showed significant difference compared to the M. anisopliae.

The living larvae hidden under leaves and cordon made cocoons and transformed into pupae. They were left to overwinter until spring at 8 ± 5 °C in room conditions.
A dult moth appeared from the pupae after 4-5 d, they emerged massively and mated. The emergence of adults of *H. cunea* treated with *B. bassiana* and *M. anisopliae* is given in Figure 3. Eggs laying continued for 10-12 d.

Figure 1. Appearance of mycosis (measured in days) of larvae of *Hyphantria cunea* treated with $10^7$ and $10^8$ conidia ml$^{-1}$ of the *Beauveria bassiana* Bb-024 and *Metarhizium anisopliae* M 7/2.

Figure 2. Mortality (%) of larvae of *Hyphantria cunea* after treatment with different concentrations of *Metarhizium anisopliae* and *Beauveria bassiana* (mean % ± SD), significant level $\alpha = 0.01$. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5 days</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>M.a. 1x10exp7</td>
<td>7.3</td>
<td>52</td>
</tr>
<tr>
<td>M.a. 1x10exp8</td>
<td>9.3</td>
<td>68</td>
</tr>
<tr>
<td>B.b. 1x10exp7</td>
<td>14.7</td>
<td>59.9</td>
</tr>
<tr>
<td>B.b. 1x10exp8</td>
<td>19.3</td>
<td>84.6</td>
</tr>
</tbody>
</table>
After 7-10 d the larvae hatched. The hatching rate was 76.3% and 70.5% for B. bassiana, and 76% and 80.5% for M. anisopliae, the hatching rate of the untreated controls was 89.5% (Figure 3). It should be noted, that in case of B. bassiana f dimorphic male emerged from showing undeveloped formed wings.

The results suggest that the B. bassiana (Bb-024) and M. anisopliae (M7/2) isolates can be used to control H. cunea.

**Acknowledgements**

This research was funded by STCU-SRNSF, grant # 5253, 2010-2012. We gratefully acknowledge Dr. Dave Moore and colleagues from CABI-UK for the molecular identification of EPF.
References


Lortkipanidze, M., Gorgadze, O., Kokhia, M., Melashvili, N. & Burdjanadze, M. 2010: Biological potential of entomopathogenic nematodes (Steinernematidae) against Hyphantria cunea (Lepidoptera: Arctiidae) in Georgia. Population dynamics, biological control, and integrated management of forest insects. Book of Abstracts: 83. IUFRO Eberswalde, Germany.


Highly effective *Beauveria* pseudobassiana strain (Dm-5) against the great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Scolytidae)

Ismail Demir¹, Seda Kocacevik¹, Ali Sevim², Mahmut Eroglu³, Zihni Demirbag¹
¹Karadeniz Technical University, Faculty of Sciences, Department of Biology, Trabzon, Turkey; ²Ahi Evran University, Faculty of Engineering and Architecture, Environmental Engineering, Kirsehir, Turkey; ³Karadeniz Technical University, Faculty of Forestry, Department of Forestry Engineering, Trabzon, Turkey

**Abstract:** The great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Scolytidae), has been a potential threat for not only Turkey, but also the entire Eurasian spruce forests for many years. Control strategies which have been applied so far are still insufficient to prevent its damage. *Beauveria* pseudobassiana strain (Dm-5) which was previously isolated from *D. micans* had 90% mortality after application of $1 \times 10^6$ ml$^{-1}$ spore suspension within 10 days towards to the larvae and adults of this pest and 90% mycosis value. In the dose-response experiments, a conidial suspension of $1 \times 10^8$ ml$^{-1}$ caused 100% mortality on both larvae and adult of *D. micans* within 5 and 6 days, respectively. Mortality values of horizontal transmission from larvae and adults which were contaminated with $1 \times 10^6$ ml$^{-1}$ spore suspension of *B. pseudobassiana* at 25% were also determined as 100% after 15 days at 20 °C under the laboratory conditions. We also determined the decrease of the damage in wood block (from spruce, 15 × 25 cm) experiments when the contamination rate of the larvae increased. Our results indicate that *B. pseudobassiana* (Dm-5) seems to be a very promising biocontrol agent against *D. micans*, this strain can spread horizontally among both larvae and adult populations of *D. micans*, and it has also a good insecticidal effect towards to larvae in the wood block.

**Key words:** *Dendroctonus micans*, entomopathogenic fungi, *Beauveria* pseudobassiana, microbial control
Laboratory testing of insect associated fungi for the control of wireworms (Agriotes sp. L.)

Jaka Razinger¹,², Matthias Lutz³, Hans-Josef Schroers², Gregor Urek², Jürg Grunder
¹Zurich University of Applied Sciences, Campus Grueental P.O. Box 335, 8820 Waedenswil, Switzerland; ²Agricultural Institute of Slovenia, Hacquetova ulica 17, Ljubljana 1000, Slovenia

Abstract: The aim of the study was to assess entomopathogenic potential of 7 isolates from 6 entomopathogenic fungal species (EPF) isolated from various substrats in Slovenia against larvae of Agriotes sp. The fungal isolates tested were Beauveria bassiana, B. brongniartii, Metarhizium anisopliae (2 isolates), M. robertsi, Purpureocillium lilacinum and Clonostachys solani. Conidia of these species were incorporated into the test substrate as a water suspension to reach a final concentration of $3.85 \times 10^6$ conidia g$^{-1}$ air-dried soil. The larval mortality was observed on a weekly basis for a total of 90 days. The mortalities observed exhibited a linear trend with slopes ranging from 0.20 to 1.23 for the fungal treatments and 0.08 to 0.18 for the control treatments. Abbott's corrected mortality at day 90 ranged from 20.7 to 76.9%. The most promising candidate biological control agent was Metarhizium anisopliae isolate 1154.

Key words: Agriotes sp., biocontrol, biological control, biopesticide, entomopathogenic fungi, pests, wireworms

Introduction

Wireworms, soil-burrowing larval stages of click beetles (Coleoptera: Elateridae), are major pests of crops including potatoes in many parts of the world (Ansari et al., 2009). They can attack a wide range of crops (Furlan et al., 2010). Wireworm tunneling in potato creates an entry point for other plant pathogens, which can cause tuber rot (Esther & Huiting, 2007). In areas highly infested with wireworms, entire batches can become unmarketable (Ansari et al., 2009). In Slovenia, Agriotes ustulatus Schall., A. lineatus L., A. obscurus L., and A. sputator L. live in grasslands and fields and thus have the potential to be agricultural pests (Gomboc & Milevoj, 2000).

Several attempts have been made to control wireworms and other pests from the click beetle family with biological agents (Tinline & Zacharuk, 1960; Esther & Huiting, 2007; Ansari et al., 2009). The experimental methodology in most of these attempts was unique. Also, the mortality rates and lethal times varied considerably. Therefore, each newly discovered EPF isolate must undergo rigorous testing, in order to determine its potential as a biocontrol agent. The aim of this study was to assess the entomopathogenic potential of several newly discovered EPF in Slovenia against wireworms.
Material and methods

Entomopathogenic fungi (EPF) isolation and culturing
The EPF were isolated from various substrates in Slovenia (Table 1). The fungal strains were routinely cultured on potato dextrose agar media at 24 °C in darkness.

Table 1: List of entomopathogenic fungal isolates tested in the study.

<table>
<thead>
<tr>
<th>Number</th>
<th>AIS ID* number</th>
<th>Genus</th>
<th>Species</th>
<th>Host organism / isolated from</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1878</td>
<td>Beauveria</td>
<td>bassiana</td>
<td>Melolontha melolontha</td>
<td>SLO</td>
</tr>
<tr>
<td>2</td>
<td>1877</td>
<td>Beauveria</td>
<td>brongniartii</td>
<td>Melolontha melolontha</td>
<td>SLO</td>
</tr>
<tr>
<td>3</td>
<td>1154</td>
<td>Metharhizium</td>
<td>anisoplia</td>
<td>soil</td>
<td>SLO</td>
</tr>
<tr>
<td>4</td>
<td>1868</td>
<td>Metharhizium</td>
<td>anisoplia</td>
<td>Agriotes sp. adult</td>
<td>SLO</td>
</tr>
<tr>
<td>5</td>
<td>1880</td>
<td>Metharhizium</td>
<td>robertsi</td>
<td>unknown</td>
<td>SLO</td>
</tr>
<tr>
<td>6</td>
<td>1797</td>
<td>Purpureocillium</td>
<td>lillacum</td>
<td>soil</td>
<td>SLO</td>
</tr>
<tr>
<td>7</td>
<td>1828</td>
<td>Clonostachys</td>
<td>solani f. nigrovirens</td>
<td>potato tuber</td>
<td>SLO</td>
</tr>
</tbody>
</table>

* Agricultural Institute of Slovenia mycological collection identification number.

Agriotes sp. larvae collection and rearing
Agriotes sp. larvae were collected in maize-wheat bait traps according to the description given by Kirfman et al. (1986) and Chabert and Blot (1992). The traps were laid out on April 14, 2012 and collected on April 28, 2012. The contents were hand-sorted and all living Agriotes sp. larvae transferred to a 15 l plastic container, containing ca. 8 kg of damp soil from the original location. The container was placed in a glasshouse on the AIS premises in Ljubljana, Slovenia. Carrot and potato slices were added regularly as food and the container was watered as needed.

Soil exposure experiment
Conidial suspensions were prepared by transferring conidia to 100 ml of sterile 0.1% Tween 80 solution. A hemocytometer was used to adjust spore concentrations. The final concentration of EPF conidia was 3.85 x 10^6 g^-1 substrate, which was air-dried for 48 h before the conidial suspension was added. The test substrate was a light commercial substrate, rich in organic matter (Bio-Presstopferde, Floragard, Oldenburg, Germany). The spore infected substrate was mixed thoroughly in a large sterile plastic bag to insure homogenous conidial distribution. 30 ml of substrate containing conidia was transferred into individual 50 ml centrifuge tube. Into each 50 ml centrifuge tube a single Agriotes sp. larva was placed. Finally, a thin slice (ca. 3 mm thick) of potato tuber was placed on top of the substrate in each tube. The tubes were loosely capped, so air could freely circulate. 15 test vessels were used for each treatment. 0.1% Tween was used for negative controls. The positive control was the insecticide ‘Marshall 25 CS’, based on Carbosulfan (24.5% active ingredient), used at a recommended concentration of 0.1%. The larval mortality was observed on a weekly basis for a total duration of 90 days. Dead or immobile larvae lacking a coat of sporulating mycelium were removed from the test vessels and placed in sterile 24-well plates to initiate growth of potentially present fungi. The experiment was carried out in an environmental chamber set to
20 °C, 80% relative humidity and total darkness. Potato slices and water was added to the test vessels as needed.

Data calculations and statistics
From the number of living larvae at each observation point, rate of mortality \((M = 100 \times \frac{\text{living}}{\text{initial larvae}})\) and Abbott’s corrected mortality \((\text{ACM} = 100 \times \frac{(X-Y)}{X})\), where \(X\) represents the percent of living larvae in the untreated control sample and \(Y\) the percent of living larvae in the treated sample. Calculation using this method eliminates errors due to deaths in the control samples, which were not due to the treatments with our selected EPFs (Abbott, 1925). Data presented are mean values. The experiment was performed twice independently. Statistical analysis was performed by computer software GraphPad Prism 5.00 and Microsoft Excel 2007.

Results and discussion
The majority of mortality curves observed in the soil experiment exhibited a linear trend, with the exception of the positive control treatment (Marshall 25 CS) (Figure 1, Table 2). The 95% confidence intervals of the EPF treatments’ slopes differed significantly from the slope of the control samples. The second experiment gave similar results with two notable differences: lower mortality was observed in the treatment with AIS 1154, and higher mortality in the treatment with AIS 1877 (not shown).

Figure 1. Mortality of Agriotes sp. larvae during a typical experiment. The experiments were followed for 90 days. The soil was amended with a concentration of \(3.85 \times 10^6\) spores g\(^{-1}\) air-dried soil at the start of the experiment. Marshall – a Carbosulfan-based insecticide, used as a positive control.
The calculated 95% confidence interval of time needed to reach a 50% mortality ($LT_{50}$) was lowest in the treatment with $M$. anisopliae strain AIS 1154 (49.9 to 64.7 days) and highest in the control treatment (118.8 to 1426 days). The positive control (treatment with Marshall 25 CS) reached a $LT_{50}$ of less than a day (Table 2). The highest ACM (at day 90) was calculated for the treatment with $M$. anisopliae strain AIS 1154 (76.9%), followed by the treatment with $M$. anisopliae strain 1880 (67.0%). The lowest ACM was calculated for the treatment with $C$. solani strain AIS 1828 and $B$. bassiana strain AIS 1878 (both 20.7%) (Table 2).

Table 2: Statistical analysis of the mortality curves and Abbott’s corrected mortalities (ACM) calculated for day 90. Slope - 95% confidence interval of the mortality slope obtained by linear regression; $r^2$ - goodness of fit of linear regression; $LT_{50}$ - 95% confidence interval of time needed to reach a mortality of 50%; Marshall - a Carbosulfan-based insecticide used as a positive control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1878</th>
<th>1877</th>
<th>1154</th>
<th>1868</th>
<th>1880</th>
<th>1797</th>
<th>1828</th>
<th>Control</th>
<th>Marshall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.270-</td>
<td>0.200-</td>
<td>0.726-</td>
<td>0.393-</td>
<td>0.497-</td>
<td>0.295-</td>
<td>0.207-</td>
<td>0.077-</td>
<td></td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.494</td>
<td>0.444</td>
<td>1.23</td>
<td>0.691</td>
<td>1.00</td>
<td>0.651</td>
<td>0.475</td>
<td>0.183</td>
<td>0.136</td>
</tr>
<tr>
<td>$LT_{50}$ [days]</td>
<td>106.4-</td>
<td>95.9-</td>
<td>49.9-</td>
<td>68.9-</td>
<td>70.2-</td>
<td>82.7-</td>
<td>95.5-</td>
<td>118.8-</td>
<td>12-</td>
</tr>
<tr>
<td>ACM at day 90 [%]</td>
<td>20.7</td>
<td>23.1</td>
<td>76.9</td>
<td>46.2</td>
<td>67.0</td>
<td>30.8</td>
<td>20.7</td>
<td>0.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

The results from the treatments with $M$. anisopliae AIS 1154 and $M$. robertsii AIS 1880 were comparable to the insecticidal activity of a $M$. anisopliae isolate reported by Kölliker et al. (2011) for $A$. lineatus. The authors obtained lower $LT_{50}$ mortality rates against $A$. sputator and higher for $A$. obscurus. They hypothesized that the pathogenicity of their isolate was species specific. Our study did not allow for differentiation of toxicity assessment against different Agriotes sp. species as we performed our experiments with field collected larvae and did not classify them to the species level. This could be overcome by rearing our own Agriotes sp. by using the protocol of Kölliker et al. (2009) and evaluating insecticidal activity for individual species. Despite these shortcomings, the isolates Metarhizium anisopliae AIS 1154 and $M$. robertsii AIS 1880 gave promising results. After successful glasshouse and field testing, they could be considered as an environmentally friendly alternative for wireworm management in conventional or organic farming systems.

Acknowledgements

We would like to thank Mrs. Cilka Bertoncelj for providing the Agriotes sp. larvae, and Ursula Löffel and Tobias Hofmann for their laboratory assistance. The research was financed by the Rectors’ Conference of Swiss Universities (call Sciex-NMS-CH, project acronym RhizoShield).
References


Laboratory and semi-field trials on the effects of Beauveria bassiana (J W-1, ATCC 74040) against soil-dwelling stages of Frankliniella occidentalis (Thysanoptera: Thripidae)

Andrea Boaria, Alberto Pozzebon, Mauro Pesce, Mauro Lorenzon, Carlo Duso
Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padova, Italy, Viale dell’Università 16, 35020 Legnaro (Padova), Italy
e-mail: andrea.boaria@studenti.unipd.it

Abstract: Beauveria bassiana (Balsamo) Vuill. is an entomopathogenic fungus used in controlling various pests. Previous research showed that B. bassiana, applied to the plant canopy, could exert a significant control of thrips populations, in particular of Frankliniella occidentalis Pergande. However, some stages (e.g., prepupae and pupae) of this species develop in the soil being less affected by control treatments applied to the canopy. The identification of biological control agents active against soil-dwelling stages of F. occidentalis is an important issue for the implementation of IPM. Here we present laboratory and greenhouse experiments carried out to evaluate the potential of B. bassiana (JW-1 ATCC 74040) in controlling soil-dwelling stages of F. occidentalis. In laboratory bioassays B. bassiana reduced significantly the emergence of F. occidentalis adults. In the greenhouse experiment, a significant control of thrips population was obtained on cyclamen potted plants.

Key words: Frankliniella occidentalis, Beauveria bassiana, soil-dwelling thrips stages, IPM, greenhouse ornamentals

Introduction

The western flower thrips (WFT) Frankliniella occidentalis Pergande is a world-wide pest of cultivated plants mainly of greenhouse ornamentals. The pest status is due to feeding activity, the transmission of Tospoviruses, and the great ability to develop resistance to pesticides. This phenomenon poses major limitation to chemically-based pest control strategies (Jénsen, 2000; López-Soler et al., 2008). Other problems in WFT control are related to its cryptic developmental stages (prepupae and pupae). Since the latter develop in soil they can escape the effects of control measures (pesticides or biocontrol agents) applied to the canopy (Tommasini & Maini, 1995; Cloyd et al., 2003). Therefore, several studies have been devoted to the development of control strategies alternative to pesticides. Many beneficial organisms resulted effective in controlling WFT populations and entomopathogenic fungi showed great potential in this framework (Brownbridge, 1995; Helyer et al., 1995; Ansari et al., 2008). Among entomopathogenic fungi, Beauveria bassiana (Balsamo) Vuill. is a well-studied biocontrol agent that has been used against various pests including WFT. This fungus is naturally occurring in the soil (e.g., Vänninnen, 1995; Quesada-Moraga et al., 2006; Meyling & Eilenberg, 2007). Some studies have investigated the effect of soil application of B. bassiana against WFT in the laboratory (Ansari et al., 2008), while less work has been done on cultivated plants. Moreover, the effects of B. bassiana in controlling WFT and other pests appear to be strain-dependent (Skinner et al., 2012). Here we tested the effect of a commercial formulation of B. bassiana (JW-1, ATCC 74040) against soil-dwelling stages of WFT in laboratory and greenhouse conditions. Preliminary results are reported here.
Material and methods

Insect rearing
Thrîps used in this study were obtained from stock cultures where insects were reared on cucumbers following a modified method described by DeGraff et al. (2009). Rearing units were kept at room temperature [24 ± 1 °C; 60-70% relative humidity, (R.H.)] with a photoperiod of 16 h (light)/8 h (dark).

Experimental procedures
In all experiments a commercial formulation of B. bassiana (strain JW-1 ATCC 74040, Naturalis®) was used. Laboratory bioassays were performed using an experimental unit constituted by a 50 ml Falcon tube containing 30 ml of peat. A piece of transparent, cellulose membrane dialysis tube, was placed on the top of the tube to avoid insect escaping and allow gas-exchange. All the material including peat was sterilized prior to the experiments. Five late second instar larvae were transferred from stock cultures to experimental units using a fine camel hair brush. Two treatments were compared: B. bassiana was applied to the soil before (2 h) larvae penetration or after (24 h) larvae penetration in soil. Sterilized water application was included as a control. Three doses of commercial formulation were used in the experiment corresponding to 3 l ha⁻¹, 9 l ha⁻¹, 27 l ha⁻¹. Each treatment was replicated 20 times. Experimental units were maintained at 24 ± 1 °C and 60% ± 5% relative humidity (R.H.). Adults emergence was monitored daily for 11 days from larvae introduction.

A greenhouse experiment was performed to evaluate the effect of soil application of B. bassiana on WFT infestation on cyclamen potted plants. Two treatments were compared: soil application of B. bassiana (two applications in 7 days) vs. water treated control. Each treatment was replicated 4 times. Each replication was placed in insect-proof cages to reduce thrips escaping. The dose of B. bassiana formulation corresponded to 9 l ha⁻¹. Plants were infested with about 10 adults and 50 juveniles two weeks prior to the first B. bassiana application. We evaluated the persistence of B. bassiana in soil samples collected from the two treatments using the “Galleria bait method” (Zimmermann, 1986). Samples of fungal mycelium present on Galleria melonella L. larvae were transferred on Petri dishes containing a selective medium and held at 25 °C for 5 days to obtain new fungal colonies. Mycelium was identified under microscope using dichotomous keys (Barnett & Hunter, 1998). The evaluation of WFT population density and structure on flowers was performed weekly until 35 d from the first B. bassiana application. Plants were kept in greenhouse at 18 ± 6 °C and 63% ± 5% R.H. Soil sampling was performed with the same timing.

Results and discussion
In laboratory the applications of B. bassiana were associated to a higher mortality compared to the control treatments (p < 0.05). In the latter natural mortality was 3% for application before larvae penetration in the soil and 16% for application after larvae penetration in the soil. B. bassiana applications exhibited an effect that ranged from 17% to 57% in terms of corrected mortality. The best results were obtained with soil applications performed after larvae penetration in soil. Mortality of soil-dwelling stages was dose-dependent, but this effect was influenced by the timing of application. In application before larvae penetration in soil, mortality was higher (p < 0.05) for 27 l ha⁻¹ (57% corrected mortality, c.m.) compared to 9 l ha⁻¹ (23% c. m.) and 3 l ha⁻¹ (17% c. m.) doses, while in application made after larvae
penetration in soil no differences were observed between 27 l ha$^{-1}$ (51% c. m.) and 9 l ha$^{-1}$ (49% c. m.) that induced higher mortality ($p < 0.05$) compared to 3 l ha$^{-1}$ dose (25% c. m.).

The greenhouse experiment showed that soil applications of $B.\ bassiana$ reduced significantly ($p < 0.05$) the WFT infestation on cyclamen potted plants (Table 1) with respect to the water treated control. The application of “Galleria bait method” revealed that $B.\ bassiana$ persisted in the soil until the end of the experiment (35 days from the first application). No symptoms and signs of $B.\ bassiana$ infection were observed on G. melonella larvae in untreated control. $B.\ bassiana$ application affected population structure of thrips: plants receiving soil applications were infested only by larvae, while control plants were infested by larvae and adults. These results demonstrate a significant effect of $B.\ bassiana$ (JW-1, ATCC 74040) strain against soil-dwelling stages of WFT.

Table 1. Effect (%) of $B.\ bassiana$ soil applications on WFT population density detected on cyclamen flowers and calculated using the formula of Henderson and Tilton (1955).

<table>
<thead>
<tr>
<th>Time after first application (days)</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduction of WFT infestation</strong></td>
<td>37.04%</td>
<td>59.09%</td>
<td>40%</td>
<td>44.83%</td>
<td>66.67%</td>
</tr>
</tbody>
</table>

In laboratory trials WFT adults emergence was reduced depending on dose and timing of application. Best results were found in treatments after larvae penetration in soil and with the highest dose. We can suggest that applying $B.\ bassiana$ after larvae penetration in soil can increase their exposure to infectious inoculum. In treatment made before larvae penetration in soil, thigmokinetic behaviour exhibited by WFT (Jensen, 2000; Ugine et al., 2005) might be responsible for limited control efficiency at lowest and intermediate doses. Results obtained here confirm the potential of entomopathogenic fungi applications against soil-dwelling stages of WFT emerged in previous investigation (Brownbridge, 1995). Ansari et al. (2008) found that applications to growing media of two other strains of $B.\ bassiana$ induced a lower emergence of adults compared to a chemical insecticide (Fipronil). The reduction in emergence of adults was comparable to that found here. Greenhouse experiment highlighted the effect of soil applications of $B.\ bassiana$ on WFT infestations in a realistic cultivation scenario. The fungus persisted in soil for 35 days and this observation is crucial for its management. No adults were found on plants receiving soil applications. This aspect can have important implications for viruses transmission: WFT acquires Tospoviruses as larvae and transmit them as adults. In previous research, soil application of an experimental strain of $B.\ bassiana$ (granular formulation) was effective in the control of WFT, while poor results were obtained with the commercial GHA strain (Skinner et al., 2012). Results obtained here confirm the potential of $B.\ bassiana$ in IPM strategies against WFT. This study stresses on the potential of the commercial strain $B.\ bassiana$ (JW-1, ATCC 74040) as valuable approach for the management of a critical point in actual WFT control strategies.
Acknowledgements

This work was supported by Intrachem Bio Italia S.p.A. (presently Biogard Division of CBC (Europe) s.r.l.

References

Prevalence of the species **Beauveria pseudobassiana** among tick-associated fungal isolates from the Republic of Moldova

Polina V. Mitkovets, Natalia V. Munteanu, Galina V. Mitina, Yuri S. Tokarev, Alexandr A. Movila, Ion Toderas, Regina G. Kleespies, Andreas Leclerque

1All-Russian Institute for Plant Protection, Chaussee Podbelskogo 3, 196608 Sankt-Peterburg, Russia; 2Institute of Zoology, Academy of Sciences of Moldova, Academiei 1, 2028 Chisinau, Rep. Moldova; 3Julius Kühn Institute (JKI) – Federal Research Centre for Cultivated Plants, Institute for Biological Control, Heinrichstraße 243, 64287 Darmstadt, Germany; 4Technische Universität Darmstadt, Institute of Microbiology, Schnittspahnstraße 10, 64287 Darmstadt

‡both first authors contributed equally to the project
*e-mail: leclerque@hotmail.com

**Abstract:** Fungal strains isolated from ixodid ticks in the Republic of Moldova were genetically characterized using the ribosomal RNA operon internal transcribed spacer (ITS) regions together with the 5.8S rRNA gene as well as the nuclear genes ef1α encoding the alpha subunit of eukaryotic translation elongation factor 1α as phylogenetic markers. On the basis of the sequence data obtained, eight out of ten isolates were assigned to the Beauveria species *B. pseudobassiana*. The two remaining isolates were consistently characterized as *Isaria farinosa* and as an *Aspergillus* species, respectively. Further work to elucidate if the prevalence of the species *B. pseudobassiana* in ticks is or not a regional phenomenon is in progress.

**Key words:** Beauveria pseudobassiana, *Isaria farinosa*, ixodid ticks, internal transcribed spacer (ITS), elongation factor 1 alpha (ef1α)

**Introduction**

As vectors of the causative agents of several severe diseases of humans and animals, e.g. Lyme borreliosis, tick-borne encephalitis, Colorado tick fever, and Rocky Mountain spotted fever, ticks pose an eminent threat to public health and set often important economic limitations to stock-farming. Tick control agents and strategies are, therefore, highly solicited.

One kind of the naturally occurring pathogens of ticks are filamentous fungi, typically of the hyphomycete genera *Metarhizium* or *Beauveria* (Kalsbeek et al., 1995; Fernandes & Bittencourt, 2008; Mitina et al., 2011). The fact that infection by these fungi is more frequent for adult female ticks as compared to males or larvae (Zhioua et al., 1999) and at sublethal levels causes decreased fecundity of infected females (Fernandes et al., 2004) makes them particularly interesting candidates for biological tick control (Samish & Rehacek, 1999; Chandler et al., 2000; Maniania et al., 2007; Hartelt et al., 2008). Therefore, and as sound taxonomic classification is a prerequisite of the registration of new biocontrol agents, fungal strains isolated from ixodid ticks in the Republic of Moldova in order to assess the degree of polymorphism in tick-associated fungal populations were genetically characterized at the genus and species level.
Material and methods

The ten investigated fungal strains, termed tick isolate MDA #1 through MDA #10, stemming from two independent samplings of Ixodes ricinus ticks at different locations of the Republic of Moldova were isolated as described by Mitina et al. (2011). The ITS and ef1α markers were amplified using primer pairs ITS4/ITS5 (White et al., 1990) and 983F/1567R (Rehner & Buckley, 2005), respectively. Sequence alignments and reconstruction of Maximum Likelihood (ML) phylogenies were performed with the ClustalX and PhyML software tools, respectively, under assumption of a gamma-distribution based model of rate heterogeneity allowing for eight rate categories. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1,000 pseudo-replicates.

Results and discussion

The ITS1-5.8SrRNA-ITS2 sequences obtained from isolates MDA #1-10 were compared to orthologous sequences from standard strains of the genera Beauveria, Isaria, Lecanicillium, Metarhizium, and – as an outgroup – Aspergillus. Consistently with its previous morphologically based classification, most isolates (MDA #2-9) clustered with Beauveria strains, whereas isolate MDA #1 appeared most closely related to Isaria strains and isolate MDA #10 clustered with the outgroup sequence (Figure 1).

![Figure 1. ITS sequence based ML cladogram for several fungal genera rooted with the Aspergillus branch. Numbers on branches designate bootstrap support percentages.](image-url)
In order to obtain a species classification for *Isaria* strain MDA#1, ITS sequences representing further *Isaria* species according to the phylogeny presented by Luangsa-ard et al. (2005) were included in the analysis. The tick-derived isolate clusters with *Isaria farinosa* strains in a maximally bootstrap supported sub-clade of the refined ITS phylogeny (Figure 2) and should on this basis be assigned to this species.

![Figure 2. ITS sequence based ML cladogram for the genus Isaria rooted with the Aspergillus flavus sequence. Numbers on branches designate bootstrap support percentages.](image)

Tick-derived *Beauveria* strains MDA#2-9 were further analyzed within the systematic framework created by Rehner & Buckley (2005). Based upon a comparison of amino-acid sequences deduced from *ef1α* partial gene sequences (Figure 3), all eight isolates should be assigned to the species *Beauveria pseudobassiana*. Importantly, within this species, the isolates from Moldova do not form a tight (presumably geographic) cluster, but are clearly assignable to different infra-specific sub-clades, a finding very much in line with an evolutionary history of the fungus-tick relationship characterized by multiple adaptation events.

**Acknowledgements**

We gratefully acknowledge support from BM BF grants MDA 08/013 and RUS 08/026.
Figure 3. EF1α peptide sequence based ML phylo- (left) and cladogram (right) for the genus Beauveria rooted with a Cordyceps ortholog. Numbers on branches designate bootstrap support percentages. The size bar denotes a 1% sequence divergence.

References


Evaluation of indigenous *Beauveria* isolates as potential agents for emerald ash borer management and the development of a diagnostic marker to monitor a post-release isolate

George Kyei-Poku, Shajahan Johny
Canadian Forestry Service, Great Lakes Forestry Centre, Natural Resources Canada, 1219 Queen Street East, Sault Ste. Marie, Ontario, Canada P6A 2E5

**Abstract:** To search for effective and safe indigenous biocontrol agents to manage emerald ash borer (EAB), we conducted a survey in 2008-2009 of entomopathogenic fungi (EPF) infecting EAB in outbreak sites in southwestern Ontario, Canada. Many *Beauveria* spp. isolates were recovered from dead and mycosed EAB cadavers residing in the phloem tissues of dead ash barks, larval frass extracted from feeding galleries under the bark of dead trees. Molecular characterization using sequences of the ITS, 5' end of elongation factor 1 alpha (EF1-α) and intergenic B loc region fragments revealed that *Beauveria* bassiana and *B. pseudobassiana* were commonly associated with EAB in the sampled sites. Initial virulence screening against EAB adults of 23 isolates representing the different clades yielded 8 isolates that produced more than 90% mortality in a single concentration assay. These isolates differed in virulence based on LC$_{50}$ values estimated from multiple concentration bioassay and based on mean survival times at a conidia concentration of 2 x 10$^6$ conidia ml$^{-1}$. *B. bassiana* isolate L49-1AA was significantly more virulent and produced more conidia on EAB cadavers compared to the other indigenous isolates and the commercial strain *B. bassiana* GHA suggesting that L49-1AA may have potential as a control agent against EAB. Studies have been developed to use auto-contamination trapping system to disseminate L49-1AA to manage EAB field populations. We targeted the EF1-α gene sequence from L49-1AA to develop an allele/strain specific primer set that will be used to monitor the introduced L49-1AA in terms of its establishment, persistence and virulence in the environment.

**Key words:** EAB, *Beauveria* spp. diversity, molecular characterization, diagnostic marker, biological control

**Introduction**

The emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae), is an invasive wood boring beetle that is decimating North America’s ash trees (*Fraxinus* spp.). To date, an estimated 30 million ash trees have succumbed to EAB infestation. The current rapid expansion of EAB poses a substantial risk to the remaining ash resources of North America. Multi-tactic approach that include the use of biological control agents has been recognized as the most suitable long term pest management strategy for invasive species (Hajek, 2009).

The goal of this study was to isolate and identify the indigenous *Beauveria* spp. infecting field population of EAB in Southern Ontario, Canada, and also evaluate the pathogenicity, virulence and conidia production of selected EAB-recovered *Beauveria* isolates against EAB. A potential *B. bassiana* isolate L49-1AA, was selected for the biocontrol of emerald ash borers and characteristics relating to the practical application in autocontamination traps have since been discussed and documented (Lyons et al., 2012).

One specific aspect that is required when microbial control agents are introduced in the environment is by monitoring and evaluating its establishment, persistence and virulence. A
molecular approach utilizing suitable genetic markers was therefore essential for identification of B. bassiana isolate L49-1AA. A molecular marker development was undertaken to allow future distinction between L49-1AA and naturally occurring B. bassiana strains within released plots.

Material and methods

Fungal-infected EAB were collected from old outbreak sites in Sarnia, Windsor and London Ontario, Canada during the summer of 2008 and 2009. Fungal isolates were recovered from dead and mycosed insects (larvae and adults) based on standard protocols (Goettel & Inglis, 1997). Recovered fungal isolates were identified by classical taxonomy following both general and specific identification keys (Humber, 2009). All the fungal isolates were also identified by molecular diagnostics using the ITS 1 and 2 spacer regions, EF1-α and Bloc sequences.

For preliminary virulence test, 23 isolates were selected from different clades based on ITS phylogeny (Figure 1) and evaluated against adult EAB using a single dose of $2.0 \times 10^7$ conidia ml$^{-1}$. From the results, eight highly virulent isolates from different clades were further tested with four different concentrations viz., $2.0 \times 10^4$, $2.0 \times 10^5$, $2.0 \times 10^6$, $2.0 \times 10^7$ conidia ml$^{-1}$. The commercial B. bassiana strain, GHA was included in the bioassay as a baseline isolate to compare virulence. Conidia production of selected Beauveria isolates were quantified by counting the conidia from mycosed cadavers obtained from the bioassay 14 day post mortality.

We used an improved Allele-Specific Polymerase Chain Reaction (AS-PCR) which is basically a conceptually simple SNP genotyping strategy. The inhibition displacement activity of AS-PCR requires only two outer common primers and one inner primer with allele-specific 3' terminus mismatch but with incorporation of an additional mismatch at the penultimate base of 3' end of allele specific inner primer. The strategy was used to design strain-specific primer set for B. bassiana isolate, L49-1AA. Beauveria spp. EF1-α gene sequences generated in this study and those archived in GenBank were aligned with BioEdit (Hall, 1999) and a segment of the aligned sequences targeted to design a strain specific primer set, EFFO × EFRO × EFF1 exclusively for L49-1AA. The PCR temperature profile for the one-pot reaction was 94 °C for 3 min initial denaturation, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 10 min.

Results and discussion

A total of 78 Beauveria isolates were retrieved from dead and mycosed EAB cadavers and gallery frass underneath the stripped ash bark at the three sampled sites viz., Sarnia, London and Windsor in Southern Ontario, Canada. The maximum likelihood (ML) phylogeny inferred from the ITS sequence alignment using MEGA 5.0 (Tamura et al., 2011) was based on 573 characters alignment comprising of 112 sequences (Figure 1). Seventeen isolates clustered together within the Beauveria bassiana (Clade A) with a strong bootstrap support (> 95%), which further split and grouped into 3 different subclades (Figure 1). The other 61 isolates clustered in Clade C of Beauveria taxonomy together with Beauveria pseudobassiana (Bloc & EF1-α trees not shown).

A single dose bioassay was conducted with a concentration of $2 \times 10^7$ conidia ml$^{-1}$ formulated from 23 different EAB-derived Beauveria isolates and the commercial isolate,
GHA. Significant difference in terms of beetle cumulative mortality was noticed between 4-6 days following treatment. Based on these results eight EAB-derived Beauveria virulent isolates belonging to different Clades on the phylogenetic tree and GHA were selected for further virulence testing (Table 1). Significant differences were observed in cumulative beetle mortality between 4 and 14 days after treatment with the four different conidial concentrations among the eight isolates derived from EAB and the commercial GHA isolate. The logarithms of LC$_{50}$ values of the eight Beauveria spp. isolates ranged from 4.58 to 5.87 (Table 1). Isolate L49-1AA had the lowest LC$_{50}$, followed by commercial isolate, GHA. Dose mortality regressions had significantly different intercepts (test of equality: $\chi^2 = 59.3$, df = 16, $P < 0.001$) but shared the same slope (test of parallelism: $\chi^2 = 8.64$, df = 8, $P = 0.374$). L49-1AA was about five times more virulent than GHA In addition, isolate L49-1AA killed EAB adults faster than all other isolates (data not shown).

Table 1. Log (LC$_{50}$) values of different Beauveria spp. against EAB adults.

<table>
<thead>
<tr>
<th>Beauveria isolates</th>
<th>Slope ± SE</th>
<th>$\chi^2$</th>
<th>Log LC$_{50}$ (95% CL)$^a$</th>
<th>Lethal Concentration Ratios (95% CI)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bassiana</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHA</td>
<td>1.38 ± 0.23</td>
<td>0.35</td>
<td>5.27 (4.88-5.88)</td>
<td>--</td>
</tr>
<tr>
<td>L491-AA</td>
<td>1.23 ± 0.24</td>
<td>1.72</td>
<td>4.58 (4.02-4.94)</td>
<td>4.9 (1.4-16.9)$^*$</td>
</tr>
<tr>
<td>L11A</td>
<td>0.98 ± 0.20</td>
<td>1.33</td>
<td>4.84 (4.20-5.25)</td>
<td>2.7 (0.7-10.3)</td>
</tr>
<tr>
<td>L19C</td>
<td>1.06 ± 0.20</td>
<td>0.77</td>
<td>4.91 (4.47-5.26)</td>
<td>2.3 (0.6-8.0)</td>
</tr>
<tr>
<td>B4B</td>
<td>1.55 ± 0.25</td>
<td>0.71</td>
<td>5.39 (5.05-5.68)</td>
<td>0.8 (0.3-2.1)</td>
</tr>
<tr>
<td>LHY48A</td>
<td>1.15 ± 0.21</td>
<td>1.85</td>
<td>5.54 (5.10-5.90)</td>
<td>0.5 (0.2-1.7)</td>
</tr>
<tr>
<td>LDY20A</td>
<td>1.76 ± 0.32</td>
<td>0.93</td>
<td>5.87 (5.52-6.14)</td>
<td>0.3 (0.1-0.7)</td>
</tr>
<tr>
<td>B. pseudobassiana</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L51D</td>
<td>1.39 ± 0.25</td>
<td>1.88</td>
<td>5.52 (5.12-5.84)</td>
<td>0.6 (0.2-1.7)</td>
</tr>
<tr>
<td>L25BC</td>
<td>1.29 ± 0.23</td>
<td>1.11</td>
<td>5.80 (5.41-6.13)</td>
<td>0.3 (0.1-0.9)</td>
</tr>
</tbody>
</table>

$^a$ Each assay included 4 different concentrations (2.0 × 10$^4$-10$^7$ conidia/ml) and a control; fifteen insects per replicate, three replication per dose.

$^b$ Lethal concentration ratio were estimated by using GHA as standard isolate based on formulas by Robertson and Preisler (1992);

LC$_{50}$ values are significantly different if the 95% CI of their lethal concentration ratios do not include 1.0 (Robertson and Preisler 1992).

*Significantly different from other isolates.

Quantitative sporulation of the Beauveria isolates on EAB cadavers obtained with a single concentration (2 × 10$^7$ conidia/ml) after 14 days of incubation was significantly different. Conidia produced by all EAB-derived isolates were significantly higher than the commercial isolate GHA ($P < 0.05$) (Figure 2).
Figure 1. Maximum-likelihood tree inferred from ITS1-5.8S-ITS2 rDNA gene sequences of Beauveria spp. using the T92 + G model of substitutions (117 taxa and 560 characters). Branch lengths represent evolutionary distance. Numbers at the nodes indicate PhyML bootstrap percentages higher than 50%. The 78 Beauveria isolates recovered from EAB in this study are in bold lettering. Isolates included in the box, clustered with L51D, belonging to B. pseudobassiana.
Figure 2. Number of conidia recovered from mycosed EAB 14 days after death and incubation in a humidified chamber. Values presented are means (± SE) from 18 insects randomly selected from three different bioassays. The letters above the error bar indicate the groups of significance (ANOVA protected Tukey’s HSD test, $\alpha = 0.05$).

PCR amplification using the AS-PCR primer set, EFFO × EFRO × EFF1 designed from alignment of all Beauveria species EF1-$\alpha$ gene sequences archived in GenBank and the target L49-1AA produced a fragment 173 bp only in L49-1AA but not from any other tested Beauveria species (Figure 3). Therefore the diagnostic tool developed in this study differentially detects and renders the discrimination of L49-1AA from naturally occurring Beauveria species and strains within our released plots.

Figure 3. Diagnostic PCR using the AS-PCR primer set EFFO × EFRO × EFF1 and different Beauveria species. Lane 1. ddH$_2$O; Lanes 2-13. Beauveria bassiana; 2) L49-1AA; 3) INRS CFL-A; 4) L11A; 5) L19C; 6) L28A; 7) B4B; 8) LHY48A; 9) LDY 20A; 10) ARSEF 8187; 11) ARSEF 8170; 12) ARSEF 8150; 13) GHA, Lanes 14-18. Beauveria pseudobassiana; 14) L51D; 15) L72B; 16) L25BC; 17) Car1; 18) 8130; and Lane 19. Beauveria brongniartii; ARSEF 8153. Top arrow-positive for B. bassiana and bottom arrow-positive for L49-1AA alone.
Conclusions

- Beauveria spp. were the predominant ‘natural’ fungal pathogens recovered from mycosed EAB and gallery frass.
- Greater than 78% of the Beauveria isolates recovered from EAB cadavers were B. pseudobassiana; we speculate that B. pseudobassiana may have a possible endophytic relationship with ash trees.
- Indigenous Beauveria isolates were comparatively virulent as GHA and interestingly produced more conidia than GHA.
- The most promising Beauveria isolate, is currently being evaluated in the field using an auto-contamination-dissemination approach.
- Since rapid detection of single-base changes is fundamental to modern molecular genotyping, a simple and cost-effective method like AS-PCR would improve the accessibility to SNP genotyping for minimally equipped laboratories when monitoring a released isolate. An important practical consideration with this approach is that it is unnecessary to prepare a high quality DNA suitable for restriction enzyme digestion or any other DNA manipulation process.

Acknowledgements

Funding was provided by of Genomics and R&D Initiative (GRDI), Canadian Forestry Service.

References

Isolation and identification of endophytic entomopathogenic fungi from dent corn

Daigo Aiuchi\textsuperscript{1}, Tatsumi Takanami\textsuperscript{2}, Sayaka Toba\textsuperscript{2}, Minehiro Ishii\textsuperscript{2}, Shin-ichiro Asano\textsuperscript{3}, Masanori Koike\textsuperscript{2}

\textsuperscript{1}National Research Center for Protozoan Disease, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 0808555, Japan; \textsuperscript{2}Department of Agro-Environmental Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 0808555, Japan; \textsuperscript{3}Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 0608589, Japan
e-mail: aigo@obihiro.ac.jp

Abstract: In this study, we sought endophytic entomopathogenic fungi from dent corn, which have potential to be developed as endophytic biopesticide with multiple roles. Dent corn samples applied to this study were collected at three locations of East Hokkaido island. Each plant sample was divided into root, stem, leaf and kernel, and then these were surface-sterilized by 70% ethanol and 0.5% sodium hypochlorite. Pieces of each tissue were placed on entomopathogenic fungi selective medium. All fungal isolates growing on this plate were transferred onto potato dextrose agar. Morphological identification to genus level was conducted by slide culture method by observing under light microscope (x100). In total, 2252 fungal isolates (greater part of isolates were \textit{Penicillium} spp. and \textit{Cladosporium} spp.) were detected on selective medium, and among them, 168 isolates were entomogenous fungi. Five genera of entomopathogenic fungi including \textit{Beauveria}, \textit{Lecanicillium}, \textit{Isaria}, \textit{Metarhizium} and \textit{Simplicillium} were detected in this study. In this study, only five plant samples were applied, but entomopathogenic fungi were detected from all locations and at all parts of plant tissue. Moreover, it is indicated that endophytic entomopathogenic fungi multiply colonize in the plant body. Although, \textit{Beauveria}, \textit{Lecanicillium}, \textit{Isaria} and \textit{Metarhizium} showed tendency to localize to some plant part, \textit{Simplicillium} tended to be ubiquitous presence in plant body. Our result can indicate that entomopathogenic fungi universally colonize into dent corn.

Key words: Endophyte, entomopathogenic fungi, dent corn

Introduction

Fungal endophytes have been detected from many agricultural crops, including tomato, cotton, corn, coffee and banana. Some fungal endophytes belong to entomopathogenic fungi such as \textit{Beauveria bassiana} Vuillemin (Quesada-Moraga et al., 2006), \textit{Metarhizium robertsii} Rehner & Humber (Ramanpreet & Bidochka, 2012), \textit{Lecanicillium} spp. (Petrini, 1981) and \textit{Isaria farinosa} (Holmsk.) Fr. (Bills & Polishook, 1991). Several species of endophytic entomopathogenic fungi have been shown to act as pathogen of pest insects, antagonists of plant pathogens and plant-growth-promoting agents (Vega et al., 2008; Ownley et al., 2008; Ramanpreet & Bidochka, 2012). Furthermore, some fungal entomopathogens have potential for dual- or multiple-control effect against several plant diseases, pest insects and plant parasitic nematodes due to its antagonistic, parasitic and disease resistance inducing characteristics (Goettel et al., 2008). In this study, we soughted endophytic entomopathogenic fungi from dent corn which have potential to be developed as endophytic biopesticide with multiple roles.
Material and methods

Plant samples
Plant samples applied to this study were collected at three locations of East Hokkaido island. Two dent corn samples (unknown, Takii & Co., Ltd.) were from "Shimizu", two dent corn samples (Ashill, Snow Brand Seed Co., Ltd.) were from "Kami Obihiro" and one dent corn sample (P7631, Hokuren) was from "Onbetsu" (Figure 1). Dent corn samples from Shimizu and Kami Obihiro were whole plant (include root, stem, leaves and ears), but sample from Onbetsu was only root and short stem.

![Figure 1. Sampling location of dent corn.](image)

Endophyte isolation and identification
Each plant sample was divided into root, stem, leaf and ear. Then, all stem nodes were cut into small pieces of 10 cm length, leaves were cut into 3 segments (10 cm long), roots were cut into 4 pieces and ears were divided into kernel (27 kernels for each ear). These pieces were surface-desinfected by 70% ethanol and 0.5% sodium hypochlorite (Arnold et al., 2001). These were then further cut into smaller segments (stem; 10 mm², leaf; 25 mm², root; rootlet, kernel; half-cut). Pieces of each tissue were placed on entomopathogenic fungi selective medium (Goettel & Inglis, 1997). Fungal growth was assessed after incubating the plates at 24 °C for 1 week. Fungal isolates grown on medium or plant tissue were replanted to fresh selective medium and incubated for 1 week. All fungal isolates growing on this plate were transferred onto potato dextrose agar. Morphological identification to genus level was conducted by slide culture method (Goettel & Inglis, 1997) by observing under light microscope (x100). Molecular based identification is now ongoing.
Results and discussion

Fungal isolates of endophytic entomopathogenic fungi isolated from dent corn are listed in Table 1. In total, 2252 fungal isolates (greater part of isolates were *Penicillium* spp. and *Cladosporium* spp.) were detected on selective medium. Among them, 168 isolates were entomogenous fungi. Five genera including *Beauveria*, *Lecanicillium*, *Isaria*, *Metarhizium* and *Simplicillium* were detected in this study. Former 4 genera include major species for fungal agents of biopesticides (Faria & Wraight, 2007), and some species of *Simplicillium* are known as parasite of mite, plant pathogen and plant parastic nematode (Fernandes & Bittencourt, 2008; Zare & Gams, 2001). In this study, only 5 plant samples were applied, but entomopathogenic fungi were detected from all locations and at all parts of plant tissue. Moreover, it is indicated that endophytic entomopathogenic fungi multiply colonize in the plant body. Although, *Beauveria*, *Lecanicillium*, *Isaria* and *Metarhizium* showed tendency to localize to some plant part, *Simplicillium* tended to be ubiquitous presence in plant body. Our result can indicate that entomopathogenic fungi universally colonize into dent corn. Future research will be conducted to reaffirm the endopytic ability of these fungal isolates by conidial inoculation to dent corn, and to reveal characteristics of these fungal isolates based on control effect of pest insects, plant diseases, and plant parastic nematodes as endophytic biocontrol agent.

Table 1. The list of endophytic entomopathogenic fungi isolated from 4 different parts of dent corn.

<table>
<thead>
<tr>
<th>Fungal genera</th>
<th>Shimizu Stem</th>
<th>Leaf</th>
<th>Root</th>
<th>Kernel</th>
<th>Kamii Obihiro Stem</th>
<th>Leaf</th>
<th>Root</th>
<th>Kernel</th>
<th>Onbetsu Stem</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Beauveria</em></td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lecanicillium</em></td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Isaria</em></td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Metarhizium</em></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Simplicillium</em></td>
<td>61</td>
<td>12</td>
<td>8</td>
<td>20</td>
<td>2</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>9</td>
<td>27</td>
</tr>
</tbody>
</table>

Acknowledgements

This work was supported by JSPS Grant-in-Aid for Scientific Research (B) 24380174. The authors thank to Tokachi Federation of Agricultural Cooperative Associations for kindly supplying dent corn samples.

References


Endophytic establishment of the entomopathogen Beauveria bassiana in Vitis vinifera plants

Yvonne Rondot, Annette Reineke
Hochschule Geisenheim University, Center of Applied Biology, Institute of Phytomedicine, Von-Lade-Str. 1, 65366 Geisenheim, Germany

Abstract: Fungal entomopathogens are important antagonists of arthropod pests and have attracted increased attention as biocontrol agents in integrated pest management programs. In addition to colonizing arthropods, evidence has accumulated that some entomopathogenic fungi like Beauveria bassiana (Bals.) Vuill. (Ascomycota: Hypocreales) can endophytically colonize a wide array of plant species. For a couple of crop plants it has been proved that endophytic B. bassiana can provide a systemic protection against damage by various insect pests or might trigger induced systemic resistance mechanisms against plant pathogens. Currently, it is unknown whether B. bassiana can exist as an endophyte in grapevine, Vitis vinifera (L.) plants and still maintains its antagonistic potential against insect pests.

In the present study, greenhouse experiments were conducted to verify endophytic establishment of the entomopathogenic fungus B. bassiana in grapevine plants after inoculation. Two different commercialized B. bassiana strains (ATCC 74040 and GHA) were used and applied as conidial suspensions or as the formulated product on the upper and lower leaf surfaces of potted grapevine plants. To determine if endophytic colonization of grapevine leaves by B. bassiana was successful, leaf disks of surface sterilized control and inoculated plants were obtained and placed on a selective medium. Verification of endophytic establishment of the respective B. bassiana strain was achieved by the amplification of strain-specific microsatellite markers. Furthermore, the antagonistic activity of endophytic B. bassiana against putative target pest insects like the vine mealybug Planococcus ficus was assessed using surface sterilized leaves for a bioassay. Possible effects of endophytic B. bassiana on the feeding preference of black vine weevil Otiorhynchus sulcatus choosing between control and inoculated plants were examined through bioassays.

Endophytic survival of B. bassiana inside leaf tissues was evident at least 28 days after inoculation, irrespective of the inoculum used. A significant effect of endophytic B. bassiana on growth but not on mortality of P. ficus was evident. Adult O. sulcatus chose significantly more often the control plants as a host plant compared to grapevine plants with endophytic B. bassiana.

Key words: endopathogenic fungi, Beauveria bassiana, endophytic growth, grapevine, Planococcus ficus, Otiorhynchus sulcatus
Effect of temperature, water activity and UV-B radiation on conidia germination and colony growth of *Beauveria bassiana* isolates from soil and phylloplane

María Fernández-Bravo, Inmaculada Garrido-Jurado, Monica Oreste, Enrique Quesada-Moraga
Universidad de Córdoba, Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, 14071 Córdoba, Spain
e-mail: o02febrm@uco.es

**Abstract:** The effect of temperature, water activity ($a_w$) and ultraviolet radiation (UV-B), key factors determining the environmental competence of entomopathogenic fungi, have been evaluated on 21 *Beauveria bassiana* isolates from soil and phylloplane of two holm oak ecosystems in Southern Spain. These isolates were molecularly characterized with base on the elongation factor 1-alpha (EF1-α) as belonging to 4 genotypes.

Effect of temperature on germination and colony growth rate was monitored in the range of 15-35 °C, with optimum temperature for germination and growth ranging from 23.9 to 30.4 °C. No significant relationship was detected between optimum and maximum temperatures for growth and habitat, soil or phylloplane.

Water activity effect on the above parameters was evaluated in a range of 9 osmotic potential conditions ($\psi$ = 0 to 200 bars) by changing the glycerol concentration in the culture media. Again, no significant relationship was detected between humidity requirements among isolates from soil and phylloplane, with maximum values of colony growth and germination rate between 0 and 5 bars. None of the isolates grew above 100 bars.

Finally, conidia of all isolates were exposed to irradiances of 920 and 1200 mW m$^{-2}$ for 2, 4 and 6 hours. In general, the delaying germination and colony growth was directly proportional to UV-B radiation dose. Three isolates belonging to a genotype including only phylloplane ones showed a particular response to UV irradiation, which may provide key ecological insights on the role of these fungi in the phylloplane.

**Key words:** ecosystem, habitat, elongation factor 1-alpha (EF1-α), osmotic potential
Viruses

Session 1
Deletion genotypes influence occlusion body potency and production in insects infected by a Spodoptera frugiperda nucleopolyhedrovirus isolate from Colombia

Gloria Barrera1,2, Trevor Williams3, Laura Villamizar2, Primitivo Caballero1,4, Oihane Simón1

1 Instituto de Agrobiotecnología, CSIC-UPNA-Gobierno de Navarra, 31192 Mutilva Baja, Navarra, Spain; 2 Corporación Colombiana de Investigación Agropecuaria (CORPOICA), Km 14 via Mosquera, Bogotá, Colombia; 3 Instituto de Ecología AC, Xalapa, Veracruz 91070, Mexico; 4 Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Navarra, Spain

Abstract: The Colombian field isolate (SfCOL-wt) of Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) is a mixture of different genotypes. To evaluate the insecticidal properties of the different variants in SfCOL-wt a plaque assay was performed and ten distinct genotypes were identified. Genotype SfCOL-A was the most prevalent (71%) and showed a PstI restriction profile identical to that of SfCOL-wt. The remaining nine genotypes presented genomic deletions of 3.8-21.8 Kb that affected the region between open reading frames (ORFs) sf20 and sf33. The potency of SfCOL-A occlusion bodies (OBs) was approximately 4-fold higher than SfCOL-wt OBs, whereas the speed of kill of SfCOL-A was similar to that of SfCOL-wt. Deletion genotype OBs were similarly or less potent than SfCOL-wt, but six deletion genotypes were faster killing than SfCOL-wt. The potency of OBs and co-ocluded mixed genotype OBs were consistently reduced in two-genotype mixtures involving equal proportions of SfCOL-A and one of three deletion genotypes (SfCOL-C, -D or -F). Speed of kill and OB production were improved only when certain genotype mixtures were co-ocluded, although OB production was higher in the SfCOL-wt isolate than in any of the genotypes or genotype mixtures that we tested. The SfCOL-wt population appears to be structured to maximize the production of OBs in each infected host suggesting this to be the principal limitation to transmission.

Key words: SfM NPV, Colombia, wild-type, genotypes, mixtures of OBs, phenotype

Introduction

Previous studies on Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) as a potential biological control agent in Colombia identified the SfCOL isolate as the most insecticidal of a total of 38 field isolates from Colombia or Nicaragua (SfNIC) (Barrera et al., 2011). SfMNPV populations have been found to be composed of different genotypes (Harrison et al., 2008; Simón et al., 2004). Previous studies have examined interactions between genotypes that determine the transmissibility of the wild-type populations (Muñoz et al., 1998; Simón et al., 2005). Evaluating interactions between genotypes can be highly advantageous during the process of selecting active material for the development of virus-based biological insecticides.

The objectives of the present study were to determine the genotypic diversity present in the SfCOL isolate and evaluate the contribution of the component genotypes to the insecticidal properties of the natural isolate.
Material and methods

Individual genotypes present within SfCOL-wt (Barrera et al., 2011) were isolated by plaque assay following the protocol described by Simón et al. (2004). Plaques were picked individually and injected into fourth instars S. frugiperda for viral amplification. OBs were purified and DNA was extracted and analyzed with the restriction endonucleases PstI. Physical maps were constructed by comparison of co-migrating and genotype-specific fragments, and confirmed by sequencing the polymorphic fragments. Relative proportion of the complete genotype SfCOL-A was determined by qPCR. The egt gene was used as an indicator gene for this genotype, as it was the only gene absent in all deleted genotypes and present only in the complete SfCOL-A genotype.

OB and co-occluded mixtures, involving equal proportions of SfCOL-A and one of three deletion genotypes (SfCOL-C, -D or -F), were produced as described by Simón et al. (2005). The insecticidal activity of the SfCOL isolate, individual genotypes and OB and co-occluded mixtures was compared with that of SfCOL isolate in a continuously renewed insect colony obtained from larvae collected in maize fields close to Bogota, Colombia. The median lethal concentration (LC$_{50}$), mean time to death (MTD) and OB productivity (OBs/larva) were determined using PoloPlus (LeOra-Software, 1987), and GLIM (Crawley, 1993). OB production was determined by counting OB content in cohorts of 24 overnight-starved second instars inoculated with the LC$_{90}$.

Results and discussion

The complete SfCOL-A genotype accounted the majority of genotypes in SfCOL-wt

Ten different genotypes (named SfCOL-A to -J) were identified by analysis of plaques using PstI endonuclease (Figure 1). SfCOL-A genotype with the complete genome showed a PstI restriction profile identical to that of SfCOL isolate in a continuously renewed insect colony obtained from larvae collected in maize fields close to Bogota, Colombia. The median lethal concentration (LC$_{50}$), mean time to death (MTD) and OB productivity (OBs/larva) were determined using PoloPlus (LeOra-Software, 1987), and GLIM (Crawley, 1993). OB production was determined by counting OB content in cohorts of 24 overnight-starved second instars inoculated with the LC$_{90}$.

![Figure 1. REN patterns of SfCOL-wt and SfCOL variants DNA digested with PstI. + indicates the polymorphic fragments of each genotype.](image-url)
All other genotypes displayed deletions of 3.8-15.1 kb affecting orfs sf20 to sf34 (Figure 2). This region of variability among the genotypes, which included ORFs that encoded non-essential proteins with auxiliary functions, was also identified in Missouri (Harrison et al., 2008) and Nicaragua (Simón et al., 2004) SfMNPV isolates.

![Figure 2. Schematic representation of the genomic variable region among SfCOL genotypes.](image)

**Deletion genotypes reduce pathogenicity but increase OB productivity**

SfCOL-A was approximately 4.4-fold more potent (in terms of concentration mortality-metrics) than SfCOL-wt, indicating that the other genotypes diminish the pathogenicity of the population. SfCOL-wt and pure genotypes SfCOL-A, -B, -D and -G were the lowest virulent viruses (in terms of mean time to death), which in the case of SfCOL-wt was related with a higher productivity. The potency of OBs and co-occluded mixtures were consistently reduced
in two-genotype mixtures involving equal proportions of SfCOL-A and one of three deletion genotypes. Speed of kill and OB production were improved only when certain genotype mixtures were co-occluded, although OB production was higher in larvae infected with SfCOL-wt isolate than in larvae infected with any of the component genotypes or mixtures thereof. Certain deleted genotypes reduced occlusion body potency but increased occlusion body production, suggesting that SfCOL-wt is structured to maximize transmissibility.

In conclusion, the SfCOL-wt field isolate comprises a high genotypic diversity of which SfCOL-A was the most pathogenic and was as virulent as SfCOL-wt. Genotypic mixtures reduced speed of kill but also reduced OB pathogenicity which is undesirable for the development of a biological insecticide. SfCOL-wt seems to be structured to maximize the likelihood of transmission by maximising OB production. SfCOL-A, due to its high OB pathogenicity is well suited to be developed as a bioinsecticide to control S. frugiperda in Colombia.

Acknowledgements

We thank N. Gorria and I. Ibáñez (Universidad Pública de Navarra, Pamplona, Spain) and C. Guzmán and A. Malagón (CORPOICA, Bogotá, Colombia) for technical assistance. This study received financial support from COLCIENCIAS project 1753-2011 and the projects AGL2008-05456-CO3-01 and AGL2011-30352-CO2-01 funded by the Spanish Ministry of Science and Technology.

References

LeOra-Software 1987: POLO-PC a user’s guide to Probit or Logit analysis. Berkeley, CA.
On the role of baculovirus photolyases in DNA repair upon UV damage of occlusion bodies

Magdalena A. Biernat¹, Primitivo Caballero², Just M. Vlak¹, Monique M. van Oers¹
¹Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands; ²Instituto de Agrobiotecnología, CSIC-Gobierno de Navarra, 31192 Mutilva Baja, Navarra, Spain

Abstract: The use of baculoviruses in insect biocontrol is hampered by their sensitivity to ultraviolet (UV) light. This irradiation induces cyclobutane pyrimidine dimers (CPDs) in DNA. CPD-photolyases repair CPDs using visible light. Plusiine baculoviruses encode photolyases, which could potentially repair UV-damage prior to infection of larvae. Whether the photolyases encoded by Chrysodeixis chalcites nucleopolyhedrovirus are involved in UV damage repair was tested by infecting larvae with UV-irradiated viral occlusion bodies (OBs) that were subsequently treated with visible light or kept in the dark. The observed mortality was the same for both treatments. We postulate that photolyases are not active as DNA repair enzymes in OBs, but may play a role in other aspects of baculovirus pathogenesis.

Key words: CPD photolyase, Chrysodeixis chalcites nucleopolyhedrovirus, DNA repair, UV sensitivity, biocontrol, circadian clock

Introduction

Sunlight is (in)directly the main source of energy for all organisms. At the same time, the ultraviolet (UV) component of sunlight can have destructive effects by causing lesions in DNA: cis-syn-cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone photoproducts (6-4PPs). CPDs are formed when two adjacent pyrimidines, usually thymines, are linked by two covalent bonds. To deal with the harmful effects of UV-irradiation, most organisms (except placental mammals) rely on lesion-specific photolyases to repair UV-induced damage in a light-dependent manner. CPD-photolyases need both redox-active flavine adenine dinucleotide (FAD) and an antenna molecule as cofactors, but do not require nucleotides and can function in an extracellular environment. This process is called photoreactivation (Brettel & Byrdin, 2010).

CPD-photolyases are conserved in a specific group of baculoviruses that infect plusiine insects (Xu et al., 2008). Baculoviruses are large, enveloped double-stranded DNA viruses that infect invertebrates, predominantly insects in the orders Lepidoptera, Hymenoptera and Diptera (reviewed by Slack & Arif, 2007) and that cause a fatal disease. The first baculovirus CPD-photolyase (phr) genes were reported in Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV) and were named Cc-phr1 and Cc-phr2 (van Oers et al., 2004; 2005). Later on, a phr gene was also identified in the genome of Trichoplusia ni single nucleopolyhedrovirus (TnSNPV) (Willis et al., 2005). The ChchNPV-encoded proteins share 45% amino acid identity. Cc-PHR2, in contrast to Cc-PHR1, possessed CPD-photolyase activity in a heterologous (bacterial) system and in vitro (van Oers et al., 2008), but there is no experimental evidence that these photolyases repair UV light-induced DNA damage in vivo, for instance in ChchNPV occlusion bodies (OBs) prior to larval infection.
Baculoviruses are applied as biocontrol agents since the 1950s as a host-specific alternative to chemical pesticides (Szewczyk et al., 2006), but quick inactivation by UV-light in the field poses a severe constraint on their use (Inceoglu et al., 2001; Sun & Peng, 2007). To limit UV inactivation expensive UV protectants are added to baculovirus formulations (Black et al., 1997). The discovery of CPD-photolyase genes in baculoviruses (van Oers et al., 2004; Willis et al., 2005) potentially provides a novel tool to reduce the UV-sensitivity of baculoviruses used for biocontrol. In this paper, we analyzed whether ChchNPV OBs could be photo-reactivated by visible light after inactivation with UV light, thereby restoring their infectivity.

Material and methods

A laboratory colony of the tomato looper, C. chalcites, was reared on artificial diet at 28 ± 1 ºC at a 16 h light/8 h dark photoperiod (Murillo et al., 2000). The Dutch isolate of ChchNPV (ChchNPV-NL) was used in these studies and has been described before (van Oers et al., 2004; 2005). At first the 90% lethal concentration (LC\textsubscript{90}) of non-radiated, wild type ChchNPV-NL was determined in insect bioassays, with in total 75 larvae per treatment and five different concentrations of OBs. These OBs were isolated by grinding virus-killed cadavers in sterile water, filtering the homogenate through muslin, followed by sedimentation at 6000 rpm for 5 min. A suspension containing 10% sucrose, 0.001% Fluorella blue, and 4.4 \times 10^5, 1.7 \times 10^4, 8.8 \times 10^3, 3.5 \times 10^3 or 7 \times 10^2 OBs ml\textsuperscript{-1} was given to 2\textsuperscript{nd} instars, which were starved for 24 h prior to droplet feeding (Hughes & Wood, 1981). An infection with dye only served as negative control. Subsequently, larvae were transferred to individual wells of 24-well plates containing diet. Mortality was recorded daily until 8 days post infection (p.i.) and the data were analyzed using Polo Plus (LeOra Software, 1987).

To determine the optimum irradiation dose, 0.5 ml suspensions of 5 \times 10^6 OBs ml\textsuperscript{-1} were irradiated in 35 mm Petri dishes (Nunc) with 250 nm UV-light at total doses of 0, 50, 100, 150, 200 or 300 J m\textsuperscript{-2} as measured by a UVX radiometer (UVP, LLC Upland, CA). The irradiated OB suspensions were kept in the dark for 6 h to keep the same set-up as in the photo-reactivation experiments described below. Subsequently, starved C. chalcites 2\textsuperscript{nd} instars (~25 insects per treatment, three-times repeated) were droplet-fed with 5 \times 10^6 irradiated OBs ml\textsuperscript{-1}. Next, ChchNPV 5 \times 10^6 OBs ml\textsuperscript{-1} OBs were irradiated at a UV dose of 0 or 200 J m\textsuperscript{-2}. The irradiated samples were either incubated in complete darkness or exposed to visible light with a regular 13 W TL-tube (Philips) at 28 ± 1 ºC for 30 min, 1 h, 2 h or 6 h. An 8 mm glass plate was used to filter out short wavelength UV-light. Two independent bioassays were performed as described above.

Results and discussion

Dose-response relationship between UV-dose and mortality

The LC\textsubscript{90} of ChchNPV for 2\textsuperscript{nd} instar C. chalcites larvae was determined as 2.8 \times 10^6 OBs ml\textsuperscript{-1} (χ²=3.14; degree of freedom: 3; heterogeneity: 1.05). A virus concentration high enough to kill approximately 100% of the larvae (5 \times 10^6 OBs ml\textsuperscript{-1}) was subsequently used to establish the dose of UV light that would reduce the mortality induced by ChchNPV OBs to ~10%. The number of death C. chalcites larvae decreased gradually with increasing UV dose and reached ~12% at 200 J m\textsuperscript{-2} (Figure 1). A UV dose of 300 J m\textsuperscript{-2} completely inactivated the OBs. Therefore a dose of 200 J m\textsuperscript{-2} was used in experiments described below.
Figure 1. UV-sensitivity of ChchNPV OBs. The mortality (%) of C. chalcites larvae infected with OBs from ChchNPV treated with different UV-light doses (J m$^{-2}$) was measured. Mean and standard deviation of triplicate samples are shown.

Photoreactivation as function of time
In order to determine whether ChChNPV ODVs carry active photolyases that can rescue virus inactivation by UV-light, ChchNPV OBs were then irradiated at a UV dose of 0 (control) or 200 J m$^{-2}$. The irradiated samples were incubated in complete darkness or exposed to visible light for various length of time. Non-irradiated OBs (0 J m$^{-2}$) were used as a positive control and resulted in 100% mortality of C. chalcites. No significant difference was found for ChchNPV exposed to visible light (photoreactivation) or kept in the dark after UV-irradiation, irrespective of how long visible light was applied (Figure 2). Hence, the bioassay indicated that the photolyases encoded in the ChchNPV genome are not present or active in OBs of ChchNPV and do not protect ChchNPV at this stage against UV-damage.

In a recent proteomic study both photolyases, PHR1 and PHR2, were not detected in the ChchNPV ODV particles (Xu et al., 2011). These data combined with the data of the current study indicate that the photolyases are not present in ODVs and hence cannot repair ODV genomes prior to infection or in the very early stages of infection before viral gene expression occurs. The fact that these proteins were not found in ODVs is in line with the presence of baculovirus early putative promoter in the motifs for the two PHR genes (CAGT for phr1; GATA for phr2) (van Oers et al., 2004; 2005). Thus the baculovirus photolyases may be expressed at an early stage of infection, which would be difficult to conceive when multiple CPDs are present in their gene sequences. Since the PHRs are routed to the nucleus to carry out their function (Xu et al., 2010), there is a possibility that these photolyases are somehow involved in a repair function during baculovirus DNA replication (Huang et al., 2012). The situation for baculovirus photolyases is therefore very different from what was reported for Fowlpox virus, which encodes a photolyase that is incorporated into mature virions, and where the enzyme was able to repair UV-induced DNA damage in extracellular virus particles (Srinivasan et al., 2001).
This leaves open the question, what the function of baculovirus photolyases could be. It is known that photolyases are homologous to cryptochromes, proteins that function in the circadian clock to regulate oscillation mechanisms, and hence, physiology, behavior and metabolism of almost all organisms (van der Horst et al., 1999). Recently, we illustrated that PHR2 can potentially function in the circadian clock by mimicking the role of a cryptochrome (Biernat et al., 2012). We postulate therefore that PHR2 could have an effect on the circadian clock of the insect host. PHR2 may thus play a role in virus-induced behavioural changes. Infected larvae become hypermobile and die at elevated positions (Goulson, 1997; Hoover et al., 2011; van Houte et al., 2012) with a putative benefit for virus transmission as OBs can be easier and more efficiently spread over the foliage. To determine whether PHRs play a role in baculovirus-induced behavior or have an effect on virus yield, e.g. by changing feeding patterns, studies in C. chalcites larvae with phr knockout baculoviruses are needed.

In conclusion, we have shown that there is no difference in UV-sensitivity of ChchNPV OBs when exposed to visible light (for photoreactivation) or not, suggesting that the PHRs may play a different role in the pathology of baculovirus-infected insect larvae. They may have a dual function, at least in the case of Cc-PHR2, as an active CPD-photolyase at some point and as a modulator of the circadian clock, with possible consequences for insect behavior and ultimately for the use of baculoviruses as biocontrol agent of pest insects. Why only plusiine baculoviruses, such as ChchNPV and TnSNPV have such genes and to what extent this relates to the behavioral consequences of this group of insects remains to be elucidated.
Acknowledgements

Magdalena Biernat was supported by a PhD grant (grant numbers ALW 2PJ/06023) from the Netherlands Organization of Scientific Research (NWO). Monique van Oers was supported in part by the Program Strategic Alliances (nr 08-PSA-BD-01) of the Royal Dutch Academy of Sciences. Rosa Murillo, Alexandra Bernal and Lara Martinez are acknowledged for useful discussions and help with conduction of bioassays.

References

LeOra Software 1987: POLO-PC a user’s guide to Probit or Logit analysis, Berkeley, CA, USA.
van Houte, S., Ros, V. I. D., Mastenbroek, T. G., Vendrig, N. J., Hoover, K., Spitzen, J. & van Oers, M. M. 2012: Protein tyrosine phosphatase-induced hyperactivity is a conserved strategy of a subset of baculoviruses to manipulate lepidopteran host behavior. PloS ONE 7: e46933.


Effect of top spray drying and freeze drying on the photostability and insecticidal activity of a Spodoptera frugiperda nucleopolyhedrovirus (SfM NPV 003) formulation

Mauricio Cruz, Martha Liliana Chaparro, Laura Fernanda Villamizar, Martha Isabel Gomez
Center of Biotechnology and Bioindustry, CORPOICA, Mosquera, Colombia
e-mail: mgomeza@corpoica.org

Abstract: The nucleopolyhedroviruses are considered as an effective biopesticide against the fall armyworm Spodoptera frugiperda. Top spray and freeze drying methods were used to prepare a wettable powder formulation based on nucleopolyhedrovirus of S. frugiperda (SfM NPV 003) and its photostability and virulence were assessed. Top spray drying method was more efficient for removing moisture content than freeze drying. No obvious differences in the insecticidal activities were observed for both drying methods although a higher photostability (88.54%) was observed in the formulation prepared with top spray drying method compared to freeze drying (77.77%) and unformulated virus (15.62%) after 6 hours of UV radiation exposure. Top spray drying method was selected as the most favorable process for being implemented in a manufacture process.

Key words: Biological control, biopesticide, entomopathogenic, fall armyworm, drying process

Introduction

The fall armyworm Spodoptera frugiperda (JE Smith, 1797) (Lepidoptera: Noctuidae), is considered as the most important pest of maize crop, reducing yields up to 35% (Fernandez, 2002). Use of entomopathogenic virus is an alternative to chemical pesticides and currently is considered as an effective strategy in the integrated pest control management. However, some concerns can limit its use, such as inactivation of virus caused by solar radiation as well as and scaling up problems during viral propagation and formulation process. A wettable powder formulation based on a Colombian Spodoptera frugiperda nucleopolyhedrovirus (SfM NPV 003) was previously developed, which showed to control the pest at 100% under laboratory conditions. The same formulation reduced the maize plant damage up to 2.5% under field conditions (Gomez et al., 2011). However, there is a limited knowledge about the drying method needed to dry the formulation, particularly when the process has been scaled up and the cost of production need to be reduced. Thus, the effect of the two drying methods (freeze and top spray drying) over photostability and insecticide activity of formulation based on SfM NPV 003 was evaluated.
Material and methods

Virus production
The viral inoculum was obtained from infected third instar larvae of S. frugiperda that exhibited symptoms of viral infection. Infected larvae were homogenized in a porcelain mortar with a sterile solution of sodium dodecyl sulfoxide (SDS) at 0.1% (w/v). The concentration of viral occlusion bodies (OBs ml⁻¹) was determined using a Neubauer counting chamber.

Formulation
A mix of 2300 ml of viral suspension containing 3.0 x 10⁹ OBs ml⁻¹ at pH of 6.36 and 3.10% solids content was used to prepare the formulation. All components including an optical brightener and other sunscreens were diluted in phosphate buffer solution at pH 7.0 and mixed with the viral suspension. The final mixture was subjected to two drying techniques, top spray drying and freeze drying.

Top spray drying
A Glatt Uni Glatt 01277 fluid bed dryer with a nozzle of 1 mm diameter was used. Three batches of 250 g of the formulated virus were evaluated. Each batch was fed into the equipment chamber at 10 ml min⁻¹. The inlet air temperature was maintained at 90 ± 2 °C, while the chamber temperature remained between 36 °C and 55 °C. Internal pressure in the chamber was 1 bar. The operation efficiency was expressed by the amount of water removed from the initial mixture per minute (g H₂O min⁻¹).

Freeze-drying
A VirTis Genesis 25ES freeze dryer was used. Three batches with 1 kg of the formulation of the virus were evaluated. Temperature in the condenser was maintained under -56 ± 3 °C at 57 ± 5 mm Torr during 24 hours. Process efficiency was determined (g H₂O min⁻¹) the same above.

Quality control
The pH in suspension and moisture content were determined by triplicate for each batch using a potentiometer Hanna 8014 and a Kern MLS 50 moisture analyzer respectively. Data were analyzed by ANOVA and Tukey test (α = 0.05).

Determination of viral LC₅₀
Neonate larvae (L1) of S. frugiperda were used to determine the mean lethal concentration (LC₅₀) for formulated and unformulated virus. Briefly, five different concentrations (1.0 x 10³ to 1.0 x 10⁷ OBs ml⁻¹) diluted in distilled water and a control without treatment were used. Fifteen larvae for each treatment were infected with virus using the droplet feeding method (Hughes & Wood, 1981). Mortality results were subjected to Probit analysis (Finney, 1952) using Polo PC (Polo LeOra Software, 1997).

Effect of the drying methods on photostability
Formulated and unformulated virus was reconstituted in distilled water adjusting a concentration of 2.0 x 10⁷ OBs ml⁻¹. Subsequently, 100 µl of each formulation was placed in a well of a 96-well standard microplate. The microplate was exposed to monochromatic ultraviolet lamp light (3VP-38) with a wavelength of 375 nm (UV-B) at 30 cm height. Time 0 (no exposure), 2, 4 and 6 hours of exposure were evaluated. After irradiation, the samples
were collected and the viral activity was assessed. Viral suspensions were used for a bioassay by the droplet feeding method (Hughes & Wood 1981). Mortality was corrected with the control (larvae without treatment) by the Schneider Orelli equation (Zar, 1999).

**Results and discussion**

Results of moisture content, pH and efficiency are described in Table 1. Significant difference (p < 0.05) were observed in final moisture content and suspension pH when drying techniques were compared (Table 1).

Table 1. Characteristics of the top spray dried and freeze dried formulations.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Final moisture content (%)</th>
<th>pH</th>
<th>Efficiency (g H₂O min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top spray drying</td>
<td>Freeze drying</td>
<td>Top spray drying</td>
</tr>
<tr>
<td>1</td>
<td>4.37a 1.54b</td>
<td>7.37a 6.06b</td>
<td>7.20 0.43</td>
</tr>
<tr>
<td>2</td>
<td>4.21a 1.73b</td>
<td>7.42a 6.06b</td>
<td>6.46 0.44</td>
</tr>
<tr>
<td>3</td>
<td>4.58a 1.75b</td>
<td>7.41a 6.05b</td>
<td>6.30 0.45</td>
</tr>
<tr>
<td>Mean</td>
<td>6.65a 0.44b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Different letters indicate significant differences by Tukey test (α = 0.05). Results of final moisture content (%) and pH were compared separately).

The efficiency was significantly higher in samples dried using top spray drying method compared with the freeze drying method (up to 16 times faster for removing the moisture). However, moisture contents obtained by freeze drying at the end of the process were significantly lower than spray drying (under 1.75%) (Table 2). The highest efficiency values obtained by top spray drying may be explained by the fact that heat and mass transfer phenomena are governed by convection, which generally have a higher dynamic compared to conductive phenomena which predominate in the freeze drying process (Al-Hakim & Stapley, 2004). Additionally, particles produced by top spray drying method have a superficial area larger than obtained by freeze drying method, this could increase transfer speeds. Significant differences found in the final pH could be attributed to the higher moisture content in the sprayed product, which is mainly buffer pH 7.0 remaining from formulation process.

The LC₅₀ for products obtained by both drying methods were not significantly different, suggesting that drying methods did not affect viral insecticidal activity. In the other hand, significant differences between dried products an unformulated virus were not found, indicating that neither the formulation process nor the drying method affected the pathogenicity (Table 2). These results are similar to the obtained by Tamez et al. (2000) with 16 sprayed formulations, where bioassays demonstrated that viral occlusion bodies were unaffected.
Table 2. Mean lethal concentration (LC\textsubscript{50}) of unformulated and formulated virus (Top spray drying and Freeze drying).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fiducial limits (OBS ml\textsuperscript{-1})</th>
<th>95%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC\textsubscript{50}</td>
<td>Lower (OBS ml\textsuperscript{-1})</td>
</tr>
<tr>
<td>Unformulated Virus</td>
<td>1.64 x 10\textsuperscript{5}</td>
<td>5.17 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td>Top spray drying</td>
<td>9.43 x 10\textsuperscript{4}</td>
<td>4.46 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td>Freeze drying</td>
<td>6.26 x 10\textsuperscript{4}</td>
<td>1.89 x 10\textsuperscript{4}</td>
</tr>
</tbody>
</table>

Regarding the photostability test, the results showed that the efficacy of unformulated virus was significantly reduced (p < 0.05) being reduce to 15.92% after 6 hours of irradiation. Dried formulations showed a significant protection from UV radiation. However, the efficacy of spray-dried product was significantly higher (88.54%) than freeze-dried (77.70%) after 6 hours of exposure (Figure 1). Spray drying method could favor coating of OBs, similar to microencapsulation, protecting them from the deleterious effects of UV radiation.

The freeze drying method demand higher energy consumption and is less efficient than top spray drying technique. Additionally, the spray drying showed a significant and positive effect on the photostability and this technique could be more favorable than freeze drying for a continuous production.

Figure 1. Effect of UV-irradiation time over efficacy of unformulated and formulated virus Different letters mean significant different according to DMS test (\(\alpha = 0.05\)).
References


Variations in the susceptibility to CpGV in populations of the codling moth, Cydia pomonella

Benoît Graillot1,2, Christine Blachere1,3, Samantha Besse2, Myriam Siegwart4, Miguel López-Ferber1

1LGEI, Ecole des Mines d’Alès, Institut Mines-Télécom. 6, Avenue de Clavières, 30319 Alès, France; 2Natural Plant Protection, Arysta LifeScience group, Avenue Léon Blum, 64 000 Pau, France; 3INRA, 6, Avenue de Clavières, 30319 Alès, France; 4INRA, UR1115 PSH, Domaine Saint-Paul, site Agroparc, 84914 Avignon Cedex 9, France

Abstract: Failure in codling moth populations control with CpGV in apple orchards has been attributed to the action of a single allele located in the Z chromosome. However, differences in the mortality patterns between genetically homogeneous susceptible and resistant insects in the laboratory strongly suggest that other mechanisms are responsible of variation in the susceptibility of insect to CpGV isolates.

Key words: Cydia pomonella, multigenic resistance

Introduction

The Cydia pomonella granulovirus, CpGV (Betabaculovirus) has been extensively used for the control of codling moth proliferation in orchards soon after its first isolation (Tanada, 1964) and characterisation (Crook et al., 1985). In 2005, a failure of control and possible development of resistance was reported in Germany (Fritsch et al., 2005), later in France (Sauphanor et al., 2006) and in other parts of Europe (Jehle & Schmitt, 2009). First investigations into the resistance of C. pomonella to CpGV-M carried out in Germany using single pair crosses clearly indicated monogenic and sex-linked resistance (Asser-Kaiser et al., 2007). Recently more detailed studies suggested that other mechanisms are required to explain the observed response of natural and laboratory populations to virus challenges (Berling et al., 2013; Jehle & Schmitt, 2009). In this study, the response of laboratory colonies to virus challenge has been analysed from this perspective.

Material and methods

Insect colonies

Three Cydia pomonella colonies were used in this analysis: i) the Sv colony, a susceptible laboratory imbred strain, derived from a field population collected at Les Vignières (Vaucluse, France) in early nineties, and reared without selection pressure at INRA (Avignon); ii) the RGV colony (Berling et al., 2009), derived from Sv by introgression of the major resistant determinant found in a french natural resistant population in St Andriol (Sauphanor et al., 2006), and the CpNPP colony, that is the susceptible colony used at industrial level for the production of Carpovirusine™. CpNPP has been reared in laboratory for more than 20 years and originally comes from northern France. All colonies were reared on artificial diet (Guenelon et al., 1981).
**Virus isolates**

Two Cydia pomonella granulovirus (CpGV) (Betabaculovirus, Baculoviridae) isolates were used in this study, the CpGV-M isolate, found in Mexico (Tanada, 1964), and the CpGV-R5, derived from NPP-R1 by selection on RGV insects (Besse et al., 2011) for 16 passages as previously described (Berling et al., 2009).

**Bioassays**

Bioassays against neonate larvae (0 to 12 h old) were carried out using a diet surface contamination method in 96-well plates containing about 200 µl of a formaldehyde-free artificial diet (Heliotothis Diet; Ward’s Natural Science, USA). A 6 µl volume of an OB suspension was deposited over the diet surface of each well (well surface area 28 mm²). The same volume of distilled water was used in control wells. Bioassays were performed using five or six CpGV concentrations, ranging from 3 to 729 OBs µl⁻¹ for the most efficient isolates (corresponding to 0.643 to 156.2 OBs mm⁻² of diet surface) and up to 3.125 x 10⁶ OBs µl⁻¹ for the RGV colony for the least efficient isolate (6.696 x 10⁵ OBs mm⁻²). One larva was placed in each well. The wells were sealed with parafilm, and the microplates were incubated in a growth chamber at 25 °C with a 16 h light/8 h dark photoperiod. Larvae dying during the first day post inoculation were excluded from the analysis. Three to five different independent replicate tests have been performed for each modality representing 600 to 1000 infected individuals per modality. Tests presenting high mortality in controls (more than 10%) or have been removed from the analysis. Mortality was recorded at 7 days postinfection. Larvae that did not react to physical stimuli were considered dead. Data were subjected to analysis using the software Polo+ (LeOra Software 2012).

**Results and discussion**

It has been proven that the major determinant of resistance to CpGV-M is located on the Z chromosome, and thus, follows a sexual transmission pattern (Asser-Kaiser et al., 2007). However, careful analysis of the available data from isogenic strains revealed inconsistencies with this predicted model (Berling et al., 2013). It has been suggested that other resistance mechanisms could exist (Jehle et al., 2011).

**Table 1. Biological efficiency of two CpGV isolates on three laboratory colonies.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Insect</th>
<th>L_D₅₀</th>
<th>95% Fiducial Limits</th>
<th>L_D₉₀</th>
<th>95% Fiducial Limits</th>
<th>Slope</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpGV-M</td>
<td>Sᵥ</td>
<td>34</td>
<td>24-46</td>
<td>125</td>
<td>86-239</td>
<td>2.271 ± 0.387</td>
<td>3.782</td>
</tr>
<tr>
<td></td>
<td>CpNPP</td>
<td>13</td>
<td>7-23</td>
<td>223</td>
<td>111-653</td>
<td>1.041 ± 0.087</td>
<td>5.9895</td>
</tr>
<tr>
<td></td>
<td>Rᵥ</td>
<td>7122</td>
<td>1196-37429</td>
<td>1.83x10⁶</td>
<td>2.15x10⁶-3.68x10⁸</td>
<td>0.531 ± 0.072</td>
<td>6.5308</td>
</tr>
<tr>
<td>CpGV-R5</td>
<td>Sᵥ</td>
<td>32</td>
<td>4-106</td>
<td>438</td>
<td>127-19402</td>
<td>0.126 ± 0.175</td>
<td>8.4499</td>
</tr>
<tr>
<td></td>
<td>CpNPP</td>
<td>7</td>
<td>3-12</td>
<td>60</td>
<td>28-279</td>
<td>1.355 ± 0.127</td>
<td>11.425</td>
</tr>
<tr>
<td></td>
<td>Rᵥ</td>
<td>22</td>
<td>14-33</td>
<td>410</td>
<td>240-845</td>
<td>1.011 ± 0.102</td>
<td>3.622</td>
</tr>
</tbody>
</table>
Table 1 presents the results of bioassays on the three *C. pomonella* colonies. As expected, the CpGV-M isolate is efficient on susceptible insects (*CpNPP* and *SV*) but not on resistant insects (*RGV*). The CpGV-R5 isolate is efficient in all three colonies.

The comparisons of efficiency between each modality indicate that each virus displays a specific action on each insect colony excluding CpGV-R5 which shows the same effect on *SV* and *RGV* (Equality hypothesis: \( P > 0.05, \chi^2 = 1.04, \text{df} = 2, \text{tail probability} = 0.596 \); Parallelism hypothesis: \( P > 0.05, \chi^2 = 0.34, \text{df} = 1, \text{tail probability} = 0.559 \)). This result could reflect the shared genetic background of *SV* and *RGV* colonies (Berling *et al.*, 2009).

When comparing the dose-response to the CpGV-M isolate of *CpNPP* and *SV*, small but significant differences are found (Equality hypothesis: \( P < 0.05, \chi^2 = 24.80, \text{df} = 2, \text{tail probability} = 0.00 \); Parallelism hypothesis: \( P < 0.05, \chi^2 = 17.56, \text{df} = 1, \text{tail probability} = 0.00 \)), whereas CpGV-R5 virus impacts these colonies on a similar way (Parallelism hypothesis: \( P > 0.05, \chi^2 = 1.02, \text{df} = 1, \text{tail probability} = 0.314 \)) but with different intensity (Equality hypothesis: \( P < 0.05, \chi^2 = 30.40, \text{df} = 2, \text{tail probability} = 0.00 \)). *CpNPP* appears to be a laboratory colony “fully susceptible” to both CpGV isolates, while *SV* and its derivate, *RGV*, have a lower susceptibility level. As seen in Figure 1, although the global trends are similar for *SV* and *CpNPP*, at low multiplicity of infection *CpNPP* is more susceptible than *SV* to both virus isolates.

![Figure 1. Dose/mortality plots for the two virus isolates CpGV-R5 (upper panel) and CpGV-M (lower panel) on three insect colonies (SV, CpNPP, and RGV).](image-url)
These differences are probably the reflect of differences in the ways the two viruses control the infection cycle in their hosts. In condition of low virus prevalence, the genetic background of $S_V$ would be enough for blocking an outbreak.

In other baculoviruses, variability on the susceptibility of natural populations to the infection with a virus isolate has been described. (Abot et al., 1996; Briese, 1986). The possibility of selection of a population with reduced susceptibility to CpGV based on this second mechanism remains open. This second mechanism would not necessarily discriminate isolates in a similar way as the resistance actually present in european orchards.

Acknowledgements

The research leading to these results have received funding from the European Community’s Seventh Framework Programme (FP7/2007-2011) under grant agreement n° 211606. B.G. is recipient of a French ANRT doctoral grant.

References


Characterisation of novel CrleGV isolates for false codling moth control - lessons learnt from codling moth resistance to CpGV

John Opoku-Debrah¹, Sean Moore¹,², Martin Hill¹, Caroline Knox³
¹Department of Zoology and Entomology, PO Box 94, Rhodes University, Grahamstown 6140, South Africa; ²Citrus Research International, PO Box 20285, Humewood 6031, Port Elizabeth, South Africa; ³Department of Biochemistry, Microbiology and Biotechnology, PO Box 94, Rhodes University, Grahamstown, 6140, South Africa

Abstract: Recently some codling moth, Cydia pomonella, populations in Europe developed resistance to CpGV. In order to prepare for the possibility of a similar occurrence with the false codling moth, Thaumatotibia leucotreta, in South Africa, a search was conducted for novel CrleGV isolates. Through overcrowding, outbreaks of novel isolates were recorded from laboratory populations of five geographically distinct host populations. The genetic novelty of these and two commercially available isolates was confirmed through restriction enzyme analysis and sequence analysis of the granulin and egt genes. Phylogenetic analysis showed the existence of two CrleGV-SA genome types. Significant differences in virulence were also shown between certain isolates against certain host populations.

Key words: Cryptophlebia leucotreta granulovirus, Thaumatotibia leucotreta, novel isolates, dose-response bioassays

Introduction

In South Africa, two Cryptophlebia leucotreta granulovirus (CrleGV-SA) products are registered for the control of the false codling moth, Thaumatotibia leucotreta (Meyrick) (Lepidoptera: Tortricidae), an important economic pest of citrus and other crops (Newton, 1998). These products, Cryptogran (River Bioscience, South Africa) (Moore et al., 2011) and Cryptex (Andermatt Biocontrol, Switzerland) (Kessler & Zingg, 2008), are arguably the most widely used mode of control for this pest in South Africa.

A recently noted risk with this sort of use of baculoviruses is host resistance. Field populations of codling moth, Cydia pomonella (L.), in Europe developed resistance to the Mexican isolate of the Cydia pomonella granulovirus (CpGV-M) (Asser-Kaiser et al., 2007). This resistance was overcome by challenging resistant insects with different virus isolates of the same species (Eberle et al., 2008; Berling et al., 2009). These trials led to commercial replacement of products containing CpGV-M with those containing genetically different CpGV isolates (Besse et al., 2011; Zingg et al., 2011).

In order to be prepared in case a similar situation should occur with T. leucotreta in South Africa, this study aimed at bioprospecting for new CrleGV isolates as alternatives to the existing commercially used ones. Additionally, virulence of isolates to cultures of different host populations was compared.
Material and methods

Host rearing
Five cultures of insect populations were established and maintained. The first population (MixC) consisted of a heterogeneous population which had been maintained continuously for over 166 generations. The other four laboratory populations (Ado, Mbl, Cit & Nels) were established from field-collected larvae from four different regions in South Africa: Addo (Eastern Cape) (Ado), Marble Hall (Limpopo) (Mbl), Citrusdal (Western Cape) (Cit) and Nelspruit (Mpumalanga) (Nels) (Opoku-Debrah, 2008). The cultures were reared on artificial diet as described by Moore (2002).

Virus acquisition and preparation
Symptomatic virus infection was induced by overcrowding in all geographic laboratory populations as described by Opoku-Debrah et al. (2013). Virus was recovered and purified according to the methods described by Moore et al. (2011) with minor modifications.

DNA characterisation and phylogenetic analysis of the granulin and egt gene sequences
Genomic DNA was extracted using a modified version of the CTAB DNA extraction protocol described by Opoku-Debrah et al. (2013). Single restriction endonuclease (REN) digest reactions were performed using BamHI, SalI, XbaI, PstI, XhoI, KpnI, HindIII and EcoRI. Digests were analysed by 0.6% agarose gel electrophoresis (AGE) in 1 x TAE buffer at 30 V for 16 h followed by ethidium bromide staining.

Granulin and egt gene sequences of all isolates were amplified by PCR using virus-specific oligonucleotides (Lange & Jehle, 2003). Phylogenetic comparisons between isolates were conducted using the nucleotide sequences of the isolates (Tamura et al., 2011).

Dose-response bioassays
The droplet feeding bioassay technique described by Pereira-da-Conceiçãoa et al. (2012) for the bioassay of neonate larvae was used. Seven-fold serial dilutions were used, ranging from $6.07 \times 10^2$ to $7.14 \times 10^7$ OBs ml$^{-1}$. Three replicates of 48 larvae per treatment and an untreated control were conducted for each population; assays were evaluated for larval mortality 7 days post inoculation.

Data were analysed by probit analysis using PROBAN (Van Ark, 1995). Median lethal dose (LD$_{50}$) and 90% lethal dose (LD$_{90}$) values were determined after pooling data from the three replicates. Multiple comparisons of probit regression lines were also conducted using the Bonferroni method and significant differences between slopes were established at $P < 0.05$.

Results and discussion

DNA characterisation
DNA profiles obtained for BamHI, SalI, XbaI and HindIII showed some differences between the seven CrleGV-SA isolates (data not shown). The clearest differences between all isolates were evident with BamHI (Figure 1). Several submolar bands were observed in the DNA profiles.

Results from this analysis showed the presence of two unique banding patterns, allowing placement of isolates into two genome groups: Cryptex, CrleGV-SA Ado, CrleGV-SA Mbl, CrleGV-SA Cit and CrleGV-SA MixC (Group One) as well as Cryptogran and CrleGV-SA Nels (Group Two) (Opoku-Debrah et al., 2013).
Comparative analysis of EGT amino acid sequences

Sequence data for the granulin gene of both Group One and Group Two CrleGV-SA isolates showed a few changes in their nucleotide sequence. There was no difference in their amino acid sequence, confirming that this gene is highly conserved (Federici, 1997).

Comparing EGT amino acid sequences, Group One CrleGV-SA showed a 98% similarity (six substitutions) to CrleGV-CV3 (GenBank ID: AY229987; Lange & Jehle, 2003) and Group Two, a 99% similarity (seven substitutions) (Opoku-Debrah et al., 2013). These differences confirmed the uniqueness of the seven isolates.

Figure 1. BamH1 restriction endonuclease digest profiles of seven CrleGV-SA isolates, analysed by 0.6% AGE at 30 V for 16 hours. Asterisks (*) indicate submolar bands.

Table 1. LD_{50} and LD_{90} (in OBs per larva) for neonate FCM larvae from the Addo population and the Mixed population in dose-response bioassays with seven CrleGV-SA inocula.

<table>
<thead>
<tr>
<th>CrleGV-SA isolate</th>
<th>Addo population</th>
<th>Mixed population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD_{50}</td>
<td>LD_{90}</td>
</tr>
<tr>
<td>Cryptex</td>
<td>2.58b</td>
<td>669.50</td>
</tr>
<tr>
<td>Cryptogran</td>
<td>1.02a</td>
<td>272.86</td>
</tr>
<tr>
<td>CrleGV-SA Ado</td>
<td>1.14a</td>
<td>358.89</td>
</tr>
<tr>
<td>CrleGV-SA MixC</td>
<td>3.12b</td>
<td>773.32</td>
</tr>
<tr>
<td>CrleGV-SA Nels</td>
<td>0.90a</td>
<td>250.11</td>
</tr>
<tr>
<td>CrleGV-SA Cit</td>
<td>0.97a</td>
<td>218.04</td>
</tr>
<tr>
<td>CrleGV-SA Mbl</td>
<td>0.83a</td>
<td>289.25</td>
</tr>
</tbody>
</table>

*Values in the same column followed by the same letter do not differ significantly (\(\alpha=0.05\)).
Dose-response bioassays
Differences in virulence were observed in both the Addo and mixed populations. For example, in assays with the Addo population both CrleGV-SA MixC and Cryptex required almost 3 virus particles per larva to elicit 50% mortality (LD<sub>50</sub>) in a given population as opposed to 1 virus particle required for the other isolates. There were no significant differences in virulence between the seven isolates against the remaining host populations.

Conclusions
These results provide us with several possible alternative CrleGV isolates in the unlikely event of T. leucotreta developing resistance to the commercial isolates. Additionally, use of different isolates against different regionally distinct host populations could be considered.

References


Elucidation of a novel mode of resistance of codling moth against Cydia pomonella granulovirus by homogenization experiments

Annette J. Sauer, Eva Fritsch, Karin Undorf-Spahn, Johannes A. Jehle
Institute for Biological Control, Julius Kühn Institute (JKI), Federal Research Center for Cultivated Plants, Heinrichstr. 243, 64287 Darmstadt, Germany

Abstract: Since 2005, codling moth (CM, Cydia pomonella) populations with a reduced susceptibility to Cydia pomonella granulovirus (CpGV, Baculoviridae) products have been reported from about 40 European orchards. The resistance could be traced back to a single, dominant, sex-linked gene. Currently, resistance management strategies are based on the application of improved CpGV products containing resistance-overcoming CpGV isolates. Recently, two CM field populations (NRW-WE and SA-GO) with a reduced susceptibility to even these improved CpGV products were found. First single pair crossing experiments between individuals of these resistant field colonies and a susceptible laboratory CM strain (CpS) indicated that the inheritance of resistance of these populations did not follow the previously described pattern of Z-linked, dominant resistance. In single-dose bioassays the susceptibility of neonates of the resistant CM colonies NRW-WE and SA-GO to different CpGV isolates (CpGV-M, -S, -V15 and -E2) was estimated. The aim of the current study was the genetic homogenization of the genetically heterogeneous field populations NRW-WE and SA-GO by two different methods: (i) repeated single pair crossings followed by family selection and (ii) successive mass crossing experiments under virus pressure. The resulting homogenous strains of NRW-WE and SA-GO with fixed resistance will be used for backcrossing experiments with CpS to elucidate the mode of inheritance of their resistance.

Key words: Cydia pomonella, CpGV resistance, biological control, genetically homogenization

Introduction

In nearly all growing regions of apple and pear worldwide, the codling moth (CM, Cydia pomonella) is the most devastating pest; CM has developed resistance to many chemical insecticides (Rodriguez et al., 2011). An alternative to the application of chemical insecticides is the use of Cydia pomonella granulovirus (CpGV). CpGV products are applied in both organic and integrated production (Huber, 1998).

From 2005 on, the first CM populations with reduced susceptibility to CpGV products were reported from south-west Germany (Fritsch et al., 2005) and France (Sauphanor et al., 2006). When CM populations of 13 German apple plantations were systematically tested for CpGV susceptibility, a resistance ratio up to 10,000 fold was determined (Asser-Kaiser et al., 2007). Meanwhile, 38 CM populations have been identified in different European countries (Schmitt et al., 2013). Single pair crossings were accomplished with a resistant field colony (CpR) to achieve a genetically homogenous, resistant CM inbred strain, referred to as CpRR1. Hybrid crossing experiments between CpRR1 and the sensitive CM strain (CpS) provided clear evidence for a monogenic, sex-linked (chromosome Z) and dominant resistance allele (Asser-Kaiser et al., 2007; 2010; Zichovà et al., 2013). CpGV-M, the so-called Mexican isolate, was the common agent used in all commercial CpGV products registered in Europe. To prevent a further selection for resistance to CpGV-M, current resistance management strategies are derived from the application of resistance-overcoming CpGV isolates.
Field observations of several CM field populations controlled with different CpGV isolates revealed two German CM populations (NRW-WE and SA-GO) with low susceptibility to both CpGV-M and the new resistance overcoming isolates, such as CpGV-S. Crossing experiments with individuals of NRW-WE and SA-GO, respectively, and the susceptible laboratory strain CpS revealed a pattern of resistance inheritance that did not follow the previously described Z-linked, dominant inheritance (Schulze-Bopp & Jehle, unpublished).

In order to provide a basic understanding of the complex baculovirus CM interaction under field conditions, it is necessary to gain a more detailed picture on the novel resistant colonies. Therefore, the genetically heterogeneous field populations NRW-WE and SA-GO need to be genetically homogenized. Two methods were applied: Larvae of NRW-WE were selected for resistance by feeding virus contaminated diet according to a method of Berling et al. (2009) and Zichová et al. (2013). Homogenization of the CM colony SA-GO was accomplished by single pair crosses and exposing larvae of the F1 generation to a discrimination concentration of CpGV-M and CpGV-S, according to the method of Asserkaiser et al. (2007). Furthermore, the control population, maintained in absence of the virus, was reared to adulthood. Hence, the selected homogenous colonies of NRW-WE and SA-GO will be used for backcrossing experiments with CpS to monitor and compare differences in the two homogeneous methods and to elucidate the novel mechanism of resistance.

Material and methods

Test insects and virus
Diapausing larvae of NRW-WE and SA-GO were first collected in 2009 from two different plantations in Germany and kept in a laboratory rearing at 26 °C, 60% relative humidity (RH) and at 16 h photoperiod before using in homogenization experiments. The susceptible strain CpS derived from Andermatt Biocontrol (Switzerland) and was frequently tested for its susceptibility to CpGV-M. The CpRR1 strain is a genetically homogenized population deriving from a resistant field colony CpR, which is identical to the resistant colony described by Fritsch et al. (2005), called “Suedbaden”.

The isolate CpGV-M used in the bioassay was a descendent from the CpGV collected in Northern Mexico (Tanada, 1964) and the isolate CpGV-S originated from the Canadian product Virosoft™, BioTEPP Inc. The isolate CpGV-V15 was developed by Andermatt Biocontrol AG (Switzerland). CpGV-E2 derived from the so-called “English Isolate” CpGV-E (Crook et al., 1985). Virus occlusion bodies (OBs) were counted with Petroff-Hauser counting chamber (depth 0.02 mm) using dark field optics.

Genetically homogenization by single pair crossing
Larvae of the resistant colony SA-GO were separated by sex in the fifth instar due to evidence of gonads visible under the skin of male individuals. After rearing to pupae and emerging of moths, 20 pairs were kept in small closed plastic boxes at 26 °C, 60% RH and at 16 h photoperiod. After mating, the deposited eggs were collected and incubated until hatch of the larvae. The offspring of each pair were divided into three cohorts. Two cohorts were tested for susceptibility to CpGV-M and CpGV-S using bioassays with the discriminating concentration of the virus. Larvae of the third cohort served as control and were kept on virus free medium until adulthood in case that the mortality in the corresponding bioassays was less than 30% after 14 days. Adults deriving from the control cohort were used for a second inbreeding step by repeating the procedure.
Genetic homogenization by mass crossing under virus selection

300 neonates of the F37 generation of the resistant laboratory colony NRW-WE were randomly selected and transferred on diet containing both CpGV-M and CpGV-S (2 x 10^5 OB ml^-1, respectively. The survivors were reared to adulthood and their progeny was again exposed to CpGV-M and CpGV-S, respectively, as described before. The selection procedure was repeated for four generations.

Bioassay

To detect resistant and susceptible individuals, larvae were subjected to a single discriminating virus concentration (5.8 x 10^4 OB ml^-1 diet) (Asser-Kaiser et al., 2007) causing > 95% mortality for the sensitive strain CpS within 7 days post exposure. Aliquots of the prepared virus suspensions were mixed into artificial diet (Ivaldi-Sender, 1974) and fed to neonates (L1) of the different CM strains. The test insects were kept at 26 °C, 60% RH with a 16 hr photoperiod and larval mortalities were recorded after 7 and 14 days.

Results and discussion

Mortality data obtained from 14-days bioassays with the CM colonies (SA-GO, NRW-WE, CpRR1 and CpS subjected to CpGV-M, -S, -V15, and -E2) are shown in Figure 1. The laboratory strain CpS was highly susceptible for CpGV-M and CpGV-S with a mortality of 100% and 92%, respectively. The resistant strain CpRR1 presented a high mean mortality (87.6%) when challenged with CpGV-S but low mortality when treated with CpGV-M. The two field populations SA-GO and NRW-WE showed low mortalities in the bioassay with CpGV-M and CpGV-S but mortalities of up to 100% in bioassays with the isolates CpGV-V15 and E2.

![Figure 1. Mortality induced in neonate larvae of different CM populations (SA-GO, NRW-WE, CpRR1 and CpS) after 14 days of incubation on the discriminating virus concentration of 5.8 x 10^4 CpGV OB ml^-1 with different CpGV isolates (CpGV-M, -S, -V15, -E2). Data show mean values of three independent replicates.](image)
Both of the novel CM colonies NRW-WE and SA-GO showed resistance to CpGV-S and -M but the isolates CpGV-V15 and CpGV-E2 overcame the resistance and mortalities up to 100% were detected. Previous analysis of these colonies demonstrated that the inheritance of their resistance did not follow the previously described pattern of Z-linked, dominant resistance (Schulze-Bopp and Jehle, unpublished). The pursued homogenization of the field colonies NRW-WE and SA-GO is essential for backcrossing experiments with CpS to detect whether there is a further mechanism of resistance. Furthermore, the two different homogenization methods as well as the two different resistant field colonies NRW-WE and SA-GO will be compared based on potential differences in their mode of resistance. The intended reciprocal backcrossing experiments followed by bioassays can also define sex-linkage of the resistance.

Acknowledgements

We thank the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) (28090E097) and the Deutsche Forschungsgemeinschaft (DFG) (Je245/14-1) for funding this research.

References


Session 2
Biological control of the box tree moth Cydalima perspectalis with Anagapha falcifera nucleopolyhedrovirus (AnfaNPV)

Jana Rose, Johannes A. Jehle, Regina G. Kleespies
Institute for Biological Control, Julius Kühn Institute (JKI) Federal Research Center for Cultivated Plants, Heinrichstr. 243, 64287 Darmstadt, Germany

Abstract: The box tree moth Cydalima perspectalis originated from East Asia. Since several years, it is a novel invasive insect pest in many European countries, causing widespread damage on box tree plants. The potential of the baculovirus Anagapha falcifera nucleopolyhedrovirus (AnfaNPV) as a potential biological control agent for the control of C. perspectalis was investigated in this study. Two AnfaNPV isolates, termed Dn10 and BI-235, were used. The infectivity of AnfaNPV Dn10 and BI-235 to C. perspectalis was evaluated by leaf disc bioassays and the median lethal concentration (LC₅₀) was determined for both isolates. In addition, light and electron microscopic analyses were performed to study the infection process. In conclusion, larvae of C. perspectalis were shown to be susceptible to both AnfaNPV isolates.

Key words: Cydalima perspectalis, biological control, baculoviruses, bioassay, pathology

Introduction

The box tree moth Cydalima perspectalis (Walker 1859) (Lepidoptera: Crambidae) is also known as Diaphania perspectalis and Glyphodes perspectalis (Mally & Nuss, 2010). It originated from East Asia but in recent years it became invasive to southwestern Germany, the Netherlands, United Kingdom, Austria, Hungary, Slovenia and Turkey (Billen, 2007; Van der Straten & Muus, 2010; Sáfián & Horváth, 2011; Seljak, 2012; Hizal et al., 2012).

This insect is susceptible to chemical insecticides, such as deltamethrin or diflubenzuron, and to Bacillus thuringiensis preparations (Korycinska & Eyre, 2011). Recently, an isolate of AnfaNPV Dn10 was identified to infect larvae of the pickleworm (Diaphania nitidalis) (Lepidoptera: Crambidae) (Jackson et al., 2009; Kleespies, unpublished results). As D. nitidalis is related to C. perspectalis we aimed to test, whether AnfaNPV is also able to infect box tree larvae.

Material and methods

Viruses

The isolate AnfaNPV Dn10 originated from diseased larvae of pickleworm D. nitidalis (Stoll) (Lepidoptera: Pyralidae) and was obtained from Prof. Said El-Salamouny in Charleston (South Carolina, USA) (Jackson et al., 2009). The isolate AnfaNPV BI-235 had been stored in the JKI baculovirus collection. It likely originated from Hostetter & Puttlser (1991).

Bioassays

AnfaNPV BI-235 and Dn10 were propagated in second to fourth instar larvae of C. perspectalis. Occlusion bodies (OBs) were isolated according to standard procedures. Leaf disc bioassays were performed to determine the median lethal concentration (LC₅₀) (Eberle et
al., 2012). In short, purified virus stocks were diluted with PBT buffer (PBS, 0.1% (w/v) BSA, 0.025% (v/v) Tween 20) and six concentrations ranging from $10^4$ to $10^8$ OBs ml$^{-1}$ were prepared. Small pieces (ca. 4 mm x 5 mm) of box tree leaves with the upper dark green surface were placed onto 3% agar in separated wells of an autoclavable 50-well tray (Licefa, Bad Salzuflen, Germany). An aliquot of 1 µl of the OB dilution was pipetted on the leaf discs and allowed to dry for 1 to 1.5 h. Thirty to fifty neonate larvae were used for each concentration and the control (PBT buffer only). One neonate larva was then placed in each well and incubated at 26 ºC and a 16 h (light)/8 h (dark) photoperiod. Larvae which consumed more than 70% of the upper surface of leaf discs were supplemented with a fresh untreated box tree leaf after three days. Other larvae were discarded. Larval mortality was recorded seven days after initial virus exposure. Each test was replicated three times for each virus isolate. Mortality data were corrected by Abbott’s formula (Abbott, 1925) and the median lethal concentration was determined using probit analysis with the software ToxRat (ToxRat Solutions, Alsdorf, Germany).

**Microscopic investigations**

For transmission electron microscopic (TEM) investigations virus infected, moribund larvae of second to fourth instar were used. Larvae were dissected, fixed in 3% glutaraldehyd for 24 h, and washed three times in veronal buffer. Post-fixation was performed with 2% osmium tetroxide for 17 h. Then, the samples were washed three times in 2.5% sucrose solution, followed by staining with uranyl acetate wolfram phosphoric acid for 5 h. After stepwise dehydrating using ethanol the specimen were embedded in methacrylate. Ultrathin sections were examined on a Zeiss 902 TEM.

**Results and discussion**

The median lethal concentrations ($LC_{50}$) of the AnfaNPV isolates Dn10 and BI-235 were determined using leaf disc bioassays and probit analysis (Figure 1). In a seven day bioassay, the $LC_{50}$ values were $7.8 \times 10^5$ OBs ml$^{-1}$ (95% fiducial limits $5.5 - 11.6 \times 10^5$ OBs ml$^{-1}$, n = 685, slope: 1.23, $Chi^2 = 29.0$) for isolate BI-235 and $2.3 \times 10^6$ OBs ml$^{-1}$ (95% fiducial limits $1.4 - 3.9 \times 10^6$ OBs ml$^{-1}$, n = 680, slope: 1.36, $Chi^2 = 17.70$) for isolate Dn10. The difference between both isolates was statistically significant on the basis of non-overlapping fiducial limits. This suggested that BI-235 was more virulent than Dn10, with a potency of 2.98.

Infection of *C. perspectalis* larvae by AnfaNPV BI-235 and Dn10 was confirmed by light microscopy (data not shown) and transmission electron microscopic studies (Figure 2). Infection of fat body, tracheal matrix and epidermis cells of box tree moth larvae were observed.

Our results clearly indicate that *C. perspectalis* is susceptible to AnfaNPV. Thus, AnfaNPV might be a candidate for developing a biocontrol agent of *C. perspectalis* on the basis of baculoviruses. Further experiments will be necessary to determine its field efficacy.
Figure 1. Probit analysis of AnfaNPV BI-235 and Dn10 activity in neonate larvae of C. perspectalis.

Figure 2. Ultrathin section of fat body of C. perspectalis infected by AnfaNPV Dn10. Note the area of virogenesis (VS); NM = nuclear membrane, VP = virus polyhedron.
Acknowledgements

We are obliged to Prof. Said El-Salamouny (Charleston, South Carolina, USA, now King Saud University, Riyadh) for providing the isolate AnfaNPV Dn10. We thank Mrs. Helga Radke (JKI Darmstadt) for excellent technical assistance in microscopy and Dr. Annette Herz and Dr. Karolin Eberle (both JKI Darmstadt) for their help and advice in insect rearing and virus purification.

References


Interactions between structural proteins of *Chilo iridescent virus*

Emine Özşahin¹, Remziye Nalcacioglu¹, Just M. Vlak², Monique M. van Oers², Zihni Demirbağ¹

¹Karadeniz Technical University, Faculty of Sciences, Department of Biology, 61080 Trabzon, Turkey; ²Wageningen University, Laboratory of Virology, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

**Abstract:** Iridoviruses infect a broad range of hosts including invertebrate (especially insects), amphibians, reptiles and fish which have ecological and economic importance. Knowledge of the viral interactome, particularly amongst structural virion proteins, has led to an emerging picture of the protein–protein interactions important for viral entry, infection assembly, and egress. Previous studies indicated that *Chilo iridescent virus* (CIV), the type member of the virus from the genus Iridovirus, contains 46 virion structural proteins. In this study we aimed to identify the protein–protein interactions among these proteins by the yeast-two-hybrid-system. The structural genes were cloned into bait and prey vectors. The *Saccharomyces cerevisiae* AH109 strain was used for transfecting these vectors. Minimal synthetic defined (SD) media were used for identification of protein–protein interactions. Up to now, we have identified five interactions (118L-415R, 232R-142R, 337L-309L, 337L-117L, 337L-295L) among CIV structural proteins. We have confirmed the interaction between 118L and 415R by a GST pull-down assay. After completing these interactome studies, we will have a better picture of the structure of the virus and may be able to predict interactions between virus and its respective hosts.

**Key words:** *Chilo iridescent virus*, structural proteins, Yeast-two-hybrid, protein-protein interaction
Natural populations of Spodoptera exigua are infected by multiple viruses: implications for the production and use of virus insecticides

Cristina Virto\textsuperscript{1,2}, David Navarro\textsuperscript{3}, M. Mar Tellez\textsuperscript{3}, Salvador Herrero\textsuperscript{4}, Trevor Williams\textsuperscript{5}, Rosa Murillo\textsuperscript{1,2}, Primitivo Caballero\textsuperscript{1,2}  
\textsuperscript{1}Instituto de Agrobiotecnología, CSIC-Gobierno de Navarra, Ctra. de Mutiliva s/n, 31192 Mutiliva B, Spain; \textsuperscript{2}Departamento Producción Agraria, Universidad Pública de Navarra, Pamplona 31006, Spain; \textsuperscript{3}IFAPA, La Mojonera 04745, Almería, Spain; \textsuperscript{4}Departamento de Genética, Universidad de Valencia, Valencia 46100, Spain; \textsuperscript{5}Instituto de Ecología AC, Xalapa 91070, Mexico

\textbf{Abstract}: Covert infections of Spodoptera exigua multiple nucleopolyhedrovirus (SeM NPV) have been detected in laboratory or field populations of the homologous host, S. exigua. Two RNA viruses belonging to the Iflaviridae family (SeIV-1, SeIV-2) were identified in transcriptome studies from different laboratory colonies of S. exigua. The three viruses are vertically transmitted and establish persistent infections. For this reason, coinfection of individual insects by these viruses is considered likely. In this study, we determined the prevalence of covert infections caused by iflaviruses and SeM NPV in order to identify virus associations in natural S. exigua populations. SeM NPV was detected in the 54\% of field-caught adults, whereas 13\% and 8\% of insects were infected by SeIV-1 and SeIV-2, respectively. The prevalence of SeIV-1 and SeIV-2 in F\textsubscript{1} adults obtained in laboratory showed higher levels than in the parental generation, whereas the prevalence of SeM NPV decreased from parents to their offspring. These findings have important implications for the in vivo production of virus based insecticides using mass-reared insects and the efficacy of these products in controlling pest populations that may be coinfected by iflaviruses.

\textbf{Key words}: SeM NPV, iflavirus, covert infection, field population

\textbf{Introduction}

Spodoptera exigua is a serious pest of several crops grown in the greenhouses of southern Spain. The larvae are susceptible to viral pathogens which are capable of producing lethal and sublethal infections. The S. exigua multiple nucleopolyhedrovirus (SeM NPV) has been observed to produce epizootics in larval populations and a number of biopesticides based on this virus have been developed and commercialized for use against this pest. SeM NPV can be transmitted either horizontally, between members of a cohort, or vertically, from parents to offspring. The latter transmission route resulted in covert infections that could be detected in field-caught adults and their progeny (Cabodevilla et al., 2011a).

Recently an analysis of the transcriptome of S. exigua larvae revealed novel RNA viruses that have been identified as belonging to the Iflaviridae family (S. exigua iflavirus-1: SeI-1; S. exigua iflavirus-2: SeI-2) (Millán-Leiva et al., 2012; Choi et al., 2012). Very little is known about iflaviruses, but they have been reported in association with NPVs in previous studies; these viruses apparently do not cause lethal infection, but result in lower larval weight gain (Vail et al., 1983). The aim of this study was to evaluate the prevalence of baculovirus and iflavirus covert infections in a field population of S. exigua and to determine their capacity for vertical transmission to the offspring of infected parents.
Material and methods

Field sampling of S. exigua insects
Field adults of Spodoptera exigua were captured with UV light-traps in the greenhouses of southern Spain. Insects were reared individually in 25 ml plastic cups and allowed to lay eggs. After two days adults were frozen at -80 ºC and twenty four neonates from each female were individualized in cups containing diet and reared through to the adult stage (F₁) in laboratory conditions. F₁ adults were frozen at -80 ºC for the subsequent analysis.

Total DNA and RNA extraction and RT-PCR and qPCR analysis
For detection of covert infections, total DNA and RNA was obtained from the adult abdomens of both field-caught and F₁ generation insects, after sexing by observation of the external genitalia. Multiplex RT-PCR and qPCR, based on SYBR fluorescence, were used to determine the prevalence of individuals infected by iflaviruses and S. exigua multiple nucleopolyhedrovirus (SeMNPV), respectively.

Results and discussion

Prevalence of covert infections in field adults
Field-caught adults showed high levels of covert infections for SeMNPV: 54% were positive by qPCR. Detection of iflaviruses was far less frequent (19%). Males and females were infected at similar frequencies for both SeMNPV (P > 0.05) and iflaviruses (P > 0.05). Previous studies carried out during 2006 and 2007 detected SeMNPV covert infection in the 16% of field-caught adults by RT-PCR (Cabodevilla et al., 2011a). However, the qPCR-based technique used in this study allowed us to increase markedly the sensitivity of the analysis. SeIV-1 seems to be frequent and easily transmitted in S. exigua laboratory colonies (Millán-Leiva et al., 2012), but this is the first time that this virus has been detected in field-caught insects. Co-infections of both virus species were relatively rare, with just 14/130 individuals harbour both SeMNPV and one or both of the iflaviruses.

Trans-generational transmission
Five females either infected or non-infected by SeMNPV were randomly selected from field-caught adults that had produced offspring. Ten adults of the offspring per F₀ female were analyzed to determine transmission rates of SeMNPV and SelVs. All three viruses were capable of vertical transmission. Overall, SeMNPV vertical transmission resulted in low levels of F₁ covert infection (10-20% depending on mating treatment), whereas SelVs prevalence increased in F₁ respect to field-caught adults. The rearing conditions are also of particular relevance, as previous studies indicate that iflaviruses quickly spread through insect cultures in laboratory conditions (Millán-Leiva et al., 2012).

No significant differences were found in numbers of descendent positive for SelVs (36%) compare to those for SeMNPV (20%) in the offspring of covertly infected insects. However the proportion of F₁ adults detected positive for SelVs was significantly higher (76%) than that for SeMNPV (10%) in the offspring from non-infected females (P < 0.05). SelV infection was also detected in the offspring of infection negative females. This may be due to an infected male linage that could contribute to virus transmission. The prevalence of SelVs in the offspring from SeMNPV covertly infected females (36%) was significantly lower than that registered for the offspring from SeMNPV-free females (76%) (P < 0.05),
suggesting that the presence of NPVs negatively affect the spreading of the iflavirus covert infection.

As iflaviruses may affect the viability of insect colonies used for the mass-production of NPV-based insecticides, particular attention should be paid to the interactions between these viruses during virus production. Future studies should also address potential differences in the susceptibility to NPV infection in insects that already harbour iflavirus infections, as this has the potential to affect the efficacy of virus based insecticidal products used for pest control.

Acknowledgements

This study was funded by the Ministerio de Ciencia e Innovación (Spain), project number AGL2011-30352-C02-01. C.V. received a predoctoral fellowship from the Universidad Pública de Navarra.

References


Estimating the importance of maternal and paternal contributions to the vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeM NPV)

Cristina Virto\(^1\), Carlos A. Zárate\(^1\), Rosa Murillo\(^{1,2}\), Primitivo Caballero\(^{1,2}\), Trevor Williams\(^3\)

\(^1\)Instituto de Agrobiotecnología, CSIC-Gobierno de Navarra, 31192 Mutilva Baja, Navarra, Spain; \(^2\)Departamento de Producción Agraria, Universidad Pública de Navarra, 31006 Pamplona, Navarra, Spain; \(^3\)Instituto de Ecología AC, Xalapa, Veracruz 91070, Mexico

**Abstract:** Vertical transmission of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeM NPV) is believed to be a common feature in field populations of *S. exigua*. To assess whether gender affects trans-generational virus transmission, four mating groups were performed using healthy and sublethally infected insects: i) healthy males (H\(^{♂}\)) × healthy females (H\(^{♀}\)); ii) infected males (I\(^{♂}\)) × healthy females (H\(^{♀}\)); iii) healthy males (H\(^{♂}\)) × infected females (I\(^{♀}\)) and iv) infected males (I\(^{♂}\)) × infected females (I\(^{♀}\)). These adults and their offspring were analyzed by qPCR to detect SeM NPV infection. Both males and females were able to transmit the infection to the next generation, although females infected a higher percentage of the offspring and female-mediated transmission was more consistent than that of males. Venereal transmission appeared to be half as effective as maternally-mediated transmission, and the main route of transmission is likely transovarial rather than transovum. The prevalence of the infection in the offspring did not vary according to gender, therefore both males and females can be infected by their parents in similar proportions. Incorporating vertically-transmitted genotypes in biological insecticides might have the potential for reducing pest densities and extending periods between virus applications.

**Key words:** SeM NPV, covert infection, gender, trans-generational transmission

**Introduction**

Baculoviruses are the most extensively studied arthropod-specific viruses due to their extremely high virulence to certain insect pests and for their biosafety characteristics for the production of recombinant proteins (Moscardi, 1999). Baculovirus survival involves two transmission pathways. Viral occlusion bodies (OBs) are responsible for horizontal transmission between larvae that is thought to be the major pathway of baculovirus transmission. Little is known about vertical transmission of viruses in insects, but this has been proposed as a survival strategy to overcome periods of host population scarcity and to facilitate the virus dispersal to geographically distant niches. Trans-generational transmission involves covertly infected adults that pass virus to their progeny via the transovum or transovarial pathway (Cory & Myers, 2003).

A previous study showed that *Spodoptera exigua* females with no evidence of a nucleopolyhedrovirus (NPV) covert infection produced virus-infected offspring after mating with field-caught males. This led us to suspect that both males and females may contribute to vertical transmission of the pathogen. However, the differential prevalence of covert infection between males and females (Cabodevilla et al., 2011), suggests a possible gender effect on the transmission process. In this study we analyzed the effect of gender on the transmission efficiency of covertly infected *S. exigua* to their progeny.
Material and methods

Insect and virus
The experiment was performed with a virus-free laboratory colony of S. exigua. A single genotype of SeMNPV, named VT-SeAl1, was used in the experiment. This genotype was previously isolated from sublethally infected insects collected in the greenhouses of Almeria (Spain) and was transmitted from parents to offspring.

DNA extraction and detection of covert infections
Total DNA was extracted from the abdomens of adults. Quantitative PCR based on SYBR fluorescence was performed to detect SeMNPV infection. Specific primers were designed to amplify a 149-bp region of the DNA polymerase gene based on the complete genome sequence of the SeMNPV strain VT-SeAl1 (unpublished data). For the standard curve VT-SeAl1 DNA was extracted from OBs, purified through CsCl gradients, quantified using a spectrophotometer and then serially diluted to the following concentrations: 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 pg µl⁻¹. Quantified viral DNA was normalized based on the total DNA concentration for each sample and measured using NanoDrop 2000.

Bioassays
To determine the influence of gender on vertical transmission, groups of adults either sublethally infected (infected males: I♂ and infected females: I♀) or virus-free adults (healthy males: H♂ and healthy females: H♀) were required. Sublethally infected insects were produced from a virus-free insect culture using 200 S. exigua fourth instars treated with 9 x 10³ OB ml⁻¹ suspension. A group of 100 larvae were treated in the same conditions using a solution without OBs. Surviving insects were reared, sexed and then classified in separate groups according to their sex and viral treatment. Once the adults emerged, the following mating treatments were performed: i) healthy males (H♂) × healthy females (H♀); ii) infected males (I♂) × healthy females (H♀); iii) healthy males (H♂) × infected females (I♀) and iv) infected males (I♂) × infected females (I♀). Five adult pairs were confined in paper bags for oviposition. Eggs batches from each treatment group were harvested and the adults frozen for subsequent analysis (F₀ generation). Egg masses from each paper bag were divided into two parts, and either soaked in a 0.25 ppm hypochlorite solution (surface decontamination) or in distilled water (no decontamination) for five minutes. Twenty-five neonates were individually reared on semi-artificial diet through to adult stage (F₁) and then frozen for subsequent analysis. The whole procedure was performed four times.

Results and discussion
Of the larvae initially treated with VT-SeAl1 OBs, 58% succumbed to virus infection, whereas no mortality was registered in mock-infected control larvae. The sensitivity for the qPCR reaction was estimated at 1 x 10⁻³ pg genomic DNA, which equates theoretically to between 6 and 7 viral genome copies.

The frequencies of qPCR positive survivors to a virus challenged were 15-85%, significantly higher than those measured in control insects (10-15%; P < 0.001). Viral load in F₀ parental adults averaged 10.3 ± 2.0 genome copies per adult (N = 72, positives for qPCR).

Sublethally infected males that mated healthy females produced offspring with 26% of infected individuals on average, compared to 8% in the offspring of the control insects. In contrast, in the mating groups in which the females were sublethally infected, the prevalence
of covert infection in offspring varied between 44% and 49%. Therefore, female-mediated vertical transmission was approximately twice as efficient as male-mediated transmission.

The prevalence of infection in F₁ adults did not differ significantly according to the surface decontamination treatment (P > 0.05). This result is in agreement with recent studies on Spodoptera exempta nucleopolyhedrovirus in which surface decontamination of eggs did not affect the detection of the virus in the offspring of infected insects (Vilaplana et al., 2008), suggesting that transovarial, rather than transovum transmission represents the most likely pathway for transmission.

Studies with Drosophila sigma virus, have indicated that transmission rates are higher in females than males (Longdon et al., 2011), although transmission has been observed to occur through both eggs and sperm. In contrast, studies on ganuloviruses (genus Betabaculovirus) demonstrated that both sexes were involved in vertical transmission for Plodia interpunctella granulovirus, with viral particles present in both testis and ovaries of sublethally infected individuals by viral transcript detection (Burden et al., 2002).

Mean values of viral load in F₁ adults did not differ significantly between mating groups (P > 0.05) i.e., the quantity of viral DNA per sublethally infected insect was independent of the parental lineage passing on the virus (male, female or both). In contrast, Longdon et al. (2011) detected lower titres of sigma virus in the embryos of two Drosophila species when the virus was paternally transmitted.

Acknowledgements

We thank N. Gorria for technical assistance. This study was funded by the Ministerio de Ciencia e Innovación (Spain), project number AGL2011-30352-C02-01. C.V. received a predoctoral fellowship from the Universidad Pública de Navarra.

References


Baculoviruses for the biological control of cutworms (Agrotis spp.)

Jörg T. Wennmann, Gianpiero Gueli Alletti, Johannes A. Jehle
Institute for Biological Control, Julius Kühn Institute (JKI) Federal Research Center for Cultivated Plants, Heinrichstr. 243, 64287 Darmstadt, Germany

Abstract: Caterpillars of the common cutworm Agrotis segetum and black cutworm A. ipsilon (Lepidoptera: Noctuidae) are wasteful feeders of various crops in agriculture. These cutworms are mainly controlled by chemical pesticides but recent attempts are aimed to control these soil pests by the application of baculoviruses. Four different baculoviruses, namely Agrotis segetum nucleopolyhedrovirus A (AgseNPV-A), Agrotis segetum nucleopolyhedrovirus B (AgseNPV-B), Agrotis ipsilon multiple nucleopolyhedrovirus (AgipMNPV) and Agrotis segetum granulovirus (AgseGV), were isolated from larvae of A. segetum and A. ipsilon and are considered as potential biocontrol agents. In natural infections, larvae of both hosts are susceptible to all four viruses and individual caterpillars of the common cutworm were observed to become infected simultaneously by AgseNPV-B and AgseGV. Co-infections may be advantageous in terms of virulence and resistance management, although the level of interaction is critical. To test for a mutualistic, neutral or antagonistic interaction and to evaluate a combined application of Agrotis-specific baculoviruses, we exemplary performed mixed infection experiments of A. segetum larvae that were exposed to AgseNPV-B and AgseGV at different concentration. For quantitative analysis of the outcome of mixed infections as well as for quality control in virus production a reliable method for detection and discriminative quantification for Agrotis-specific baculoviruses is required. We established a multiplex PCR analysis based on highly specific oligonucleotides which also permit quantification by quantitative PCR. As a prerequisite of these studies the genome of AgseNPV-B was completely sequenced by 454 sequencing technique. Comparative genome sequence analyses gave a detailed insight into the molecular setup of the three Agrotis-specific NPVs and confirmed that they can be regarded as three different but close related species. Our results will help to develop and evaluate Agrotis-specific baculoviruses as biocontrol agents and to understand the evolutionary co-existence of viruses that are highly adapted to the same hosts.

Key words: cutworms, Agrotis, baculoviruses, pest control, co-infection

Introduction

Caterpillars of several noctuid moths that live in soil and feed on roots of plants are described as cutworms. The term cutworms includes moth of the genera Agrotis, Euxoa, Noctua, Peridroma and Xestia (Bourner & Cory, 2004). They are worldwide distributed and known as wasteful feeders on various agricultural plants. Two highly harmful cutworms are known from the genus Agrotis (Noctuidae): the common cutworm Agrotis segetum (Denis & Schiffermüller) and the black cutworm Agrotis ipsilon (Hufnagel). To date, cutworms (Agrotis spp.) are mainly controlled by chemical pesticides (e. g. pyrethroids). Baculoviruses are considered as biological control agents for a sustainable control of the common and black cutworm in agriculture and have been already successfully tested for the control of these wasteful soil pests (Bourner et al., 1992). In the past, three different nucleopolyhedroviruses (NPV) and one granulovirus (GV) were isolated and characterized from A. segetum (Agse) and A. ipsilon (Agip) larvae: AgseNPV-A (Jakubowska et al., 2005, 2006), AgseNPV-B (Alaway & Payne, 1983), AgipMNPV (Boughton et al., 1999; Harrison, 2009) and AgseGV
As only limited genetic information of AgseNPV-B was available, its genome was completely sequenced and whole genome comparisons of Agrotis-specific NPVs were performed. Bioassays revealed that A. ipsilon and A. segetum are susceptible to more than one Agrotis baculoviruses (El-Salamouny et al., 2003; Bourner & Cory, 2004). Co-infections between AgseGV and AgseNPV-B are frequently observed, although their type of interaction is not yet well understood. However, in terms of resistance management a combined application of two baculoviruses is considered as beneficial and occurring interactions may be critical for a successful application. Mainly three different types of interactions are conceivable: mutualism, neutralism and antagonism. To investigate and optimize the potential application of the four known Agrotis baculoviruses for cutworm control, the susceptibility of A. segetum and A. ipsilon larvae to these viruses needs to be determined not only in single but also in mixed infections. In this study, the level of interaction was investigated for AgseNPV-B and AgseGV in simultaneously infected A. segetum larvae. The present results help to understand the Agrotis baculovirus complex, how closely related baculoviruses evolved in the same host genera, how they differ on the molecular level and how they interact in co-infections.

**Material and methods**

**Whole genome sequencing**

Purified genomic DNA of AgseNPV-B was completely sequenced by 454 whole genome sequencing technique. Reads were assembled to a consensus sequence and open reading frames and homologous repeat (hr) regions were annotated by GeneQuest Software (DNASTAR Lasergene v8.1.4). Baculoviruses share 30 core genes that were found in all completely sequenced and published virus genomes. The concatenated sequences of all 30 core genes of AgseNPV-B and other selected baculoviruses were used to infer their phylogenetic relationship by maximum parsimony analysis.

**PCR based detection, discrimination of Agrotis baculoviruses**

Based on the complete polyhedrin and granulin sequences of AgseNPV-A, AgseNPV-B, AgipM NPV and AgseGV, four highly specific different pairs of oligonucleotide primers, one pair for each virus, were designed. To discriminate between all four viruses, the resulting PCR products differed in size. The primers also allowed a multiplex PCR amplification, which allowed detecting all four baculoviruses within a single PCR reaction.

**Bioassays**

Bioassays for AgseNPV-B, AgipM NPV and AgseGV were performed for neonate A. segetum larvae. For each virus, larvae were fed on semi-artificial diet containing distinct concentrations of virus occlusion bodies (OB). Fifty L1 larvae of A. segetum were used for each concentration and mortality was scored after 14 d post infection (p.i.). The bioassays were repeated in triplicate. Median lethal concentrations (LC\textsubscript{50}) and 10% mortality (LC\textsubscript{10}) were calculated by probit analyses.

**Mixed infection studies**

The LC\textsubscript{50} and LC\textsubscript{10} (14 d p.i.) of AgseNPV-B and AgseGV were used for co-infection experiments. Neonate A. segetum larvae were exposed to combined lethal concentrations that were previously determined by bioassays (14 d p.i.): LC\textsubscript{50}:LC\textsubscript{50}, LC\textsubscript{50}:LC\textsubscript{10}, LC\textsubscript{10}:LC\textsubscript{50},
Each treatment comprised 25 neonate larvae and was repeated six times. Mortality was scored after 14 days and cadavers were individually collected. Viral genomic DNA was isolated from dead larvae and the production of AgseNPV-B and AgseGV per larva was determined by quantitative (q) PCR using the designed, highly specific PCR primers for AgseGV and AgseNPV-B.

Results and discussion

The completely sequenced AgseNPV-B genome showed a high similarity in GC content, number of detected ORFs and genomic length to AgseNPV-A (Table 1). Despite these overall similarities, the DNA sequence of AgseNPV-B appeared to me more similar to that of AgipMNPV than to that of AgseNPV-A. This was proven by maximum parsimony phylogenetic analysis based on the concatenated 30 baculovirus core genes. According to this analysis AgseNPV-B is more close related to AgipMNPV than to AgseNPV-A. Whole genome comparisons of AgseNPV-A, AgseNPV-B and AgipMNPV also revealed that the arrangement of ORFs and homologous repeat regions (hr) were highly similar to each other.

Table 1. Characteristics of the genomes of AgseNPV-B, AgseNPV-A, AgipNPV and AgseGV.

<table>
<thead>
<tr>
<th></th>
<th>length (bp)</th>
<th>ORF</th>
<th>% GC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgseNPV-B</td>
<td>148,986</td>
<td>154</td>
<td>45.69</td>
<td>this study</td>
</tr>
<tr>
<td>AgseNPV-A</td>
<td>147,544</td>
<td>153</td>
<td>45.71</td>
<td>Jakubowska et al. (2006)</td>
</tr>
<tr>
<td>AgipMNPV</td>
<td>155,122</td>
<td>163</td>
<td>48.57</td>
<td>Harrison (2009)</td>
</tr>
<tr>
<td>AgseGV</td>
<td>131,680</td>
<td>132</td>
<td>37.31</td>
<td>GenBank (NC_005839)</td>
</tr>
</tbody>
</table>

In mixed infections using different concentrations of AgseNPV-B and AgseGV no increase of mortality was observed compared to single infections (data not shown). It could be observed that the amount of co-infected larvae was dependent on the applied AgseNPV-B concentration and that a higher AgseNPV-B concentration reduced the production of AgseGV per larva. However, the AgseGV concentration in mixed virus treatments did not seem to affect the amount of produced AgseNPV-B per larva. It could be concluded from the data, that no mutualism was found, rather a competition for resources.

Based on the polyhedrin and granulin gene sequences of the four Agrotis baculoviruses oligonucleotides to be used in PCR were designed. The oligonucleotides did not show any undesired binding in multiplex PCR control reactions and were also fully functional for qPCR analyses. Bioassays showed that A. segetum larvae are less susceptible to AgipMNPV (LC$_{50} = 7.3 \times 10^3$ OB ml$^{-1}$) and AgseGV (LC$_{50} = 27.0 \times 10^3$ OB ml$^{-1}$) than to AgseNPV-B (LC$_{50} = 3.3 \times 10^3$ OB ml$^{-1}$) (Table 2). Furthermore, the speed of killing of AgseGV was rather low and resulted in a high LC$_{50}$ value after 7 and 14 d p.i.
Table 2. Median lethal concentration (LC<sub>50</sub>) of AgseGV, AgseNPV-B and AgipMNPV in 7-day bioassays in neonate A. segetum larvae.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (95% CL) [OB ml&lt;sup&gt;-1&lt;/sup&gt;] (x10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>LC&lt;sub&gt;10&lt;/sub&gt; (95% CL) [OB ml&lt;sup&gt;-1&lt;/sup&gt;] (x10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Slope</th>
<th>df</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgseGV</td>
<td>27 (5-131)</td>
<td>0.1 (0.001-0.7)</td>
<td>0.53</td>
<td>3</td>
<td>24.53</td>
</tr>
<tr>
<td>AgseNPV-B</td>
<td>3.28 (2.62-4.00)</td>
<td>0.34 (0.21-0.50)</td>
<td>1.30</td>
<td>3</td>
<td>9.56</td>
</tr>
<tr>
<td>AgipNPV</td>
<td>7.29 (5.90-8.90)</td>
<td>0.55 (0.36-0.79)</td>
<td>1.15</td>
<td>3</td>
<td>8.94</td>
</tr>
</tbody>
</table>

Acknowledgements

We thank Tim Köhler for helping to conduct the mixed virus experiments.

References


Posters
Insecticidal activity of a spray dried formulation based on a Colombian Spodoptera frugiperda nucleopolyhedrovirus

Judith Elena Camacho¹, Martha Isabel Gómez², Mauricio Cruz², Laura Fernanda Villamizar²
¹Doctorado en Biociencias, Universidad de La Sabana, Chía, Colombia; ²Center of Biotechnology and Bioindustry, CORPOICA, Mosquera, Colombia

Abstract: A Colombian Spodoptera frugiperda nucleopolyhedrovirus (SfMNPV 003) with high potential for the development of an efficient biopesticide was microencapsulated by top spray drying with a pH dependent polymer (Eudragit® S100) and its insecticidal activity was evaluated under laboratory and greenhouse conditions. Significant differences between LC₅₀ values of the microencapsulated virus, the dried virus and the virus without any treatment were not detected under laboratory conditions, suggesting that microencapsulation by top spray drying did not affect the virus insecticidal activity. Three different microencapsulated batches showed the same efficacy under greenhouse conditions and significant differences between formulated and unformulated virus were not detected (p > 0.05). In conclusion, SfMNPV003 insecticidal activity was not affected by formulation process and developed biopesticide demonstrated its potential for S. frugiperda control and could be included in programs of integrated pest management (IPM).

Key words: Microencapsulation, insecticidal activity, baculovirus, armyworm

Introduction

Baculoviruses have been widely studied due to their high pathogenicity for a large number of species of insects considered as pests (Miller, 1997; Moscardi, 1999; Caballero et al., 2001). However, one limitation for its use is the activity losses observed under field conditions (Caballero et al., 2001), being necessary to develop formulations able to reduce this inactivation. The microencapsulation constitutes a promising technique to obtain stable baculovirus formulations (Villamizar et al., 2010). Microencapsulation with polymeric materials is very useful for biopesticides development because this process can protect viral particles from environmental conditions as UV light or temperature. Moreover, these are resistant to rain and dew and can be easily dispersed in the air to be consumed by insect larvae (Winder et al., 2003).

S. frugiperda J E Smith (1797) (Lepidoptera: Noctuidae) known as armyworm causes important economic losses in different crops as sorghum, rice and maize (García & Del Pozo, 1999). A native S. frugiperda nucleopolyhedrovirus (SfMNPV) with high potential as biopesticide (Gomez et al., 2010) was microencapsulated by oil-in-oil emulsion (O/O) solvent evaporation method and formulation improve virus photostability (Villamizar et al., 2010). Considering the risks of working with organic solvents for microencapsulation by the developed method a new formulation process by using top spray drying was proposed. Top spray drying allows to process extremely heat-sensitive materials as baculoviruses, due to the short drying times and the low product temperatures. Moreover, this technique offers attractive advantages for producing microcapsules in a relatively simple, inexpensive and continuous operation. (Horazeck & Viernstein, 2004). The present study was conducted to
determine the effect of the new formulation process by spray drying over the SfMNPV 003 insecticidal activity.

**Material and methods**

**Virus production**

Virus occlusion bodies (OBs) production was conducted by inoculating third instar larvae of *S. frugiperda* with a SfMNPV 003 suspension using the droplet feeding method (Hughes & Wood, 1981). OBs were recovered from dead larvae by mixing and filtering and virus was then dried in a fluid bed with an internal pressure of 1 bar, a flow rate of 6.18 ml min\(^{-1}\), an inlet temperature of 92 ± 5 °C and an opening gate inlet air angle of 25° at the start of process and 35° at the end.

**Microencapsulation by top spray drying**

Microencapsulated product was prepared by spraying an aqueous suspension of dried virus (1.35% w/v) and Eudragit® S100 (6.0% w/v) in a Glatt Uni Glatt 01277 fluid bed dryer, equipped with a nozzle to 1.0 mm to adjust the airflow. The operation conditions were an inlet temperature of 80 ± 5 °C, an internal pressure of 2.23 bars, a flow rate of 4.12 ml min\(^{-1}\) and an opening gate inlet air angle of 25°.

**Insecticidal activity under laboratory conditions**

The bioassay was carried out following the methodology described by Hughes & Wood (1981). Suspensions were prepared and adjusted to five concentrations between 2.0 x 10\(^3\) to 2.0 x 10\(^8\) OBs ml\(^{-1}\) for the three microencapsulated product batches. Control treatment consisted in non-treated larvae. Experimental design was completely randomized (CRD) with factorial arrangement and three replications per treatment, each one with 30 larvae. Mortality was determined seven days after inoculation and results were analyzed by Probit analysis (Finney, 1952) in order to determine LC\(_{50}\) values (Biostat, 2007).

**Insecticidal activity under greenhouse conditions**

Plants of maize (*Zea mays* L.) ICA 508 variety (special for cold weather) were grown in soil under greenhouse conditions. Randomized complete block design (RCBD) with four replicates was used. Treatments were three batches of microencapsulated product and unformulated dried virus both adjusted to 1 x 10\(^7\) OBs ml\(^{-1}\) and a control without any application. The experimental unit consisted in a row of 1.5 m long with 10 plants and 15 cm of planting distance. The crop was subjected to usual irrigation, fertilization and weed control conditions. Thirty days after sowing, plants were sprayed with 2 ml of treatments by using a handheld sprayer. One hour after application, two second instar larvae of *S. frugiperda* were placed per plant. After two days, 10 larvae from each replicate per treatment were collected. Larvae were placed in separate plastic cups containing artificial diet and incubated at the laboratory at 28 ± 2 °C and at 60% of relative humidity. Mortality rate was assessed after seven days and efficacy was determined using the Schneider-Orelli’s formula (Zar, 1999). The normality of the data was estimated by Shapiro-Wilk test and homogeneity of variance using Bartlett’s test. Differences between treatments were evidenced by ANOVA and Tukey test (\(\alpha = 0.05\)) with the program Statistic 8.1.
Results and discussion

Microencapsulation by top spray drying

Three microencapsulated product batches presented a mean viral concentration of $1.05 \times 10^{9}$ OBs g$^{-1}$, a particle size of 18.84 µm and a moisture content of 10.38%.

Insecticidal activity under laboratory conditions

Treated larvae with microencapsulated product and unformulated dried virus showed typical signs of infection as change of color from pink to dark brown, development delay and reductions in feeding and mobility (Moscardi, 1999). Diseased larvae present a brittle tegument which is easily broken delivering a brown fluid mainly corresponding to OBs (Caballero et al., 2001).

The LC$_{50}$ values for three different batches of microencapsulated product are presented in Table 1. The comparison of the confidence limits (95%) did not reveal significant differences between the LC$_{50}$ of three batches suggesting repeatability during the manufacture process. These results were compared with the LC$_{50}$ determined previously for unformulated and purified virus and even the lethal concentration for microencapsulated product is lower than obtained for unformulated virus, fiducial limits comparison suggest that both treatments has the same pathogenicity and microencapsulation process by top spray drying with Eudragit® S100 did not affect the insecticidal activity of viral isolate SfMNPV 003, even internal temperature during spraying drying processes was 42.15 ± 5 °C, which did not significantly inactivated the virus.

Table 1. Mean lethal concentrations of microencapsulated and unformulated virus.

<table>
<thead>
<tr>
<th>Batch</th>
<th>LC$_{50}$ (OBs ml$^{-1}$)</th>
<th>95% fiducial limits (OBs ml$^{-1}$)</th>
<th>p</th>
<th>X$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$1.3 \times 10^4$</td>
<td>$2.8 \times 10^2$</td>
<td>$6.4 \times 10^5$</td>
<td>0.53</td>
</tr>
<tr>
<td>2</td>
<td>$3.1 \times 10^4$</td>
<td>$9.1 \times 10^2$</td>
<td>$1.0 \times 10^6$</td>
<td>0.63</td>
</tr>
<tr>
<td>3</td>
<td>$3.1 \times 10^4$</td>
<td>$4.0 \times 10^3$</td>
<td>$1.4 \times 10^5$</td>
<td>0.94</td>
</tr>
<tr>
<td>Average</td>
<td>$2.5 \times 10^4$</td>
<td>$1.7 \times 10^3$</td>
<td>$5.9 \times 10^5$</td>
<td>0.70</td>
</tr>
<tr>
<td>Unformulated virus (Gómez et al., 2010)</td>
<td>$2.3 \times 10^5$</td>
<td>$5.4 \times 10^4$</td>
<td>$4.7 \times 10^6$</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Insecticidal activity under greenhouse conditions

Two days after treatments applications the damage caused by the larvae was lower in plants applied with the viral treatments compared with the control (no application). The main damage caused by S. frugiperda occurs on the whorl leaves, being higher in not treated plants (control) (Figure 1).

Young larvae of S. frugiperda make scratches on the soft parts of the leaves, which then appear as small translucent areas. When larvae are in advanced instars begin to make perforations or areas feed when are opened the leaves. In this phase is characteristic observe larval wastes (Negrete & Morales, 2003). Symptomatic larvae were only observed in plants from virus applied treatments. Efficacies of three batches of microencapsulated product applied at $1 \times 10^7$ OBs ml$^{-1}$ were 82.36%, 87.40% and 62.22% respectively with an average
value of 77.32% and unformulated virus reached an efficacy of 77.33%. Differences between means were detected using Tukey's test (95%), which did not detect significant differences between all viral treatments (p>0.05) confirming the repeatability between product batches and suggesting that the developed formulation and top spray drying process did not affect viral activity.

The microcapsules produced by the method of microencapsulation by oil-in-oil emulsion (O/O) solvent evaporation improved virus photostability, but presented residues of organic solvent in the formulation (Villamizar et al., 2010), while microcapsulation by top spray drying avoided this residues and microcapsules showed a smaller particle size (18.84 µm) than obtained with the solvent evaporation method (198 µm). In conclusion, top spray drying method demonstrated high potential for being used as formulation process in order to obtain microcapsules based on SfMNPV 003, biopesticide that could be included in integrated pest management programs.

Figure 1. Recent damage produced by S. frugiperda in maize plants, (a) untreated plant (b) treated plant.

References


Cydia pomonella granulovirus knockout mutants: The potential role of pe38 in overcoming codling moth resistance

Manuela Gebhardt, Karolin E. Eberle, Johannes A. Jehle
Institute for Biological Control, Julius Kühn Institute (JKI), Federal Research Center for Cultivated Plants, Heinrichstraße 243, 64287 Darmstadt, Germany

Abstract: The Cydia pomonella granulovirus (CpGV) belongs to the genus Betabaculovirus of the family Baculoviridae. CpGV is a worldwide used biological agent to control the infestation of apples, pears and walnuts by codling moth (C. pomonella L.). In 2005, the first resistance of field populations of C. pomonella (CM), with up to 1000-fold reduced susceptibility to CpGV products containing the isolate CpGV-M (found 1964 in Mexico), was discovered in Europe. Since then, several CpGV isolates (e.g. CpGV-I12, -S) have been found that were able to overcome the resistance of CM under laboratory conditions. Molecular analysis of different CpGV isolates have shown that the only genomic differences, which all resistance overcoming isolates have in common, are an insertion of 24 nucleotides in the early gene pe38 (Eberle & Jehle, unpublished). Preliminary results suggest that recombinant CpGV with a knockout of pe38 loses their ability to infect susceptible or resistant CM larvae. The aim of this work is to confirm the role of pe38 in overcoming the resistance of CM by creating knockout and rescue mutants based on an already existing CpGV-M bacmid. According to the source of pe38 of either resistance overcoming isolate (e.g. CpGV-S) or non resistance overcoming isolate (e.g. CpGV-M), we assume that the recombinant viruses should be infective against susceptible larvae only - in the case of pe38 from CpGV-M - or against both susceptible and resistant larvae - in the case of pe38 from e.g. CpGV-S. Results of the strategy of elucidating the viral mechanism of overcoming CpGV resistance will be presented.

Key words: resistance, baculovirus, codling moth
Sequence analysis of CpGV-R5 isolate, able to efficiently control CpGV-M resistant insects: relation between biological activity and genome

Benoit Graillot1,2, Samantha Besse2, Christine Blachère-Lopez1,3, Jérôme Olivares4, Myriam Siegwart4, Miguel López-Ferber1

1 LGEI, Ecole des Mines d’Alès, Institut Mines-Telecom, 6, Avenue de Clavières, 30319 Alès, France; 2 NPP (Arysta LifeScience), 35 Avenue Léon Blum, 64000 Pau, France; 3 INRA, 6, Avenue de Clavières, 30319 Alès, France; 4 INRA, Agroparc, 84914 Avignon Cedex 9, France

Abstract: The CpGV-R5 isolate is able to overcome resistant populations of codling moth to the CpGV-M isolate. The complete sequences of CpGV-R5 and the CpGV-M used for industrial production at Natural Plant Protection have been determined. Among the differences found, some are specific to the R5 isolate, others are common to various isolates able to overcome the resistance, like modification on the p38 gene product.

Key words: Granulovirus, CpGV, resistance, sequence analysis

Introduction

The first isolate of the Cydia pomonella granulovirus, CpGV, was found in Mexico (CpGV-M (Tanada, 1964). CpGV is used in biological control against codling moth. In Europe, all commercial formulations of CpGV are derived from the same CpGV-M isolate (Lacey et al., 2008). In 2005, first cases of resistance were detected in Germany (Fritsch et al., 2005) and France (Sauphanor et al., 2006). Researches conducted in various laboratories and companies resulted in the characterization of various virus isolates that could control the CpGV-M resistant insects (Berling et al., 2009; Eberle et al., 2009; Rezapanah et al., 2008; Zingg, 2011). Among them, the CpGV-R5 has been selected for commercialization by Natural Plant Protection (Arysta LifeScience) (NPP) (Besse et al., 2011). The complete sequence of CpGV-M is known (NC_002816.1) (Luque et al., 2001). Comparison between the sequences of these new isolates can allow the identification of the virus genes involved in resistance by-passing, in a first step to unveil the modification in the host-virus relationships in CpGV-M resistant insects. This approach has been used with various CpGV isolates (Eberle et al., 2009).

In this study the sequences of the CpGV-M isolate presently used by NPP (CpGV-MNPP) and the CpGV-R5 isolate have been determined and compared to the reference sequence of CpGV-M1 (Luque et al., 2001).

Material and methods

Virus amplification and DNA extraction

All viruses used were provided by Natural Plant Protection (Arysta LifeScience) (NPP). NPP-R1 was previously described by Berling and coworkers (Berling et al., 2009). NPP-R5 was derived from NPP-R1 through selection by passing on RGV resistant insects. CpGV-MNPP is the stock used for Carpovirusine™ production. It comes from the original Mexican isolate
All viruses were amplified on SV larvae as previously described. Occlusion bodies were purified and viral DNA extracted as described in Berling et al. (2009).

**Sequencing**
Sequencing was carried out by the GENTYANE platform of genotyping (INRA UMR 1095, Clermond-Ferrand, France) using a shotgun approach. For closing the remaining sequencing gaps, a primer walking strategy was used. Purified PCR amplicons were sequenced by Eurofins MWG (Ebersberg, Germany).

**PCR amplification and gel purification of amplicons**
Specific primers were designed close to the borders of the gaps in the DNA sequences, using the sequence of CpGV-M previously published (Luque et al., 2001) as a reference. PCR reactions were carried out using standard protocols. The presence of the amplicon was controlled on a 1% agarose gel stained with ethidium bromide. The amplicons were gel purified using the QIAquick gel extraction kit (Quiagen) following manufacturer instructions.

**Genome assembly and sequence analysis**
The sequences were assembled and analyzed using Clone manager V9 (SCI-ED software). Web available programs were used for sequence comparison.

**Results and discussion**

Table 1. Main differences observed between CpGV-M1 and CpGV-R5. △ indicates deletions and ▼indicates insertions.

<table>
<thead>
<tr>
<th>CpGV-R5 ORF</th>
<th>CpGV-M1 ORF name</th>
<th>position in CpGV-M1</th>
<th>length (nt)</th>
<th>length (aa)</th>
<th>position and type of aa differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>7</td>
<td>ie-1</td>
<td>3392 &lt; 4858</td>
<td>493</td>
<td>E367D, ▼VPHW463, R466I</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>pe38</td>
<td>18574 &lt; 19722</td>
<td>374</td>
<td>△312-319, K355Q, Q355K</td>
</tr>
<tr>
<td>32</td>
<td>32</td>
<td>27331 &gt; 28671</td>
<td>1341</td>
<td>447</td>
<td>△14-21, L71M, ▼TEEELQQNT120, V250A, V264A, M429T</td>
</tr>
<tr>
<td>46</td>
<td>46</td>
<td>mp-nase</td>
<td>36835 &lt; 38472</td>
<td>546</td>
<td>V85L, E177D, ▼G472</td>
</tr>
<tr>
<td>87</td>
<td>87</td>
<td>lef-5</td>
<td>68491 &lt; 69219</td>
<td>242</td>
<td>A87L, E88R, N89T, P90T</td>
</tr>
</tbody>
</table>
Sequence comparison between CpGV-M1 and CpGV-MNPP

The CpGV-MNPP sequence is similar to the CpGV-M1 sequence (Luque et al., 2001). Thirty one changes affecting ORFs were found, some contributing to fuse two contiguous ORFs. The most important change is located in ORF32, with a series of substitutions. All SNPs in polh and lef8 of CpGV-MNPP are conserved (Eberle et al., 2009). Fourteen other differences are detected in non-coding regions. A 16 nt insertion is found between ORFs 15 and 16, and a variability region is located between ORFs 50 and 51.

Figure 1. Partial alignment of the predicted amino acid sequences of various isolates of CpGV at the ORF7 (IE-1) and ORF24 (PE38).

Sequence comparison between CpGV-M1 and CpGV-MNPP

IE-1 CpGV-M1        351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVFVFNMKVRDNTQRYRINCF
IE-1 CpGV-MNPP      351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVFVFNMKVRDNTQRYRINCF
IE-1 CpGV-R1.8      351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVFVFNMKVRDNTQRYRINCF
IE-1 CpGV-R5        351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVFVFNMKVRDNTQRYRINCF

IE-1 CpGV-M1        401 RMDSVHVWNSSVMYSDVQFNLKKMIQRHRWGTHHILQFDYMNSMMSKL
IE-1 CpGV-MNPP      401 RMDSVHVWNSSVMYSDVQFNLKKMIQRHRWGTHHILQFDYMNSMMSKL
IE-1 CpGV-R1.8      401 RMDSVHVWNSSVMYSDVQFNLKKMIQRHRWGTHHILQFDYMNSMMSKL
IE-1 CpGV-R5        401 RMDSVHVWNSSVMYSDVQFNLKKMIQRHRWGTHHILQFDYMNSMMSKL

IE-1 CpGV-M1        451 HAEVSKLIRYV----LSRRSFDTLQNDCSKLKSLHVKYK1VYE
IE-1 CpGV-MNPP      451 HAEVSKLIRYV----LSRRSFDTLQNDCSKLKSLHVKYK1VYE
IE-1 CpGV-R1.8      451 HAEVSKLIRYV----LSRRSFDTLQNDCSKLKSLHVKYK1VYE
IE-1 CpGV-R5        451 HAEVSKLIRYV----LSRRSFDTLQNDCSKLKSLHVKYK1VYE

PE38 CpGV-M1        101 PRVQTAERNFYEGAHPMEAEQESPANPEADYNSMMDDMINN
PE38 CpGV-MNPP      101 PRVQTAERNFYEGAHPMEAEQESPANPEADYNSMMDDMINN
PE38 CpGV-R1.8      101 PRVQTAERNFYEGAHPMEAEQESPANPEADYNSMMDDMINN
PE38 CpGV-R5        101 PRVQTAERNFYEGAHPMEAEQESPANPEADYNSMMDDMINN

Perfect match between all five sequences from 150 to 300

PE38 CpGV-M1        301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKLKRR
PE38 CpGV-MNPP      301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKLKRR
PE38 CpGV-R1.8      301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKLKRR
PE38 CpGV-R5        301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKLKRR

PE38 CpGV-M1        351 YLNKQKFSIQYIKSLTDSLRTAKKPIKY
PE38 CpGV-MNPP      351 YLNKQKFSIQYIKSLTDSLRTAKKPIKY
PE38 CpGV-R1.8      343 YLNKQKFSIQYIKSLTDSLRTAKKPIKY
PE38 CpGV-R5        343 YLNKQKFSIQYIKSLTDSLRTAKKPIKY

CpGV-R5 appears to be different from the 4 other classes of CpGV isolates proposed in function of the nucleotide variability at granulin (polh) and lef-8 genes (Eberle et al., 2009). CpGV-R5 is similar to B class (as CpGV-E2) on granulin and to A class when considering lef-8.
The isolate CpGV-R5 has 124 differences in respect to CpGV-M1 (Luque et al., 2001). Eberle (2010) has compared CpGV-M, CpGV-I12 and CpGV-S. Two thirds of the 66 ORFs conserved between CpGV-M1 and CpGV-S are also conserved in CpGV-R5. Only one third presents differences, and among these only two ORFs (ORF25 and ORF26) harbours major changes. The most significant differences are presented in table 1. The detail of the PE38 and IE-I sequences for each virus is presented in Figure 1. Previous work (Eberle, 2010) suggested an association between PE38 (ORF24) variation and virus ability to bypass the resistance. Figure 1 details the differences found at the PE38 (CpGV-M1 ORF 24) between the various isolates analyzed in this paper. The main deletion previously described for CpGV-I01, is also conserved in CpGV-R1.8 and its derivative, CpGV-R5. Two specific amino acid changes differentiate these last isolates from all the others, K 355Q and Q 362K.

Acknowledgements

The research leading to these results have received funding from the European Community’s Seventh Framework Programme (FP7/2007-2011) under grant agreement no. 211606. B.G. is recipient of a French ANRT doctoral grant.

References


Functional characterization of serine/threonine protein kinase gene (AMV197) of Amsacta moorei entomopoxvirus

Hacer Muratoglu¹, Remziye Nalcacioglu¹, Srin Perera², Basil Arif², Zihni Demirbag¹
¹Karadeniz Technical University, Faculty of Sciences, Department of Biology, 61080 Trabzon, Turkey; ²Laboratory for Molecular Virology, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada

Abstract: We report here the functional characterization of a serine/threonine (Ser/Thr) protein kinase gene (ORF AMV197) of Amsacta moorei entomopoxvirus (AMEV). A recombinant virus lacking AMV197 (AmΔPK/gfp) initiated viral DNA replication 6 hour earlier than parental AMEV. However, the recombinant virus yielded five-fold lower progeny virus. Expressed AMV197 gene of AMEV by Bac-to-Bac expression system yielded a 72 kDa homodimeric protein. Protein kinase substrate profiling by peptide microarray indicated that 80 of 1248 substrates belong to 28 protein kinase family were phosphorylated by expressed protein. While AMV197 was known to phosphorylate both serine and threonine putatively, expressed protein kinase also phosphorylated probes with tyrosine residues. The results indicate that AMV197 is an active protein kinase and phosphorylates several known substrates. However, further experiments are needed to identify the exact role of this protein in AMEV replication.

Key words: Amsacta moorei entomopoxvirus (AMEV), protein kinase, peptide microarray
Transcriptional analysis of CpGV isolates in Cydia molesta

Dönüs Toy1,2, Diana Schneider1, Zihni Demirbag2, Johannes A. Jehle1
1Institute for Biological Control, Julius Kühn Institute (JKI) Federal Research Center for Cultivated Plants, Heinrichstraße 243, 64287 Darmstadt, Germany; 2Department of Biology, Karadeniz Technical University, 61080 Trabzon, Turkey

Abstract: The oriental fruit moth Cydia molesta is an insect of the Tortricidae family. Its origin is in China but today it is a major pest in nearly all stone fruit growing areas (Asia, Europe, South and North America, the Middle East, New Zealand and Australia). The principal host plant of the fruit moth is peach but the larvae feed also on apple, quince, plum, cherry, nectarine and pear. Cydia molesta is also a closely related species to the codling moth Cydia pomonella, a major pest in apple production. The codling moth is controlled by the Cydia pomonella granulovirus (CpGV), which is of great importance for codling moth control in both organic and integrated pome fruit production. Though C. molesta and C. pomonella are closely related, the infection success of C. molesta by conventional CpGV-M, is not high. Recently a CpGV isolate, termed V22 (MadexTwin, Andermatt Biocontrol), had been selected and showed improved efficacy to C. molesta. For a better understanding of the infection process of conventional CpGV-M and the improved CpGV-V22 in C. molesta, a comparative transcription analysis of these two viruses in C. molesta was performed. The transcription of selected genes (granulin, ie-1, lef-8, mcp) of CpGV-M was analyzed by reverse transcription quantitative PCR (RT-qPCR). It was found that transcription levels are low compared to those of CpGV-M in C. pomonella. Further experiments will compare the transcriptional level of these genes of CpGV-V22 in both C. pomonella and C. molesta.

Key words: CpGV, Cydia pomonella, Cydia molesta, transcriptome
An examination of stress-related activation of SeM NPV in covertly infected Spodoptera exigua

Cristina Virto\textsuperscript{1,2}, David Navarro\textsuperscript{3}, María Mar Tellez\textsuperscript{2}, Rosa Murillo\textsuperscript{1,2}, Trevor Williams\textsuperscript{4}, Primitivo Caballero\textsuperscript{1,2}

\textsuperscript{1}Instituto de Agrobiotecnología, CSIC-Gobierno de Navarra, Ctra. de Mutilva s/n 31192 Mutilva baja, Spain; \textsuperscript{2}Departamento Producción Agraria, Universidad Pública de Navarra, Pamplona 31006, Spain; \textsuperscript{3}IFAPA, La Mojonera, 04745 Almería, Spain; \textsuperscript{4}Instituto de Ecología AC, Xalapa 91070, México

Abstract: The aim of this study was to evaluate the effect of different stress factors on covertly infected Spodoptera exigua larvae in terms of nucleopolyhedrovirus (NPV) activation. For this, adult survivors that had ingested occlusion bodies of S. exigua multiple nucleopolyhedrovirus (SeM NPV) were mated and the subsequent generation (F\textsubscript{1}) tested for virus activation in the second instar in both laboratory and field conditions. In the laboratory, a number of treatments were tested including chemical stressors, inoculation with heterologous NPV species and Bacillus thuringiensis spores and crystals. Both, parental and F\textsubscript{1} adults were confirmed to harbor the infection by qPCR. Virus activation was observed in insects treated with 0.1% copper sulphate, 1% iron sulphate, and 1 ppm sodium selenite, resulting in 12%, 15%, and 41% mortality due to SeM NPV, respectively, whereas no larvae with symptoms of viral infection were registered in virus-free controls. No effect on NPV-induced mortality was detected after inoculation with heterologous virus. Field trials were carried out by artificial infestation of pepper crops in experimental greenhouses using sublethally infected S. exigua larvae to evaluate copper sulfate and sodium selenite as activation factors. Very little NPV-induced mortality (< 5%) was observed in those larvae treated in field conditions.

Key words: NPVs reactivation, stress factors, Spodoptera exigua multiple nucleopolyhedrovirus

Introduction

Recently studies on baculovirus transmission reported a high prevalence of sublethal or covert infections in lepidopteran populations such as Spodoptera exigua (Cabodevilla et al., 2011a). Spontaneous nucleopolyhedrovirus (NPV) outbreaks might explain the initiation of natural epizootics in host populations. However, very little is known about the mechanisms, which triggers covert infections to become patent fatal infections. Virus activation has been related to stress conditions for larvae that experience high densities during rearing (Fuxa et al., 1999), extreme temperatures or certain chemical treatments (Ilyinykh et al., 2004). Investigating the factors involved in virus reactivation may contribute to the development of new strategies for biological control using NPV-based biopesticides. The aim of this study was to evaluate the effect of different types of treatments as activation factors in covertly infected S. exigua larvae, in both laboratory and field conditions.
Material and methods

Insect and virus
A virus-free colony of S. exigua maintained in the insectary of Universidad Pública de Navarra was used for this experiment. The VT-SeAl1 strain of SeMNPV was used to inoculate S. exigua larvae (Cabodevilla et al., 2011a).

Covert infection induction and qPCR virus detection
Covert infections were established in S. exigua virus-free cultures according to the methods described by Cabodevilla et al. (2011b). Briefly, fourth instar (L4) virus-free larvae were sublethally infected with occlusion bodies (OBs) of the vertically transmitted isolate VT-SeAl1. A group of larvae were treated similarly except that the inoculum did not contain OBs; this lineage was used as control. Adult survivors to the virus challenge were mated and the subsequent generation (F1) tested for virus activation in the second instar (L2) by treating covertly infected larvae with chemicals or entomopathogens. Groups of 24 larvae were dosed by droplet-feeding with one of the following groups of treatment: i) chemical stressors: copper sulfate (1%-0.1%), 1% iron sulfate, hydroxyamine (1-0.1%), 2% Tinopal, 1ppm sodium selenate, or 1 ppm paraquat dichloride; ii) inoculation with: Chrysoideixis chalcites NPV (non-permissive), Mamestra brassicae NPV (permissive), SeMNPV-US2, Bacillus thuringiensis spores, Bt crystal, mixed Bt spores & crystals (1:1); and iii) rearing temperatures of 18 ºC and 28 ºC. NPV mortality was registered by checking cadavers for the presence of OBs using a phase-contrast microscope. To confirm transgenerational transmission of infection a group of F1 larvae were reared to adults and tested for amplification of the viral specific gene DNA polymerase by qPCR using a SYBR based method (Cabodevilla et al., 2011b).

Field trials
Treatments that had proved to be effective activation factors in the laboratory experiments were tested in field conditions. Three experimental greenhouses of 100 m² area in the installations of IFAPA (Instituto de Investigación y Formación Agraria y Pesquera, Almería, Spain) were planted with pepper crops using a plantation frame 0.5 × 1 m. Each greenhouse was split into four plots in which one of the following four treatments was applied i) 0.1% copper sulphate, ii) 1 ppm sodium selenate, iii) Bt-based insecticide (FlorBac, Bayer), and iv) water control. The offspring of sublethal infected adults (100% positive for qPCR) were used for artificial infestations. Egg masses were placed on the three central plants of each plot at a rate of 200 eggs per plant. Once most of the larvae reached second instars, treatments were applied to plants using a hand-held sprayer. After 48 h post treatment a total of 30 larvae per plot were collected from the three central plants and confined individually in 25 ml plastic cups provided with diet and reared in the laboratory until death or pupation.

Results and discussion

Reactivation of SeMNPV by stressor factors in laboratory conditions
All of the F1 tested adults (n = 27) were confirmed to harbor the virus by qPCR, suggesting a high prevalence of persistent infection in larvae subjected to activator treatments. NPV mortality was observed in 0.1% copper sulfate, 1% iron sulfate, and 1-ppm sodium selenate treatments that resulted in 12%, 15%, and 41% virus mortality, respectively, whereas no larvae with symptoms of viral infection were registered in virus-free controls. None of the
remaining chemical treatments cased virus activation. Ilyinykh et al. (2004) reported similar results on the activation of occult virus by feeding Lymantria dispar larvae on diet containing 0.6% copper sulfate. Copper iron and selenium are essential microelements required for body functions and the immune system (Chaturvedi et al., 2006). They have been described as involved in immuno modulation that influence the course of the outcome of a variety of viral infections. Therefore the chemicals used here that resulted in activation of the virus seem to be acting as physiological stressors.

No effect of entomopathogen inoculation was observed in subletally infected larvae, since only those viruses to which S. exigua is a permissive species (MbM NPV and SeM NPV) resulted in 64% and 17% of NPV mortality, respectively. However, numerous studies have reported the triggering effect of heterologous viruses in the activation of occult NPV infections, including S. exigua (Murillo et al., 2011).

** Reactivation of SeM NPV by chemical stressors in field conditions **

Fourth instar larvae collected from plants treated with copper sulphate or sodium selenate showed very low levels of NPV-induced mortality; 1.4-2.4%, respectively. A Bt-based pesticide was included as a control since this pesticides used is highly extended in biological control crop systems in Almeria. This pathogen resulted in 2.8% NPV mortality in greenhouse treated larvae. No virus mortality was observed in larvae from control plants.

** Acknowledgements **

We thank N. Gorría for technical assistance. This work has been funded from the Ministerio de Ciencia e Innovación (Spain) by the project number AGL2011-30352-C02-01. C.V. received a pre-doctoral fellowship from the Universidad Pública de Navarra.

** References **


Functional analysis of Chilo iridescent virus zinc-binding matrix metalloproteinase gene

Aydın Yesilyurt, Hacer Muratoglu, Zihni Demirbag, Remziye Nalcacioglu
Karadeniz Technical University, Faculty of Sciences, Department of Biology, 61080 Trabzon, Turkey

Abstract: Basement membranes that surround the tissues of lepidopterous larvae act as physical barrier to the movement of viruses. Therefore, one of the potential approaches is using the enzymes that disrupt the basement membrane proteins in biological control of agricultural pest insects. Matrix metalloproteinases are zinc-dependent endopeptidases that have the combined capacity to degrade all the components of the extracellular matrix. Chilo iridescent virus (CIV) genome encodes a 264 amino acid protein (ORF 165R) containing zinc-dependent matrix metalloproteinase (MMP) domain with over 40% amino acid sequence identity to a large group of organisms including primarily variety of Drosophila species. The CIV-MMP homolog was cloned and a recombinant AcMNPV bacmid that expresses CIV-MMP under the Autographa californica multiple nucleopolyhedrovirus polyhedrin promoter was constructed. Recombinant bacmid was produced and transferred to Sf-9 cell line for high-level expression of recombinant protein. Expressed protein was purified from Sf-9 cells after 96 hour post infection. Western blot analysis of the protein resulted in a 34 kDa protein band. CIV-MMP protein digested dye-impregnated collagen (Azocoll). The enzymatic activity was inhibited by metalloproteinase inhibitor EDTA. These results suggest that the CIV-MMP gene homolog encodes a functional metalloproteinase which can be utilized in biological control of lepidopteron pests.

Key words: Chilo iridescent virus, CIV, metalloproteinase, bacmid, gene expression
Soil pests
Latest field results on the biological control of *Diabrotica virgifera virgifera* with nematodes

Ralf-Udo Ehlers  
e-nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany  
e-mail: Ehlers@e-nema.de

**Abstract:** The entomopathogenic nematode *Heterorhabditis bacteriophora* has been tested successfully against larvae of the western corn rootworm (*Diabrotica virgifera virgifera*) for the last 5 years in Hungary, Austria and Italy. When applied at a dose of $1.5 \times 10^9$ nematodes ha$^{-1}$ the results have been comparable to those obtained with chemical seed dressing with neonicotinoids or application of granular insecticides containing the pyrethroid Tefluthrin. At higher dose of $2 \times 10^9$ nematodes ha$^{-1}$ the results were more stable at control between 70 and 90%. Although the differences are remote, in comparison to chemical insecticides the nematodes usually provided higher reduction of adults whereas less root damage was recorded for chemical insecticides. The effect of nematodes is equally high whether applied during sawing of the maize or at occurrence of the larvae approximately 6 weeks later. Different application techniques have been tried. Seed dressing and granular application often caused problems under commercial conditions. Liquid applications into the drill with 200-400 litre water have provided optimal conditions for nematode establishment and persistence until the occurrence of the larvae. Article 55 of the new EU regulation (EC) No 1107/2009 on the placement of plant protection products on the market explicitly implies the promotion of the use of non-chemical and natural alternatives. Directive 2009/128/EC (SUD) aims to achieve the sustainable use of pesticides. Article 14 lines out that “the Member States shall take all necessary measures to promote low pesticide-input pest management, giving wherever possible priority to non-chemical methods, so that professional users of pesticides switch to practices and products with the lowest risk to human health and the environment”. Biological control industry is preparing to supply the markets with the necessary amounts of the entomopathogenic nematode *H. bacteriophora*. In 2011, the first product (Dianem®) based on this nematode was introduced. Although EU member states should give priority of non-chemical management of *Diabrotica* in accordance with the SUD, member states provided emergency authorisations (Article 53, Regulation (EC) No 1107/2009) for chemical insecticides to control the pest. Article 53 allows use only “where such a measure appears necessary because of a danger which cannot be contained by any other reasonable means”. In conclusion, we suggest implementing and executing of EU regulations/directives in accordance with consumer demands. Consistent enforcement of European legislation would lead to preference for non-chemical control, prevent the use of problematic pesticides and promote low pesticide-input management thus contributing to implementation of IPM in maize production.

**Key words:** western corn rootworm, *Heterorhabditis bacteriophora*, application technique, regulation
Development of new formulations for soil pest control

Miriam Hanitzsch, Michael Przyklenk, Bianca Pelzer, Anant Patel
Department of Engineering Sciences and Mathematics, University of Applied Sciences Bielefeld, Wilhelm-Bertelsmann-Str. 10, 33602 Bielefeld, Germany

Abstract: In formulation science, there are few systematic investigations on encapsulation of agro-biologicals with regard to materials, methods and technology for mass production. The aim of this work was to develop novel mechanically stable capsule systems with increased persistence in soil. To this end, we tested different methods with several biopolymers, combinations of biopolymers and lignin as capsule additive. Capsule systems were prepared by ionic gelation, thermal gelation, complex coacervation and additional bead coating. In selected capsules, lignin was incorporated. Capsules based on single biopolymers were able to form stable spherical capsules, e.g. alginate, pectin derivates and gelatin. Capsules based on combinations of polymers also showed stable capsule formation, e.g. alginate/gelatin, alginate/lignin and SEC/PDADMAC. Additionally, lignin was used successfully as additive in SEC hollow beads. First experiments indicate significant differences in biological degradability and thus persistence in soil with different capsule systems. These novel capsule systems with increased persistence are suitable for delivery of BCAs into the soil.

Key words: formulation, encapsulation, immobilization, entomopathogenic fungi, beads, hollow beads, biological control, agrobiological, Metarhizium anisopliae, bio-insecticide

Introduction

The EU-funded project INBIOSOIL will explore in detail the recently discovered synergistic effects between entomopathogenic fungi (EPFs), entomopathogenic nematodes (EPNs), and semiochemicals by developing innovative co-formulations, making use of strategies derived from nature. The formulations to be developed first aim at overcoming the classic drawbacks in the application of biocontrol agents (BCAs) like EPF, e.g. handling, low shelf life and poor establishment in soil. Formulation methods such as encapsulation offer a solution to these problems.

For the encapsulation of BCAs only conventional alginate beads were produced according to standard or uneconomic methods. To date nobody investigated e.g. the effect of all 20 different available alginates on efficacy. Besides, capsules based on other biopolymers, hollow beads and coated capsules (Vemmer & Patel, unpublished) have not been tested successfully so far. Also, the novel formulation trend of mixing polymers with different physicochemical and biochemical properties into one formulation with improved characteristics such as improved re-swelling at high mechanical strength was not investigated.

EPF such as Metarhizium anisopliae or Beauveria bassiana have been formulated by encapsulation in conventional alginate beads supplemented with nutrients (e.g. Burges, 1998; Pereira & Roberts, 1991; Moore & Caudwell, 1997; Gerdng-Gonzalez et al., 2007) but establishment in soil is still slow and biomass content to high, making the formulations uneconomic. That is why we aim at developing novel capsules containing EPFs in the scope of the EU-funded project INBIOSOIL. The aim of this work was to develop novel mechanically stable capsule systems with increased persistence in soil. To this end, we tested several biopolymers, combinations of biopolymers and lignin as capsule additive.
Material and methods

Ionic Gelation
Beads were formed by dripping polymer solutions with a concentration of 2% into a cross-linking solution containing 2% Ca\(^{2+}\) ions. Differing polymer concentrations were used with Gelatin Bloom 280 in combination with alginate and with xanthan. Here, concentrations of 2%, 5% and 0.5% were used, respectively.

Thermal gelation
Beads were formed by dripping a warm biopolymer solution into a cold CaCl\(_2\) solution. Concentration of Gelatin Bloom 280 was 20% and of Gelatin Bloom 280 in combination with alginate were the same as used above. Guar gum and gellan gum were used at a concentration of 1%.

Complex coacervation
For the production of hollow beads a solution of a polyelectrolyte was dripped into a solution of another polyelectrolyte with counter charges.

Bead coating
Coated Ca-alginate beads with a second layer of alginate were produced by dripping the Ca-alginate beads into a 0.8% alginate solution.

Results and discussion

Polymer screening
Capsule formation by ionic gelation was evaluated for 14 screened biopolymers and 2 polymer combinations. Thermal gelation was evaluated for 3 biopolymers and one polymer combination. Polymers showing instable or no capsule formation with this method were further evaluated using complex coacervation.

Eight of the screened biopolymers were able to form stable spherical capsules with either ionic or thermal gelation (Figure 1 A). All three biopolymer combinations were also able to form stable spherical capsules, demonstrating that these gelation methods can also be used to produce capsules based on two different polymers (Figure 1 B).

Differences in gelation properties and functional groups of the biopolymers are responsible for the formation of different assembly levels. The functional groups of the tested biopolymers are typical for each biopolymer. In short, alginate has carboxylic groups with a negative charge, so it is able to form capsules by using ionic gelation. Chitosan on the other hand has amine groups which are positively charged, so this polymer is not able to form capsules with any counter ion, but with the complex coacervation method. Capsule formation via complex coacervation clearly showed two biopolymer combinations which are able to form stable and spherical hollow beads based on two polymers with different physiochemical characteristics (Figure 2).

All biopolymers have characteristics beyond their capsule forming properties. For example, pectin derivates may serve as capsule matrix but at the same time as nutrient source and may additionally improve reswelling of dried capsules (data will be shown).
Figure 1. Evaluation of capsule formation using ionic and thermal gelation. (A) single biopolymers, (B) polymer combinations. Legend for capsule formation: 1 = no capsule formation, 2 = instable capsule formation, 3 = no spherical capsule, 4 = spherical capsule formation. VEG = degree of esterification.

Figure 2: Evaluation of capsule formation using complex coacervation. A: 2% SEC/2% PDADMAC, B: 3% SEC/5% PDADMAC, C: 2% SEC/2% PDADMAC + lignin, D: 2% chitosan/2% SEC, E: 1% chitosan/5% lignin, F: 2% PDADMAC/2% SEC, G: 2% CMC/2% PDADMAC. Samples were prepared by dripping a solution of the first polymer into a solution of the second one. Legend for capsule formation: 1 = no capsule formation, 2 = instable capsule formation, 3 = no spherical capsule, 4 = spherical capsule formation.
Capsule systems with properties like good mechanical stability and drying properties can be achieved by combining different biopolymers like alginate and gelatin and additives. Furthermore, chitosan with antimicrobial and lignin with poor biodegradability are candidates for an effective capsule system to increase the persistence of encapsulated EPF in the soil. First experiments indicate significant differences in biological degradability and thus persistence in soil with different capsule systems (data will be shown).


Figure 4: Influence of drying on capsule size. Capsules were dried 24 h at room temperature.
Drying of hydrogel capsules results in a reduction of size correlated to the loss of water. Depending on the used biopolymers the shrinking of the capsules differs in form and degree (Figure 3). For each of the different capsule systems screened in Figure 1 the diameter of moist and dry capsules (full beads) was measured. 13 of 15 capsules lost about 60% of their volume after drying, with the exception of the hollow beads containing SEC. They dried into thin films retaining 75% of their diameter (Figure 4). Here, fillers will be investigated.

To conclude, these novel capsule systems with increased persistence are suitable for delivery of fungal BCAs into the soil. In further experiments promising polymers and additives will be analysed by measuring characteristic properties like particle size, particle size distribution, diffusion properties, mechanical stability, reswelling, redissolvability, biological degradability and toxicity. Additionally, we will develop encapsulated EPF with a reduced biomass content, high survival, long shelf-life, slow or controlled release in soil from a “depot” resulting in increased establishment in soil, longer persistence and decreased number of applications. The formulated biomass will be characterized by viability assays. Data will be shown. Furthermore, within the INBIOSOIL project the formulated EPF will be tested for efficacy against target insects such as wireworms, western corn root worm larvae and black wine weevil larvae. Additionally the influence of the formulated EPF on non-target beneficial insects will be evaluated.

Acknowledgements

The work presented here was done in the EU-funded project INBIOSOIL, grant agreement no: 282767.

References

Click beetles disperse widely across farmland: what else do we need to know?

Rod Blackshaw¹, Robert S. Vernon²

¹Centre for Agricultural and Rural Sustainability, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK; ²Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, P.O. Box 1000, Agassiz, British Columbia, V0M 1A0, Canada

Abstract: For many years wireworm research focussed on the distribution of larvae and their control in field crops. The development of sex pheromone lures has facilitated the study of adult male distributions over greater spatial scales but the assumption that these would provide a simpler monitoring technique has not been fulfilled and it is now clear that we cannot be confident about what the trap counts actually mean. This secondary focus on adult males neglects the critical issue of female behaviours in agricultural landscapes. Understanding these is essential to the development of new management strategies in an era of declining insecticide availability. In this paper we briefly review what is known about the movement of click beetles across farmland and identify research gaps that need to be filled if we are to develop area-wide management strategies.

Key words: Elateridae, Agriotes, click beetles, wireworms, dispersal, spatial distribution, area-wide management

Introduction

The history of wireworm pest research shows that the focus has been on controlling the larvae in a crop. However, research into wireworm control has been restricted by the twin challenges of larval identification and their cryptic soil habitat. Recently, progress has been made in the routine identification of larvae through the use of molecular methods (Ellis et al., 2008; Staudacher et al., 2011). This has encouraged consideration to be given to the ecology of individual species within regional pest complexes. The development of species-specific sex pheromones for a range of click beetles (Toth et al., 2003 and subsequent papers) opened up the possibility that a simpler method than monitoring wireworms was available that would overcome the problems of directly estimating wireworm numbers in the soil. However, this approach was challenged by Blackshaw et al. (2008) on three grounds. Firstly, for any adult cohort to reflect that of the wireworm population each annual cohort should be approximately equal in size. The second assumption is that the distribution of adult sex pheromone trap catches should be related to that of the wireworms themselves. Finally, unless activity/density responses in relation to the trapping system are similar for the different species then species-specific relationships would need to be developed to guarantee robust predictions. None of these three assumptions survived the comparison with data (Blackshaw et al., 2008) and a subsequent study revealed substantial interspecific differences in sex pheromone trapping rates of Agriotes lineatus, A. obscurus and A. sputator (Hicks & Blackshaw, 2009) which implied potential differences in walking behaviours for these three species.

Since wireworm movement through soil is limited (Schallhart et al., 2011) colonisation of new areas, and addition of a new generation to an existing population, will depend on dispersal by the adult click beetles. Thus differences in walking behaviours are potentially important to the spread across and distribution in agricultural landscapes. In this paper we
summarise the evidence for dispersal and then address the issue of what we need to know in order to contemplate an area-wide management strategy for wireworms.

**Click beetle dispersal**

There has only been one published study to date that directly investigated how far away click beetles can be found from the site of larval feeding. Schallhart et al. (2009) captured an adult male *A. obscurus* in a sex pheromone trap at least 80 m from where it had to have fed as a larva. This distance was similar to the maximum estimated for *A. obscurus* (51 m) and *A. lineatus* (82 m) over 45 days in a mark-release-recapture (MRR) study (Hicks & Blackshaw, 2008). Thus, it appeared that adult male dispersal was somewhat limited.

This view supported the conclusion reached by Blackshaw & Vernon (2006) that there was, generally, spatial stability of *A. lineatus* and *A. obscurus* populations at the landscape scale over three years. At field scales, however, differences in the spatial distributions of these two species became apparent with dynamic change over the adult activity period (Blackshaw & Vernon, 2008). At the time this was attributed to advection of the sex pheromone and the progressive overlapping of individual trap attraction zones, with the greater walking speed of *A. lineatus* (as reported by Hicks & Blackshaw, 2008) contributing to an earlier breakdown of population spatial structure.

Results showing that the attraction zones of *Agriotes* sex pheromones might be limited to a few metres (Sufyan et al., 2011) suggest that it was not the pheromones per se that were causing the breakdown in spatial structure reported by Blackshaw & Vernon (2008) but that there was an additional intrinsic factor arising out of adult dispersal behaviours. One possible explanation is that adult male click beetles are moving much further than previously thought and that sex pheromone traps capture beetles originating from a range of distances when they happen to be in the immediate vicinity. This would also explain the lack of directional effect in MRR studies reported by Hicks & Blackshaw (2008) for *A. lineatus*, *A. obscurus* and *A. sputator* and by Kishita et al. (2003) for *Melanotus okinawensis*; the direction from which individuals are caught may not be the same as the direction from their release point.

If this is the case, then it can be expected that adult click beetles will be found widely distributed across agricultural landscapes and not restricted to areas where wireworms can be recovered. Blackshaw & Hicks (2012) reported a study into the distribution of *A. lineatus*, *A. obscurus* and *A. sputator* in an agricultural landscape using transects of sex pheromone traps at 100m spacing. The sampled area covered a range of crops and land cover and showed that all three species were present in each field but that there were interspecific differences in their distributions. In a separate, but related study (Benefer et al., 2012) each field was sampled to assess wireworm numbers. In the fields covered by the transects, wireworms were restricted to permanent and temporary (ley) grass and none were found in any of the cultivated soils. Furthermore, no *A. lineatus* larvae were recovered despite there being more of this species than either of the others. This provides strong evidence to support the contention that adult click beetles are highly mobile even when dispersing by walking.

A survey of adult male (using sex pheromone traps) and wireworm (using soil sampling) distributions in 97 organic fields across six farms in the UK also showed that there were no strong spatial associations between adults and larvae of the same species (Benefer et al., 2012).

A second conclusion to be drawn from Benefer et al. (2012) is that not all the beetles captured in a field originated there. This implies that there are refugia in the landscape and that the spatial dynamics of these pests might be of the source: sink model with cropping land
the recipient of inflowing beetles/eggs. This hypothesis is entirely consistent with the findings of Blackshaw & Vernon (2006) and the observation of probable edge effects in sex pheromone trap counts attributable to the movement of male click beetles into the crop from the field margin (Blackshaw & Vernon, 2008).

The potential for uncropped areas to act as reservoirs for crop invasions was tested using MRR in two experimental sites at Agassiz, British Columbia. Each was 72 m x 72 m with one in wheat and the other kept as bare fallow. In each field, a number of pitfall traps were deployed and marked A. obscurus male beetles released from several locations along each of the four sides of each field. Traps were checked and emptied after different time periods. Individuals were caught 1 m from the field edge within 1 h of release and captures some 30 m from the release point were observed after 19 h. Unmarked (naturally occurring) beetles of both sexes were also recovered from traps across both fields. Generally there were more males captured than females and they were caught earlier in the season.

Concurrent releases of marked male A. lineatus and A. obscurus beetles were made from the centre of the field and a significant difference in their respective trap counts recorded. This reinforces the view that there are behavioural differences that influence their dispersal across farmland. More importantly, we also found significant differences between male and female A. obscurus releases from the field centre, suggesting that we cannot necessarily infer knowledge of female dispersal and spatial distributions from that of males.

Conclusions

Male click beetles are widespread in agricultural land and substantially more mobile than previously thought. There is evidence for interspecific differences in behaviour which influence the rate at which they disperse. Uncropped areas will act as refugia in the landscape and adults move out of these into adjacent farmland, whether it has a potential host or not. Although not yet conclusive, emerging evidence indicates that there are intersexual differences in dispersal behaviour which may affect field colonisation rates.

To date, field studies have concentrated on adult males largely because of the availability of sex pheromones that enable them to be easily trapped at a location and for large numbers to be collected for MRR studies. Given that wireworms do not move far through the soil (Schallhart et al., 2011), the critical behaviour for where they are to be found will be that of the ovipositing female. We have shown that we cannot necessarily extrapolate from male to female dispersal behaviours but we also know very little about when mating occurs, the timing of oviposition, the oviposition period and adult longevity or even the timing of emergence. Furthermore, even if we knew all this, we still lack the ability to recover eggs from the soil in order to test hypotheses.

This knowledge of female behaviour is essential if we are to develop new management strategies that act to limit pest numbers in field crops through area-wide management. One potential approach that could be considered would be to disrupt mating through the widespread use of sex pheromones – but only if we can get the timing right. Development of a female specific lure would also allow us to contemplate a push-pull strategy to separate males from females at the critical mating period.
References


Ellis, J. S., Blackshaw, R. P., Parker, W., Hicks, H. & Knight, M. 2009: Genetic identification of morphologically cryptic agricultural pests. Agric. For. Ent. 11: 115-121.


Distribution and abundance of *Agriotes ustulatus* L. adults on pheromone traps in four regions in Croatia

Antonela Kozina, Maja Čačija, Renata Bažok
University of Zagreb, Faculty of Agriculture, Department for Agricultural Zoology, Svetošimunska 25, 10000 Zagreb, Croatia

Abstract: During several years of investigations, the distribution and the abundance of *Agriotes ustulatus* in four different regions of Croatia were researched with the aim to correlate the abundance with the prevailed climatic conditions in each region. *Agriotes ustulatus* was captured by pheromone traps (Csalomon) on 17 fields distributed at seven localities in four different regions in Croatia according to the climatic data. The highest dominance indices of *A. ustulatus* were recorded in the warmest county, County of Vukovar-Srijem and species was classified as eudominant. *Agriotes ustulatus* was subdominant at locality Čazma where the average temperature was the lowest comparing to the other localities.

Key words: abundance, *Agriotes ustulatus* L., Croatia, distribution, pheromone traps

Introduction

Genus *Agriotes* belongs to the family Elateridae (Coleoptera), which is characterized by a large number of genera. In Croatia, the most important species are: *Agriotes lineatus* L., *Agriotes sputator* L., *Agriotes obscurus* L., *Agriotes brevis* Cand. and *Agriotes ustulatus* Schall. (Maceljski, 2002). *A. ustulatus* is the largest one and overwinter only as larvae. The larvae develop over two years, pupate during May, and adult forms occur between May and September (Bažok, 2007; Furlan, 1996; Honeki & Furlan, 1995). It requires two or three calendar years for full development in Croatia (Maceljski, 2002). In Croatia, the maximum of flight is in late June and early July (Bažok & Igrč Barčić, 2010; Štrbac, 1983). *A. ustulatus* is the Mediterranean species, encountered in central, southern and Eastern Europe (Camprag, 1997). In Croatia, this species dominates on the fields of Slavonia and Baranja, especially in the eastern part (Maceljski, 2002; Štrbac, 1983). Bažok (2007) states, that this species is represented in Northwest Croatia at medium to high population density. The main aim of these investigations was to determine the distribution and abundance of *A. ustulatus* in four different regions of Croatia and correlate the abundance with the prevailed climatic conditions in each region.

Material and methods

Field data

From 2001 to 2005, pheromone traps targeting the five most important *Agriotes* species (*A. lineatus*, *A. sputator*, *A. obscurus*, *A. brevis* and *A. ustulatus*) were set in two fields in the region of Zagreb (localities Oborovo and Čazma). From 2007 to 2010, pheromone traps targeting the same species were set in fields in region of Koprivnica-Križevci (five fields in Ferdinandovac) and region of Virovitica-Podravina (three fields in Terezino Polje and two
fields in Bankovci). During the years 2007 and 2008, all five species were monitored in region of Vukovar- Srijem (two fields in Bošnjaci and three fields in Tovarnik). Altogether 17 fields were involved in the investigation and according to the climatic and edaphic data fields were grouped into four main regions (counties) and seven different micro-regions (localities).

**Pheromone traps**
To collect *A. brevis*, *A. lineatus*, *A. sputator* and *A. obscurus* funnel traps were used and for *A. ustulatus* Varb3 traps were used. The monitoring period of *A. brevis*, *A. sputator*, *A. lineatus* and *A. obscurus* was from the 18th to the 32nd week of the year, and that of *A. ustulatus* was from the 23rd to the 32nd week of the year. Traps were inspected once a week. During each weekly observation period all beetle specimens were collected from the traps and counted. Pheromone vials were replaced every six weeks.

**Data analysis**
Adult population densities at trapped localities were classified according to provisional categories set by Furlan et al. (2001) as follows: High = more than 500 adults per trap per season; Medium = between 50 and 500 adults per trap per season; Low = less than 50 adults per trap per season; NO = no specimens. These limit values are not considered as economic thresholds. Based on the total individual number of five species and the individual number of each particular species the dominance was calculated for each field and year. The dominance was calculated with Balogh’s formula (cit. Balarin, 1974). The results (eudominant, dominant, subdominant, recedent, subrecedent) were classified according to Tischler and Heydeman (cit. Balarin, 1974). Climatic conditions about average air temperature and rainfall for each year were taken from the nearest meteorological stations. Data on click beetle capture, dominance indices and values of collected meteorological elements were analyzed by ANOVA (ARM 7 GDM software) with mean separation using Duncan multiple Range test (DMR). If necessary, the data were transformed by log (x+1) transformation before the analysis.

**Results and discussion**
The significant differences in climatic conditions, among all four counties, are notable (Table 1).

Table 1. Characteristics of the climatic conditions at different localities (micro-regions) during the period of investigation.

<table>
<thead>
<tr>
<th>County</th>
<th>Micro-region (locality)</th>
<th>Period of investigation</th>
<th>Average air temperature (°C) ± SD</th>
<th>Total amount of rainfall ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zagreb</td>
<td>Oborovo</td>
<td>2001-2005</td>
<td>11.38 ± 0.61 b</td>
<td>1018.58 ± 211.64 a</td>
</tr>
<tr>
<td></td>
<td>Čazma</td>
<td></td>
<td>11.24 ± 0.63 b</td>
<td>885.02 ± 111.08 ab</td>
</tr>
<tr>
<td>Koprivnica-Križevci</td>
<td>Ferdinandovac</td>
<td>2007-2010</td>
<td>11.33 ± 0.36 b</td>
<td>860.5 ± 224.07 abc</td>
</tr>
<tr>
<td>Virovitica-Podravina</td>
<td>TerezinoPolje Bankovci</td>
<td>2007-2010</td>
<td>11.5 ± 0.52 b</td>
<td>903.68 ± 281.32 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.48 ± 0.43 b</td>
<td>909.95 ± 281.32 ab</td>
</tr>
<tr>
<td>Vukovar-Srijem</td>
<td>Bošnjaci Tovarnik</td>
<td>2007-2008</td>
<td>13.05 ± 0.07 a</td>
<td>742.15 ± 155.21 bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.05 ± 0.07 a</td>
<td>645.85 ± 184.06 c</td>
</tr>
<tr>
<td>LSD P =0.05</td>
<td></td>
<td></td>
<td>0.557</td>
<td>217.586</td>
</tr>
</tbody>
</table>
County of Vukovar-Srijem was the county with the highest average temperature (13.1 °C) and the lowest amount of rainfall, especially the locality Tovarnik. The highest amount of rainfall was established in the County of Zagreb, locality Oborovo. The total amount of rainfall in the County of Koprivnica-Križevci and the County of Virovitica-Podravina was between ones mentioned above. The average temperatures in the County of Zagreb, the County of Koprivnica-Križevci and the County of Virovitica-Podravina were lower comparing to the County of Vukovar-Srijem.

There was a significant difference in the average capture of *A. ustulatus* among counties and localities (Table 2). In the County of Zagreb, locality Oborovo, the number of *A. ustulatus* individuals captured per pheromone trap was high (over 500 beetles per trap per season) with the exception in 2005, when that number was medium. In the same County, but at the locality Čazma, the number of *A. ustulatus* individuals was low, except in 2003, when it was medium. Mean average capture of *A. ustulatus* in County of Zagreb was 1295.21 beetles per field at locality Oborovo and 30.66 beetles/field at locality Čazma. In other two counties (County of Koprivnica-Križevci, County of Virovitica-Podravina) the population was classified as medium, with mean average capture between 131.68 and 243.78 individuals per locality. In the County of Vukovar-Srijem at both localities during the year 2007 high capture was established, while in 2008 the population was medium. The mean average capture per field was higher at Tovarnik locality (519.08 individuals) than at Bošnjaci (176.07 individuals).

Table 2. Classification of *A. ustulatus* population density according to Furlan et al. (2001) based on the average capture of adults on pheromone trap/field.

<table>
<thead>
<tr>
<th>County</th>
<th>Micro-region (locality)</th>
<th>Mean average capture/field</th>
<th>Classification of adult population level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2001</td>
<td>2002</td>
</tr>
<tr>
<td>Zagreb</td>
<td>Oborovo</td>
<td>1295.21 a*</td>
<td>H**</td>
</tr>
<tr>
<td></td>
<td>Čazma</td>
<td>30.66c</td>
<td>L</td>
</tr>
<tr>
<td>Koprivnica-Križevci</td>
<td>Ferdinandovac</td>
<td>131.68 b</td>
<td>M</td>
</tr>
<tr>
<td>Virovitica-Podravina</td>
<td>TerezinoPolje</td>
<td>243.78b</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>Bankovci</td>
<td>142.11 b</td>
<td>M</td>
</tr>
<tr>
<td>Vukovar-Srijem</td>
<td>Bošnjaci</td>
<td>176.07 b</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>Tovarnik</td>
<td>519.08 ab</td>
<td>H</td>
</tr>
<tr>
<td>LSD P = 0.05%</td>
<td></td>
<td>0.542 t***</td>
<td></td>
</tr>
</tbody>
</table>

* Means followed by same letter are not significantly different according to Duncan Multiple Range (DMR) test (P = 0.05); **H – High = more than 500 adults/ trap/season; M – Medium = between 50 and 500 adults/trap; L – Low = less than 50 adults /trap /season; ** = LSD is reported in transformed data units (data were transformed using of log( x+1) transformation; x- no data available
The highest dominance indices of A. ustulatus were recorded in the warmest county, County of Vukovar-Srijem (Figure 1) what corresponds with statement of Maceljski (2002) and Štrbac (1983). These results partially correspond with the results of the other authors (Furlan, 1996; Furlan, 1998; Tackenberg et al., 2011) that the species prefers higher temperatures. Despite lower average temperatures, in the County of Virovitica-Podravina species was classified as eudominant. This county is characterized with sandy soils and Furlan (1998) states that A. ustulatus prefers sandy soil with little clay. In spite of very high capture, at locality Oborovo the dominance index was 47%. The capture of other species at this locality was very high as well. Although the temperatures were lower than in eastern part of Croatia, Oborovo was characterized with high amount of rainfall, what is another key factor for this species (Furlan 1996; Furlan, 1998). A. ustulatus was subdominant at locality Čazma where the average temperature was the lowest comparing to the other localities.

References


![Figure 1. The dominance indices of A. ustulatus at different localities (micro-regions) in Croatia, LSD (P = 0.05) = 29.096.](image-url)


Efforts to develop female-targeted attractants for click beetles - a summary

Miklós Tóth¹, Lorenzo Furlan², József Vuts¹,⁵, Éva Bálintné Csonka¹, István Szarukán³, Teodora B. Toshova⁴, Mitko Subchev⁴, Dimitar I. Velchev⁵, Christine M. Woodcock⁵, John C. Caulfield⁵, Patrick Mayon⁵, John A. Pickett⁵, Michael A. Birkett⁵
¹Plant Protection Institute MTA ATK, Herman O. u. 15. Budapest 1022, Hungary; ²Veneto Agricoltura, Viale dell’Università, 14 – Agripolis Legnaro (Pd) 35020, Italy; ³Debrecen University, Ctr. Agric. Sci., Debrecen, Pf. 58, 4001, Hungary; ⁴Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Science, Sofia, 2 Gagarin str., 1113, Bulgaria; ⁵Maize Research Institute, Kneza, 5835, Bulgaria; ⁶Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom

Abstract: An overview is given on recent research efforts to develop attractant combinations capable of attracting female click beetles.

Key words: click beetles, Coleoptera, Elateridae, Agriotes, female attractant, floral lure, pheromone

Introduction

Moni­toring populations of adults of pest click beetles is largely based on the use of female-produced sex pheromones (e.g. Furlan et al., 2007). This has the disadvantage that “trap catches of males must usually be interpreted in terms of the behaviour of the females, thus adding to the complexity of that interpretation” (Wall, 1985). Capture of female insects would provide a better opportunity for: 1) more precise monitoring, leading to more accurate decision making on timing of control strategies against a given pest species; 2) more efficient mass trapping by catching gravid females for direct population reduction; 3) a more efficient application of the lure-and-kill method; 4) an opportunity to assess egg content, fecundity and fertility of captured females.

Material and methods

In the course of the experiments, internationally established and widespread methods were used. Please refer to descriptions of material and methods in the references cited.

Results and discussion

Flower-visiting species

Insects locating a flower are aided by an array of visual and chemical stimuli. In the case of flower-visiting click beetles chemical communication between flowers and adult beetles could be exploited to defining female-targeted attractants. Indeed, in Agriotes ustulatus, which can frequently be seen feeding on flowers, (E)-anethol is attractive to females (and males), and this attraction can be significantly increased by the addition of cinnamate based
compounds (Tóth et al., 2011). Further results indicate that the number of female beetles captured increased dramatically when this floral attractant was applied together with the pheromone in the same trap, compared to the catch in traps with the floral lure only (Tóth et al., 2009).

It was interesting that female numbers caught increased with increasing dose of the pheromone in dual-baited traps (Figure 1), giving more evidence that the pheromone unequivocally has a favourable influence on female captures of _A. ustulatus_. Female-targeted traps for catching _A. ustulatus_ equipped with a dual pheromone plus floral lure are already available on the market for use by both growers and experts.

**Figure 1.** Catches (mean + SE) of _Agriotes ustulatus_ beetles in traps baited with the pheromone only, the floral lure only, and dual baited traps with the floral lure plus different doses of the pheromone in a trapping test in Knezha, Bulgaria. (Data from M. Tóth, T. B. Toshova, M. Subchev, D. I. Velchev, unpublished). Columns with the same letter within one diagram are not significantly different by ANOVA, Student-Newman-Keuls, P = 0.05.

Non-flower-visiting species

Similar perspectives are open in the study of chemical communication between green plant material and species which feed on green leaves of plants. In the development of a female-targeted lure for _A. brevis_ (which cannot be observed feeding on flowers, instead, feeding on green leaves of weeds), the initial idea was given by a commonly used method called "forage traps" for collecting both sexes of adults of click beetle species (Furlan, 2004). Using 40 x 40 cm plastic sheets put on bare soil in areas known to be infested with wireworms, and the sheets being covered with fresh foliage of different Gramineae and/or Leguminosae plants, adult beetles congregate below the foliage and can easily be collected on the sheets. This same method was reported to be efficient to collect _A. brevis_ with sheets covered with _Lolium italicum_ (Gramineae) or _Medicago sativa_ (Leguminosae) foliage, most abundant constituents of the typical habitat type of _A. brevis_ in Italy (L. Furlan, pers. comm.). Assuming that plant-derived volatiles are responsible for the aggregation of beetles under the foliage, at least partially, preliminary field tests with YF traps (Furlan et al., 2004; this trap type is in wide use in pheromone trappings of click beetles in Europe) baited with a couple of shoots of
M. sativa or L. italicum were set up. Traps containing shoots of either plant species caught significantly more A. brevis than empty control traps (Vuts et al., 2011).

Consequently, volatiles were collected from N. sativa and L. italicum shoots, and structure elucidation of compounds eliciting responses from the antennae of A. brevis in GC-FID/EAD studies was attempted. So far we identified 5 compounds in volatile collections from L. italicum, and 9 compounds from M. sativa. The compounds (Z)-3-hexenyl acetate (which was the dominant constituent) and methyl benzoate were present in collections from both plant species.

![Image of Figure 2](image-url)

**Figure 2.** Catches (mean + SE) of Agriotes brevis in traps baited with blends of compounds identified from volatiles of cut Lolium italicum and Medicago sativa plants formulated in different dispensers in trapping tests in Veneto, Italy (Data from M. Tóth, L. Furlan, J. Vuts, unpublished). Disp-1 = polyethylene capsule; disp-2 = polyethylene bag with dental roll; disp-3 = polyethylene capsule with piece of dental roll. Columns with the same letter within one diagram are not significantly different by ANOVA, Student-Newman-Keuls, P = 0.05.

In preliminary field tests in 2009, a blend of the 5 Lolium compounds formulated in two different types of dispensers caught significantly more A. brevis than unbaited traps, and a considerable percentage of the capture were females (Figure 2, grey pie chart area). This was confirmed in 2010 by using a reduced blend of only 4 components occurring partly in the L. italicum or M. sativa volatiles (or both), when again higher catches were recorded in baited traps with both dispenser types tested. In 2011, traps baited with the synthetic plant-derived lure caught again higher numbers than unbaited traps, however, only ca. half of the numbers in traps baited with natural L. italicum shoots.

In conclusion, although the first results are highly promising, further optimization and improvement is needed to develop a lure based on synthetic green-leaf semiochemicals for practical use in click beetle control.
Acknowledgements

The research presented was partially supported by OTKA grant K81494 to MT and by grant DO02-244/2008 of the Bulgarian National Scientific Fund to MS.

References


New perspectives for wireworm control based on an improved understanding of their feeding ecology

Michael Traugott, Karin Staudacher, Nikolaus Schallhart, Corinna Wallinger
Mountain Agriculture Research Unit, Institute of Ecology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria

Abstract: Wireworms, the soil-dwelling larvae of click beetles (Coleoptera: Elateridae), are found throughout the world and they damage a wide spectrum of arable and vegetable crops. Unfortunately, these insects are hard to control using insecticidal-, biological-, and cultivation-based control measures, as their behaviour and occurrence in the soil column is hard to predict. Developing alternative management tactics is thus rooted in a sound understanding of the biology and ecology of these insects. In this context, the knowledge of the feeding ecology of wireworms is a key aspect. Fortunately, within the last few years considerable progress has been made in this research area as novel technology allowed examining the feeding behaviour of wireworms in both mesocosm and fielding experiments. This work examined, which plant species are preferentially attacked, when feeding occurs, and how environmental parameters affect wireworm feeding behaviour. In this talk we will synthesize the current understanding of the feeding ecology of Agriotes wireworms and identify, how this knowledge can be employed to improve the control of these pests.

Key words: Elateridae, pest control, Agriotes spp., diet
Posters
Exploratory use of geometric morphometrics in the identification of wireworm species

Darija Lemić¹, Katarina Mikac², Hugo A. Benitez³, Maja Ćaćija¹, Antonela Kozina¹, Renata Bažok¹
¹University of Zagreb, Faculty of Agriculture, Department for Agricultural Zoology, Sveštismunska 25, 10000 Zagreb, Croatia; ²Institute for Conservation Biology and Management, University of Wollongong, Australia; ³Faculty of Life Sciences, University of Manchester, UK

Abstract: Wireworms are click beetle larvae from the genus Agriotes (Coleoptera: Elateridae) that cause considerable damage to field crops. Five of the 18 Agriotes species recorded in Central Europe are found in arable land in continental Croatia and can cause significant economic yield losses. The identification of these larvae to the species level is difficult using classical taxonomic measurements. Our study explores the use of species-specific morphological characters (i.e. specific spiracles placed on the ninth abdominal segment, certain structures of the mandible) that will enable the use of geometric morphometric methods for diagnostic purposes. Geometric morphometrics (GM) is the quantitative measurement, analysis and interpretation of shape variation in organisms. The application of GM in taxonomy and systematic is novel and has the potential to provide information on shape variation through the relative position of anatomical landmarks. GM has been previously used in WCR population analyses, Bactrocera dorsalis species complex, Tortricidae and Geometridae species analyses and other important agricultural insect pests. The aim of this study was to explore the use of landmark-based morphometric analyses as a simple method to discriminate among species in mixed wireworms populations. Approximately 10 landmarks were used in species discrimination of five Agriotes species, including A. sputator, A. lineatus, A. brevis, A. obscurus, A. ustulatus, randomly collected on arable land across Croatia. Each landmark was digitised and imported in MorphoJ software for further statistical analyses. Statistical procedures used in this study were: Generalized Procrustes Analyses, Discriminant Function Analyses, Principal Component Analyses and Canonical Variates Analyses. Morphometric results will be verified by polymerase chain reaction (PCR) analyses of Agriotes species using diagnostics primers published by Staudacher et al. [Bull. Entomol. Res. 101: 201-210 (2011)]. We demonstrate that GM techniques hold promise as a diagnostic tool for discriminating between morphologically cryptic taxa of the Agriotes species complex.

Key words: wireworms, geometric morphometrics, species identification
Development of novel biocontrol encapsulation techniques for garlic extracts: first results

Bianca Pelzer, Miriam Hanitzsch, Anant Patel
Department of Engineering Sciences and Mathematics, University of Applied Sciences Bielefeld, Wilhelm-Bertelsmann-Str. 10, 33602 Bielefeld, Germany

Abstract: The aim of the work presented here is to develop novel eco-efficient encapsulation techniques for bioactive ingredients used in biological pest control for the EU project INBIOSOIL. A process for production of small-scale alginate beads containing garlic extract by air atomization with self-constructed technical encapsulation equipment was developed. The encapsulation protects the active ingredients against oxygen and other outside influences, thus enhancing shelf life and allows a slow release effect. The capsule size produced with this technology can be varied - depending on the desired product - between 4 and 600 µm. Particles are stable and spherical.

Key words: alginate beads, garlic extract, air atomization, encapsulation, formulation, capsules, confuse and kill, biological control, semiochemicals, bioinsectide, wireworms, western corn rootworm

Introduction

Recent studies have shown that, beneath other beneficial effects, garlic (Allium sativum) has bioinsecticidal activity and thus can be used for insect pest control (Feng-Lian et al., 2009). Some of the active components contained in garlic (e.g. allicin) are sensitive against several external factors (e.g. oxygen, light), highly volatile and insoluble in water. It follows that slow or controlled release systems that can stabilize sensitive components of garlic extracts, e.g. novel capsule formulations, are needed.

To this end investigations of release kinetics (e.g. slow or controlled release and release from a depot) are crucial. The release of active ingredients can be either constant or triggered by environmental conditions (e.g. temperature, humidity etc.) and encapsulation material properties. Hereby efficacy can be enhanced and application costs can be reduced due to a decreased number of applications. Preliminary studies on lab scale have shown that garlic juice can be encapsulated in beads to slow down the release of active ingredients to control Phytophthora (Slusarenko et al., 2008).

The aim of this work was to develop a novel process for the production of garlic capsules based on biodegradable materials.

Material and methods

Encapsulation of garlic extract

A solution of 1% sodium alginate and garlic extract provided by Neem Biotech was atomised through a nozzle with self-constructed technical encapsulation equipment. The droplets fell into a calcium chloride solution, where they hardened. Particle sizes from 4 to 600 µm were obtained, thus offering a wide range of application options. When dried, particle size can be further reduced, offering the option of reaching nano scaled products.
Results and discussion

As encapsulation material for the garlic extract, containing the oxygen-sensitive substance allicin, the hydrophilic biopolymer hydrogel calcium alginate was considered as encapsulation matrix due to its low oxygen permeability, low toxicity and gelling properties (e.g. Heuskin et al., 2012).

Air-atomization method fits the properties of the sensible garlic extract since heat effects are avoided and reaction conditions are gentle. In order to optimize both particle shape and particle size distribution, several hollow cone nozzles were tested. The one providing the finest capsules with the best particle size distribution was used.

Further optimization of particle size distribution was achieved by installing the nozzle horizontally rather than vertically. This way, droplets of larger size, having a higher weight than smaller droplets, could be sorted out, as shown in Figure 1.

Figure 1. Optimized air-atomization method, offering reduced particle size distribution by fractioning.

The following pictures (Figure 2) show the effect of these first optimisations regarding particle size distribution for calcium alginate beads.
Also, the distance between nozzle and calcium chloride solution was increased, in order to offer a proper time of spherical droplet formation. By this, an optimal particle shape was obtained. Figure 3 shows the effect of a lower distance between nozzle and CaCl$_2$ solution, as well as a stable particle of spherical form as a result.

For the formation of small-scaled particles (about 4 µm), a tenside was added to the CaCl$_2$ solution, since otherwise particles were unable to penetrate the solution surface due to low weight and size.

Apart from the physical properties, sensory evaluation showed that typical “garlic odour” was decreased by encapsulation compared to pure garlic extract indicating a slow release effect of allicin, one of the lead components.

Future experiments will deal with measurements of exact particle size, particle size distribution and physical stability of capsules. Furthermore, drying of capsules down to nano-scale will be investigated.

With regard to application, novel co-formulations with semiochemicals and other pesticidal agents will be developed, implementing a “confuse and kill” or “attract and kill” strategy. Efficacy of the capsules obtained with the developed methods will be tested by the work partners of the EU project INBIOSOIL against wireworms on potato, western corn rootworm on maize, black vine weevil on strawberries and sciarids in growing media.
Acknowledgements

This research was funded by the EU project IOBC, Grant agreement no. 282767.

References

The project ATTRACT: Protection of crops from soil-borne insect pests with a novel attract and kill strategy

Marina Vemmer¹, Wilhelm Beitzen-Heineke², Hubertus Kleebeg³, Edmund Hummel³, Stefan Vidal⁴, Anant Patel¹
¹University of Applied Sciences, Department of Engineering and Mathematics, Engineering and Alternative Fuels, 33602 Bielefeld, Germany; ²BIOCARE GmbH, Dorfstr. 4, 37574 Einbeck, Germany; ³TRIFOLIO-M GmbH, Dr.-Hans-Wilhelmi-Weg 1, 35633 Lahnau, Germany; ⁴Georg-August University Goettingen, Department for Crop Sciences, Agricultural Entomology, Grisebachstrasse 6, 37077 Goettingen, Germany

Abstract: The project ATTRACT targets the development of a novel attract-and-kill strategy for the protection of crops from soil-borne insect pests. The aim is the design of a plant protection product with an innovative formulation based on CO₂ emitting sources as an attractive compound and environmentally friendly insecticidal compounds.

Key words: attract-and-kill, pest control, wireworm, western corn rootworm, black vine weevil

Introduction

Larvae of herbivorous insects (e.g. wireworms, western corn rootworm, black vine weevil) cause severe losses in many crops (potato, maize, strawberry). A control of these pests with soil insecticides is severely restricted or has recently been abandoned. The project ATTRACT aims at developing innovative attract and kill formulations which can be produced in technical scale and can then be used as novel control strategies against soil-borne insect pests in conventional as well as organic farming systems. By attracting larvae to the capsules containing a kill compound (Figure 1) insecticide applications or other control strategies can be replaced, the amount of insecticides can be minimized and the environment and health of farmers and consumers can be protected.

Outlook

In the project ATTRACT novel formulations (capsules, granules) based on CO₂ emitting sources will be developed and tested under practical conditions in order to lure larvae away from plant roots. In these attract formulations plant-based environmentally friendly insecticidal compounds, such as neem and quassin will be incorporated in multiphase or multilayer systems with additives. These formulations will be optimized in efficacy tests in lab, greenhouse and field experiments. First data on encapsulation and efficacy will be shown.
Figure 1. Larvae use CO$_2$ to locate the roots of living corn plants (A). Larvae are attracted by CO$_2$ emitting sources and are killed by an environmentally friendly insecticidal compound (B).

Acknowledgements

The project is supported by funds of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.
IPM (Fungi/Bacteria)

IPM microbial control based strategies
Combined use of entomopathogenic fungi and their extracts to improve the control of the cotton leafworm *Spodoptera littoralis* (Boisduval) (*Lepidoptera: Noctuidae*)

Inmaculada Garrido-Jurado, Gloria Resquín-Romero, Enrique Quesada-Moraga
Universidad de Córdoba, Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, 14071 Córdoba, Spain
e-mail: g72gajui@uco.es

**Abstract:** This work has evaluated the activity of entomopathogenic fungi and their extracts against *Spodoptera littoralis* larvae, a very harmful polyphagous pest. Twenty-six *Metarhizium* spp. and *Beauveria* spp. isolates and their crude extracts were evaluated against second instar *S. littoralis* larvae. Among *Beauveria* isolates, the higher mortality rates and lower survival times were in the range of 75-80% and 8.7-9.6 days respectively. Among *Metarhizium* isolates, the higher mortality rates and lower survival times were in the range of 55-60% and 9.0 days respectively. The crude extracts from the most virulent isolates, 6 from *Beauveria* and 2 from *Metarhizium*, were obtained in Adamek's liquid medium and bioassayed against second instars in alfalfa leaf disc experiments. The extracts of *Metarhizium* isolates EAMb 09/01-Su and EAMA 01/58-Su caused the highest mortality rates, 80.0 and 66.6%, and lowest AST values, 5.1 and 4.4 days, respectively. Combined treatments of fungal suspensions of isolates EAMb 09/01-Su and EAMA 01/58-Su and their extracts caused higher mortality rates than the single ones, in a dose-dependent manner, with mortality rates reaching 100% for EAMb 09/01-Su isolate and its extract at 1 mg ml\(^{-1}\) and 76.0% mortality for EAMA 01/58-Su, and its extract at 1 mg ml\(^{-1}\). These results highlight the potential of a *S. littoralis* integrated control strategy based on the combined use of entomopathogenic fungi and their extracts.

**Key words:** biological control, synergism, metabolites, *Metarhizium, Beauveria*
Insecticidal activity of a semi-purified extract from Metarhizium brunneum (Ascomycota: Clavicipitaceae) against the red palm weevil Rhynchophorus ferrugineus (Coleoptera: Curculionidae)

Inmaculada Garrido-Jurado¹, Óscar Dembilio², Josep Anton Jacas², Lola Ortega¹, Carlos Campos¹, Enrique Quesada-Moraga³
¹Universidad de Córdoba, Departamento de Ciencias y Recursos Agrícolas y Forestales, ETIAM, 14071 Córdoba, Spain; ²Universitat Jaume I (UJI), Unitat Associada d’Entomologia Agrícola – Institut Valencià d’Investigacions Agràries (IVIA), Departament de Ciències Agràries i del Medi Natural, Campus del Riu Sec, Av. de Vicent Sos Baynat, s/n. 12071 Castelló de la Plana, Spain

Abstract: The red palm weevil, Rhynchophorus ferrugineus (Olivier) (Coleoptera: Curculionidae), is currently considered the most damaging pests of palms worldwide. It has spread extensively from its origin mainly by trading of infested palm trees and offshoots. In Europe, many preventative and curative procedures, mostly chemical, have been implemented with variable degrees of success to eradicate and contain R. ferrugineus. However, these techniques have been hampered by environmental concerns related to the use of pesticides and legislation restricting their use. For this reason, there is an increasing interest in the use of natural enemies of R. ferrugineus with emphasis in entomopathogenic fungi (EPF), which have provided encouraging results as microbial control agents of this pest. Nevertheless, EPF have also shown to be a poorly studied source of insecticidal compounds of natural origin. The aim of this study was to determine the insecticidal activity of the crude extract of Metarhizium brunneum EAMb 09/01-Su strain against R. ferrugineus and to purify the active fractions. The crude extract containing low-molecular-weight secondary metabolites was separated into different fractions by adjusting the acetonitrile/water ratio in the gradient elution buffer of the semi-preparative HPLC. Subsequently, this extract was evaluated per os against R. ferrugineus adults and larvae. The F5B and F6 fractions showed high oral toxicity and this is the first evidence of insecticidal activity of fungal compounds against R. ferrugineus.

Key words: entomopathogenic fungi, Curculionidae, metabolite, per os activity, biological control
Subterranean control of an arboreal pest: EPNs and EPFs for FCM

Sean Moore1,2, Candice Coombes2, Aruna Manrakhan1, Wayne Kirkman1, Martin Hill2, Ralf-Udo Ehlers3, John-Henry Daneel1, Jeanne de Waal4, Jo Dames2, Antoinette Malan5

1Citrus Research International, PO Box 20285, Humewood 6031, Port Elizabeth, South Africa; 2Department of Zoology and Entomology, PO Box 94, Rhodes University, Grahamstown 6140, South Africa; 3Institute for Phytopathology, Department of Biotechnology and Biological Control, Christian-Albrechts-University Kiel, Hermann-Rodewald Str. 9, 24118 Kiel, Germany; 4Dow AgroSciences, PO Box 355, Paarl 7620, South Africa; 5Department of Conservation Ecology and Entomology, Stellenbosch University, P/Bag X1, Matieland, 7602 Stellenbosch, South Africa

Abstract: Control measures against the false codling moth (FCM), Thaumatotibia leucotreta, have traditionally ignored the soil-borne pupal stage. Recent trials with entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPFs) have targeted this life-stage. Application of Heterorhabditis bacteriophora to a citrus orchard floor, reduced T. leucotreta infestation of fruit by up to 81%. Conservation of H. zealandica through non-usage of a nematicide also resulted in dramatically lower fruit infestation. Dose-response and exposure time-response bioassays identified the three most promising fungal isolates against pupating T. leucotreta. Orchard trials showed persistence of these fungi in orchard soil for at least six months.

Key words: Thaumatotibia leucotreta, entomopathogenic nematodes, Heterorhabditis, entomopathogenic fungi, Beauveria bassiana, Metarhizium anisopliae

Introduction

False codling moth (FCM), Thaumatotibia (= Cryptophlebia) leucotreta (Meyrick) (Lepidoptera: Tortricidae), is an important pest of citrus and other crops in South Africa (Newton, 1998). It has traditionally been controlled by targeting the above-ground life-stages. These includes the egg, which is laid on the fruit (e.g. using egg parasitoids and insect growth regulators); the larval stage, between hatching and penetration into the fruit (e.g. using granulovirus and chemicals); and the nocturnally active moth (e.g. using mating disruption, attract and kill, and the sterile insect technique) (Moore & Hattingh, 2012). Recently, research has focussed on targeting the previously ignored soil-borne pupal stage. This has been done with EPNs and EPFs. Research with EPNs is in the final stage of field work, whereas work with EPFs is still relatively new. However, both groups of pathogens are showing strong potential as additional weapons against T. leucotreta.

Material and methods

Entomopathogenic nematodes: introduction

This trial was conducted on Klawervlei Farm in the Western Cape Province of South Africa (32°21′26″S 18°55′82″E). Four Palmer Navel orange orchards of approximately 1 ha each, with micro sprinkler irrigation, were used. Commercially formulated, semi-desiccated Heterorhabditis bacteriophora infective juveniles (IJs) (e-nema, Germany) were applied on
27 September 2011 (spring) in three of the orchards and one was left as an untreated control. EPNs were applied to the soil using a spray machine in two of the orchards (1450 l water per ha: 10 and 20 IJs cm$^{-2}$ respectively) and through the irrigation system at 20 IJs cm$^{-2}$ (in 200 l water) in the other – all followed by 6 h irrigation.

Before application of EPNs and at 1, 4 and 8 weeks after application, monitoring was conducted to determine presence of EPNs in the soil. Six small cages, each with 20 T. leucotreta (sentinel) larvae, were planted per treatment. Cages were removed after one week and T. leucotreta larvae were counted, recorded as alive or dead and infested (with EPNs) or not.

T. leucotreta pheromone traps were hung and monitored weekly from 6 October 2011 until the trial was terminated on 15 March 2012. Weekly from 22 December 2011 to 15 March 2012, all fruits which had dropped from 10 data trees in the middle of each treatment block were retrieved and assessed (dissected and inspected) for T. leucotreta infestation.

**Entomopathogenic nematodes: conservation**

On Crocodile Valley Estate in Mpumalanga Province (25º28’39”S 31º03’59”E), the natural occurrence of H. zealandica in the soil was determined to be high. Rugby (cadusafos) (100 g/l ME (EW)) (FMC Chemicals, USA), a locally available nematicide, was applied to the soil in a 1 ha block of Washington Navel orange trees; an adjacent block of the same size was left untreated and used as a control. Rugby was applied to the soil underneath trees at a rate of 20 ml (a.i.) m$^{-2}$ and followed by irrigation. Natural occurrence of EPNs and the impact of the cadusafos application on these EPNs was determined as described for the previous trial. This was done before application and 2, 4 and 8 weeks post-application. Monitoring of fruit infestation by T. leucotreta larvae was also initiated immediately after application (3 February 2012), as described above, and continued until 25 April 2012.

**Entomopathogenic fungi: bioassays**

Goble et al. (2010) identified 62 potentially useful EPF isolates from in and around citrus orchards in the Eastern Cape Province of South Africa. Twelve isolates were identified by Goble et al. (2011) as having potential for control of T. leucotreta and fruit flies.

In this study, eight of these isolates (and two commercial isolates) were further investigated in the form of concentration dose-response bioassays, using three concentrations ($1 \times 10^4$, $1 \times 10^5$ and $1 \times 10^6$ conidia ml$^{-1}$). Fungal suspension (5 ml) was mixed with autoclaved sand (50 g) in petri dishes. Twenty fifth-instar larvae, ready to pupate within the next 24 hours, were placed on the sand and incubated at 26°C (12:12 h, L:D). After 7 days, the pupae were removed and placed on sterile sand and incubated as before. Ten days after first emergence, the number of dead pupae as well as emerged and dead adults was recorded. Dead adults and pupae were surface sterilised in 70% ethanol and placed on SDA plates so that mycosis could be observed. The procedure was replicated four times for each isolate. PROBAN (Van Ark, 1995) was used to determine the LC$_{50}$ and LC$_{90}$ values for each fungal isolate investigated.

For exposure time-response bioassays, two concentrations were investigated (LC$_{50}$ and $1 \times 10^7$ conidia ml$^{-1}$). For each concentration, fifth-instar larvae were exposed to the fungus-inoculated soil for different time periods (1, 3, 5 and 7 days). An untreated control was used for each time period. Logit analysis was used to determine the LT$_{50}$ and LT$_{90}$ value for each fungal isolate investigated. The commercial isolates were not included.
Entomopathogenic fungi: field persistence
Rice overgrown with each of three fungal isolates (0.5 g per isolate) was separately mixed per isolate with 100 g autoclaved orchard soil. This was then placed into net bags and buried a few centimetres below the soil under citrus trees at Mosslands Farm in the Eastern Cape Province (33°24′S 26°26′E). Each month for six months, four bags of each isolate and a control were collected. Laboratory assays were conducted using 50 g soil from each bag and from the control, following the procedure described above. In addition, CFU (colony forming unit) counts were performed for each net bag including the control. Non-normal data were analysed using the Kruskal-Wallis non-parametric test after transformation and then subjected to a multiple mean rank test. Normally distributed data were analysed by ANOVA followed by Tukey’s post-hoc test. Linear regression analysis was used to test for correlation between the monthly CFU count and mycosis percentage.

Results and discussion

Entomopathogenic nematodes: introduction
The lowest *T. leucotreta* trap catches and fruit infestation were recorded in the two treatments where EPN persistence was good, i.e. the 10 IJs cm$^{-2}$ spray and the 20 IJs cm$^{-2}$ irrigation. Relative to the untreated control, FCM infestation over this period was reduced by 63.64% and 54.55% by the 10 IJs cm$^{-2}$ treatment and the irrigation treatment, respectively. Relative to the 20 IJs cm$^{-2}$ spray treatment (which can be considered as a second untreated control because no survival of EPNs was recorded beyond one week) infestation was reduced by 80.95% and 76.19%, respectively. EPN survival in this orchard was poor, as the irrigation system in the orchard was deficient, resulting in inadequate soil-moisture levels after EPN application. Trap catches and fruit infestation were significantly higher in this second control (the high-dose spray treatment) than the other two EPN treatments (P<0.05). Trap catches and fruit infestation between the micro sprinkler-applied treatment and the untreated control did not differ significantly (P > 0.05).

Entomopathogenic nematodes: conservation
Before application of the nematicide, EPN levels in the two orchards were similar. At two and four weeks after application, mean (± SE) percentage infestation of sentinel *T. leucotreta* larvae with *H. zealandica* in the untreated control was 19.5 ± 8.9% and 24.7 ± 12.3%, respectively, while in the nematicide-treated block it was 1.9 ± 1.9% and 3.4 ± 2.2%, respectively. Rugby significantly reduced the level of EPNs in the soil, but these had fully recovered by eight weeks after treatment.

Consequently, mean (± SE) number of fruit infested with *T. leucotreta* larvae per tree per week for the 13 weeks from immediately post-application of the nematicide to harvest (3 February to 25 April 2012), was 0.09 ± 0.03 in the control block and 0.22 ± 0.06 in the treated block. Although infestation was more than 2.4 times higher in the nematicide-treated than untreated block, the difference was not statistically significant (P > 0.05).

Entomopathogenic fungi: bioassays
The three isolates which generally caused the lowest *T. leucotreta* eclosion, highest pupal mortality and lowest LC$_{50}$ and LC$_{90}$ values are listed in Table 1. The commercial products did not fare well in comparison (Table 1), however, neither of them are registered or recommended for use against *T. leucotreta*. 
Table 1. Lethal concentrations (LC\textsubscript{50} and LC\textsubscript{90}) for the three most promising fungal isolates and two commercial isolates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Lethal Concentration [conidia ml\textsuperscript{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{LC}<em>{50} ) \quad \text{LC}</em>{90} \</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>G 11 3 L6</td>
<td>( 6.26 \times 10^5 ) \quad 1.91 \times 10^7 \</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>FCM Ar 23 B3</td>
<td>1.92 x 10\textsuperscript{6} \quad 1.67 \times 10^8</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>G Ar 17 B3</td>
<td>1.98 x 10\textsuperscript{5} \quad 1.02 \times 10^7</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Eco-Bb\textsuperscript{®}</td>
<td>2.16 x 10\textsuperscript{6} \quad 1.92 \times 10^10</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>ICIPE 69</td>
<td>2.60 x 10\textsuperscript{7} \quad 2.08 \times 10^10</td>
</tr>
</tbody>
</table>

Logit analysis indicated that it would require a minimum of 5 and a maximum of 14 days to obtain an LT\textsubscript{50} and LT\textsubscript{90} respectively at the LC\textsubscript{50} concentration whilst at the higher concentration, \( 1 \times 10^7 \) conidia ml\textsuperscript{-1}, a minimum exposure time of 4 and a maximum of 9 days was required to obtain a LT\textsubscript{50} and LT\textsubscript{90}, respectively.

**Entomopathogenic fungi: field persistence**

For all isolates, including the commercial isolates tested, a large initial decrease was recorded in the number of CFU per gram of soil over the first month. CFU numbers stabilised thereafter. After six months in the field, all fungal isolates were still present, although at relatively low numbers, within the soil (G 11 3 L6 – \( 1.14 \times 10^4 \) CFU g\textsuperscript{-1}; FCM Ar 23 B3 – \( 1.46 x 10^3 \) CFU g\textsuperscript{-1}; G Ar 17 B3 – \( 2.71 \times 10^4 \) CFU g\textsuperscript{-1}; Eco-Bb\textsuperscript{®} – \( 2.93 \times 10^1 \) CFU g\textsuperscript{-1} and ICIPE 69 – \( 9.42 \times 10^2 \) CFU g\textsuperscript{-1}). The greatest decrease in CFU g\textsuperscript{-1} was obtained for Eco-Bb\textsuperscript{®} with the least for G 11 3 L6.

Average percentage mycosis varied greatly for all isolates over the six month period. In some cases, even though a decrease in the number of CFU g\textsuperscript{-1} was recorded, the average percentage mycosis still increased. For example, for isolate G 11 3 L6, even though the CFU count recorded for T\textsubscript{1} was lower than that recorded for T\textsubscript{0}, FCM percentage mycosis still increased significantly from 59.9% to 92.3%.

**References**


Do plant-associated insect toxin producing pseudomonads have the potential for the biocontrol of insect pests?

M. Maurhofer¹, B. Ruffner¹, P. Flury¹, M. Péchy-Tarr², E. Fischer³, P. Kupferschmied², C. Keel²
¹Plant Pathology/Institute of Integrative Biology, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland; ²Department of Fundamental Microbiology, University of Lausanne, 1015 Lausanne, Switzerland; ³Natural Resources Sciences, University of Applied Sciences ZHAW, 8820 Wädenswil, Switzerland

e-mail: monika.maurhofer@usys.ethz.ch

Abstract: The excellent root colonizers Pseudomonas protegens and Pseudomonas chlororaphis were so far mainly known for their ability to produce antifungal compounds and to suppress soil-borne plant diseases. In contrast to other biocontrol pseudomonads these two species are also able to produce a potent insect toxin (Fit) and to display oral and injectable activity against insects. Pseudomonas based products against bacterial and fungal plant diseases are already on the market. If certain Pseudomonas biocontrol agents could be used against both, plant diseases and plant pests this would of course be highly interesting for the development of a new kind of biocontrol product. We are therefore currently evaluating the potential of Fit toxin producing pseudomonads for the biological control of insect pests. The Fit producing model strains P. protegens CHA0 and P. chlororaphis PCL1391, which are highly effective against fungal diseases, display also high oral activity against larvae of different lepidopteran insect pests. CHA0 is also orally active against the aphid Acrithosiphon pisum, but is neither toxic to the large earth bumblebee Bombus terrestris an important pollinator nor to larvae of the mosquito Aedes aegypti. CHA0 can establish high populations in larvae of the root weevil Otiorhynchus sulcatus and of the European cockchafer (Melolontha melolontha) and change the composition of the larvae's own bacterial flora. When fed to larvae of O. sulcatus, CHA0 survives the pupal stage and can be recovered from hatched adults. Additional experiments showed that CHA0 is compatible with the biocontrol fungus Metarhizium anisopliae. Taken together our results suggest that Fit producing pseudomonads can establish and survive very well in insects, but that high virulence might be specific for certain insect genera.

Key words: Pseudomonas protegens, Pseudomonas chlororaphis, Fit insect toxin
Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays

P. Flury¹, B. Ruffner¹, M. Péchy-Tarr², P. Kupferschmied², C. Keel², M. Maurhofer¹
¹Institute of Plant Pathology, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland; ²Department of Fundamental Microbiology, University of Lausanne, 1015 Lausanne, Switzerland

Abstract: Pseudomonas bacteria demonstrate in a stunning way the metabolic and ecological diversity of bacterial lifestyles. Their ecology may differ dramatically, even between closely related strains and include pathogenic and beneficial bacteria. The Pseudomonas fluorescens group harbors many root-associated biocontrol agents that suppress soil-borne fungal diseases of many different crops. Remarkably, strains of the species Pseudomonas protegens and Pseudomonas chlororaphis also display potent oral insecticidal activity towards lepidopteran insect larvae. Insecticidal activity is triggered in part by the Fit insect toxin and unknown GacA regulated traits. The main aim of the presented study is to discover the traits enabling plant-associated pseudomonads not only to antagonize fungal pathogens but also to infect, colonize and kill insects. To this end, 15 strains of fluorescent pseudomonads were characterized for their biocontrol activity against root pathogens, as well as their oral insecticidal activity and their ability to multiply in insect larvae. While most strains were able to colonize insect larvae upon ingestion, the lethal oral activity was restricted to strains of P. protegens and P. chlororaphis. The experimental approach was complemented by a comparative genomic survey of the 15 fluorescent pseudomonads, to yield an improved understanding of insect pathogenicity and the contributing virulence factors. We found that a remarkable 37% of predicted protein coding genes shared by P. protegens and P. chlororaphis, but absent in other strains of the P. fluorescens group, could be potentially associated with insect virulence. Our combined approach of genomic survey and bioassays reveals intriguing aspects on insect association and insect pathogenesis of plant-associated pseudomonads and identifies several Pseudomonas strains with potent dual activity against root pathogens and insect pests.

Key words: Pseudomonas species, plant association, genomics, insect virulence
**Colorado potato beetle** (*Leptinotarsa decemlineata* Say) - control strategies in organic farming using biological insecticides (azadirachtin, *Bacillus thuringiensis* var. *tenebrionis*, pyrethrum and spinosad)

**Stefan Kühne**, **Uta Priegnitz**, **Benjamin Hummel**, **Frank Ellmer**

1. Federal Research Centre for Cultivated Plants (Julius Kühn Institute, JKI), Institute for Strategies and Technology Assessment in Plant Protection, 14532 Kleinmachnow, Germany; 2. Humboldt University Berlin, Faculty of Agriculture and Horticulture, Albrecht-Thaer-Weg 5, 14195 Berlin

**Abstract:** Field experiments using different control agents for Colorado potato beetle control showed that all tested application strategies were effective. Time-shifted application of neem (NeemAzal T/S) and *Bacillus thuringiensis* var. *tenebrionis* (B.t.t.) (Novodor FC) as well as double treatment with B.t.t. achieved under optimal weather conditions gave an effectiveness level of over 80% and increased yields. Surprisingly, a single application of spinosad (SpinTor) also proved to be very effective (> 80%) in the three years studied in spite of the difficult study conditions in 2009. Due to lower cost and a high efficacy of Spinosad it is likely farmers would prefer this plant protection product. Considering resistance of the Colorado potato beetle it is recommended to change the insecticides every year.

**Key words:** Colorado potato beetle, organic farming spinosad, *Bacillus thuringiensis*, neem

**Introduction**

In Germany, the increasing demand for organically grown potatoes could not be met although the acreage has been increased to more than 8200 hectares (ZMP, 2008). Two of the main reasons for this deficit are yield losses caused by early summer drought and heavy infestation by the Colorado potato beetle. Recent increases in potato beetle occurrence in some regions of Germany can be attributed to increased area size, regional concentration of crops with close crop rotation, and increasing resistance of the potato beetle to pyrethroids (Nauen, 2005). In many areas, preventive measures do not suffice to prevent potato beetle damage. Insecticides can and should be used to prevent economic losses in these cases - even in organic farming. In order to develop a strategy for sustainable control of the Colorado potato beetle in organic farming, the Julius Kühn Institute launched a series of field tests at an EU-certified organic farming site (Control No. D-ST-043-4829) located in Dahnsdorf, Germany. These studies focused on the efficacy, combinability, optimal timing of application of biological insecticides approved for use in organic farming. This article is a continuation of results that have been published by Kühne et al. (2012).

**Material and methods**

The studies were conducted in accordance with the specifications in EPPO guideline PP 1/12 (3). The tests were conducted using a block design with four repetitions of each treatment...
variant and one untreated control. The plot size for each treatment variant was 6 × 34 m in all years. The number of potato beetles and the percentage of feeding damage was determined weekly on the same 10 randomly selected and marked potato plants per treatment variant. This made it possible to estimate the variance attributable to local Colorado potato beetle occurrence and to perform an analysis of infestation dynamics per plant.

In practice, treatment is usually necessary in potato fields when the average number of beetles detected is one egg mass or 10 larvae (L1, L2) per plant on 5 plants from 5 sampling lines distributed across a given field. In addition to scouting, simulations with SIMLEP3 (Simulation: Leptinotarsa), a computer-aided decision support tool developed by the Central Office of the German states (ZEPP), were performed in all the years studied. SIMLEP3 can be used to model the population dynamics (maximum occurrence of development stages) of the Colorado potato beetle in order to determine the optimal timing of treatment. SIMLEP3 uses a temperature-sum method to calculate the population dynamics of the potato beetle from the first sign of egg-laying to the emergence of first instar larvae. The date of first egg-laying in the field was used as the input value for model calculation of the time of maximum number of first instar larvae. Local weather data are also needed for the forecast. Our calculations were based on weather data obtained from the weather station located directly on the test site in Dahnsdorf.

Table 1. Biological insecticides used to control the Colorado potato beetle

<table>
<thead>
<tr>
<th>Active substance concentration</th>
<th>NeemAzal-T/S</th>
<th>Novodor FC</th>
<th>SpinTor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azadirachtin (neem)</td>
<td>10 g l⁻¹</td>
<td>20 g l⁻¹</td>
<td>480 g l⁻¹</td>
</tr>
<tr>
<td>Bacillus thuringiensis var. tenebrionis (B.t.t.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larvae (L1 to L2)</td>
<td>3.0</td>
<td>5.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Larvae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X i – Irritating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N – Dangerous for the environment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water pollution control</td>
<td>No minimum distance to surface waters required</td>
<td>No minimum distance to surface waters required</td>
<td>Maintain minimum distance to surface waters; Very toxic to fish, fish bait and algae</td>
</tr>
<tr>
<td>Bee protection</td>
<td>Non-toxic to bees</td>
<td>Non-toxic to bees</td>
<td>Toxic to bees</td>
</tr>
<tr>
<td>Beneficial insects (BIs)</td>
<td>Not harmful to BIs except hoverflies (Episyrphus balteatus)</td>
<td>Slightly harmful to seven-spot ladybirds (Coccinella septempunctata)</td>
<td>Slightly harmful to seven-spot ladybirds (Coccinella septempunctata); Harmful to egg parasitoids (T. dendrolini)</td>
</tr>
<tr>
<td>Application volume (l/ha)</td>
<td>2.5</td>
<td>3.0 to 5.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Water volume (l/ha)</td>
<td>400</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>Price per unit</td>
<td>55 € l⁻¹</td>
<td>21 € l⁻¹</td>
<td>375 € l⁻¹</td>
</tr>
<tr>
<td>Treatment costs</td>
<td>16 € ha⁻¹</td>
<td>16 € ha⁻¹</td>
<td>16 € ha⁻¹</td>
</tr>
</tbody>
</table>
The different biological insecticide treatment variants (Table 1) were applied according to the manufacturer's instructions at the optimal application times and under optimal weather conditions (no direct sunlight, wind speed < 1 m/s, and temperature < 20 °C) each year except 2009, during which the treatments had to be postponed because the potato beetle counts did not reach the threshold level during the optimal treatment time frame predicted by SIMLEP3.

The first egg masses were found on 26 May 2009, but treatment was not necessary until 5 weeks later (5 July 2009). The reason for this delay was a cold snap at the beginning of egg-laying in late May 2009, which prolonged the oviposition period by several weeks. This also resulted in extension of the hatching period, and the beetle counts did not reach the treatment threshold until nearly 3 weeks later than in the previous years of the study. Consequently, the 2009 treatments were not administered until the 7th and 10th of July, which was far outside the predicted optimal treatment time frame set by SIMLEP3. Furthermore, a rain shower occurring shortly after the second treatment date severely impaired the efficacy of the treatment products. Because the 2009 plant surveys were also performed later than in other years.

Late blight (Phytophthora infestans) was treated as needed using a copper-based product (Cuprozin Flüssig, 750 g ha⁻¹ copper per treatment) throughout the entire test site in all years. The amount of pure copper used was 2.25 kg ha⁻¹ in 2008 and 2009, 1.5 kg ha⁻¹ in 2010. Sufficient control of late blight was achieved in all years except 2009.

Results and discussion

The results of our studies showed that all application strategies were effective to control the Colorado potato beetle (Table 2).

Table 2. Control of the Colorado potato beetle by biological insecticides at the test site in Dahnsdorf, Germany (Brandenburg), degree of effectiveness (%) based on the estimated leaf area loss attributable to feeding by the potato beetle 24-25 days after the first treatment and increase in yield (dt ha⁻¹) relative to the yields in the untreated controls (UC). * Statistically significant difference to the untreated controls (Tukey; P < 0.05). Active ingredient (a.i.): Bacillus thuringiensis var. tenebrionis (B.t.t.) 20 g l⁻¹ (Novodor FC), neem 10 g l⁻¹ (NeemAzal-T/S), and spinosad 480 g l⁻¹ (SpinTor).

<table>
<thead>
<tr>
<th>Year</th>
<th>1st treatment (T1)</th>
<th>Dose  (l ha⁻¹)</th>
<th>2nd treatment (T2)</th>
<th>Dose  (l ha⁻¹)</th>
<th>Time of 2nd treatment</th>
<th>Effectiveness (%)</th>
<th>Increase in yield (dt ha⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>B.t.t.</td>
<td>3</td>
<td>B.t.t.</td>
<td>5</td>
<td>T1 + 4 days</td>
<td>78 *</td>
<td>54 *</td>
</tr>
<tr>
<td>2009</td>
<td>B.t.t.</td>
<td>3</td>
<td>B.t.t.</td>
<td>5</td>
<td>T1 + 3 days</td>
<td>37 *</td>
<td>40 *</td>
</tr>
<tr>
<td>2010</td>
<td>B.t.t.</td>
<td>3</td>
<td>B.t.t.</td>
<td>5</td>
<td>T1 + 4 days</td>
<td>88 *</td>
<td>52</td>
</tr>
<tr>
<td>2008</td>
<td>Neem</td>
<td>2.5</td>
<td>B.t.t.</td>
<td>3</td>
<td>T1 + 4 days</td>
<td>82 *</td>
<td>70 *</td>
</tr>
<tr>
<td>2009</td>
<td>Neem</td>
<td>2.5</td>
<td>B.t.t.</td>
<td>5</td>
<td>T1 + 3 days</td>
<td>43 *</td>
<td>53 *</td>
</tr>
<tr>
<td>2010</td>
<td>Neem</td>
<td>2.5</td>
<td>B.t.t.</td>
<td>5</td>
<td>T1 + 4 days</td>
<td>82 *</td>
<td>21</td>
</tr>
<tr>
<td>2008</td>
<td>Spinosad</td>
<td>0.05</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>82 *</td>
<td>103 *</td>
</tr>
<tr>
<td>2009</td>
<td>Spinosad</td>
<td>0.05</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>83 *</td>
<td>37 *</td>
</tr>
<tr>
<td>2010</td>
<td>Spinosad</td>
<td>0.05</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>87 *</td>
<td>17</td>
</tr>
</tbody>
</table>
Time-shifted application of neem (NeemAzal T/S) and B.t.t. (Novodor FC) leads to the relatively rapid cessation of feeding following ingestion. Neem should always be administered before B.t.t. whenever the time-shifted combination strategy is used. This approach achieved an effectiveness level of over 80% and increased yields by 42 to 70 dt ha\(^{-1}\) in three out of four years studied (2006 to 2008) (Kühne et al., 2008). Two-time treatment with B.t.t. also achieved comparably good results. However, a second application of the same active ingredient within a given year is not recommended in practice due to the risk of resistance development (Kühne et al., 2012).

Surprisingly, a single application of spinosad (SpinTor) also proved to be very effective (> 80%) in the three years studied in spite of the difficult study conditions in 2009. Although the degree of effectiveness was very high (2009) in the spinosad group (83%) compared to 43% (neem/B.t.t.) and 37% (B.t.t./B.t.t.) in the other two groups (statistically significant), this did not result in higher yields than in the other two groups. The reason for this was the rapid spread of late blight, which could not be adequately managed in 2009, resulting in yield loss. However, the increase in yield of the different treatment variants compared to the untreated controls was statistically significant (range: 37 to 53 dt ha\(^{-1}\)). In 2010, the yield differences between the untreated control and the treatment variants are not statistically secured. The reason lies in the severe drought, the high block variances meant.

The cost of treatment was lower when using Spinosad by 36 € ha\(^{-1}\), compared to neem and B.t.t. by 277 € ha\(^{-1}\) and shifted B.t.t. application by 203 € ha\(^{-1}\).

**Acknowledgements**

I would like to thank my co-worker and assistant, Mrs. Britta Friedrich, who has given me advice and support for many years. Thanks also to the students of the Humboldt University in Berlin and the University of Applied Sciences in Eberswalde for their commitment and passion in preparing their theses and to Dr. Eckhard Moll for assistance in performing the statistical analysis of the field studies.

**References**


Nematodes
Update on life cycle of entomopathogenic nematodes

Ralf-Udo Ehlers
e-nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany
e-mail: ehlers@e-nema.de

Abstract: Entomopathogenic nematodes (EPN) are within the genera Heterorhabditis and Steinernema, which are closely associated with enterobacteria of the genera Photorhabdus and Xenorhabdus, respectively. The life cycle of the nematodes will be reviewed. With the help of a video the development of Heterorhabditis bacteriophora in larvae of the corn rootworm will be followed (e-nema, 2013). The adult nematodes (hermaphrodites) first lay eggs into the insect cadaver from which offspring develop into a second adult generation of amphimictics (female and male) and dauer juveniles (DJs). On day 6 after infection, juveniles hatch inside the uterus and develop to dauer juveniles (DJ), feeding on the maternal body content (endotokia matricida). The resulting DJs have to load themselves with cells of their symbiont. In the past, we suggested that the pre-dauer stages loaded themselves in the moment the intestine of the adult nematode dissolved (Johnigk & Ehlers, 1999). The hermaphrodite is still alive ingesting haemolymph with a mix of micro-organisms. This theory was disproven. Ciche et al. (2008) investigated transmission of cells of the symbionts in detail. Already during the J4 stage the symbiont cells are forming a biofilm on the surface of the rectal intestinal cells and are then transferred into the rectal gland cells. Within the gland cell vacuoles the symbiont cells seem to divide and fill the vacuoles. The J1, which have hatched inside the uterus, destroy the uterus tissue and then get access to the mother’s body cavity. Almost at the same time the rectal gland cells lyse and release the vacuoles and the symbiont cells colonize the body cavity of the mother. The J1 are thus exposed only to symbiont cells and are able to load themselves much before the intestine of the hermaphrodite lyses during endotokia matricida.

EPN are able to grow and reproduce on non-specific symbiotic bacteria, but they do not transmit them in the dauer juvenile. Other than heterorhabditids DJs, which retain the bacteria in the anterior part of their intestine, DJs of the genus Steinernema form a receptacle (formerly vesicle) to harbour cells of their specific symbiont. Heidi Goodrich-Blair and co-workers have investigated the relation between steinernematids and Xenorhabdus in detail (Bhasin et al., 2012). Few bacterial cells enter into the receptacle during DJ formation and then outgrow to an initiating population to 30 to 200 bacterial cells, entirely filling the receptacle. Cells first adhere to a nematode-derived anucleate cluster of spherical bodies called the intravesicular structure (IVS). Nematode intestine localization (nil) factors A, B and C, have been identified in X. nematophila that contribute to specificity and are not detected in other Xenorhabdus spp. NilB is a surface exposed outer membrane protein whose expression is repressed by NilR and growth in nutrient-rich medium. Mutant NilB strains were not retained in the receptacle. Studies with GFP bacteria revealed that cells first colonize the anterior intestinal cells during development of the first juvenile stages at the pharyngeal-intestinal valve (PIV) anterior to the intestinal epithelium. The intestine then constricts while X. nematophila cells remain at the PIV. The anterior intestine constriction then relaxes again and colonizing bacteria occupy the receptacle. Similar to H. bacteriophora, specific binding to nematode epithelia is also reported from S. carpocapsae and S. feltiae, however, binding to
the posterior intestine cells has not been reported in steinernematids and transfer to the juveniles during endotokia matricida is not yet understood in steinernematids. New findings have been reported by the group of Nelson Simoes on EPN virulence mechanisms (Jing et al., 2010; You-Jin et al., 2009; 2010). EPN virulence is depending on the ability of the nematodes to invade hosts and to overcome insect defences. Host invasion is supported by the release of enzymes. Transcripts analysis of S. carpocapsae DJs identify genes encoding for proteases (Jing et al., 2010; You-Jin et al., 2009; 2010), which have been detected to play a key role in nematode penetration into insects. Among the genes predicted to be involved in insect immune suppression, effectors were identified, which are able to destroy humoral and cellular defences of the host insect enabling the DJs and their released symbionts to establish in the haemocel (Balasubramanian et al., 2009; 2010).

**Key words:** life cycle, symbiosis, virulence mechanisms, Heterorhabditis bacteriophora, Photorhabdus luminescens, Steinernema carpocapsae, S. feltiae, Xenorhabdus nematophila, X. bovienii

### References


e-nema 2013: Biological control of the Western Corn Rootworm Diabrotica v. virgifera with the entomopathogenic nematode Heterorhabditis bacteriophora. [http://www.youtube.com/watch?v=0K75WVXDSHc](http://www.youtube.com/watch?v=0K75WVXDSHc).


Aiming to eradicate small hive beetle Aethina tumida using entomopathogenic nematodes

Andrew G. S. Cuthbertson, James J. Mathers, Lisa F. Blackburn, Gay Marris, Mike A. Brown, Giles E. Budge
The Food and Environment Research Agency, National Bee Unit, Sand Hutton, York YO41 1LZ, UK

Abstract: The small hive beetle (Aethina tumida) is an endemic parasitic pest and scavenger of colonies of social bees indigenous to sub-Saharan Africa. In this region the beetles rarely inflict severe damage on strong colonies since the bees have developed strategies to combat them. However, A. tumida has since ‘escaped’ from its native home and has recently invaded areas such as North America and Australia where its economic impact on the apiculture industry has been significant. Commercially available entomopathogenic nematodes were screened for their potential to control beetle larvae. The nematodes Steinernema kraussei and S. carpocapsae provided excellent control with 100% mortality of larvae being obtained. Delayed applications of the nematodes following larvae entering sand to pupate also provided excellent control for up to 3 weeks. The information gained supports the development of contingency plans to deal with A. tumida should it occur in the UK or Europe.

Key words: Aethina tumida; Apis mellifera; biological control; entomopathogenic nematodes

Introduction

In its native range the small hive beetle (Aethina tumida Murray, Coleoptera: Nitidulidae) (SHB) is an occasional parasite and scavenger of honey bee colonies indigenous to sub-Saharan Africa (Neumann & Elzen, 2004). However, as an invasive species it has caused much economic damage, and since 1996 has become established in North America and Australia. The SHB has yet to be reported in Europe, South America or Asia (Cuthbertson et al., 2013).

The SHB lifecycle consists of a pupation stage that occurs outside the beehive in the surrounding soil. Both larvae and pupae can be found in the soil. Therefore, there is an opportunity for control measures to be applied at this stage that will not have any impact upon the bees in the hive. Beekeepers have traditionally used pesticides containing permethrin to control larvae and pupae in the soil (Hood, 2004). However, continued use of this can give rise to resistance and undesirable side effects on both honey bees and humans (Cuthbertson & Brown, 2009). Therefore, there is much demand to improve the range of products available for the control of the larvae and pupae stages. Such alternative control agents include entomopathogenic nematodes (EPN), which have successfully been used against other invertebrate pests. In regards to A. tumida, the infectivity of three species of nematodes towards wandering larvae (the larval stage that is actively seeking a pupation site) was shown previously to be moderate (Cabanillas & Elzen, 2006).
Material and methods

Insect rearing
Aethina tumida were cultured and maintained as described by Cuthbertson et al. (2008) under strict quarantine conditions. Final instar (wandering) larvae were used for all experimental trials. The control agents used are all commercially available products in the UK and across Europe and comprised 3 EPN’s: Steinernema feltiae (Nemasys), S. kraussei (NemasysL), S. carpocapsae (Capsanem). The impact of direct and in-direct exposure along with delayed application of the agents showing most potential were investigated in separate experiments (Cuthbertson et al., 2012).

Direct exposure of larvae to control agents
For direct exposure trials, individual wandering larvae were dipped in recommended dose rates of the nematode products (10,000 infective juveniles ml⁻¹) for 3 seconds.

Indirect exposure of larvae to control agents
For indirect exposure, 7 cm diameter by 15 cm tall plastic containers were filled with sand (8% moisture content). 50 ml of control product (nematode) was added over the surface of the sand at the same dose rates as in the direct trials. Once the solution had soaked down into the sand, ten wandering larvae were added to the surface. The containers were then sealed. Treatments were maintained for 6 weeks in order to allow adult beetles to emerge. Mortality was calculated as the number of beetles that failed to emerge.

Delayed application of nematodes against beetle larvae
Delayed application trials using the two nematode species that gave the best indirect control were conducted. Ten wandering SHB larvae were added to a container. Following 24 h the first batch of nematode solution was added. Then at weekly intervals, nematode treatments were added to larvae infested containers. Control containers received 50 ml water.

Results and discussion
Direct exposure demonstrated a significant treatment effect on the wandering larvae when compared to the control (P < 0.001) (Figure 1). The nematodes showed promise; S. carpocapsae achieved significantly higher mortality than S. kraussei and S. feltiae (P < 0.05), which in turn achieved significantly higher mortality than the control. Upon dissecting the larvae, nematodes freely emerged from the body cavity confirming their ability to infect the larvae. It has been stated that susceptibility of insects to control agents generally declines with increasing insect size. This has been demonstrated with mermithid nematodes against mosquito larvae. However, as nematodes enter through the natural openings of the larvae, Gaugler & Molloy (1981) showed that susceptibility was a function of larval size with larger larvae being more susceptible to nematode infection, simply due to the fact that it was easier for nematodes to enter the natural openings.
Figure 1. Impact of direct exposure of control agents on Aethina tumida wandering larvae after 2 weeks. Error bars represent the 95% confidence intervals (Cuthbertson et al., 2012).

Treating the sand before adding the larvae exposes the SHB to the biocontrol agents during pupation, and more closely simulates how beekeepers might apply such products in the field. Indirect exposure demonstrated a significant treatment effect on SHB mortality when compared to the control ($P < 0.001$). Treating the sand produced excellent results for S. kraussei and S. carpocapsae where total mortality of A. tumida was achieved. No adults emerged from either of these two treatments. Also, S. feltiae achieved significantly higher mortality than the control ($P < 0.05$; Figure 2).

Figure 2. Impact of in-direct exposure of control agents on Aethina tumida wandering larvae. Error bars represent the 95% confidence intervals (Cuthbertson et al., 2012).
Following delayed application of the nematodes, significant reduction in adult beetle emergence was obtained ($P < 0.001$; Figure 3) for up to 3 weeks following larvae entering the sand to pupate (Cuthbertson et al., 2012).

![Graph showing beetle emergence](image)

Figure 3. Impact of delayed applications of entomopathogenic nematodes on Aethina tumida wandering larvae following their submergence in sand pots. Beetle emergence assessed after 6 weeks following larvae being added to sand. Error bars represent the 95% confidence intervals (Cuthbertson et al., 2012).

The data from our screening trials are consistent with those of Cabanillas & Elzen (2006) and Ellis et al. (2010), who demonstrated that A. tumida larvae and pupae are susceptible to entomopathogenic nematodes. In our study nematode efficacy varied with nematode species. Our trials demonstrate that commercially available entomopathogenic nematodes in the UK can infest and kill A. tumida wandering larvae. Furthermore, these products are available across Europe, and so have the potential to be used as control agents should the small hive beetle expand its range to this continent (Cuthbertson et al., 2010; 2012; 2013).

Acknowledgements

The Aethina tumida research is funded by the Department for Environment, Food and Rural Affairs (UK). The nematodes were supplied by Koppert UK Ltd. and Becker Underwood Ltd.

References


The development of mollusc-parasitic nematode Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae) in different substrates

Jiří Nermut1, Vladimír Půža1
1Biology Centre ASCR v. v. i., Institute of Entomology, Branišovská 31, 37005 České Budějovice, Czech Republic

Abstract: The effect of different growing substrates on the development of the facultative slug parasite Phasmarhabditis hermaphrodita has been studied in a series of laboratory experiments. Wild, laboratory and Nemaslug strains of P. hermaphrodita were reared in agar plates on homogenized pig kidney, the homogenized bodies of Deroceras reticulatum, Arion lusitanicus, and Galleria mellonella, the faeces of D. reticulatum and A. lusitanicus, or leaf compost. Development time, yield, lipid reserves, and the body length of females and dauer larvae were assessed. All P. hermaphrodita strains were able to grow and reproduce on all tested substrates. However, yields were markedly higher on animal substrates. Lipid content and body size varied across the substrates, however, even plant tissue produced normal sized individuals with normal lipid content. It thus seems that the quality of the substrate is expressed mainly in yield. High and less variable yields and faster development of the wild and Nemaslug strains, in comparison with the laboratory strain, were probably due to different bacterial associates. The dramatic differences in yields on animal substrates, in comparison to those on plant tissue, illustrate the evolutionary advantage of the association of nematodes with invertebrates.

Key words: slug parasitic nematodes, lipid reserves, progeny production, development time

Introduction

Phasmarhabditis hermaphrodita is a bacteriophagous nematode that does not live in a strict association with only one species of bacteria as entomopathogenic nematodes (EPNs) do, but is associated with, and able to feed on, many bacterial species (Wilson et al., 1995a, Rae et al., 2010) that are common in its habitat. Bacterial species significantly influence P. hermaphrodita progeny production and dauer juveniles (DJs) quality (Wilson et al., 1995a) and are responsible for the pathogenicity of nematode-bacteria complexes towards their hosts (Wilson et al., 1995b).

Apart from the parasitic cycle P. hermaphrodita also has a necromenic life cycle (Mengert, 1953), and has been shown to reproduce on earthworms (Rae et al., 2009), leaf litter (MacMillan et al., 2009) and slugs or slug faeces homogenates (Tan & Grewal, 2001).

As found previously growing substrate influenced the reproductive capacity and progeny production of P. hermaphrodita (Tan & Grewal, 2001; Rae et al., 2009). The influence of growing substrate has been thoroughly studied in EPNs and other nematodes. Its nutritional composition (e.g. lipids, proteins etc.) and origin (plant vs. animal) is an important factor in nematode growth and reproduction and may determine the final yield (Friedman, 1990), body size (Hooper et al., 1999; Yang et al., 1997) and lipid content of infective juveniles (Abu Hatab & Gaugler, 1999) or development (Ehlers & Shapiro-Ilan, 2005).

In the present study we assessed the influence of different growing substrates and bacteria on three P. hermaphrodita strains and the differences between them. We focused on the development time, yield, size, and lipid reserves of females and dauer larvae.
Material and methods

Three strains of *P. hermaphrodita* were used in this study, the Nemaslug® strain, the laboratory strain derived from the commercial Nemaslug® strain, and the wild strain isolated from an *Arion* sp. cadaver. Slugs of the species *Arion lusitanicus* and *Deroceras reticulatum* were collected in České Budějovice (Czech Republic) and *Galleria mellonella* larvae were obtained from laboratory culture.

The influence of different substrates on three *P. hermaphrodita* strains was observed on 2% pure agar plates in Petri dishes, diameter 55 mm. Substrates used were as follows: homogenized pig kidney, sterilized slug faeces, the homogenized bodies of *A. lusitanicus*, *D. reticulatum*, and *G. mellonella*, and leaf compost. The homogenized slugs and *G. mellonella* were prepared from individuals that were autoclaved and homogenized with sterile water.

A 0.02 g piece of each substrate was placed on the plate and twenty DJs of *P. hermaphrodita* in 20 µl water were pipetted onto the substrate. The experiment was performed at 15 °C, and in two series, each consisting of 10 replicates. The first series was checked daily, and the development of the population observed under a stereomicroscope, and the first appearance of mature females was recorded. Twenty mature females from each dish were collected for lipid staining (Patel et al., 1997) and measurement of length. The other series was observed every day, and the development was recorded, until the substrate was depleted and the new DJs emerged. The DJs were collected and counted, and 20 individuals from each dish were used for the measurements of the lipid content and body length. The whole experiment was repeated twice in time.

The associated bacteria of all tested nematode strains were isolated both from surface sterilized DJs (Wilson et al., 1995a) and from homogenized sterile pig kidney 3 days after inoculation with surface sterilised DJs. Bacteria were identified in the specialized laboratory at the CCM (Czech Collection of Microorganisms).

Results and discussion

All the tested substrates supported the growth and reproduction of all three tested strains of *P. hermaphrodita*. These results are in accordance with the statement that *P. hermaphrodita* is able to live and reproduce on various organic materials (Mengert, 1953; Tan & Grewal, 2001; Rae et al., 2006).

Yield in our study was clearly affected by substrate. Similarly to the results of studies with EPNs (Yang et al., 1997) *P. hermaphrodita* in our study produced up to 20-30 fold more progeny on substrates based on animal tissue than on faeces and compost. These differences clearly illustrate the benefit of the association of the nematode with invertebrates. In studies on EPNs medium composition has been shown to affect the amount and quality of symbiotic bacteria and in turn nematode yield (Abu Hatab & Gaugler, 2001). The same explanation can be applied to our observations.

In EPNs the lipid content has been shown to vary with the quality of the substrate (Abu Hatab & Gaugler, 1999). In our study we found negligible differences in the lipid content of the females and, to a lesser extent, the DJs of all tested strains across the substrates. This observation suggests that unlike EPNs (Yang et al., 1997) *P. hermaphrodita* can produce full quality DJs on a variety of substrates.

There was an apparent variation in female and larval length on different substrates. This finding fits well with the studies performed on EPNs (Yang et al., 1997). Similarly,
P. hermaphrodita females reared on dead slugs were usually bigger than females reared on bacterial culture (Hooper et al., 1999). Yang et al. (1997) have shown that the IJs of EPNs grow larger on animal substrates. Similarly, we have shown that, in general, larvae reared on pig kidney and homogenised Galleria were larger, while in slug faeces and compost shorter females and DJs were produced, especially in the Nemaslug strain.

There were apparent differences in the response of the tested strains to different substrates. We assume that at least some of these differences could be due to differences in the composition of bacterial associates. As expected the Nemaslug strain contained Moraxella osloensis only. The laboratory strain lost its original bacterial associate during repeated culturing. Its present associates Acinetobacter sp., Alcaligenes faecalis, Bacillus cereus and Stenotrophomonas sp. probably originate from non-sterile slugs used for nematode propagation in the laboratory. The wild strain harboured a quite similar bacterial assemblage, though having Pseudomonas putida in addition.

We suppose, the lower yield and slower development of the laboratory strain in comparison with the Nemaslug are probably due to the associated bacteria. The same differences between the laboratory and wild strain are questionable as these nematodes have very similar bacterial assemblages with the only difference being the presence of P. putida in the wild strain. These bacteria have been shown to support well the growth of P. hermaphrodita (Wilson et al., 1995b). However, further research is necessary to show the role of bacterial associate in these experiments.

Figure 1. Dauer juveniles yield of the wild, laboratory and Nemaslug strains of Phasmarhabditis hermaphrodita on different substrates.
Legend: p.k. pig kidney, h.g. homogenised G. mellonella, h.d. homogenised D. reticulatum, h.a. homogenized A. lusitanicus, f.d. faeces of D. reticulatum, f.a. faeces of A. lusitanicus, co. leaf compost.
Acknowledgements

We would like to thank Mrs Lenka Kropáčková for her assistance. This research was supported by Czech Science Foundation, project: GA ČR P504/12/2352.

References

New nematodes associated to *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae): preliminary description

Monica Oreste¹, Francesca De Luca², Elena Fanelli², Alberto Troccoli², Eustachio Tarasco¹

¹DISSPA - Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti. Università degli Studi di Bari “Aldo Moro”, Via Amendola 165/a, 70126 Bari, Italy; ²Istituto per la Protezione delle Piante del CNR, Sezione di Bari, Via Amendola 122/D, 70126 Bari, Italy

Abstract: Several nematodes, juveniles and adults, were found dissecting some *Rhynchophorus ferrugineus* pupae and adults from infested *Phoenix canariensis* exemplar in Bari (Italy). Insect was intact externally but inner tissues were completely liquefied. Nematodes were collected using the water trap method and total DNA was extracted from each individual. The 18S rDNA, the ITS containing region and the mitochondrial cytochrome oxidase I (COI) were amplified and sequenced. ITS-RFLP analysis were also obtained. BLAST search revealed that nucleotides sequences are similar (93%) to *Koerneria* sp. RS1982 (Nematoda: Diplogastridae). Nematodes belonging to Diplogastridae are commonly associated with insects, with different types of association depending on diplogastrid genera. *Koerneria* spp. are frequently associated with stag and dung beetles. Characterization studies are now still in progress for the species identification. Our future purpose is to clarify the kind of association between this specie and the Red Weevil and the eventual role as natural control agent.

Key words: *Rhynchophorus ferrugineus*, nematode, *Koerneria* sp., nucleotide sequence, identification
The role of bacterial symbionts in the competition of entomopathogenic nematode species

Vladimír Půža, Jiří Nermut, Zdeněk Mráček
Institute of Entomology, Biology Centre, ASCR, v.v.i., Branišovská 31, 37005 České Budějovice, Czech Republic

Abstract: Competition between entomopathogenic nematode (EPN) species is still a largely neglected topic. Previous research has shown that in the competition within one insect host, nematode Steinernema affine strongly dominates over S. kraussei and suggested a possible role of symbiotic bacteria in the competition. In present study, S. affine and S. kraussei and their symbionts were reared in different combinations on Wouts agar plates, and nematode development was observed. Resulting progeny from these combinations was harvested and body size and lipid content of infective juveniles (IJ)s were assessed. S. affine was able to develop, mature and produce viable progeny on the symbiont of S. kraussei. Interestingly, there was no difference in the duration of the cycle or reproduction potential, IJ size and lipid content between S. affine reared on their own symbiont and symbiont of S. kraussei. On the other hand, S. kraussei developed and reproduced well only on its own symbiont. These experiments explained the previously observed dominance of S. affine over S. kraussei. Research with more EPN species is planned to further clarify the topic.

Key words: Steinernema, Xenorhabdus, competition

Introduction

Interspecific competition of entomopathogenic nematodes (EPNs) is still an understudied area despite its implications for the use of EPNs in biological control. Laboratory experiments revealed, that EPNs do not avoid co-infecting hosts together with heterospecifics (Koppenhöfer et al., 1995; Koppenhöfer & Kaya, 1996; Půža & Mráček, 2009) and natural multiple infection has been also observed (Bovien, 1937). Thus there is a potential for the inter-specific interactions between sympatric entomopathogenic nematode species. It is believed that the outcome of the competition intensity and its impact on the competitors depends on the inoculum size of nematodes. However, S. affine has been shown to suppress S. kraussei in co-infected G. mellonella regardless the inoculum size or the ratio between both nematodes (Půža & Mráček, 2009) or host species (Půža & Mráček, 2010), suggesting a possible role of bacterial symbiont in this interaction.

In present study, the role of symbiotic bacteria in the competition between S. affine and S. kraussei was investigated on wouts agar plates and infections of G. mellonella.

Material and methods

Nematode and bacteria preparation and rearing
S. affine and S. kraussei originating from one locality were selected for the experiments. A xenic 1st instar larvae of both strains were prepared according to (Kaya & Stock, 1997).
Symbiotic bacteria of both strains were isolated from haemolymph of the freshly infected G. mellonella that was streaked on NBTA agar plates. Single colonies were then transferred to the liquid YS medium and incubated 2 d on orbital shaker at 25 °C prior to experiments.

Then the larvae of both strains were separately and in mixture reared on single or mixed symbiotic bacteria on Wouts agar plates. The plates were checked daily and the development of the nematodes was recorded.

The assessment of the progeny quality
IJs were harvested from the plates and their body length and maximal body width was measured under light microscope. The lipid content was assessed according to Patel et al. (1997).

Infections
IJs harvested from heteroxenic combinations were surface sterilised according to Han and Ehlers (1998) and the retention of the bacteria was tested by the infections of G. mellonella and by placing the IJs to the sterile YS medium for 48 h.

Mixed infections of S. affine IJs from monoxenic and heteroxenic combinations with monoxenic S. kraussei were performed, and the outcome of the infection was assessed.

Results and discussion

Wouts agar plates
The results from the Wouts agar tests are summarised in the Table 1. As expected, both nematodes grown and reproduced on their original bacterial symbionts. Differences were, however, observed, when the nematodes were reared on the each other’s symbiont. Whereas S. affine was able to grow and reproduce on the symbiont of S. kraussei, the same was not true for the latter. When reared on the symbiont of S. affine, only a part of S. kraussei larvae developed to pigmy adults that died after several days without further reproduction.

<table>
<thead>
<tr>
<th>nematode</th>
<th>bacteria</th>
<th>adults</th>
<th>Reproduction</th>
<th>IJs</th>
<th>duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. affine</td>
<td>X b A</td>
<td>yes</td>
<td>Yes</td>
<td>yes</td>
<td>12</td>
</tr>
<tr>
<td>S. affine</td>
<td>X b K</td>
<td>yes</td>
<td>Yes</td>
<td>yes</td>
<td>12</td>
</tr>
<tr>
<td>S. affine</td>
<td>both</td>
<td>yes</td>
<td>Yes</td>
<td>yes</td>
<td>12</td>
</tr>
<tr>
<td>S. kraussei</td>
<td>X b K</td>
<td>yes*</td>
<td>Y es</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>S. kraussei</td>
<td>X b A</td>
<td>yes*</td>
<td>Y es</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>S. kraussei</td>
<td>both</td>
<td>yes</td>
<td>No</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>both</td>
<td>both</td>
<td>yes</td>
<td>yes</td>
<td>yesa</td>
<td>12a</td>
</tr>
</tbody>
</table>

*a only dwarf adults; * only S. affine

When both bacteria were available, S. kraussei mainly developed to adults, but subsequent reproduction was occasional with no IJ production. It thus seems that the symbiont of S. affine is toxic for S. kraussei. This explains the universal dominance of the
latter species in mixed infections. On the other hand, S. affine is unaffected by the symbiont of S. kraussei (see further). The mixed treatment with both bacteria and nematodes simulated the infection of the host, where also two nematodes and two bacteria are present. Again, only S. affine reproduced.

**Progeny assessment**
No differences were observed in body size and lipid content of S. affine IJs grown symbiotically and aposymbiotically (Figure 1) and in the duration of the cycle (Table 1). IJs from Wouts agar plates were in general smaller in comparison to larvae reared in G. mellonella (Figure 1).

![Figure 1. Lipid reserves (A) and body length and width (B) of S. affine IJs grown on their original symbiont (XbA), symbiont of S. kraussei (XbK) and reared in G. mellonella (G.m.).](image-url)
Infections of G. mellonella
As was expected, incubation in YS medium and experimental infection of G. mellonella showed no retention of S. kraussei symbiont in S. affine IJs. Interestingly, in the mixed infection of S. affine IJs from heteroxenic combination with monoxenic S. kraussei, where the symbiont of S. affine was missing, S. affine still dominated (data not shown). This fact suggests also a role of the nematode in the competition. However, further research with more EPN species is needed to clarify this topic.

Acknowledgements

The research was supported by the grant GA CR P504/12/2352.

References

Research and development for a nematode-based biological control solution for western corn rootworm in maize

Stefan Toepfer, Ulrich Kuhlman
CABI, Rue des Grillons 1, 2800 Delemont, Switzerland
e-mail: s.toepfer@cabi.org

Abstract: 10 years of joint efforts in research and development have led to a nematode-based biological control solution for one of the most destructive maize pests, the western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae).

Key words: entomopathogenic, insect parasitic nematodes, inundative biological control

Introduction

The western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), is one of the most destructive pests of maize in North America. It is a univoltine species with eggs that overwinter in the soil. After maize has germinated, the eggs hatch, and its three larval life stages feed on maize roots, often causing plant lodging and yield losses. Adults can occasionally reduce yields through intensive silk feeding. Over the last 25 years, it has moved into Europe causing major problems in maize.

Between 2004 and 2008, CABI, the University of Neuchâtel, the farmer association Landi REBA in Basel, the Plant Protection Directorate in Hodmezovasarhely, Agroscope Reckenholz-Tänikon, the University of Kiel, and the nematode producers e-nema at Schwentinenthal and Andermatt Biocontrol at Grossdietswil, laid the scientific base for nematode-based biological control products against WCR (CABI, 2008). Between 2007 and 2008, a number of institutions reviewed biological control options against rootworms and proposed them to the European Commission (Diabr-Act, 2007; CABI, 2008). Between 2009 and 2012, the Landwirtschaftliches Technologiezentrum Stuttgart, Cult-tec Ltd. in Freiburg, the Austrian Agency for Health and Food Safety in Vienna, the Cereal Research Station in Szeged, SAGEA Centro di Saggio S.r.l., CABI, and others improved application technologies aiming for the farmer-friendliest and least-costly method (CABI, 2012).

Achievements of research so far

Review of biological control options against WCR
Several natural enemy species or groups appeared promising candidates for control strategies with different ecological rationales. Research proposed to pursue: (1) developing inundative biological control products, particularly mass-produced entomopathogenic nematodes and fungi; (2) understanding specific natural enemies of Diabroticina throughout the Americas including potential classical biological control agents; and (3) enhancing natural enemies through cultural practices. Details in Kuhlmann et al. (1998); Diabr-A ct (2007); Toepfer et al. (2009); Pilz et al. (2008).
**Nematode screening in laboratory**

Screenings experiments in petri dishes on filter paper or in sand, as well as bioassays in containers with sand or soil and maize revealed that Heterorhabditis bacteriophora, H. megidis, Steinernema feltiae, S. arenarium, and S. kraussei are highly virulent against WCR larvae. St. abassi was found intermediate. S. carpocapsae and S. glaseri appeared less virulent. Details in Toepfer et al. (2005); Kurtz et al. (2009), Hiltpold et al. (2010).

**Nematode screening in the field**

Plant scale field experiments with artificial WCR infestation and into-soil applications of fluids of different nematode species during sowing or later in June, revealed that H. bacteriophora and H. megidis are highly effective against WCR larvae (i.e. up to 81%), and in preventing damage to maize roots (i.e. up to 80%), and this largely to the same extent as pesticides. S. feltiae appeared slightly less effective. Details in Toepfer et al. (2008); Pilz et al. (2009); Hiltpold et al. (2010).

**Scientific pre-requisites**

Instar susceptibility of target

Bioassays with different life stages of WCR and different nematodes revealed that all larval instars and even pupae are effectively killed by H. bacteriophora, H. megidis and St. feltiae. Adults appeared less susceptible. Details in Kurtz et al. (2009).

Orientation of nematodes to target

Nematodes were found to orient towards WCR-damaged maize roots using the root-emitted organic volatile compound (E)-β-caryophyllene as an orientation cue to find and attack WCR larvae. Caryophyllene might be particularly important for H. megidis and less for H. bacteriophora. Other authors mention that caryophyllene is of little to no importance for nematodes. Details in Rasmann et al. (2005); Hiltpold et al. (2008); Anbesse et al. (2013).

Maize hybrid importance

There are hardly any hints that the choice of maize hybrids is important for biological pest control with nematodes. Some hybrids have lost the capability to emit the nematode-attracting (E)-β-caryophyllene; however, most European maize hybrids emit caryophyllene upon larval feeding. Details in Rasmann et al. (2005); Hiltpold et al. (2008; 2010).

Establishment and persistence of nematodes

Field experiments revealed that applied nematodes establish at relatively low rates in the soil of maize fields; but, that they survive more than two months, which is long enough to effectively kill all three larval instars. Nematodes were found to propagate on WCR larvae in the field, a big advantage over pesticides. Details in Kurtz et al. (2007); Pilz et al. (2011a).

Soil importance

Field trials showed that H. bacteriophora can effectively kill WCR larvae in a wide range of soils in maize fields. As WCR larvae are usually most damaging in dense soils, also control efficacies of nematodes were found higher in dense soils than in light, e.g. sandy, soils. Details in Grabenweger et al. (2010); Toepfer et al. (2010d); Pilz et al. (2011a).

Non-target effects

Entomopathogenic nematodes are restricted to arthropods, thus there is no danger to plants or humans. Nematodes are known to be slightly host specific on insect groups. However, field
experiments revealed only minor effects of treatments on non-target populations, suggested to be a result of the generally poor arthropod diversity in soils of intensive field crops such as maize, as well as of the application of nematodes into relatively narrow soil areas and close to the target. Details in Babendreier et al. (2014); Gaugler (2002).

**Application of nematodes**

Where?
Nematodes were successfully applied through fluid solid stream sprays, micro-granules or seed coating into soil at sowing, or through fluid solid stream sprays or granules into soil next to young maize plants, or through fluid narrow flat sprays applied with lots of water over rows of small plants. Details and more options in Toepfer et al. (2010abc).

When?
Nematodes were successfully applied into soil at sowing (Mid April to early May in Central Europe), this is, a few weeks before WCR egg hatching; as well as into or onto soil along rows of young maize plants (mid to late May in Central Europe). Field applications against adults, i.e. in July or August, have never been attempted. Details in Toepfer et al. (2010c).

Formulation
Nematodes can be applied against WCR larvae preferably just diluted in water. Micro-granules, seed coatings, capsules and other options need further research. Details in Toepfer et al. (2010abc); Hiltpold et al. (2012).

Need of water
Field experiments revealed that the need of water during application is variable and depends on the soil type, whether conditions, and application techniques. Currently a minimum of 200 to 400 l water ha⁻¹ are advised for fluid stream sprays of nematodes into soils, and a minimum of 800 to 1000 l ha⁻¹ for narrow stream sprays onto the soil or plants. Details through SAGEA Centro di Saggio S.r.l. (2010, pers. comm.); Toepfer et al. (2010abc).

**Farmer friendly application techniques**
Fluid and micro-granular applications as well as seed coating with nematodes appeared technically possible with available farmer machineries; and all achieved control of WCR larvae as well as root damage prevention. Currently most promising and most used is the fluid stream spray application into the soil at sowing, using sowing machines with applicators that apply nematodes behind the sowing wheel and prior the soil-closing wheels. Details in Toepfer et al. (2010c); SAGEA Centro di Saggio S.r.l. (2010, pers. comm.); Cult-tec (2012).

**Field scale efficacy and dosage-efficacy response**
Field scale trials using farmer machinery revealed that H. bacteriophora is able to reach control efficacies of WCR larvae to the same extent as soil insecticides and insecticide seed coatings. On multiple year, site and machinery average, control efficacies are approximately between 30 and 80%. Nematodes can also significantly prevent root damages, on levels close to soil insecticides and insecticide seed coatings. A dose-efficacy response curve is not finally established, but preliminary results suggest that the optimal dose of nematodes might be somewhat between 2 and 3 billion ha⁻¹ maize field. Details in Pilz et al. (2009; 2011b); Toepfer et al. (2010b); SAGEA Centro di Saggio S.r.l. (2010, pers. comm.).
Products
H. bacteriophora and H. megidis products are available from several biocontrol companies, and can be applied, without restrictions, in countries where entomopathogenic nematodes do not need registrations and where the products consider species that are native, e. g. currently in Germany. One of the products (Dianem™) is, for example, registered in Austria.

Legislation
With the banning of several insecticides for seed coatings due to their bee toxicity, and with recent discussions on a number of soil pesticides in maize, farmers need alternative products. Moreover, the European Directive on sustainable use of pesticides requests from EU member countries to prefer alternative pest control options. Entomopathogenic nematodes are exceptionally safe biocontrol agents; thus they are exempted from registration in many European countries, in others they need registration. Details in Ehlers (2003); European Commission, (2008; 2009); Delos et al. (2011); Gill et al. (2012); Cressey (2013).

Conclusions
Joint research and development efforts have led to a nematode-based biological control solution for western corn rootworm in maize, which is now ready for use and safe.

Acknowledgements
The successful development of a biological control solution for western corn rootworm was a 10-year collaborative effort of many partners (please refer to: CABI (2008; 2012). It largely relied on public funding (Swiss Commission for Technology and Innovation of Federal Office for Professional Education and Technology; a Specific Support Action ‘Policy Oriented Research’ through the ECs 6th RTD Framework Programme; the Ministry for Rural Areas and Consumer Protection of the State of Baden-Württemberg, Germany; and the Federal Ministry of Agriculture of Germany), and to some extent on funding from Landi REBA AG Switzerland, e-nema GmbH Germany, in-kind contributions of farmers, and many others.

References


Hiltpold, I., Hibbard, B. E., French, B. W. & Turlings, T. C. J. 2012: Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. Plant and Soil 358: 11-25.


Development of a method to establish entomopathogenic nematodes (EPN) in arable soils by using farm-suitable field equipment

Wolfgang Büchs
Federal Research Center for Cultivated Plants, Institute for Crop and Soil Science, Bundesallee 50, 38116 Braunschweig, Germany

Abstract: One major problem of the utilization of entomopathogenic nematodes (EPN) against pests in arable crops is that topical spraying with usual spray equipment is often not effective because of the risk of drying up of the EPN before they can act against the relevant pests. Furthermore, the stages active on the crop plants are in many cases less susceptible against EPN, have a short activity period and are often protected by leaves so that they are not hit by spraying. Many pests however stay more or less inactive for a longer time (e.g. pupation, overwintering, estivation) in arable soils. In this period of inactivity they are considerably susceptible regarding attacks by EPN and/or entomopathogenic fungi (EPF). The goal is to find a technique, suitable for normal field application which helps to establish in particular EPN-populations for longer term in arable soils so that they are present when the pest organisms enter the soil for pupation, overwintering or aestivation. To achieve this we tested the applicability of the so called Cultan-technique. This technique was developed to inject a concentrated ammonium solution by high pressure into the soil with the effect that a subterranean ball-like deposit is formed from which nitrogen is slowly released into the adjacent soil.

Our idea was, that this technique could be used to apply in particular EPN into the upper soil layers so that they are better protected against drying up and are able to revert to more alternative food items in period the relevant life stages of pests are not present. Thus, covered by soil, it can be assumed that they presumable have a better chance to survive and establish in comparison to application by normal spray equipment. For the injection of EPN into the soil we modified the Cultan-technique by using a water solution of with the same concentration of EPN which is recommended for normal spray application for products which are already on the market.

In the first preliminary tests which are demonstrated here the technique was applied in organic winter oilseed rape, because it is the arable crop with the most pest organisms with at least one life stage staying for a longer period in the soil. The injection-technique was tested as autumn and spring application either alone or as combination. It was compared in a randomized block designed field experiment with a topical spray application of EPN (Steinernema feltiae), EPF (Beauveria bassiana) and Spinosad, and an untreated control. First results of these preliminary tests and experiences with this technique are reported.

Key words: Steinernema feltiae, Beauveria bassiana, Cultan-technique, application, oilseed rape

Acknowledgements

It is thankfully appreciated that the tests were supported by E-nema GmbH, Raisdorf, Germany, by providing Steinernema feltiae, and by Intrachem Bio Italia Spa., Grassobbio, Italy by providing Beauveria bassiana.
Biosafety analysis of the Bacillus pumilus 15.1 strain through a Caenorhabditis elegans pathogenicity assay

Juan F. Caña Roca¹, Diana C. García¹, Juan I. Vilchez-Morillas², Maximino Manzanera², Tania Domínguez¹, Antonio Osuna¹, Susana Vílchez¹,³

¹Biological Control of Pest and Vectors Research Line, Institute of Biotechnology, Faculty of Sciences, Campus Fuentenueva 180, University of Granada, Granada, Spain; ²Water Research Institute. Cl. Ramón y Cajal 4, 18071, University of Granada, Granada, Spain; ³Department of Biochemistry and Molecular Biology I, Faculty of Sciences, Campus Fuentenueva 18071, University of Granada, Granada, Spain

Abstract: Using a Caenorhabditis elegans pathogenicity assay we evaluated the biosafety of the B. pumilus 15.1 strain, a recently isolated bacteria active against larvae of the Mediterranean fruit fly Ceratitis capitata. In the study we evaluated the toxicity of this strain toward the nematode together with other B. pumilus strains and compared its toxicity with a non pathogenic strain (Escherichia coli OP50) and a pathogenic one (Burkholderia cepacia). After this study, we concluded that B. pumilus 15.1 is a safe strain and could not represent a problem to be used as a biological control agent.

Key words: Caenorhabditis elegans, pathogenicity assay, Ceratitis capitata, Bacillus pumilus

Introduction

Bacillus pumilus 15.1 strain has been recently described as active against larvae of the Mediterranean fruit fly Ceratitis capitata, one of the worst pest for fruits and vegetables world-wide. The toxicity mechanism of this strain is completely unknown, so studies for elucidating it are on progress. B. pumilus 15.1 could be useful in the future for developing a bioinsecticide against this pest. For that reason and although B. pumilus species is not considered as a health risk, we decided to evaluate the biosafety of this strain through a C. elegans pathogenicity assay.

C. elegans (Maupas, 1900) is a soil nematode that feeds on microorganism and is a very well studied organism model for the study of microbial pathogenicity and toxicity mechanisms (Aballay & Ausubel, 2002; Smeesters et al., 2011), given it is a eukaryotic organism with a very simple biological cycle and easy to maintain under laboratory conditions.

Here we describe the pathogenicity assays performed on C. elegans fed on the strain B. pumilus 15.1 and other bacterial strains with known pathogenicity in order to evaluate the biosafety of the strain.

Material and methods

Bacterial strains and culture conditions
Bacillus pumilus 15.1, B. pumilus M1, Bacillus thuringiensis var. kurstaki, Escherichia coli OP50, and Burkholderia cepacia were cultured on LB plates at 30 °C. When cultured in
liquid medium, 3 ml of LB were placed in a 15 ml test tube and incubated overnight in an orbital shaker (240 rpm) at 30 °C.

Pathogenicity assay
C. elegans was grown on the strain E. coli OP50 cultured on PDA (Potato Dextrose Agar, Sigma, 15 g l\(^{-1}\) agar, 20 g l\(^{-1}\) dextrose and 4 g l\(^{-1}\) potato extract) plates (Navas et al., 2007). When nematodes were needed for the assay, PDA plates were disrupted and washed with 1 l of running water and filtered through a 90 µm sieve. Nematode suspension was allowed to sediment for 3 h in a decantation flask. Then, the suspension was filtered through a 28 µm sieve to obtain the nematodes. Nematodes were transferred into a 50 ml concentration flask with 17 ml of water supplemented with 1 µg ml\(^{-1}\) ampicillin and incubated for 2 h in order to eliminate the E. coli OP50 strain. Once concentrated, 1 ml of nematode suspension was transferred to a Fujiwara slide and observed under a Nikon SMZ800 microscope and preadults nematodes were selected. Five preadults were placed on PDA plates with a lawn of the test strain. To get this bacterial lawns, 24 h before the pathogenicity assay, 50 µl of an overnight culture of the bacterial under study was evenly distributed on the surface on a 5.5 cm PDA plate and incubated overnight at 30 °C. Once the 5 preadults were placed on the bacterial lawn, plates were incubated at 20 °C. In each assay, every strain was tested on 5 different plates and the assay was repeated twice.

Nematode counting
Every day, each plate was observed under the microscope and the number of eggs, juveniles per preadults and adults was counted and registered. At the end of the experiment, the total population of nematodes in each assay was determined. For that, agar from plates were disrupted, washed and filtered through a 90 µm sieve as previously described. Nematodes were recovered in a final volume of 20 ml of water. One millilitre of the suspension was used to determine the number of individuals under the microscope and total population of nematodes in each plate was extrapolated.

Results and discussion
In order to determine the biosafety of the B. pumilus 15.1 strain, we performed a C. elegans pathogenicity assay previously described by Ruiz-Diez et al. (2003). Together with B. pumilus 15.1 strain, the strain E. coli OP50 was used as negative control of pathogenicity and Burkholderia cepacia as positive control (Palleroni & Holmes, 1981; Yabuuchi et al, 1992). In addition, several other strains were included in the assay, as B. thuringiensis var. kurstaki, and two other B. pumilus strains. In the study we followed the population dynamic of C. elegans by counting the number of eggs, juveniles/preadults and adults every 24 h (Tables 1, 2 and 3).

In addition, we recorded the total number of individuals 7 days after the beginning of the assay (Figure 1).
Table 1. Number of eggs (means and standard deviation, SD) registered in PDA plates along time.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h Mean</th>
<th>24 h SD</th>
<th>48 h Mean</th>
<th>48 h SD</th>
<th>72 h Mean</th>
<th>72 h SD</th>
<th>96 h Mean</th>
<th>96 h SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pumilus 15.1</td>
<td>1.25</td>
<td>2.58</td>
<td>10.62</td>
<td>6.30</td>
<td>64.125</td>
<td>26.88</td>
<td>293.75</td>
<td>155.56</td>
</tr>
<tr>
<td>B. pumilus 15.1C</td>
<td>0</td>
<td>0</td>
<td>23.1</td>
<td>15.41</td>
<td>63</td>
<td>37.06</td>
<td>206</td>
<td>138.86</td>
</tr>
<tr>
<td>B. pumilus M1</td>
<td>1.25</td>
<td>1.71</td>
<td>14.12</td>
<td>9.97</td>
<td>84.375</td>
<td>47.46</td>
<td>219</td>
<td>109.14</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>0</td>
<td>0</td>
<td>3.75</td>
<td>0</td>
<td>2.875</td>
<td>4.67</td>
<td>2.875</td>
<td>4.67</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>0.125</td>
<td>0.33</td>
<td>2</td>
<td>0</td>
<td>161.5</td>
<td>115.82</td>
<td>239</td>
<td>143.3</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Number of juvenile per preadults (means and SD) registered in PDA plates along time.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h Mean</th>
<th>24 h SD</th>
<th>48 h Mean</th>
<th>48 h SD</th>
<th>72 h Mean</th>
<th>72 h SD</th>
<th>96 h Mean</th>
<th>96 h SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pumilus 15.1</td>
<td>3.25</td>
<td>2.98</td>
<td>10.25</td>
<td>8.65</td>
<td>58.12</td>
<td>20.24</td>
<td>352.25</td>
<td>60.43</td>
</tr>
<tr>
<td>B. pumilus 15.1C</td>
<td>3.87</td>
<td>4.98</td>
<td>16.12</td>
<td>5.62</td>
<td>71.25</td>
<td>26.77</td>
<td>255.12</td>
<td>131.62</td>
</tr>
<tr>
<td>B. pumilus M1</td>
<td>8.50</td>
<td>8.83</td>
<td>12.62</td>
<td>5.65</td>
<td>73.87</td>
<td>24.33</td>
<td>427.5</td>
<td>245.80</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>1.00</td>
<td>1.50</td>
<td>1.24</td>
<td>1.61</td>
<td>2.75</td>
<td>3.63</td>
<td>2.75</td>
<td>3.86</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>2.25</td>
<td>2.77</td>
<td>25.12</td>
<td>16.18</td>
<td>129.25</td>
<td>84.70</td>
<td>379.25</td>
<td>41.03</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>0.50</td>
<td>0.70</td>
<td>1.12</td>
<td>1.53</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Number of adults (means and SD) registered in PDA plates along time.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h Mean</th>
<th>24 h SD</th>
<th>48 h Mean</th>
<th>48 h SD</th>
<th>72 h Mean</th>
<th>72 h SD</th>
<th>96 h Mean</th>
<th>96 h SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pumilus 15.1</td>
<td>5.60</td>
<td>0.80</td>
<td>5.00</td>
<td>1.35</td>
<td>14.75</td>
<td>4.99</td>
<td>103.12</td>
<td>32.59</td>
</tr>
<tr>
<td>B. pumilus 15.1C</td>
<td>5.00</td>
<td>0</td>
<td>14.87</td>
<td>14.97</td>
<td>25.12</td>
<td>24.07</td>
<td>88.00</td>
<td>41.98</td>
</tr>
<tr>
<td>B. pumilus M1</td>
<td>5.60</td>
<td>1.20</td>
<td>5.37</td>
<td>1.67</td>
<td>17.75</td>
<td>6.19</td>
<td>86.62</td>
<td>36.13</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>5.00</td>
<td>0.63</td>
<td>1.12</td>
<td>0</td>
<td>1.50</td>
<td>2.34</td>
<td>0.62</td>
<td>1.31</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>5.00</td>
<td>0</td>
<td>10.5</td>
<td>15.67</td>
<td>27.75</td>
<td>9.76</td>
<td>83.75</td>
<td>62.77</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>5.00</td>
<td>0.63</td>
<td>0.87</td>
<td>0.74</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results showed that under the conditions used, E. coli OP50 support a nematode population of 1208 (± 299) individuals per plate after 7 d. In the same way, B. pumilus 15.1, B. pumilus 15.1C, and B. pumilus M1, supported a high nematode population of 725 (± 193), 734 (± 312), and 825 (± 246) nematodes per plate, respectively. B. thuringiensis, a strain considered as safe and extensively used as biological control, supported a nematode population of 62 (± 23) individuals per plate, while, B. cepacia, considered as a pathogen did
not support nematode proliferation. After this study we concluded that the strain *B. pumilus* 15.1 is a non-pathogenic strain and could potentially be used as a biological control agent in a safe way.

Figure 1. *Caenorhabditis elegans* pathogenicity assay. Total numbers of individuals of *C. elegans* 7 days after the beginning of the assay.

**Acknowledgements**

We thank the Ministry of Education and Science (MEC) for partially funding this project (CGL2008-02011) and supporting J. F. Caña-Roca and I. Vilchez through the FPI and FPU Programs respectively. We thank the Consejería de Ciencia, Innovación y Empresa of Junta de Andalucía for supporting T. Dominguez through the project reference P10-AGR-6409 and SENACYT-Ecuador for supporting D. García.

**References**


The indigenous entomopathogenic nematode searching results at different agrocenosis of Georgia

M. Chubinishvili, Ts. Chkhubianishvili, M. Kakhadze, I. Malania, I. Rijamadze
NLE Agricultural University of Georgia, Kanchaveli Institute of Plant Protection

Abstract: Some kind of soil is a habitat for entomopathogenic nematodes (EPNs), which may be considered as potential biological control agents to various pest insects in different agroecosystems of Georgia. The investigations of soil samples for searching and isolation of local EPN strains have been conducted. Determination of invasive ability of isolated nematodes has been carried out according to generally accepted methods in insect nematology. The infectivity of isolates on laboratory insect cultures, the greater wax moth, Galleria mellonella, and the meal worm, Tenebrio molitor, has been approved. As a result of multiple researches, the new model of nematode direct migration has been elaborated, which gives possibility to obtain more infective juveniles (IJ$s) from soil during a short period. The experiments were continued on establishment of new isolates invasive ability. 100 IJ$s of strain I, 100-150 IJ$s of strain II, and 100-120 IJ$s of strain III were used for contamination of 10 G. mellonella larvae of average size. The last instars of 10 larvae of T. molitor were infected by 150 IJ$s of all experimental strains. A typical pattern of nematode pathology has been obtained and IJ$s were applied to the test insects in next trials of bioassays. The comparative virulence has been determined between strain I, strain II and strain III. The preliminary results show perceptivity of new approach isolation nematodes for searching of EPN local strains, which are produced in vivo for identification.

Key words: Entomopathogenic nematodes, virulence, Galleria mellonella, Tenebrio molitor
Field evaluation of entomopathogenic nematodes for controlling fall webworm Hyphantria cunea (Lepidoptera: Arctiidae) in West Georgia

Oleg Gorgadze¹, Manana Lortkipanidze¹, Patrick Tailliez², Medea Burjanadze³, Madona Kuchava¹

¹Institute of Zoology, Ilis State University, Tbilisi, Georgia; ²INRA & Universite Montpellier 2, Unite Division Génomes & Interactions Microorganismes – Insectes, France; ³Agricultural University of Georgia, Tbilisi, Georgia

Abstract: The present work deals with results of application of entomopathogenic nematodes of the genus Steinernema (S. carpocapsae, S. thesami and Steinernema sp.) against the harmful pest of the forest and agricultural crops Hyphantria cunea (Lepidoptera: Arctiidae) distributed in Georgia. Field experiments were carried out in August of 2012 on private plots of Guria region of the West Georgia in hazelnut plantations diseased with pest’s larvae. A high percentage of mortality ranging from 93.6% to 98.3% was observed in all experiments as a result of entomopathogenic nematode application. Among the species used, the efficiency of a new Steinernema species was specially noticed. High efficiency of the treatment was also promoted by optimum climatic conditions (Temperature = 28 °C and hygrometry = 99%).

Key words: entomopathogenic nematodes, bioformulation, Hyphantria cunea

Introduction

The American white webworm or fall webworm (FWW) Hyphantria cunea Drury (Lepidoptera: Arctiidae) is a very harmful quarantine pest. It is distributed in west Georgia. The pest is a polyphag insect. It has been established that the species damages more than 400 plant species in Georgia (Edilashvili, 2002). As FWW is also an urban insect, the control of this pest needs special bioformulations (entomopathogenic fungi, viruses, bacteria, nematodes and other organisms) which are safe for human and environment. Numerous experiments have been carried out using the mentioned bioformulations, which show that their efficiency fluctuates within the range 55-98% (Burjanadze et al., 2012; Chkhubianishvili et al., 2011; Gorgadze, 2000; Edilashvili, 2002; Lortkipanidze et al., 2010).

The objective of the present investigation was to study the efficiency of entomopathogenic nematodes belonging to the genus Steinernema (S. carpocapsae, S. thesami and Steinernema sp.) against fall webworm at optimum conditions in the field.

S. carpocapsae introduced to Georgia is associated with a specific symbiotic bacteria Xenorhabdus nematophila, whereas local forms, such as S. thesami and Steinernema sp., which belong to the S. affine/intermedium group are associated with the symbiotic bacteria Xenorhabdus bovienii. Species of bacteria associated with local nematodes have been identified at the Laboratory of Diversity, Genome, and Microorganisms-insects Interactions (DGIMI, INRA) of the National Institute of Agronomical Research of Montpellier University (France).
Material and methods

Infective juveniles (IJ’s) of S. carpocapsae, S. thesami and Steinernema sp. were reared on larvae of Galleria melonella and Bombyx mori (Veremchuk, 1986; Dutky, 1964). Field experiments were carried out in the second decade of 2012 in Guria region (west Georgia) in hazelnut plantations diseased with FWV larvae. The pest produces two generations during the season in the mentioned region – in May and August. The warmest month August was chosen for experiments. Concentrated nematodes were transported in icebox in order to prevent mortality of nematodes due to transfer to long distance (300 km). Before starting the experiments and treatment of beforehand chosen experimental and control plants, the number of pests per m² area of branches was evaluated. The number of pests per m² fluctuated from 65 to 289. Experiments were carried out as follows: One control plant without any nematode application and 3 experimental plants, one with S. carpocapsae, a second one with S. thesami and the third one with Steinernema sp.). All suspensions used in trials contained equal concentration of nematodes (2500 ± 120 nematodes ml⁻¹ water). Treatment of experimental plants by nematode suspension was performed using the hand apparatus of the OBX-14 type in evening hours, in cloudy weather at 28 °C temperature and 99% relative humidity. Monitoring of treated plants and accounting of dead pests was made on 3rd, 5th and 7th days after spraying according to the method by Abbott (Abbott, 1925).

Results and discussion

Checking of sprayed plants 14 hours after treatment showed that the nematode suspension was not dried out on leaf surfaces, especially on the lower sides of leaves, where the pests were assembled in colonies. While examining such leaves only living and active forms of invasive juveniles were revealed. None of individuals of larvae was dead. Larvae were in passive condition, though reacted on irritant.

Twenty hours after treatment with entomopathogenic nematodes, damage on leaves caused by pests was reduced, while mortality rate of pests was significantly increased from the 3rd day post-treatment.

Only three days after treatment, more than 90% of the pest larvae were dead whatever the nematode species used. Where suspension of S. carpocapsae was used for spraying, 94.3% mortality of pest’s larvae has been stated on the experimental plant; on the 5th day mortality reached 98.1%; and on the 7th day the mortality rate of pests was almost not changed. The average mortality rate in this variant of experiment was 96.8% (Figure 1).
Figure 1. Mortality of Hyphantria cunea 3rd - 4th instar larvae after application of entomopathogenic nematode suspension of the genus Steinernema (2500 ± 120 nematodes/ml water) under field conditions (temperature: 28 °C and humidity: 99%).

Similar results of mortality were obtained using S. thesami for biocontrol. The highest mortality rate of 93% was reached 5 days after treatment.

When Steinernema sp. was tested against FWW, the mortality rate was 95% on the 5th day after application, similar to the other tested nematodes S. carpocapsae and S. thesami. In the untreated control experiments, no mortality of the pest was observed. When checking under the binocular microscope, 22-36 individuals of developed 4th - 5th instar Steinernema nematodes were observed in each dead larva of FWW.

In all experiments where S. carpocapsae, S. thesami and Steinernema sp. were used for the biological control of FWW, high mortality levels were observed. It is worth to note the special efficiency of a new species Steinernema sp. against the pest. High efficiency of the used formulations seems to be favoured by optimal climatic conditions (temperature, humidity, etc.) during the experiment. These parameters are of great importance for the activity and efficiency of entomopathogenic nematodes.

Acknowledgements

We gratefully acknowledge the financial support from the Shota Rustaveli National Science Foundation & STCU, Project #5630

References


Lortkipanidze, M., Gorgadze, O., Kokhia, M., Melashvili, N. & Burdjanadze, M. 2010: Biological potential of entomopathogenic Nematodes (Steinernematidae) against Hyphantria cunea (Lepidoptera: Arctiidae) in Georgia. Population dynamics, biological control, and integrated management of forest Insects. Book of Abstracts: 83. IUFRO Eberswalde, Germany.

Veremchuk, G. V. 1986: Methodical recommendations of laboratory rearing on Honeycomb Moth (Galleria melonella) and application of entomopathogenic nematodes. Leningrad: 1-38 (in Russian).
Feeding activity and survival of slug Arion lusitanicus (Gastropoda: Arionidae) exposed to the rhabditid nematode, Phasmarhabditis hermaphrodita (Nematoda: Rhabditidae)

Dinka Grubišić¹, Tina Hamel¹, Tanja Gotlin Čuljak¹, Ana Loparić¹, Mirjana Brmež²
¹Faculty of Agriculture University of Zagreb, Department of Agricultural Zoology, Svetosimunska cesta 25, 10000 Zagreb, Croatia; ²Faculty of Agriculture in Osijek, Department for plant protection, Petra Svačića 1d, 31000 Osijek, Croatia

Abstract: Slugs are important pests of cultivated plants in Croatia. In many sites slug species Arion lusitanicus (Mabille, 1868) (Gastropoda: Arionidae) has become the most frequent species, which is very hard to control by chemical molluscicides. Since 1996 a biological molluscicide based on nematode Phasmarhabditis hermaphrodita (Schneider, 1859) (Nematoda: Rhabditidae) has been formulated as an effective product for slug control. In order to establish feeding activity and survival of adult A. lusitanicus specimens exposed to parasitic nematode P. hermaphrodita and to compare its efficiency to efficiency of chemical molluscicides, a laboratory experiment was performed. Adult specimens of A. lusitanicus (fed on lettuce leaves in flower pots) were exposed to P. hermaphrodita, metaldehyde and methiocarb treatments. Food consumption of slug specimens was measured daily. Survival of slugs was observed to their death, up to 30th day. In the first week of investigation, chemical molluscicide treatments were found to differ significantly from the biological product and control. At the treatments treated by nematodes, daily leaf area consumption was also reduced and was significantly different from the control treatment. Food consumption was reduced on both chemical and biological treatments but the most of adult specimens of A. lusitanicus survived and continued to feed. To the end of the second week of investigation, food consumption decreased on all treatments and was mainly uniform with no significant differences between treatments. During the experiment, the slugs were dying within the period of 3 to 30 d at the treatments treated by nematode P. hermaphrodita or in the period of 9 to 24 d at the treatments treated by metaldehyde and methiocarb. Because the tolerance level to slug damages in lettuce market is effectively zero, these results indicate a failure of biological product based on P. hermaphrodita in control of adult specimens of A. lusitanicus as well as a failure of chemical molluscicides. These data point at a great need for integrated control of slug damage which must include cultural and different alternative control measures, not only chemical control.

Key words: Arion lusitanicus, molluscicides, parasitic nematodes, Phasmarhabditis hermaphrodita, slugs, slug control

Introduction

Arion lusitanicus (Mabille, 1868) is the slug species which has spread with an alarming rate in many sites in Croatia, where it is considered as a serious pest. It damages many vegetables and ornamentals but also oilseed rape, maize and sunflowers. In Central Europe, A. lusitanicus is the major pest slug species and the most sales of molluscicides in the home and garden market can be attributed to this species (Weidema, 2006). Current control methods for A. lusitanicus in Croatia rely on chemical molluscicides, which are often ineffective and can harm non-target organisms. Phasmarhabditis hermaphrodita (Schneider, 1859) is a nematode that parasites many slugs and snails. In 1994 it was formulated into biological molluscicide (Glen et al., 1996) which is used as inundative biocontrol agent. The nematode
is applied onto soil, where it seeks out slugs and infects them. Infection rapidly leads to feeding inhibition and later kills the slugs. There are some indications that in the large A. rionid species susceptibility to P. hermaphrodita decreases with body size and that P. hermaphrodita cannot kill or inhibit feeding of A. lusitanicus individuals of >1 g weight (Grimm, 2002; Speiser et al., 2001). In order to establish the efficacy of P. hermaphrodita in control of adult specimens of A. lusitanicus, the laboratory experiment was performed in Zagreb in autumn 2009.

**Material and methods**

Laboratory experiment was performed in period October 26 to November 9, 2009 in Zagreb-Maksimir, Croatia. There were six treatments in the experiment: (1) untreated control, (2) metaldehyde pellets (5% active ingredient, recommended rate), (3) methiocarb pellets (4% active ingredient, recommended rate), (4) P. hermaphrodita (30 nematodes cm\(^{-2}\), recommended rate), (5) P. hermaphrodita (15 nematodes cm\(^{-2}\)) and (6) P. hermaphrodita (15 nematodes cm\(^{-2}\)) + P. hermaphrodita (15 nematodes cm\(^{-2}\), applied one week following the first application). There were four replicates of each treatment. Every replicate was represented by flower pot (21 cm in diameter) repleted by potting soil. One adult specimen of A. lusitanicus (weight 2 g) was placed along with one lettuce leaf in every flower pot. Lettuce leaves were changed every day. Commercial formulation of P. hermaphrodita (Phasmarhabditis - System; supplier: Biobest N.V., Belgium) was used in the experiment. The nematodes were stirred in water and were applied to the flower pots using watering can “Rose”. Pellets of molluscicides were broadcasted by hand on the soil surface in flower pots. Slug feeding was assessed by measuring of the lettuce leaf area eaten by slugs (using millimeter paper) after 14 d For the purpose of interpreting the results, air temperature data measured on measurement station in Zagreb - Maksimir were used. All data were subjected to ANOVA and Duncan's New MRT (P = 0.05). Simultaneously, monitoring of appearance, behavior changes and survival of slugs was conducted. After measurement of slug feeding after 14 d the survival of slugs in each replicate was observed to their death, up to day 30.

**Results and discussion**

Overview data about food consumption of slug species A. lusitanicus in a laboratory experiment measured for 14 d are presented in Figure 1. Food consumption caused by slugs was evidenced on all treatments. The data obtained on biological product treatments are different then the data reported by Glen et al. (2000) and Grewal et al. (2001, 2003) indicated that slugs infected by nematodes cease to feed. From the 2\(^{nd}\) to the 6\(^{th}\) day, food consumption of A. lusitanicus on treatments treated by chemical molluscicides was found to differ significantly from biological product treatments and control (Figure 1). It was to be expected because nematodes need a few days to begin parasitization and disabling the host. At the nematode treatments, daily leaf area consumption, in the first week of experiment, was also reduced and was significantly different from the control treatment. From the day 7 to day 14 of food consumption assessment statistically significant differences were determined on days 8, 10 and 11, when significantly less food consumption was measured on all chemical and biological treatments compared to the untreated control. Towards the end of the experiment, from day 12 to 14, poor feeding of slugs on lettuce leaves was evident in all treatments. Food consumption was often uniform and there were no significant differences between treatments.
Exposure of slugs to low temperatures may lead to reduced feeding, therefore measurements were completed after 14 d.

Monitoring of the survival of slugs (feeded by lettuce) was continued from day 14 to day 30. During the experiment, the slugs died between day 3 to day 30 when treated with P. hermaphrodita and between day 9 and day 24 when treated with metaldehyde and methiocarb. Glen et al. (2000) and Grewal et al. (2001, 2003) reported that slugs infected by nematodes die within 4 to 20 d, depending on temperature and abundance of the nematode population, what is comparable with results in this investigation. In a 14-day experiment, Grimm (2002) reported significant mortality (about 30-50%) of A. lusitanicus specimens of 0.24 g or smaller, while the mortality of bigger specimens (0.45 g) was less than 10%. Mean daily temperatures measured in Maksimir were 11.7 °C in October 2009 and 8.0 °C in November. The measured temperatures were low, which could be the reason why, when compared to literature data, a bit longer time was needed for slugs to die after the application of nematodes. The optimum temperature for the development of nematode P. hermaphrodita is about 17 °C (Glen et al., 1996) so it is obvious that the temperature conditions during the experiment were not optimal. However, nematodes develop in the temperature range of 5-20 °C, indicating that nematodes in slugs were developing slower during the experiment. This could be the reason, why the achieved efficiency in reducing of food consumption was lower. During the experiment, changes on the body of adult A. lusitanicus specimens in form of a swollen mantle and damages on the epidermis have been noticed. This was also reported before (Tan & Grewal, 2001; Grimm, 2002). Grimm (2002) and Speiser et al. (2001) reported that P. hermaphrodita can not kill or inhibit feeding of A. lusitanicus of more than 1 g weight. Their findings are not in accordance with the results obtained in this investigation. According to the measured food consumption, it is evident that none of the treatments prevented feeding.
of adult forms of A. lusitanicus effectively. This has been observed by many producers of lettuce and other vegetables. In accordance with our results, the use of chemical or biological molluscicides need to be complemented by cultural methods, such as physical or other alternative methods of slug control.

References


New insights to insect response to the infection by nematobacterial complex

Pavel Hyršl¹, Pavel Dobeš¹, Badrul Arefin², Lucie Kučerová³, Robert Markus², Zhi Wang², Michal Žurovec³, Ulrich Theopold²

¹Department of Animal Physiology and Immunology, Institute of Experimental Biology, Masaryk University, Kotlářská 2, 61137 Brno, Czech Republic; ²Department of Molecular Biosciences, The Wenner-Gren Institute, University of Stockholm, 10691 Stockholm, Sweden; ³Biology Centre of the AS CR, Institute of Entomology, Branišovská 1160/31, 37005 České Budějovice, Czech Republic

Abstract: Entomopathogenic nematodes (EPNs) of the genera Heterorhabditis are obligate and lethal insect parasites. In recent years they have been used increasingly as biological control agents. These EPNs are symbiotically associated with bacteria of the genera Photorhabdus. The bacterial symbionts are essential to kill the host (within 24-48 hours) and digest its tissues to provide nutrients for themselves as well as for expanding nematodes. Drosophila larvae are suitable insect hosts and part of the tripartite model system we used before to show the importance of haemolymph clotting and eicosanoids during the infection.

We used the well-established tripartite model (Drosophila, nematodes, bacteria), DNA chips and bioinformatic tools to compare gene expression in non-infected and infected fly larvae. We focused on the early time point of nematode infection and therefore infected Drosophila larvae using H. bacteriophora harbouring GFP-labelled P. luminescens bacteria. Infected (GFP positive) larvae were collected 6 hours after infection.

We detected approximately 650 genes whose expression was significantly influenced by nematobacterial infection caused by H. bacteriophora and P. luminescens. Most of them are upregulated upon infection including mainly the genes involved in antimicrobial response and development. Based on Gene Ontology annotation we identified several pathways, which could be involved in sealing and repairing the wound caused by invading nematodes.

We compared these results with the available data for other infection types caused by bacteria and parasitic wasps. Small group of genes were common for all three types of infection and approximately 25 genes were overlapping in each pairwise comparison. We focused on the genes expressed in the haemocytes and fat body, respectively, and we subjected selected candidate genes to functional tests. We tested the effect of mutations or knockdown of selected genes for the susceptibility of flies to the nematobacterial infection. The overlap between the protective genes and genes induced by the nematobacterial infection was not complete. Therefore, we assume that only a fraction of the genes involved in the protection of infected larvae from death are induced by the nematobacterial infection.

Our research is supported by research grants from the Swedish Research Council (VR-NT 2010-5118), the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) and by a grant from Ministry of Agriculture of Czech Republic (NAZV-KUS QJ 1210047).

Key words: Drosophila, immunity, Heterorhabditis, Photorhabdus
Compatibility of five different entomopathogenic nematode (Nematoda: Rhabditida) species with registered insecticides and fungicides under laboratory conditions

Žiga Laznik1*, Stanislav Trdan1
1University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Chair of Phytomedicine, Agricultural Engineering, Crop Production, Grassland and Pasture Management, Jamnikarjeva 101, 1111 Ljubljana, Slovenia
* Author for correspondence: tel.: +386 1 320 3226, e-mail: ziga.laznik@bf.uni-lj.si

Abstract: To increase our knowledge on the susceptibility of entomopathogenic nematode (EPN) species to agrochemicals, the compatibility of the infective juveniles (IJs) of the Steinernema feltiae, S. carpocapsae, S. kraussei, Heterorhabditis bacteriophora and H. downesi with 6 chemical, one plant-based and one bio-insecticide, and 13 synthetic organic and two inorganic fungicides were investigated under laboratory conditions. The effect of direct IJ exposure to insecticides for 6 and 24 hours was tested in Petri dishes at 15, 20 and 25 °C. In our experiment we determined the best compatibility of S. feltiae with active ingredients azoxystrobin, azadirachtin, Bacillus thuringiensis var. kurstaki and imidacloprid. The present study showed that S. carpocapsae and S. kraussei are sensitive to all tested insecticides, while H. bacteriophora is sensitive only to abamectin and lufenuron. Nematode H. downesi significantly suffered the highest mortality when infective juveniles were mixed with active ingredients (a. i.) tebuconazole, spiroxamine, and triadimenol. Based on our research, we conclude that compatibility is not only a species-specific but also a strain-specific characteristic.

Key words: entomopathogenic nematodes, compatibility, insecticides, fungicides

Introduction

Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) can be used as biological control agents to suppress a variety of economically important insect pests (Laznik & Trdan, 2011). EPN are often applied to sites and ecosystems that routinely receive other inputs that may interact with nematodes, including chemical pesticides, fertilizers, and soil amendments (De Nardo & Grewal, 2003). It is often desirable to know if a pesticide can be tank-mixed or applied simultaneously with another pesticide to save time and money and for compatibility with integrated pest management (IPM) and integrated production (IP) systems (Grewal, 2002).

EPN infective juveniles (IJs) can tolerate short-term exposure (2-24 h) to many chemical and biological insecticides, fungicides, herbicides, fertilizers, and growth regulators and can thus be tank-mixed and applied together (De Nardo & Grewal, 2003; Laznik et al., 2012). However, generalizations cannot be made because the nematodes` susceptibility depends on several factors, such as species, strain, agrochemical formulations and application dose (Grewal, 2002; Laznik et al., 2012).

To increase the knowledge of the EPN species and strain susceptibility to agrochemicals (insecticides and fungicides) and to explore the effect of their mechanisms on the viability of these organisms, the aim of this study was to select some commercial insecticides and fungicides currently used in Slovenia for integrated crop protection and to evaluate their effects on the survival of IJs from native Slovenian EPN strains of Steinernema feltiae.
(Filipjev), S. carpocapsae Weiser, and Heterorhabditis bacteriophora Poinar and commercial strains [Becker Underwood: S. feltiae, S. carpocapsae and S. kraussei (Steiner); Koppert B.V: S. Feltiae] at different temperatures under laboratory conditions, thereby determining their suitability in IPM programs.

Material and methods

Pesticides
In the present study, 8 commercial insecticides and 15 commercial fungicides registered against different insect pests and pathogens in Slovenia were evaluated (Table 1).

Table 1. Trade names, active ingredients, and concentrations (concentr.) of the formulations tested in this study.

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Active ingredient</th>
<th>Test Concentr.</th>
<th>Trade Name</th>
<th>Active ingredient</th>
<th>Test Concentr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliette flash</td>
<td>Phosethyl-Al</td>
<td>3.7 g l⁻¹</td>
<td>Sabithane</td>
<td>Dinocap</td>
<td>0.4 ml l⁻¹</td>
</tr>
<tr>
<td>Bellis</td>
<td>Boscalid &amp; Pyraclostrobin</td>
<td>0.8 g l⁻¹</td>
<td>Tattoo</td>
<td>Mancozeb &amp; Propamocarb</td>
<td>4 ml l⁻¹</td>
</tr>
<tr>
<td>Clarinet</td>
<td>Fluquinconazole &amp; Pyrimethanil</td>
<td>1.5 ml l⁻¹</td>
<td>Teldor SC 500</td>
<td>Fenhexamid</td>
<td>2 ml l⁻¹</td>
</tr>
<tr>
<td>Cuprabelau-Z</td>
<td>Copper hydroxide &amp; Zinc</td>
<td>4 g l⁻¹</td>
<td>Vetricene</td>
<td>A bamectin</td>
<td>1.25 m l⁻¹</td>
</tr>
<tr>
<td>Dithane M-45</td>
<td>Mancozeb</td>
<td>2.5 g l⁻¹</td>
<td>Match 050 EC</td>
<td>Lufenuron</td>
<td>2 ml l⁻¹</td>
</tr>
<tr>
<td>Falcon EC-460</td>
<td>Tebuconazole &amp; Spiroxamine &amp; Triadimenol</td>
<td>0.4 ml l⁻¹</td>
<td>Delfin WG</td>
<td>B. thuringiensis var. kurstaki</td>
<td>0.75 g l⁻¹</td>
</tr>
<tr>
<td>Folpan 80 WDG</td>
<td>Folpet</td>
<td>150 ml l⁻¹</td>
<td>Chess 50 W G</td>
<td>Pymetrozine</td>
<td>0.6 g l⁻¹</td>
</tr>
<tr>
<td>Pepelin</td>
<td>Sulphur</td>
<td>6 g l⁻¹</td>
<td>Neemazal-T/S</td>
<td>A zadirachtin</td>
<td>3 ml l⁻¹</td>
</tr>
<tr>
<td>Polyram DF</td>
<td>Metiram</td>
<td>1.2 g l⁻¹</td>
<td>Confidor 200 SL</td>
<td>Imidacloprid</td>
<td>0.75 m l⁻¹</td>
</tr>
<tr>
<td>Previcur 607 SL</td>
<td>Propamocarb</td>
<td>2.5 ml l⁻¹</td>
<td>Karate Zeon 5 CS</td>
<td>Lambda-cibltozin</td>
<td>0.15 ml l⁻¹</td>
</tr>
<tr>
<td>Ridomil Gold Plus 42.5 WP</td>
<td>Copper hydroxide &amp; Metalaxyl-M</td>
<td>4 g l⁻¹</td>
<td>Pirimor 50 W G</td>
<td>Pirimicarb</td>
<td>0.6 g l⁻¹</td>
</tr>
<tr>
<td>Quadris</td>
<td>Azoxyostrobin</td>
<td>1 ml l⁻¹</td>
<td>control</td>
<td>Distilled water</td>
<td></td>
</tr>
</tbody>
</table>

Nematodes
EPNs were reared and prepared as described elsewhere (Laznik et al., 2012). Six EPN strains were included in the insecticidal experiment. The commercial preparations Nemasys (a. i. Steinernema feltiae), Nemasys C (a. i. S. carpocapsae) and Nemasys L (a. i. S. kraussei) were obtained from Becker Underwood (Littlehampton, United Kingdom). All other strains
S. feltiae B30, S. carpocapsae C101 and Heterorhabditis bacteriophora D54 were isolated from the soil in Slovenia (Laznik & Trdan, 2011). Four EPN strains were included in fungicidal experiment. The commercial preparation Entonem (a. i. S. feltiae) was obtained from Koppert B.V. (Berkel en Rodenrijs, The Netherlands). All of the other strains were isolated from the soil. S. feltiae C76 and S. carpocapsae C67 were isolated in Slovenia (Laznik & Trdan, 2011), while Heterorhabditis downesi 3173 was isolated in Hungary (Tóth, 2006).

**Compatibility test**
Compatibility test was made according to Laznik et al. (2012).

**Statistical analyses**
Statistical analyses was made according to Laznik et al. (2012).

**Results and discussion**

In our experiment we determined the best compatibility of S. feltiae with active ingredients azoxystrobin, azadirachtin, B. thuringiensis var. kurstaki and imidacloprid (Table 2 and Table 3). The present study showed that S. carpocapsae and S. kraussei are sensitive to all tested insecticides, while H. bacteriophora is sensitive only to abamectin and lufenuron (Table 3). Nematode H. downesi significantly suffered the highest mortality when infective juveniles were mixed with a. i. tebuconazole, spiroxamine, and triadimenol (Table 2).

Most previous studies of the compatibility of nematodes with chemicals have been conducted as laboratory bioassays with direct exposure of nematodes to pesticides (Rovesti & Deseo, 1990; Gordon et al., 1996; Laznik et al., 2012). The large variability between pesticides from the same chemical group in their compatibility with EPN make the extrapolation of data between products unreliable (Rovesti & Deseo, 1990); thus, each candidate product for an IPM system should be tested individually. Similar extrapolation of compatibility data between different nematode species or even strains is also undesirable (Laznik et al., 2012).

The results of the present study and those of previous investigations (De Nardo & Grewal, 2003; Laznik et al., 2012) in which the compatibility of plant protection products with EPN was evaluated revealed that compatibility is species-specific. The present study revealed that azadirachtin and pirimicarb did not affect the viability of S. feltiae and H. bacteriophora nematodes. However, previously mentioned active ingredients did affect the viability of S. carpocapsae and S. kraussei. Laznik et al. (2012) recently reported that compatibility of EPN with pesticides (fungicides) is not only a species-specific but also a strain-specific characteristic. Similar conclusions were also obtained in the present study. Namely, the active ingredients azadirachtin, B. thuringiensis var. kurstaki and imidacloprid did not affect the viability of the domestic strain S. feltiae B30. In contrast, the before-mentioned insecticides significantly reduced the number of living IJs of a commercial product of the same tested EPN species.
Table 2. Percent change in the survival of different EPN strains after incubation with 15 different fungicides at 15 °C, 20 °C and 25 °C for 24 h.

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Change in nematode survival after exposure to chemicals for various durations at different temperatures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. feltiae strain C76</td>
</tr>
<tr>
<td>Allierette flash</td>
<td>-59.9b</td>
</tr>
<tr>
<td>Bellis</td>
<td>-58.6b</td>
</tr>
<tr>
<td>Clarinet</td>
<td>-68.2b</td>
</tr>
<tr>
<td>Cupraflau-Z</td>
<td>-65.1b</td>
</tr>
<tr>
<td>Dithane M-45</td>
<td>-98.2cd</td>
</tr>
<tr>
<td>Falcon EC-460</td>
<td>-100.0d</td>
</tr>
<tr>
<td>Folpan 80 WDG</td>
<td>-61.4b</td>
</tr>
<tr>
<td>Pepelin</td>
<td>-60.8b</td>
</tr>
<tr>
<td>Polyram DF</td>
<td>-63.7b</td>
</tr>
<tr>
<td>Previcur 607 SL</td>
<td>-72.8bcd</td>
</tr>
<tr>
<td>Ridomil Gold Plus 42.5 WP</td>
<td>-77.2bcd</td>
</tr>
<tr>
<td>Quadris</td>
<td>-13.9a</td>
</tr>
<tr>
<td>Sabithane</td>
<td>-70.7bc</td>
</tr>
<tr>
<td>Tattoo</td>
<td>-52.2b</td>
</tr>
<tr>
<td>Teldor SC 500</td>
<td>-59.3b</td>
</tr>
<tr>
<td>Control (water)</td>
<td>100.0a</td>
</tr>
</tbody>
</table>

*Values were significantly different (P ≤ 0.05) in Tukey's multiple range tests. Small letters indicate that statistically significant differences were observed between the control treatment and fungicide treatments with the same EPN strain at the same temperature.*
Table 3. Corrected mortality rates (%) of insecticidal-treated EPN strains at different temperatures 6 hrs after exposure. Means within a row followed by different letters are significantly different (P < 0.05, Tukey's test). SfB30 – Steinernema feltiae strain B30; SfBU – Steinernema feltiae strain Becker Underwood; ScC101 – Steinernema carpocapsae strain C101; ScBU – Steinernema carpocapsae strain Becker Underwood; SkBU – Steinernema kraussei strain Becker Underwood; HbD54 – Heterorhabditis bacteriophora strain D54.

<table>
<thead>
<tr>
<th>EPN strain</th>
<th>Temp. (°C)</th>
<th>Abamectin</th>
<th>Azadirachtin</th>
<th>B. t. var. kurstaki</th>
<th>Imidacloprid</th>
<th>Lambda-cyhalothrin</th>
<th>Lufenuron</th>
<th>Pirimicarb</th>
<th>Pymetrozine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>54.2 b</td>
<td>0.0 a</td>
<td>7.4 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>10.5 a</td>
<td>0.0 a</td>
<td>9.2 a</td>
</tr>
<tr>
<td>SfB30</td>
<td>20</td>
<td>20.8 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>3.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>31.4 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>86.3 g</td>
<td>25.0 e</td>
<td>25.8 e</td>
<td>28.3 e</td>
<td>10.2 c</td>
<td>14.5 d</td>
<td>4.4 b</td>
<td>39.1 f</td>
</tr>
<tr>
<td>SfBU</td>
<td>20</td>
<td>86.5 c</td>
<td>1.1 a</td>
<td>4.6 b</td>
<td>7.6 b</td>
<td>0.0 a</td>
<td>0.2 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>90.3 d</td>
<td>8.3 b</td>
<td>30.1 c</td>
<td>6.4 b</td>
<td>0.2 a</td>
<td>0.0 a</td>
<td>1.4 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.0 b</td>
<td>32.9 c</td>
<td>25.1 c</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>1.2 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>ScC101</td>
<td>20</td>
<td>0.2 a</td>
<td>13.1 b</td>
<td>12.5 b</td>
<td>0.5 a</td>
<td>15.1 b</td>
<td>23.6 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>19.5 c</td>
<td>58.4 f</td>
<td>6.5 b</td>
<td>42.2 e</td>
<td>28.3 d</td>
<td>54.4 f</td>
<td>26.5 cd</td>
<td>39.7 e</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.3 a</td>
<td>19.2 bc</td>
<td>18.3 b</td>
<td>14.5 b</td>
<td>16.5 b</td>
<td>22.2 bc</td>
<td>30.8 c</td>
<td>16.5 b</td>
</tr>
<tr>
<td>ScBU</td>
<td>20</td>
<td>0.0 a</td>
<td>9.9 b</td>
<td>25.2 c</td>
<td>5.5 ab</td>
<td>18.3 b</td>
<td>8.4 b</td>
<td>0.0 a</td>
<td>10.2 b</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>7.4 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>33.6 d</td>
<td>13.5 b</td>
<td>22.2 c</td>
<td>2.7 a</td>
<td>28.5 c</td>
<td>49.4 f</td>
<td>27.4 c</td>
<td>43.4 e</td>
</tr>
<tr>
<td>SkBU</td>
<td>20</td>
<td>25.9 d</td>
<td>3.6 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>14.9 b</td>
<td>19.9 c</td>
<td>13.3 b</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>21.4 d</td>
<td>0.0 a</td>
<td>8.4 b</td>
<td>18.3 cd</td>
<td>19.5 cd</td>
<td>0.0 a</td>
<td>8.2 b</td>
<td>14.6 c</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>30.3 c</td>
<td>0.0 a</td>
<td>26.9 c</td>
<td>14.5 b</td>
<td>0.5 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>HbD54</td>
<td>20</td>
<td>16.7 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.4 a</td>
<td>19.0 b</td>
<td>2.4 a</td>
<td>0.0 a</td>
<td>16.1 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>13.7 b</td>
</tr>
</tbody>
</table>

Acknowledgements

The present work was performed within project V4-1067, which was granted by the Slovenian Research Agency, and by the Ministry of Agriculture and Environment of the Republic of Slovenia. A portion of the present research was funded by Horticulture No. P4-0013-0481, a program funded by the Slovenian Research Agency, and a portion of the research was funded within Professional Tasks from the Field of Plant Protection, a program funded by the Ministry of Agriculture and Environment of the Republic of Slovenia. We thank Polona Pust and Sanja Ljubi for technical assistance and Dr. Tamás Lakatos and Dr. Timea Tóth for providing the Slovenian strains of EPN. We would like to thank Gareth Martin (Becker & Underwood) and Frans Weber (Koppert B.V.) for providing the commercial EPN strains.
References


Susceptibility of *Phytodecta fornicata* (Coleoptera: Chrysomelidae) to *Heterorhabditis bacteriophora*

Ivana Majić¹, Emilija Raspudić¹, Marija Ivecić¹, Mirjana Brmež¹, Ankica Sarajlić¹, Andrea Mirković¹,²

¹Faculty of Agriculture, Josip Juraj Strossmayer University of Osijek, Kralja Petra Svačiće 1d, 31000 Osijek, Croatia; ²Dow AgroSciences, Petra Hektorovića 2, 10000 Zagreb, Croatia

Abstract: The infectivity of *Heterorhabditis bacteriophora* for adult stage of *Phytodecta fornicata* was evaluated in the laboratory. Two different nematode concentrations (1000 and 2000 infective juveniles (IJ)s per adult) were tested at temperatures of 22 °C and 30 °C. Mortality of insects was checked at 3 d post infection (p.i.); emergence of IJs from cadavers was noted at 8 and 11 d p.i.. For the first time, the experiment demonstrated that *H. bacteriophora* applied in rate of 1000 IJs per beetle is causing 100% mortality of *P. fornicata*, and that IJs developed inside adult insect cadavers. The effects of temperature and concentration of nematode preparation were of less importance. The harvest of IJes from cadavers was obtained but with low population level. The time of emergence of IJes was temperature dependent. In order to develop cost effective and sustainable control measure, further mortality studies on individuals, with other species and strains, and assays with more realistic environments are needed.

Key words: *Phytodecta fornicata*, *Heterorhabditis bacteriophora*, mortality, emergence

Introduction

In southern Europe, damages on lucerne (*Medicago sativa*) caused by the lucerne leaf beetle *Phytodecta (Gonioctena) fornicata* (Brüggemann) (Coleoptera: Chrysomelidae) have been reported since early 1910s and 1920s (Jablonskowski, 1921; Knell, 1922). Lately, literature published regarding this pest has been mainly reported from Bulgaria, Italy, Serbia and Croatia. *P. fornicata* is a monophagous pest, feeding on leaf, leaf buds and stems of lucerne. This pest causes defoliating of the plants resulting in major crop losses. Kereši & Sekulić (2005) reported yield loss of 30-50% in the first mowing of lucerne. Some agro-technical measures, such as earlier mowing can be applied in order to reduce crop loss or insect population. Recently, new biotechnological approaches have been proposed to combat this pest (Ninković et al., 2007). Insecticidal application is often advised since several chemical compounds were reported as effective. However, environmentally friendly measures are needed. Entomopathogenic nematodes (EPNs) have proven their efficiency in control of Coleopteran pests in different crops and in lucerne (Shields et al., 1999; 2009). EPN are resistant to widely used pesticides (Koppenhöfer et al., 2000), and this characteristic is an advantage as it provides a great potential for biopesticide development and a sustainable plant protection measure. So far, no reports are published regarding susceptibility of *P. fornicata* to entomopathogenic nematodes.

The aim of this paper is to determine infectivity of entomopathogenic nematode *Heterorhabditis bacteriophora* against lucerne leaf beetle.
Material and methods

Beetles of *Phytodecta fornicata* were collected in lucerne fields at the Agricultural Institute Osijek, Croatia in April 2011. Experiment was conducted in laboratory between April 29 until May 9, 2011. Ten beetles were placed on wet filter paper with lucerne leaves in a Petri dish. Suspensions of 10000 and 20000 infective juveniles (IJs) ml$^{-1}$ (i.e. dose of 1000 and 2000 IJs per beetle), respectively, were pipetted in each Petri dish. Experiment was done at two temperature regimes: in a climate chamber at 30 °C and at room temperature (22 °C). Each treatment was replicated four times and included untreated control dishes. *H. bacteriophora* was obtained from Koppert B. V. (Berkel en Rodenrijs, The Netherlands). To estimate mortality, insects were checked on day 3 post EPN application (p.i.). Ten cadavers were placed on four White traps (White, 1927) depending on the nematode treatment. Two traps were kept in climate chamber at 30 °C and other two traps at room temperature (22 °C). From White traps, the emerging IJs were harvested and counted on at 8 and 11 d p.i.. Analysis of variance (PROC GLM) and means separation with Tukey’s test (SAS 9.2; SAS Institute, Carey, NC, USA) were applied for data of mortality of insects. Data were arcsine transformed prior to analysis.

Results and discussion

Mortality rates of lucerne leaf beetle are presented in Table 1. In both nematode treatments, all beetles were found to be dead and mortality was 100% already at lower nematode dose. In control dishes mortality of leaf beetles was 20 and 22.5%, respectively, depending on the temperature regime. Statistically significant difference was observed between control and nematode treatments. Insects had similar mortality rates under both temperature regimes, and did not statistically differ. The laboratory mortality study using *P. fornicata* showed that *H. bacteriophora* is a candidate for use in lucerne integrated pest management. Since, there are no reports on *P. fornicata* these are the first findings. Mortality studies using variety of steinernematid and heterorhabditid species have been done with success on other chrysomelids (Ellers-Kirk et al., 2000; Trdan et al., 2008; Laznik et al., 2010) and other *Phytodecta* species (Tomalak, 2009).

Table 1. Mortality of *Phytodecta fornicata* caused by *Heterorhabditis bacteriophora*.

<table>
<thead>
<tr>
<th>Treatment (IJ beetle$^{-1}$)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td>Control</td>
<td>22.5a</td>
</tr>
<tr>
<td>1000</td>
<td>100b</td>
</tr>
<tr>
<td>2000</td>
<td>100b</td>
</tr>
</tbody>
</table>

Values in columns marked with different letter are statistically different (P < 0.05).

Counts of harvested IJs from White traps revealed the effect of temperature regime on emergence of nematodes from cadavers (Figure 1). Eight days post treatment maximum number of IJs (42 IJs in total) was obtained from the traps kept at 30 °C, while only 5 IJs were
recovered from traps kept at room temperature. Three days later, no IJs were recorded from traps in climate chamber while 50 IJs in total were recovered from cadavers kept at room temperature. The nematode behavioural ecology, changing conditions in insect cadaver and environment as well as food availability are factors that determine IJs development and emergence (Lacey & Georgis, 2012).

Figure 1. Harvested IJs from lucerne leaf beetle cadavers.

The development and emergence of IJs from cadavers were expected in higher numbers. Results indicate harvest of IJs should have been done after longer period of incubation of cadavers on White traps since the population of EPN in the insect cadaver increases with time, and/or in different environmental conditions. Emergence of IJs is usually expected within 8 days p. i. (O’Leary et al., 1998). These results may indicate that only a small portion of nematodes was infectious to penetrate and reproduce in cadavers, i.e. there might have been a higher percentage of non-infectious EPN in the preparation. This might be the reason why we collected low level of IJs from the cadavers.

For the first time, the experiment demonstrated that H. bacteriophora applied in rate of 1000 IJs per beetle is causing 100% mortality of P. fornicata and that IJs develop inside adult cadavers of P. fornicata. The effect of concentration of nematode preparation was of lesser importance, and future studies should involve lower concentration of nematode preparation. In order to develop cost effective and sustainable control measures, further mortality studies on individuals, with other nematode species and strains, and assays with more realistic environments are needed.
References


Laznik, Ž., Tóth, T., Lakatos, T., Vidrih M. & Trdan S. 2010: Oulema melanopus (L.) (Coleoptera: Chrysomelidae) adults are susceptible to entomopathogenic nematodes (Rhabditida) attack: results from a laboratory study. J. Plant Dis. Protect 117: 30-32.


Tomalak, M. 2009: Inoculative release of entomopathogenic nematodes for control of Phytodecta quinquepunctata (Coleoptera: Chrysomelidae) and other tree pests in urban green space. IOBC-WPRS Bull. 45: 391-394.


The susceptibility of mulberry moth to infection by entomopathogenic nematodes, Heterorhabditis bacteriophora and Steinernema carpocapsae

Nona Mikaia
Sokhumi State University, 9, Anna Politkovskaya Str., 0186 Tbilisi, Georgia

Abstract: The mulberry moth, Glyphodes pyloalis, is considered as an urban pest and therefore the application of environmentally safe means for mulberry trees protection is recommended. Among the entomopathogenic nematode (EPN) species Heterorhabditis bacteriophora and Steinernema carpocapsae are important as a biological control agents. The susceptibility of G. pyloalis to infection by H. bacteriophora and S. carpocapsae infective juveniles (IJ) was tested under laboratory conditions. Individuals of IV instar larvae were collected from mulberry trees in Georgia, Tbilisi (village Digomi). Nematode suspensions at a concentration of 1500 IJs/ml were used for treatment of mulberry leaves. After 72 h, the mortality of G. pyloalis caused by H. bacteriophora was 54%, whereas S. carpocapsae caused 76% mortality. The results suggest that nematode suspensions of H. bacteriophora and S. carpocapsae can be used to control G. pyloalis in urban plots.

Key words: Mulberry moth, Glyphodes pyloalis, Heterorhabditis bacteriophora, Steinernema carpocapsae

Introduction

The mulberry moth Glyphodes pyloalis (Walker) (Lepidoptera: Pyralidae) have been found on the leaves of mulberry trees in Kakheti (East Georgia) (Kanchaveli et al., 2009). The pest insect is distributed in USA (Florida, Mississippi, and Virginia States), Mexico, India, Japan, Iran, in republic of Central Asia and Azerbajian. It is specialized as a monoplane damaging the leaves of mulberry trees (Figure 1). The G. pyloalis is considered as an urban pest and therefore the application of environmentally safe means for mulberry trees protection is recommended. Among entomopathogenic nematodes (EPNs) the species Heterorhabditis bacteriophora and Steinernema carpocapsae are important biological control agents (Glazer et al., 2000). The strains of H. bacteriophora and S. carpocapsae were introduced in Georgia from Germany, e-nema company and then EPN has been mass produced successfully at Botany-Zoology laboratory in Tbilisi. Insect-parasitic nematodes of the families Heterorhabditidae and Steinernematidae have been known for decades as effective biological agents of insect pests. These nematodes can actively locate, infect and kill a wide range of insect species. Only the third stage juvenile (infective or dauer stage) can survive outside the insect host and move from one insect to another. Insect mortality, due to nematode infection, is caused by a symbiotic bacterium (Photorhabdus luminescens and Xenorhabdus nematophilus). The infective juveniles (IJ s) carry the symbiotic bacteria in their intestines and release them into the insect haemolymph. The bacteria cells proliferate and eventually kill the insect host (usually within 72 h) (Kaya et al., 1997).
Figure 1. G. pyloalis larvae on mulberry leaves.

**Material and methods**

100 individuals of IV instars larvae were collected from mulberry trees in Tbilisi (village Digomi) and transferred to the Botany and Zoology Laboratory of the Department of Natural Sciences and Health Care at conditions of 24-25 °C and 70-74% relative humidity. Nematodes suspensions of 1500 IJs ml⁻¹ was used for the treatment of mulberry leaves (Figures 2 & 3). The cultivation of the EPN H. bacteriophora and S. carpocapsae was performed under controlled condition at 25 °C and 74% relative humidity on caterpillars of last instar wax moth, Galleria mellonella, using standard techniques (Kaya et al., 1997). Insect mortality was determined after 48 and 72 h. The mortality of G. pyloalis was corrected for control mortality using the formula of Abbott (1925). Dead larvae of G. pyloalis were transferred from Petri dishes into the special traps, where the reproduction of H. bacteriophora and S. carpocapsae started (White, 1927). Controls were treated with sterile water. The preliminary experiments on the susceptibility of G. pyloalis to H. bacteriophora and S. carpocapsae have been carried according to Salame et al. (2010) (Figures 2-6).

**Results**

Data of EPNs H. bacteriophora and S. carpocapsae concerning mulberry pyralid – Glyphodes pyloalis are presented. The invasive larvae were detected after 48 h. Mortality of Glyphodes pyloalis larvae after 72 h was 54% when treated with H. bacteriophora and 76% when treated with S. carpocapsae (Figure 7).
Figure 2. *G. pyloalis* larvae infected with *H. bacteriophora*.

Figure 3. *H. bacteriophora* isolated from *G. pyloalis* larvae.

Figure 4. *H. bacteriophora* isolated from *G. pyloalis* larvae – detail.

Figure 5. *G. pyloalis* larvae infected with *S. carpocapsae*.

Figure 6. *S. carpocapsae* isolated from *G. pyloalis* larvae.

Figure 7. The mortality (%) of *G. pyloalis* larvae infected with *H. bacteriophora* and *S. carpocapsae* after 24, 48 and 72 h.
Conclusions

These preliminary investigations propose the possibility of invasion of G. pyloalis larvae by H. bacteriophora and S. carpocapsae. This gives the possibility to use the nematodes H. bacteriophora and S. carpocapsae to control G. pyloalis in urban plots in the future.

Acknowledgements

I would like to thank to all our staff of the Botany and Zoology Laboratory of the Faculty of Natural Sciences and Health Care of Sokhumi State University for technical assistance.

References

Attract and kill against western corn rootworm larvae with entomopathogenic nematodes

Mario Schumann¹, Felicitas Kaemena¹, Anant Patel², Stefan Vidal¹
¹Georg-August University, Department of Crop Sciences, Agricultural, Entomology, Grisebachstrasse 6, 37077 Göttingen, Germany; ²University of Applied Sciences, Department of Engineering and Mathematics, Engineering and Alternative Fuels, Wilhelm-Bertelsmann-Straße 10, 33602 Bielefeld, Germany

Abstract: The western corn rootworm (WCR) Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) is a serious maize pest in the US Corn Belt and in Eastern/Central Europe. Biological control with entomopathogenic nematodes (EPNs) against the soil dwelling larval stages has proven to be effective under field conditions. The implementation of this approach is, however, currently hampered by higher costs compared to chemical control options.

Attract and kill mechanisms may increase the chance for a contact between the target and a kill substance and have been shown to improve efficacy of the killing agents. This mechanism might also help in reducing nematode application densities, whilst maintaining high control levels, and would reduce costs for biological control of WCR larvae.

In this study we used a combination of CO₂, an attractant semiochemical known to be used by WCR larvae in host plant location, and the entomopathogenic nematode Heterorhabditis bacteriophora as the killing agent (provided by e-nema, Schwentinental). For the release of the attractant an artificial CO₂ source was encapsulated (CO₂ emitting capsules) to ensure a long and slow release of the semiochemical. The nematodes were manually mixed with the capsules.

A non-destructive observation device was used to examine the spatial infection pattern of 2nd instar WCR larvae by EPN and to assess the EPN infection rate over a period of 7 days. This device consists of a thin soil layer (45 cm x 30 cm x 6 mm) embedded between two glass sheets, which were divided into 60 grids with 10 vertical and 6 horizontal layers (each grid: 4.5 cm x 5 cm) to localize and quantify larval infections. A maize plant was grown for 4 weeks in the device and WCR larvae were then placed 7 cm deep into the soil 15 cm away from the maize stem. The attract and kill components (capsules and EPNs) were inserted 30 cm away from the maize stem. To assess the efficacy of this attract and kill approach, a conventional treatment was also set up with the current application scenario of EPNs by applying them directly at the maize stem.

The results showed that EPNs infect WCR larvae more than 5 cm from the application point of the EPNs in an attract and kill and a conventional treatment. This indicates that WCR larvae either avoid or emigrate out of EPN infected soil parts, thus recognizing EPNs either upon contact or through volatiles released by EPNs. In both treatments the first infection with EPNs was measured 2 days after release of WCR larvae in the device and was significantly higher after 7 days through a combination of EPN with the CO₂ capsules compared to a conventional application of EPNs. Consequently a combination of EPNs and semiochemicals used in host finding could help to reduce application rates and costs of biological control of WCR larvae with EPNs, making this strategy more competitive with regard to chemical control options.
Key words: Heterorhabditis bacteriophora, Diabrotica virgifera virgifera, encapsulation, application technique
Cloning strategy for recovering phage-displayed Cry1Aa13 mutants from phages with affinity towards proteins present in the gut of Ceratitis capitata

Tania Domínguez1, Juan F. Caña Roca1, Diana C. García1, Antonio Osuna1, Susana Vílchez1,2
1Biological Control of Pest and Vectors Research Line Institute of Biotechnology, Faculty of Sciences, University of Granada, Spain; 2Department of Biochemistry and Molecular Biology I, Faculty of Sciences, University of Granada, Spain

Abstract: Using the phage display technique, a pool of phages from a library of bacteriophages expressing Cry1Aa13 toxins with modified loops 2 at the domain II was selected that showed affinity toward proteins present in the guts of the Mediterranean fruit fly, Ceratitis capitata. The sequences of the hypervariable regions of the in vivo selected phages were analysed and an almost identical sequence was obtained in all of the selected phages. Those phages bearing toxins different from the wild type toxin at the loop 2 were selected in order to recover the Cry1Aa13 mutant toxins. Here we describe the cloning strategy designed and used to clone the toxins from the phage genome in order to be expressed.

Keywords: Ceratitis capitata, phage display, Cry toxins, in vitro evolution

Introduction

Cry toxins from Bacillus thuringiensis, have been widely studied and used for their ability to control pest and vector insects. Cry toxins are very specific to the target insect and they are a good alternative to chemical control of pests and vectors (Frederici, 2005). Although a high number of natural Cry toxins are reported (Crickmore, 2013), there is not an active toxin for all insects of interest. In addition, the resistance phenomenon has been observed in some insects toward Cry toxins (Bravo et al., 2012), requiring new toxins with novel specificities.

One of the most powerful techniques for in vitro evolution of proteins is the phage display of mutant libraries. In this system, the variants are expressed on the surface of a phage allowing them to interact with other proteins and be retained on immobilized target molecules. Those virus particles that do not bind are removed by washing, and those attached can be recovered and amplified in E. coli (Nelson, 2004).

Using this molecular tool, we selected a pool of phages from a library of mutant toxins (courtesy of Prof. D. J. Ellar, University of Cambridge), displaying on their surface variations in loop 2 of domain II of a Cry1Aa13 toxin (Pigott, 2006; Pigott et al., 2008) with affinity to proteins present in the gut of C. capitata adults (Domínguez et al., 2011). Here, we report the cloning strategy used to obtain the mutant toxins present in the selected phages, in order to express the novel toxins.
Material and methods

Obtaining DNA phages
Phages were amplified by propagation in liquid culture (Dominguez et al., 2011). This process was repeated until we obtained a phage suspension with $10^{10}-10^{11}$ plaque forming units (pfu). Bacteriophage DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen).

Cry gene PCR amplification
The gene coding for the complete Cry toxin was obtained by PCR using primers TD1 (5’-AATTAGATCTAGACGAAAGGGCATCGC-3’) and TD2 (5’-AATTCCGGGCTATTCTAAATCATATTC-3’) (Figure 2). The amplified fragment also contained the P_{lac} promoter (inducible by IPTG). Amplification conditions were: 10 min at 95 °C, 30 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Cloning and selection of positive clones
The TD1-TD2 fragments were introduced into the pGEM-T plasmid according to the manufacturer’s instructions. Transformants were plated on LB + 0.5 mM IPTG + 80 mg ml\(^{-1}\) Xgal + 100 mg ml\(^{-1}\) Ampicillin. White colonies were picked onto a fresh LB plate with X-gal, IPTG and Amp to double check that they were white. White colonies were screened by colony PCR using primers A2F (5’-CCCGTACTTGTCATTAAACTGG-3’) and A2R (5’-GGAGGCAAATTTGCGTAGG-3’) and using the following conditions: 10 min at 95 °C, 30 cycles 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, final extension at 72 °C for 10 min.

Figure 1. Hypervariable regions at loop 2 of the Cry1A a13 mutants from the in vivo selected phages with affinity to proteins present in the gut of C. capitata.
Conformative PCR
To confirm the presence of the complete toxin on the positive clones, plasmids were extracted using a Mini prep kit (Qiagen) and used as template in a PCR with TD1-A2R (10 min at 95 °C, 30 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min 30 s, and final extension at 72 °C for 10 min) and A2F-TD2 (10 min at 95 °C, 30 cycles of 95 °C for 50 s, 56 °C for 50 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min).

Results and discussion

After cloning and sequencing the hypervariable region of the selected phages from the CC2 library (Pigott, 2006), we observed that most of the toxins present in the selected phages showed the same amino acid sequence (Figure 1). Three phages were chosen as representatives of the Cry1A a13 mutant toxins selected: Fly 4, Fly 12 and Fly 31.

All the sequences obtained from the selected phages were completely different from the Cry1A a13 wild type. From the 29 sequences analysed, 5 showed a stop codon inside of the hypervariable region. 21 sequences showed the same hypervariable sequence (GARDGGPGPPLDC), with the exception of one (Fly 31). Seven out of the 20 (Fly 25, Fly 30, Fly 32, Fly 33, Fly 35, Fly 37, Fly 40) showed a mutation outside the hypervariable region. After the analysis, phage Fly 4 (GARDGGPGPPLDC), Fly 31 (GARDGGPGPPPDC) and Fly 12 were selected for cloning.

Figure 2. Cloning strategy of the mutant cry genes into pGEM-T from selected phages.
Obtaining mutant toxins from the selected phage

The cry mutant genes from Fly 4, Fly 12 and Fly 31 phages were obtained by PCR using TD1-TD2 primers and total phage DNA as a template following the strategy shown in Figure 2. The primers were designed to amplify the complete cry1Aa13 gene including the inducible P_{lac} promoter. The amplified fragments were analysed in a 1% agarose gel and in all cases showed the expected size of 2000 bp (Figure 3).

**Figure 3.** A agarose gel (1%) of the TD1-TD2 PCR fragments amplified from different phages: lanes 1, 7, 12: molecular weight marker (pb = base pairs), lanes 3 and 8: EMBL phage (negative control), lanes 4 and 9: CP2 phage (positive control) containing wild Cry1Aa13 toxin, lanes 5: Fly 12 phage, lane 11: phage Fly 4, lane 13: phage Fly 31.

Cloning and screening of the Cry1Aa13 mutant toxins

The amplified TD1-TD2 fragments were introduced into plasmid pGEM-T following the strategy detailed in Figure 2. White colonies were screened by colony PCR with primers A2F and A2R to confirm that they were positive (data not shown). The positive clones were selected for further analysis.

**Figure 4.** A agarose gel (1%) of the TD1-A2R (lanes 2-7) and A2F-TD2 (lanes 9-14) PCRs. Lanes 2 and 9: master mix; lanes 3 and 10: pGEM - T recircularced (negative control) lanes 4 and 11: CP2 phage (positive control); lanes 5 and 12: pFly 4 clone; lanes 6 and 13: pFly 12 clone; lanes 7 and 14: pFly31 clone; lanes 1 and 8: molecular weight markers (pb = base pairs)
Clone analysis
To confirm that the positive clones contained the complete cry gene, two PCRs were performed, one with the pair of primers TD1-A2R, that amplified a fragment of 1400 bp containing the P lac promoter, the N-terminal end of the mutant toxin and the loop 2 of the domain II of cry gene, and another one with the pair of primers A2F-TD2, that amplified a 800 bp fragment containing the beginning of the loop 2 until the C-terminal end of the toxin. Amplicons obtained from the clones showed the same size as in the positive control, indicating that the plasmids pFly 4, pFly 12 and pFly31 contained the complete cry gene (Figure 4).

Acknowledgements

We thank the Ministry of Education and Science (MEC) for funding this project (CGL2008-02011) and supporting J. F. Caña-Roca through the FPI Program. We thank the Consejería de Ciencia, Innovación y Empresa of Junta de Andalucía for supporting T. Domínguez through the project reference P10-AGR-6409 and SENACYT-Ecuador for supporting D. García.

References


Efficacy evaluation of different Bacillus thuringiensis sv kurstaki strain EG 2348 formulations against Malacosoma neustrium (Lepidoptera: Lasiocampidae)

Luca Ruiu¹, Achille Loi¹, Giovanni Falchi¹, Edith Ladurner², Andrea Braggio², Pietro Luciano¹
¹ Dipartimento di Agraria, University of Sassari, Via E. de Nicola, 07100 Sassari, Italy; ² CBC (Europe) S.r.l., BIOGARD Division, Via XXV Aprile, 44, 24050 Grassobbio (BG), Italy

Abstract: Cork oak forest protection and management require continuous monitoring of defoliator moth species. Among these, the European tent caterpillar, Malacosoma neustrium L., can cause widespread and extensive defoliation of host plants, and the implementation of an appropriate management program becomes necessary. Sustainable control methods may include the use of entomopathogenic microorganisms, such as Bacillus thuringiensis serovar kurstaki (Btk). However, the formulation of the microbial control agents can be a key factor for the success of application programs. The results of an efficacy trial with different formulations of Btk strain EG 2348 against larvae of M. neustrium conducted in a cork oak forest in Sardinia (Italy) are reported. In the trial, a commercial and an experimental formulation of Btk strain EG 2348 were tested (henceforth Rapax® and Rapax Experimental) in comparison to two Btk-based reference products (Foray 48B® and Delfin®). Both formulations of Btk strain EG 2348 proved to be effective in controlling the pest.

Key words: Malacosoma neustrium, Bacillus thuringiensis, microbial control, forest

Introduction

Malacosoma neustrium L. (Lepidoptera: Lasiocampidae) is a univoltine species overwintering in egg masses. Larvae hatch from eggs in spring. Early-instar larvae feed gregariously and gather on plant foliage to construct white webbings (tents) at major branch forks, while later-instar larvae are solitary and feed all over the crown (Verdinelli et al., 2004). During outbreaks, larvae can cause widespread and extensive defoliation of host plants, and the implementation of an appropriate management program becomes necessary (Luciano & Lentini, 2007).

Sustainable management strategies of this pest may include the use of entomopathogenic microorganisms, such as Bacillus thuringiensis serovar kurstaki (Btk)-based products (Martin & Bonneau, 2006). The active ingredient is represented by a mixture of bacterial spores and parasporal crystals containing insecticidal Cry toxins acting by ingestion and differing among strains for their potential against different target insects (Crickmore, 2006). In addition to strain characteristics, the formulation of the microbial control agent can be a key factor for the success of application programs, especially in forests because of the need of ensuring thorough coverage on big-sized trees and over wide areas (Satinder et al., 2006).

The results of an efficacy trial with two different formulations of Btk strain EG 2348 against larvae of M. neustrium, conducted in a cork oak forest in Sardinia, are reported. The trial was conducted in compliance with Good Experimental Practice (GEP) guidelines established by the European and Mediterranean Plant Protection Organization (EPPO PP 1/210(1), ref. Efficacy evaluation of insecticides – Defoliators of forest trees).
Material and methods

Tested treatments
In the trial, two formulations of Btk strain EG 2348 were tested: a commercially available Suspension Concentrate (Rapax®) and an experimental Aqueous Flowable formulation (Rapax Experimental), both from CBC (Europe) Srl, Italy. Both formulations were tested in comparison to two commercial Btk-based reference products, respectively Foray 48B® (Valent Bioscience Corporation) and Delfin® (Certis USA), and an untreated control. The two Btk strain EG 2348-based formulations were tested at two different application rates (respectively 1.0 and 1.5 l ha⁻¹), while the Btk-based reference products were applied at the recommended label rates (Table 1). For all tested products, a preliminary laboratory bioassay against early-instar M. neustrium larvae was conducted to verify their efficacy.

Table 1. Tested treatments and applied rates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient (strain)</th>
<th>Concentration¹ a. i. (%)</th>
<th>Formulation²</th>
<th>Applied rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rapax® Btk EG2348</td>
<td>7.5</td>
<td>SC</td>
<td>1.5 l/ha</td>
<td></td>
</tr>
<tr>
<td>Rapax® Btk EG2348</td>
<td>7.5</td>
<td>SC</td>
<td>1.0 l/ha</td>
<td></td>
</tr>
<tr>
<td>Rapax Exp. Btk EG2348</td>
<td>7.5</td>
<td>AF</td>
<td>1.5 l/ha</td>
<td></td>
</tr>
<tr>
<td>Rapax Exp. Btk EG2348</td>
<td>7.5</td>
<td>AF</td>
<td>1.0 l/ha</td>
<td></td>
</tr>
<tr>
<td>Delfin® Btk SA11</td>
<td>6.4</td>
<td>WG</td>
<td>750 g/ha</td>
<td></td>
</tr>
<tr>
<td>Foray 48B® Btk HD1</td>
<td>2.1</td>
<td>AF</td>
<td>3.0 l/ha</td>
<td></td>
</tr>
</tbody>
</table>

¹ a. i. = active ingredient, ² SC, Suspension Concentrate; AF, Aqueous Flowable Formulation; WG, Water Dispersible Granule

Experimental design and assessments
The trial was conducted in 2012 in a cork oak forest nearby Ploaghe-Chiaramonti (Northern Sardinia, Italy). The actual presence of M. neustrium in the study forest was verified the previous winter via monitoring and counts of egg masses.

The experimental design consisted in a completely randomized block design with 4 replicates per treatment (plot size: 1 tree). All cork oak trees used in the trial were uniform in size (approximately 5 m in height and with 7 m foliage projection diameter), and showed a comparable initial M. neustrium infestation level (mean number of M. neustrium tents/tree: 3).

All treatments were applied on 11 May, when the majority of larvae were in an early developmental stage (almost exclusively 2nd-3rd-instar larvae) using a motorized knapsack sprayer for experimental trials (M3 series, Cifarelli SpA, Italy).

To verify whether pest distribution was homogeneous among treatments just before treatment application, in each plot, the number of M. neustrium larvae present in the tents and in groups on branches was counted. After treatment application, instead, the larvae had
already switched to a solitary behaviour on foliage. Therefore, to estimate the efficacy of the
different treatments, the number of larvae present on eight 30 cm long outer branches,
randomly selected on each tree, was counted one and two weeks after treatment application.
Furthermore, two weeks after treatment application, defoliation caused by M. neustrium
larvae was estimated by assigning percent defoliation values according to the following scale:
2% with tree defoliation < 5%, 7.5% with defoliation ranging from 5-10%, 15.5% with
defoliation ranging from 11-20%, 25.5% with defoliation ranging from 21-30%, 38.0% with
defoliation ranging from 31-45%, 53% with defoliation ranging from 46-60%, 70.5% with
defoliation ranging from 61-80%, and 90.5% with defoliation > 80%.

The number of larvae/tree (preliminary assessment), the number of larvae/8 branches and
the percentage of defoliation were compared across treatments using 1-way ANOVAs
followed by Student-Newman-Kuels' test for post-hoc comparisons of means.

**Results and discussion**

All tested products proved to be effective in the laboratory against M. neustrium larvae (data
not reported). This efficacy was confirmed under open field conditions: all tested treatments
significantly reduced the number of M. neustrium larvae in comparison to the untreated
control (Table 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N. larvae/tree 11 May</th>
<th>N. larvae on 8 branches/tree 18 May</th>
<th>N. larvae on 8 branches/tree 25 May</th>
<th>Defoliation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>150.8 ± 19.6 a</td>
<td>17.8 ± 0.8 a</td>
<td>22.3 ± 1.4 a</td>
<td>71.8 ± 10.8 a</td>
</tr>
<tr>
<td>Rapax® (1.5 l/ha)</td>
<td>179.0 ± 36.0 a</td>
<td>2.0 ± 0.6 bc</td>
<td>2.0 ± 0.4 c</td>
<td>5.4 ± 3.4 b</td>
</tr>
<tr>
<td>Rapax® (1.0 l/ha)</td>
<td>169.5 ± 31.3 a</td>
<td>5.3 ± 1.3 b</td>
<td>2.5 ± 0.7 c</td>
<td>12.0 ± 4.5 b</td>
</tr>
<tr>
<td>Rapax Exp. (1.5 l/ha)</td>
<td>104.5 ± 24.3 a</td>
<td>1.3 ± 0.6 c</td>
<td>1.8 ± 0.5 c</td>
<td>2.0 ± 0.0 b</td>
</tr>
<tr>
<td>Rapax Exp. (1.0 l/ha)</td>
<td>247.5 ± 55.3 a</td>
<td>5.5 ± 1.7 b</td>
<td>5.3 ± 0.9 b</td>
<td>11.5 ± 2.3 b</td>
</tr>
<tr>
<td>Delfin®</td>
<td>181.3 ± 24.4 a</td>
<td>3.0 ± 0.4 bc</td>
<td>2.5 ± 0.3 c</td>
<td>4.8 ± 1.6 b</td>
</tr>
<tr>
<td>Foray 48B®</td>
<td>150.8 ± 20.2 a</td>
<td>2.0 ± 0.4 bc</td>
<td>1.8 ± 0.3 c</td>
<td>2.0 ± 0.0 b</td>
</tr>
</tbody>
</table>

Both one and two weeks after treatment application, the lower rate (1.0 l ha⁻¹) of Rapax
Experimental showed significantly higher infestation levels than the higher rate (1.5 l ha⁻¹),
while no significant dose-response effect emerged for Rapax. Except for Rapax Experimental
at the lower rate at the final assessment, the efficacy in reducing the number of M. neustrium
larvae of both formulations of Btk strain EG 2348 was always comparable to that of the Btk-
based reference products. However, this lower efficacy and the significant dose-response
effect observed for the aqueous flowable formulation should be considered with caution
because of the slight though not significantly higher mean initial infestation level on trees
treated with the lower rate of Rapax Experimental (Table 1).
Percent defoliation levels two weeks after treatment application were significantly higher in untreated control plots than in treated plots, with differences among treated plots not being significant (Table 2). Mean percent defoliation exceeded 70% in the untreated control, while it was equal to or below 5% for Rapax® and Rapax Experimental at 1.5 l ha⁻¹ and for Foray 48B® and Delfin®. Slightly though not significantly higher defoliation values were recorded for Rapax and Rapax Experimental at 1.0 l ha⁻¹ (mean = 12%).

Bt-based formulations have been successfully used to control tent caterpillars already in the past (Van der Laan & Wassink, 1962). Over time different formulations with improved efficacy have been developed by the industry (Lord, 2005). Ladurner et al. (2011) investigated the efficacy of different formulations of Btk strain EG 2348 against the tomato leaf miner, Tuta absoluta, on tomato, and in their studies the suspension concentrate proved to be more effective than the wettable powder. Under our trial conditions, the suspension concentrate and the aqueous flowable formulation, applied as a broadcast foliar spray from the ground, seemed to show comparable and high efficacy against M. neustrium. However, further research is needed to evaluate the same formulations also with aerial application equipment, most commonly used on large forests and where also other Lepidopteran defoliators can be found (Smitley & Davis, 1993; Luciano & Lentini, 2007).

Acknowledgements

This study was financially supported by Sardegna Ricerche (Project Bioinnovation: Incentivo Ricerca Polaris 2010).

References


Development of a new bio-insecticide for controlling lepidopteran pests

Kazım Sezen, Remziye Nalçacioglu, Ismail Demir, Hüseyin Tepe, Islam Yildiz, Zihni Demirbağ
Karadeniz Technical University, Faculty of Sciences, Department of Biology, Trabzon, Turkey
e-mail: sezen@ktu.edu.tr

Abstract: Turkey has been used to be a self-sufficient country in terms of agricultural products, but today it is importing agricultural products from many other countries. One of the most important reasons for this is to not be reliant on the effective control of pests of economically important plants. The insects belonging to the order Lepidoptera are one of the most harmful insect groups in our country. Members of this group cause serious damages in agricultural and forested areas as well as in warehouses. So far, efforts to control lepidopteran pests have mainly involved the use of chemical insecticides, particularly insect growth inhibitors. However, these agents can have undesirable side-effects on humans, plant and other animal species, particularly predators and parasitoids of lepidopteran pests. Therefore, it is necessary to find alternative and environmentally friendly control methods. In this study, we propose to develop a biological preparation (bio-insecticide) against lepidopteran pests using an insecticidal isolate of Bacillus thuringiensis subsp. kurstaki. Our results showed that the isolate has maximum growth at 30 °C, at pH 7 in Tryptic soy broth containing 1% NaCl. Its sporulation was supported in synthetic medium and the bacterial cell suspension was produced in pilot fermenter. A powder bio-pesticide was produced using this cell suspension and necessary formulation materials in the spray dryer. The physical and biological properties like wettability, suspensibility, particle size, moisture content, and viable spores of the formulated powder were determined and noted as 30 s, 80%, 25 µm, 8% and 3 × 10^{11} CFU g^{-1} dw, respectively. Insecticidal activity of the product against Thaumetopoea pityocampa, Plodia interpunctella and Lobesia botrana larvae in laboratory conditions were investigated. Mortality results were identified as 48% against T. pityocampa, 90% against L. botrana and 90% against P. interpunctella. Toxicity/pathogenicity assays of the dried powder on eukaryotic hosts were performed on rats. Subsequently, blood, feces and lung samples of rats were investigated for the presence of B. thuringiensis spores.

Key words: Bacillus thuringiensis, Lepidoptera, bio-pesticide
Bioluminescence determination of antibacterial activity of Bombyx mori and Galleria mellonella haemolymph

Libor Vojtek\(^1\), Pavel Dobes\(^1\), Ender Buyukguzel\(^2\), Pavel Hyrsl\(^1\)
\(^1\)Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic; \(^2\)Department of Biology, Faculty of Arts and Science, Bulent Ecevit University, 67100 Zonguldak, Turkey

Abstract: We describe an antibacterial assay based on bioluminescence of two Gram negative bacteria Photorhabdus luminescens and transformed Escherichia coli, which can be used for a real-time measurement of antibacterial activity. We observed a significant dose-dependent decrease of bioluminescence using both bacterial species during one hour after exposure to Bombyx mori or Galleria mellonella haemolymph. The humoral origin of the antibacterial activity observed in whole haemolymph was confirmed in haemolymph plasma without haemocytes. Antibacterial activity operating against Gram negative bacteria was measured in unaffected insect larvae as well as after septic injury; increased antibacterial activity in haemolymph was detected in the latter case which confirms inducibility of antimicrobial agents. This method can be widely used for determination of antibacterial activity in insects and supposedly in other invertebrates.

Key words: antibacterial activity, Galleria mellonella, Bombyx mori, bioluminescent bacteria

Introduction

Bioluminescence is the production and emission of light by living organisms; it is a naturally occurring form of chemoluminescence where energy is released by enzymatic (luciferase) reaction in the form of light (in bacteria maximum 490 nm).

The genus Photorhabdus includes terrestrial Gram negative bacteria, which are mainly found in association with entomopathogenic nematodes Heterorhabditis spp. Upon entering an insect host nematodes release bacterial cells from their intestinal tract which quickly establish a lethal septicaemia in the host (ffrench-Constant et al., 2003).

Similarly transformed Escherichia coli K12 are capable of light production. It contains plasmid with the complete luxABCDEamp operon originating from Photorhabdus leading to the expression of bacterial luciferase, which uses along-chain aldehyde as substrate in the generation of light (Atosuo et al., 2012).

Insect immunity involves both humoral and cellular aspects. Cellular activities in the insect rely on haemocytes which perform phagocytosis, encapsulation and nodulation. Humoral factors include especially highly potent antimicrobial peptides (AMPs) and the enzyme phenoloxidase. Several of the cellular reactions (clotting, nodule formation and encapsulation) activate phenoloxidase and therefore a visible melanisation.

The aim of this study was to analyse antibacterial activity of insect haemolymph using direct real-time measurement of changes in bioluminescence produced by P. luminescens or E. coli K12.
Material and methods

Bacterial suspensions
Photorhabdus luminescens, subsp. kaiyaii was inoculated in LB medium after isolation from fresh surface sterilized cadavers of Galleria mellonella infected by the entomopathogenic nematode Heterorhabditis bacteriophora. Transformed E. coli K12 resistant to ampicillin with luxABCDEamp genes was used. All E. coli media contained 100 µg ml⁻¹ ampicillin. Bacterial stocks were prepared after cultivation in liquid broth medium by adjusting to optical density 1.0 (at 400 nm) for P. luminescens and 0.25 (at 620 nm) for E. coli using Spekol 11 (Carl Zeiss).

Luminometry
Bioluminescence of bacterial suspensions after exposition to insect haemolymph was measured during one hour using luminometer LM01-T (Immunotech) at 25 °C (P. luminescens) or 37 °C (E. coli). The light emission during reaction is positively correlated with bacterial viability (Atosuo et al., 2012). Results are expressed in relative light units (RLU).

Bacterial viability measurements
Colony forming units (CFU) were counted in fresh E. coli suspension and then 30 min after addition of haemolymph. Both bacteria suspension and bacteria treated with haemolymph were diluted logarithmically and plated on dishes with nutrient agar. Number of colonies was determined after overnight incubation at 37 °C.

Haemolymph collection
Bombyx mori larvae (5th instar, 5 days old) were reared on mulberry leaves. G. mellonella larvae (7th instar, 3-4 days old) were obtained from laboratory cultures maintained on an artificial diet (Haydak, 1936) at 29 ± 1 °C in constant darkness. Haemolymph was collected by proleg amputation and pooling into cooled Eppendorf tube with several crystals of phenylthiourea (PTU) as anticoagulant.

Antimicrobial peptides induction
B. mori larvae were pricked laterally through the proleg with needle dipped in sterile insect saline or bacterial suspension (E. coli or P. luminescens). Untreated larvae were used as a control. After five hours of incubation haemolymph was collected for measurement of antibacterial activity as described above.

Results and discussion
Insect haemolymph shows high antibacterial activity. We observed a significant decrease of the bioluminescence signal in samples containing B. mori haemolymph. Concentrations of haemolymph ranging from 10% to 40% were tested against both E. coli (Figure 1a) and P. luminescens (Figure 1b). For subsequent experiments 20% was selected as an optimal dilution of haemolymph, it suppresses approximately 50% of bacteria in 30 min.

To verify that antibacterial activity is based on humoral factors, three different preparations of 20% haemolymph samples were tested. Freshly collected haemolymph was compared to haemocyte-free haemolymph and haemolymph stored at -20 °C for 30 days. All tested samples showed comparable antibacterial activity (bioluminescence signal differs within 3% in experiments using E. coli and 10% with P. luminescens in all three haemolymph
samples) both against E. coli (Figure 2a) and P. luminescens (Figure 2b) leading to the assumption that the measured antibacterial activity was caused by humoral factors acting against Gram negative bacteria.

Figure 1. Dependence of antibacterial activity against bioluminescent E. coli (a) or P. luminescens (b) on B. mori haemolymph concentration expressed as a decline of bioluminescence in relative light units (RLU).
Figure 2. Antibacterial activity against E. coli (a) or P. luminescens (b) in 20% fresh, centrifuged and frozen haemolymph of B. mori expressed as a decline of bioluminescence in relative light units (RLU).

Haemolymph of G. mellonella was also used. Similarly to B. mori, haemolymph from G. mellonella showed strong antibacterial activity against both E. coli and P. luminescens, approximately 50% decrease in bacterial bioluminescence signal in 30 min was observed.

To highlight the practical use of the assay, untreated larvae were compared to larvae pricked with a bacterial suspension to induce antibacterial activity. B. mori larvae pricked by E. coli or P. luminescens showed higher antibacterial activity five hours after pricking. The increase of antibacterial activity was reflected in decreased E. coli bioluminescence that was significantly different compared to untreated control or larvae pricked with insect saline (Figure 3; 45% bioluminescence decline in 30 min). Insects treated with E. coli had stronger
antibacterial response (76% bioluminescence decline in 30 min) than larvae treated by natural insect pathogen *P. luminescens* (63% bioluminescence decline in 30 min).

CFU counts showed approximately 30% decrease in viability of bacteria 30 min after treatment with haemolymph, whereas decrease in luminescence signal was about 50%, suggesting that antibacterial factors in haemolymph have partly only bacteriostatic effect.

![Figure 3. Influence of septic injury on antibacterial activity against *E. coli* of 20% *B. mori* haemolymph. *B. mori* larvae were pricked by sterile needle (saline), suspension of *E. coli* and *P. luminescens*. Decline of bioluminescence is expressed in relative light units (RLU) ± SD.](image)

Insects do not have a complement as vertebrates thus mostly AMPs are likely to be responsible for bactericidal effect. Most of the AMPs detectable in the haemolymph upon microbial infection are produced within a few hours by the fat body, haemocytes and other specific tissues (Lemaitre & Hoffmann, 2007). Apart from induced AMPs synthesis there is also constitutive level of AMPs present in haemolymph. Bioluminescence can be used as a new, fast and real-time method for assessment of haemolymph antibacterial activity.

**Acknowledgements**

Our research is supported by research grant from the Ministry of Agriculture of the Czech Republic NAZV – KUS (Q)1210047. We are grateful to Esa-Matti Lilius (University of Turku), Ulrich Theopold (Stockholm University) and Natraj Krishnan (Mississippi State University) for providing useful comments on draft of this article.

**References**

Insect pathogens and entomoparasitic nematodes
pp. 339-342

Intramolecular cleavage at the loop between $\alpha_3$-helix and $\alpha_4$-helix is critical for cytotoxic activity of Cry8Da

Takuya Yamaguchi, Hisanori Bando, Shin-ichiro Asano
Hokkaido University, Department of Applied Bioscience, Laboratory of Applied Molecular Entomology, N9W9 Kita-ku Sapporo, Hokkaido, Japan

Abstract: Cry8Da from Bacillus thuringiensis galleriae SDS-502 has the toxicity against both larvae and adult P. japonica. Cry8Da is processed into three fragments (64 kDa, 54 kDa and 8 kDa) by gut juice of P. japonica. Fragments of 54 kDa and 8 kDa are derived from the cleavage of 64 kDa fragment at the loop between $\alpha_3$-helix and $\alpha_4$-helix in Domain I. Binding assays showed that the 54 kDa fragment bound to both larvae and adult P. japonica brush-border membrane vesicles while the 64 kDa and 8 kDa fragments did not. We constructed a protease-resistant mutant, 8Da-R163A, in which R163 on the loop was changed to A163. To directly investigate whether intramolecular cleavage is critical for insecticidal activity of Cry8Da, we performed cytotoxic assays against midgut epithelial cells (MECs) prepared from adult P. japonica using purified uncleaved (64 kDa) and intramolecular cleaved (mixture of 54 kDa and 8 kDa) Cry8Da toxin. Cytotoxic assay showed MECs were destroyed by only intramolecular cleaved Cry8Da toxin. Intramolecular cleaved Cry8Da toxin also formed oligomeric structure after incubation with MGCs. These results strongly support our idea that the cleavage at the loop between $\alpha_3$-helix and $\alpha_4$-helix is critical for toxicity of Cry8Da.

Key words: Cry8Da, intramolecular cleavage, P. japonica, MECs, 54 kDa

Introduction

Bacillus thuringiensis (Bt) is a rod shaped, Gram-positive, spore forming bacterium. Bt produces parasporal crystal (Cry) proteins during sporulation. Since the crystal proteins often show insecticidal activity to specific species within the orders Lepidoptera, Diptera and Coleoptera, especially larvae of these insects, Bt is widely used in pest control agents. Control of Coleopteran pests such as larvae of family Scarabaeeidae, which damage the roots of turf grass and other horticultural and agricultural plants, is difficult, because they are living in soil where sprayable Bt formulation is hard to reach the target insects. Therefore, it is desired to find a Bt Cry protein that effectively controls both larvae and adults of target beetles. We reported that Cry8Da and Cry8Db have toxicity against not only larvae but also adults of Japanese beetle (Popillia japonica Newman).

Cry8Da is processed to 64 kDa, 54 kDa and 8 kDa fragments by adult P. japonica gut juice. The fragments of 54 kDa and 8 kDa are derived from intramolecular cleavage of the 64 kDa fragment at the loop between $\alpha_3$-helix and $\alpha_4$-helix of Domain I. Binding assays showed that the 54 kDa fragment bound to both larvae and adult P. japonica brush-border membrane vesicles (BBMV) while 64 kDa and 8 kDa fragments did not (Yamaguchi et al., 2010). This intramolecular cleavage induces specific binding of Cry3A toxin and BBMV of target insects (Walters et al., 2008). Thus, intramolecular cleavage at the loop is common feature among only beetle active Cry proteins and is seems to be important for showing toxicity.
In this paper, we constructed a protease-resistant mutant 8Da-R163A, which is altered from R$^{163}$ to A$^{163}$. We also performed cytotoxic assay against MECs from adult P. japonica using intramolecular cleaved or none intramolecular cleaved Cry8Da toxin.

**Material and methods**

**Preparation of midgut epithelial cell (MECs)**
MECs were prepared from dissected midguts of adult P. japonica. Midguts from excised to remove the peritrophic membrane and food contents were washed with 10 mM glucose in PBS, pH 7.4. Midguts were placed in a Petri dish containing 10 mM glucose in PBS, pH 7.4 with 1000 U ml$^{-1}$ collagenase (Wako Pure Chemical Industries). After gently shaking for 1 h at 25 °C, dissociated MECs were centrifuged for 3 min at 500 ×g and the resulting pellet was resuspended in PBS. The cells were washed by centrifugation several times until the supernatant was clear.

**Cytotoxic assay**
Cytotoxicity of intramolecular cleaved Cry8Da toxin (54+8 kDa) and uncleaved Cry8Da toxin (64 kDa) was assessed by measuring ATP amounts of live MECs. MECs and Cry8Da toxins were incubated in a well of 96 well-plate (Iwaki) for 120 min at 25 °C. After the incubation, ATP amount of live MECs were measured with CellTiter-Glo™ Luminescent Cell Viability Assay (Promega) and GloMax™ 96 Microplate Luminometer (Promega) according to manufacturer’s protocol. Also Cry8Da toxin treated MECs were observed under the microscopy.

**Detection of oligomer**
To confirm Cry8Da toxin form oligomer when toxin kills MECs, we tried to detect oligomer of Cry8Da toxin. MECs were incubated with intramolecular cleaved Cry8Da toxin for 60 min at 25 °C in PBS. After the incubation, MECs were harvested by centrifugation (20,000 xg, 5 min, 4 °C) and washed two times with PBS. MECs were suspended with 0.5% Triton X-100 in PBS and solubilized for 10 min at 25 °C. After the incubation, soluble and insoluble materials were fractionated by centrifugation (20,000 xg, 15 min, 4 °C). Insoluble fraction was suspended with 6 M urea, 2 M thiourea, 2% CHAPS in 40 mM Tris-HCl, pH 6.8. Soluble and insoluble fractions were subjected to 8% SDS-PAGE and proteins were transferred to PVDF membrane. Cry8Da proteins on membranes were detected by mouse polyclonal antibody of Cry8Da toxin and SuperSignal West Pico Chemiluminescent substrate (Thermo).

**Results and discussion**

**Cytotoxicity of Cry8Da toxins against MECs**
To directly investigate intramolecular cleavage at the loop between α3-helix and α4-helix is critical for insecticidal activity of Cry8Da, we performed cytotoxic assay against MECs prepared from adult P. japonica using purified uncleaved (64 kDa fragment) and intramolecular cleaved (54 kDa and 8 kDa fragments) Cry8Da toxin. Cry toxins cause death of midgut epithelial cells by making pores on plasma membrane of midgut epithelial cells of target insects, which lead to intoxication of target insects. Therefore cytotoxic assay against MECs can demonstrate intramolecular cleavage of Cry8Da toxin is critical for insecticidal activity of Cry8Da. Cell viability of MECs incubated with Intramolecular cleaved Cry8Da
toxin (54+8 kDa) reduced to 20% (Figure 1). Also microscopic observation showed cell blebs and cell burst, which is typical symptom of pore forming toxin attack, when MECs incubated with intramolecular cleaved Cry8Da toxin. Otherwise uncleaved Cry8Da toxin did not show significant reduction of cell viability of MECs and cytopathic effects such as bleb and burst. These results clearly showed intramolecular cleavage at the loop between \( \alpha_3 \)-helix and \( \alpha_4 \)-helix is critical for insecticidal activity of Cry8Da.

Figure 1. Purification of Cry8Da toxins and cytotoxic assay against MECs prepared from adult \( P. \) japonica. A) Gut juice treated Cry8Da were purified by anion exchange chromatography and gel filtration chromatography. Purified toxins were subjected to Tricine-SDS-PAGE. B) MECs prepared from adult \( P. \) japonica were treated with uncleaved (64 kDa fragment) or cleaved (mixture of 54 kDa and 8 kDa) Cry8Da toxin and cell viability were recorded. Black bars indicate standard deviation.

Detection of oligomer
Cry toxins have been characterized as pore forming toxins. Common mode of action of pore forming toxins is binding to the receptor molecule(s) on target cell, oligomerization, and insertion of part of the toxin into the plasma membrane. Cry1Ab toxin binds to its first receptor, cadherin like protein, and then \( \alpha_1 \)-helix of Cry1Ab toxin is removed by a membrane bound proteinase. Loss of \( \alpha_1 \)-helix induced oligomerization of Cry1Ab toxin. Oligomerized Cry1Ab toxin detach cadherin like protein and bind to a second receptor, GPI anchored APN or ALP followed by insertion to plasma membrane to form pore (Bravo et al., 2011). Oligomeric structure of Cry8Da toxin was detected from Triton X-100 insoluble fraction of MECs after incubation like in the case of Cry1Ab toxin (Figure 2). Previous studies showed that fragments of 54 kDa and 8 kDa still form a toxin complex after activation, however, only the 54 kDa fragment of Cry8Da toxin binds to \( P. \) japonica BBMV. The 8 kDa fragment responding to \( \alpha_1-\alpha_3 \)-helix of Domain I did not. This suggests that the removal of the 8 kDa fragment from the 54 kDa fragment is a trigger of oligomerization like Cry1Ab. \( P. \) japonica MECs starts blebbing after toxin exposure within 30 min. This means that the oligomer of Cry8Da toxin makes toxic pores in the plasma membrane of MECs. Thus, the mode of action
of Cry8Da has similarity with other Cry toxins, such as Cry1Ab. We showed a receptor difference between larvae and adult P. japonica (Yamaguchi et al., 2010). Cry1Ab toxin requires complicated receptor interaction as described above. Receptor identification will lead to understand overall mode of action of Cry8Da against P. japonica.

In this study we showed that the intramolecular cleavage at the loop between $\alpha_3$-helix and $\alpha_4$-helix of Domain I is critical for insecticidal activity of Cry8Da. Also, Cry8Da makes oligomers on MECs prepared from adult P. japonica.

![Figure 2. Detection of Cry8Da oligomeric structure. Cry8Da treated (+) or not treated (-) MGCs were collected and solubilised with Triton X-100. Soluble fractions (S) and insoluble fractions (P) were subjected to SDS-PAGE, Western blotting followed by detection of anti Cry8Da serum.](image)

**References**


Electron microscope and genetic analysis of an intracellular bacterium associated with the common rough woodlouse, *Porcellio scaber* (Isopoda, Porcellionidae)

*Regina G. Kleespies, Andreas Leclerque*

Federal Research Centre for Cultivated Plants, Julius Kühn Institute (JKI), Institute for Biological Control, Heinrichstraße 243, 64287 Darmstadt, Germany

Abstract: The common rough woodlouse, *Porcellio scaber*, is a common and widespread isopod species of Western and Northern Europe. A previously unknown intracellular bacterium has been identified in a diseased *Porcellio* larva. Microscopic studies revealed the subcellular structures characteristic of infection by *Rickettsiella*-like bacteria. Molecular phylogenetic analysis based on the 16S ribosomal RNA encoding *rrs* gene demonstrated that the woodlouse pathogen belongs to the taxonomic genus *Rickettsiella* (Gammaproteobacteria; Legionellales). Moreover, genetic analysis makes it likely that this new pathotype should be considered a member of the “*Rickettsiella armadillidii* complex”, i.e. a group of *Rickettsiella* bacteria found mainly in terrestrial isopods. *R. armadillidii* is currently placed in synonymy with the nomenclatural type species, *Rickettsiella popilliae*. The present study does not lend support to this synonymization.

Key words: *Porcellio scaber*, `*Rickettsiella armadillidii*`, *Rickettsiella popilliae*, intracellular pathogens, entomopathogenic bacteria, transmission electron microscopy (TEM), 16S ribosomal RNA

Introduction

The gamma-proteobacterial genus *Rickettsiella* (Philip) comprises intracellular pathogens of a wide range of arthropods that typically multiply in vacuolar structures within fat body cells and are frequently associated with protein crystals. Currently, four recognized species - i.e. the nomenclatural type species *Rickettsiella popilliae* (Dutky & Gooden) as well as *Rickettsiella grylli* (Vago & Martoja), *Rickettsiella stethorae* (Hall & Badgley), and *Rickettsiella chironomi* (Weiser) - and numerous pathotypes are distinguished within the genus (Fournier & Raoult, 2005).

*Rickettsiella*-like bacteria from numerous insects and arachnids have been described morpho- and histopathologically, and intracellular bacteria from, e.g., crickets, ticks, aphids, as well as coleopteran and dipteran insects have genetically been demonstrated to belong to the genus *Rickettsiella* (Fournier & Raoult, 2005 and references therein). Moreover, *Rickettsiella*-like bacteria have been reported to occur in crustaceans as, e.g., in woodlice (Vago et al., 1970; Cordaux et al., 2007) and freshwater amphipods (Federici et al., 1974; Larsson, 1982). Infection of hemocytes and midgut glands (hepatopancreas) appears to be the rule in crustaceans, as is the absence of well-defined protein crystals. 16S rRNA based molecular taxonomic analyses have motivated assignment of several isopod pathogens to the genus *Rickettsiella* (Gammaproteobacteria) (Cordaux et al., 2007), whereas *Rickettsiella*-like bacteria from further crustaceans, including woodlice, have genetically been assigned to the genus *Coxiella* (Cooper et al., 2007) and the orders Rickettsiales or Chlamydiales (Kostanjsek et al., 2004).
Porcellio scaber (Latreille, 1804) (Isopoda, Porcellionidae) is the most common species of woodlouse, known from Central and Western Europe, the United Kingdom, and has also colonized North America, South Africa and Australia. The common rough woodlouse occurs in a wide range of habitats and is chiefly found under stones, and on rotting wood. Generally, only little is known about natural diseases of woodlice. The present work describes an infection with an intracellular bacterium of the genus Rickettsiella in P. scaber.

Material and methods

Specimens of the common rough woodlouse, Porcellio scaber, were collected in March 2012 from a garden at Maintal, Frankfurt/Main region, Germany, where alive and dead specimens were found under stones and wooden boards distributed over an area of about 500 square meters. In a dead hypertrophied larva of this isopod species, infection with Rickettsiella-like bacteria was detected by light and electron microscopy. For measurements of this pathogen, negatively stained preparations, using 2.0% sodium phosphotungstate in aqua bidest were examined by electron microscopy. The average sizes of negatively stained Rickettsiella bacteria were determined using “ImageSP software” (Troendle, M oorenwies, Germany).

The almost complete 16S rRNA encoding gene was amplified from infected woodlouse tissue and sequenced. Alignment with orthologous sequences, pairwise p-distance matrix construction as well as phylogenetic reconstruction using Neighbor Joining (NJ) or Minimum Evolution (ME) algorithms were performed by means of the MEGA 4 software, whereas the corresponding Maximum Likelihood (ML) phylogeny was reconstructed using the PhyML program. Irrespective of the method of phylogenetic reconstruction employed, a gamma-distribution based model of rate heterogeneity allowing for eight rate categories was assumed and tree topology confidence limits were explored in non-parametric bootstrap analyses over 1,000 pseudo-replicates. A consensus tree was generated from the different phylogenies using the PhyLP 3.6 software tool.

Results and discussion

In squash preparations of infected tissues of P. scaber, tiny bacteria dancing in rapid Brownian movement were observed with phase contrast microscopy as is typical of Rickettsiella disease. Negatively stained bacteria in electron microscopy were rod-shaped with an average size of app. 590 nm in length and 270 nm in width (Figure 1), i.e. a cell dimension similar to that measured for Rickettsiella bacteria from insects. In contrast to other known Rickettsiella-infections, no well-defined associated crystals could be detected in any tissues of this isopod. The typical appearance of infections with Rickettsiella-like bacteria appears consistent with the main cytological features of the life cycle as described by Huger & Krieg (1967) for insect pathogenic Rickettsiella bacteria. The absence of structurally well-defined membrane-bounded crystals in P. scaber that are a characteristic feature of infection by insect Rickettsiella (Kleespies et al., 2011), have also been observed in previous studies of infections by Rickettsiella-like bacteria from isopods, e.g., the pill bug, Armadillidium vulgare (Latreille, 1804) (Isopoda, Armadillidiidae) (Vago et al., 1970; Federici et al., 1974).
Three independent amplification experiments from infected *P. scaber* generated the identical 16S rRNA gene sequence (GenBank accession number JX406180). When this consensus sequence was used as BlastN query, 16S rRNA gene sequences from a number of *Rickettsiella* bacteria were identified as best hits. Consistently, the Maximum Likelihood phylogeny (Figure 2A) reconstructed from the alignment of 16S rRNA genes from selected bacteria firmly places the new bacterium among the previously described gamma-proteobacterial *Rickettsiella* species and pathotypes. Importantly, the clade comprising all considered *Rickettsiella* bacteria receives maximal (100%) bootstrap support in the ML tree as well as in the corresponding ME and NJ phylogenies (data not shown). A comprehensive view is given by the extended majority rule consensus tree combining the results from these alternative approaches (Figure 2B). Thus, the new bacterium can consistently be assigned to the taxonomic genus *Rickettsiella*.

Concerning the relative taxonomic position of this new specimen within the genus *Rickettsiella*, it is obvious from the phylogenies presented in Figure 2 that the strain under study clusters tightly with several pathotypes from terrestrial isopods. This presumed representation of the “*R. armadillidii* complex” receives maximum bootstrap support and is located in a sister position with respect to the species *R. popilliae*. In order to explore the infrageneric taxonomic position of the *P. scaber* pathogen in more quantitative terms, pairwise rrs sequence identities were established by construction of a p-distance matrix. Pairs of sequences from within the “*R. armadillidii* complex” were found >99% identical, whereas the respective values with respect to further *Rickettsiella* bacteria, including the *R. popilliae* pathotypes, ranged between 97% and 98% (Figure 2). In terms of a sequence identity threshold of 98.5% as applied for species delineation within the order Chlamydiales these values would have to be translated into a co-speciation of the isopod pathogens.
In conclusion, the results of the present study firstly demonstrate that the bacterial pathogen associated with P. scaber belongs to the taxonomic genus Rickettsiella (Gammaproteobacteria, Legionellales) and appears both morphologically and genetically closely related to further woodlouse-associated bacteria forming the "R. armadillidii complex". In contrast, the currently accepted inclusion of this pathotype complex into the recognized species R. popilliae is not supported by our data. However, a respective taxonomic assignment should not be based on 16S rRNA gene data alone.

References


Miscellaneous

Poster
Impact of various oilseed rape productions on biological potential of endogaeic active ground beetles (Coleoptera: Carabidae)

Ivan Juran¹, Tanja Gotin Čuljak¹, Wolfgang Büchs², Dinka Grubišić¹, Ivan Sivčev³
¹University of Zagreb, Faculty of Agriculture, Department of Agricultural Zoology, Sveštimunska 25, 10000 Zagreb, Croatia; ²Federal Research Center for Cultivated Plants, Institute for Crop and Soil Science, Bundesallee 50, 38116 Braunschweig, Germany; ³Institute for Plant Protection and Environment, Department of Plant Pests, Banatska 33, 11080 Zemun, Serbia

Abstract: Ground beetles are one of the most important family of arthropod predators in arable crops. Compared to central European countries that have most frequently listed predator species in oilseed rape fields, in Croatia nothing is known about ground beetles and their activity. A trial was set up on three different oilseed rape productions: conventional (ploughing, full seed dressing, intensive application of pesticides and fertilizers), integrated (mulching, no seed dressing, reduced input of pesticides and fertilizers, 3 m width trap crop strip along each side of the field) and organic (ploughing, no seed dressing, peas as pre-crop, entire field rounded with 3 m width trap crop strip, no fertilizers and pesticides applied). The aim of this research was to investigate endogaeic activity and density of ground beetles and the impact of different oilseed rape production systems on their appearance. Endogaeic traps were used for monitoring endogaeic activity and sampling predatory arthropods. Eight traps were put on each production system and on integrated and organic trap crop strip were put four additional traps. During 2011, monitoring was conducted from February 10 till June 30. Samples were taken every two weeks. Results showed that the level of endogaeic activity of ground beetles was highest in organic production with 26.5 individuals per trap in the centre of the field and 27.25 individuals per trap in the trap crop strip. In the centre of the conventional field number of ground beetles individuals per trap was 21.63. In integrated system level of endogaeic activity was the lowest with 7.5 individuals per trap in the centre of the field and 17.25 individuals per trap in the trap crop strip. This presentation provides results about endogaeic active ground beetles in different managed oilseed rape fields in Croatia.

Key words: endogaeic traps, ground beetles, oilseed rape, Croatia

Acknowledgement: It is thankfully appreciated that the tests were supported by Trece Inc., Adair, Oklahoma, USA by providing us endogaeic traps.
Ground beetles (Coleoptera: Carabidae) in sugar beet fields as the base for conservation biological control

Tomislav Kos, Renata Bažok, Zrinka Drmić, Željka Graša
University of Zagreb Faculty of Agriculture, Department for Agricultural Zoology, Svetošimunska street 25, 10000 Zagreb, Croatia

Abstract: The fauna and abundance of ground beetles (Coleoptera, Carabidae) in arable crops can be an indicator of influence of different agricultural measures on biodiversity. The aim of our study was to determine ground beetle fauna abundance and frequency in two fields with different herbicide and insecticide application practice, and to determine differences in total number of species and individuals, collected with two capturing methods. The study was conducted in 2012 in the eastern part of Croatia (County of Vukovar-Srijem). Beetles were collected in a period of twenty-one weeks (April-September) by setting four modified pitfall traps aimed to collect above ground fauna and four probes (WB PROBE II® Trap, Trece inc.) aimed to collect endogeic fauna in each field. Nine different species and eight genera were identified in the study. Most abundant were *Pseudoophonus rufipes* (De Geer 1774) and *Bembidion* sp. (Latreille 1802). Both are classified as eudominant. The most frequent species was *P. rufipes* classified as constant (71.42%) and the most frequent genus was *Bembidion* sp. (38.04%) classified as accessory. There was no significant difference between fields among total number of established species and/or genus no matter if they were captured by pitfall trap or probe. Significantly more individuals were captured in pitfall traps on the field No. 1 (33.3) than on the field No. 2 (8.8), respectively. Opposite, significantly fewer individuals were captured with probe on the Field No. 1 (0.5) than on the field No. 2 (6.6), respectively.

Key words: Abundance, agricultural practice, Croatia, frequency, ground beetles, sugar beet

Introduction

Among many other arthropod species, ground beetles are usually considered as important indicative organisms for assessment ecological effects of different agricultural measures. They are known as important predatory organisms of the soil living pests (Lövei & Sunderland, 1996; Sunderland, 2002). Sugar beet is highly sensitive crop to weeds, pests and diseases. Comparing to other arable crops, the amount of pesticides used for sugar beet pest control is higher. Pest control practice and pesticide application practice depends on field condition and farmers experience. We hypothesized that the number of ground beetle species and individuals depend on the pesticide application practice on each particular field. The aim of our study was 1) to determine and to analyze ground beetles abundance and frequency in two fields with different herbicide and insecticide application practice, and 2) to determine differences in total number of species and individuals, collected with two capturing methods.
Material and methods

The study was conducted during the vegetation season 2012 on two sugar beet fields (Table 1) located in the eastern part of Croatia, village of Tovarnik (County of Vukovar-Srijem).

Table 1. Basic information on experimental fields.

<table>
<thead>
<tr>
<th>Field No</th>
<th>Field size</th>
<th>Precrop</th>
<th>Hericides</th>
<th>Insecticides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Active ingredient</td>
<td>Dose g/ha or ml/ha</td>
</tr>
<tr>
<td>1</td>
<td>130</td>
<td>wheat</td>
<td>desmedifam fenmedifam klopiralid triflusulfuron</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>184</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>135</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>wheat</td>
<td>desmedifam fenmedifam etofumesat klopiralid</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

Above ground arthropods were sampled by using pitfall traps. Each trap consisted of a plastic cup 11 cm in diameter and 9 cm deep. A clay ring was placed around each trap to facilitate soil dwelling entomofauna into traps. Water solution (5%) of sodium benzoate was casted to the pitfall trap for conservation of captures and to prevent escape or cannibalism. The endogeic fauna was collected with perforated probe (Ø = 35 mm, h = 440 mm, size of perforations: 4 mm x 2 mm) (WB PROBE II® Trap, Trece inc.). Four pitfall traps and four probes per plot were placed diagonally across each field, starting at least 20 m from the field boundary to minimize edge effect. The distance between pitfall trap and endogeic trap was 2 m and the distances among pitfall traps were at least 30 m. Samples were taken every week between April 19 and September 11. All ground beetles were identified to the genus level when possible to the species level, using the following identification keys: Auber (1965); Bechyne (1974); Harde & Severa (1984); Casale & Kryzhanovskij (2003). Based on the total number of collected individuals and the individual number of each particular species the dominance was calculated. The results (eudominant, dominant, subdominant, recedent, subrecedent) were classified according to Tischler & Heydeman (cit. Balarin, 1974). The frequency was calculated with the Balogh’s formula (cit. Balarin, 1974). Total number of species and total number of individuals captured in pitfall traps and probes were subjected to ANOVA analysis (one-way, P = 0.05) to determine differences between fields. For mean separation Duncan’s multiple range test was used.

Results and discussion

Seventeen taxa were identified throughout the study, with nine identified to species level and eight to genus level (Table 2). Pseudophonus rufipes was eudominant and the most abundant species in the study. Genus Bembidion sp. was also eudominant and the second most abundant
species. Frequency of P. rufipes was considered as constant, while Bembidion sp. was classified as accessory. All other taxa were classified as accidental.

Established species of ground beetles correspond with those identified in previous studies in Croatia (Balarin, 1974; Sekulić et al., 1973; Durbešić, 1987; Durbešić et al., 2006; Bažok et al., 2007; Kos et al., 2010).

Table 2. Total number of captures, abundance and frequency of ground beetle in sugar beet fields, Tovarnik, 2012.

<table>
<thead>
<tr>
<th>No</th>
<th>Name of species/ genus</th>
<th>C*</th>
<th>A**</th>
<th>F***</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nebria sp. (Latreille, 1802)</td>
<td>3</td>
<td>2.27 c</td>
<td>9.52 d</td>
</tr>
<tr>
<td>2.</td>
<td>Acupalpus (Acupalpus) parvulus (Sturm, 1825)</td>
<td>3</td>
<td>2.27 c</td>
<td>4.76 d</td>
</tr>
<tr>
<td>3.</td>
<td>Agonum (Agonum) muelleri (Herbst, 1784)</td>
<td>6</td>
<td>4.55 c</td>
<td>9.52 d</td>
</tr>
<tr>
<td>4.</td>
<td>Agonum (Europhilus) fuliginosum (Panzer, 1809)</td>
<td>7</td>
<td>5.30 b</td>
<td>23.80 d</td>
</tr>
<tr>
<td>5.</td>
<td>Amara sp. (Bonelli, 1810)</td>
<td>10</td>
<td>7.58 b</td>
<td>14.28 d</td>
</tr>
<tr>
<td>6.</td>
<td>Bembidion sp. (Latreille, 1802)</td>
<td>26</td>
<td>19.70 a</td>
<td>38.09 c</td>
</tr>
<tr>
<td>7.</td>
<td>Calosoma (Campalita) auropunctatum auropunctatum (Herbst, 1784)</td>
<td>3</td>
<td>2.27 c</td>
<td>9.52 d</td>
</tr>
<tr>
<td>8.</td>
<td>Carabus (Oreocarabus) glabratras glabratras (Paykull, 1790)</td>
<td>3</td>
<td>2.27 c</td>
<td>14.28 d</td>
</tr>
<tr>
<td>9.</td>
<td>Chlaenius sp. (Bonelli, 1810)</td>
<td>6</td>
<td>4.55 c</td>
<td>19.04 d</td>
</tr>
<tr>
<td>10.</td>
<td>Cylindera (Cylindera) germanica (Linne, 1758)</td>
<td>2</td>
<td>1.52 c</td>
<td>9.52 d</td>
</tr>
<tr>
<td>11.</td>
<td>Dyschirius sp. (Bonelli, 1810)</td>
<td>4</td>
<td>3.03 c</td>
<td>4.76 d</td>
</tr>
<tr>
<td>12.</td>
<td>Platynus sp. (Bonelli, 1810)</td>
<td>1</td>
<td>0.76 d</td>
<td>4.76 d</td>
</tr>
<tr>
<td>13.</td>
<td>Poecilus (Poecilus) cupreus (Linne, 1758)</td>
<td>6</td>
<td>4.55 c</td>
<td>1904 d</td>
</tr>
<tr>
<td>14.</td>
<td>Pseudoophonus (Pseudoophonus) rufipes (De Geer, 1774)</td>
<td>40</td>
<td>30.30 a</td>
<td>71.42 b</td>
</tr>
<tr>
<td>15.</td>
<td>Pterostichus (Morphnosoma) melanarius (Illiger, 1798)</td>
<td>4</td>
<td>3.03 c</td>
<td>19.04 d</td>
</tr>
<tr>
<td>16.</td>
<td>Tachyta sp. (Kirby, 1837)</td>
<td>7</td>
<td>5.30 b</td>
<td>19.04 d</td>
</tr>
<tr>
<td>17.</td>
<td>Tachys sp. (Dejean, 1821)</td>
<td>1</td>
<td>0.76 d</td>
<td>4.76 d</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>132</td>
<td>100.00</td>
<td>-</td>
</tr>
</tbody>
</table>

C* (Total capture on both fields); A** (Abundance (Percent and rank: a-eudominant (10% < ); b-dominant (5%-10%); c-subdominant (1%-4.99%); d-recedent (0.5%-0.99%); e-subrecedent (0.01%-0.49%)); F*** (Frequency (Percent and rank (a-euconstant (75%-100%); b-constant (50%-75%); c-accessory (25%-50%); d-accidental (0.1%-25%).

Investigation did not provide significant differences between fields in species richness (Table 3). A verage number of individuals captured in pitfall trap was significantly higher in the field No. 1 than in the field No. 2. Although seed treatment and insecticide treatments against sugar beet weevil were similar on both fields, in the field No. 2 insecticides were applied twice on the whole surface while in the field No. 1, first application of insecticides was conducted only on the edges. The more intensive tillage was carried out in the field No. 1 and herbicides were applied in three applications vs. two applications in the field No. 2. Soil
tillage and weed control were discussed by Thiele (1977) as the main factor of the numerousness and richness of the beneficial fauna.

Table 3. Results of ANOVA for number of species and individuals per trap for pitfall trap and probe, Tovarnik, 2012.

<table>
<thead>
<tr>
<th></th>
<th>Number of taxa per trap</th>
<th>Number of individuals per trap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pitfall trap</td>
<td>Probe</td>
</tr>
<tr>
<td>Field No. 1</td>
<td>5.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Field No. 2</td>
<td>5.0</td>
<td>1.5</td>
</tr>
<tr>
<td>LSD</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pitfall trap</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field No. 1</td>
<td>33.3 a*</td>
<td>0.5 b</td>
</tr>
<tr>
<td>Field No. 2</td>
<td>8.8 b</td>
<td>6.8 a</td>
</tr>
<tr>
<td>LSD</td>
<td>10.97</td>
<td>5.73</td>
</tr>
</tbody>
</table>

* Means followed by the same letter are not significantly different according to Duncan Multiple Range test (P = 0.05).

Acknowledgements

The study was conducted within the frame of the project “Technology transfer in sugar beet production: improvements in pest control following the principles of integrated pest management (IPM)” founded by Croatian Science Foundation.

References


Impact of Entomophaga maimaiga on gypsy moth populations in Bulgaria

Plamen Mirchev\(^1\), Andreas Linde\(^2\), Daniela Pilarska\(^3\), Plamen Pilarski\(^4\), Margarita Georgieva\(^1\), Georgi Georgiev\(^1\)

\(^{1}\)Forest Research Institute, Bulgarian Academy of Sciences, Sofia, 132 St. Kliment Ohridski Blvd., Sofia 1756, Bulgaria; \(^{2}\)University of Applied Sciences Eberswalde, Alfred-Moeller-Str. 1, 16225 Eberswalde, Germany; \(^{3}\)Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1, Tsar Osvoboditel, 1000 Sofia, Bulgaria; \(^{4}\)Institute of Plant Physiology and Genetics, Acad. G. Bonchev Str., Bldg, 21, 1113 Sofia, Bulgaria; \(^{5}\)Czech University of Life Science, Prague, Czech Republic

Abstract: The entomopathogenic fungus Entomophaga maimaiga Humber, Shimazu and Soper (Entomophthorales) (Entomophthoraceae) was introduced into three populations of gypsy moth (Lymantria dispar L., Lepidoptera: Erebidae) in Bulgaria in 1999. After the first strong epizootics in 2005, the species was introduced in six outbreak populations of gypsy moth in different regions of the country from 2008 to 2011. Due to the resulting fungal epizootics, the calamities of the pest in Bulgaria were totally suppressed. The pathogen increased its impact by a natural range extension and it is now present in nearly all regions of the country in which L. dispar occurs.

Key words: gypsy moth, Entomophaga maimaiga, Bulgaria, biological control

Introduction

The gypsy moth (Lymantria dispar L., Lepidoptera: Erebidae) periodically causes severe damage in deciduous forests in several Central and Eastern European countries, as well as in the USA where it was introduced in the end of the 19\(^{th}\) century. In Bulgaria, oak stands of different age were infested over long periods of time (Georgiev et al., 2007). Repeated defoliations and decrease growth cause a physiological weakening of the host plants, thereby increasing their susceptibility to infestations of wood borers and plant pathogenic fungi. To reduce the pest density and control gypsy moth populations, broad spectrum chemical insecticides and the bacterial pathogen Bacillus thuringiensis var. kurstaki (Btk) were used. Due to a lack of host specificity, these methods affect aquatic organisms and many other species within the order Lepidoptera, and thus reduce biodiversity in forest ecosystems (Miller, 1990).

The entomopathogenic fungus Entomophaga maimaiga Humber, Shimazu & Soper (Entomophthorales: Entomophthoraceae) was described as a host specific pathogen of L. dispar from Japan (Soper et al., 1988). It was introduced into the USA in the beginning of the 20\(^{th}\) century. Since then, it successfully reduced gypsy moth density in several states. In 1999, E. maimaiga was successfully introduced into Bulgaria from the USA (Pilarska et al., 2000). Thereafter it caused epizootics and mortality in four outbreak populations of gypsy moth, located 30-70 km from the introduction sites (Pilarska et al., 2006).

In this paper we present results of recent introductions of E. maimaiga in gypsy moth populations in Bulgaria and on the impact of the fungus on the pest.
**Material and methods**

From 2008 to 2011, six introductions of *E. maimaiga* were performed in outbreak populations of *L. dispar* in oak forests in different parts of the country (Table 1). Two of the introductions were conducted during the spring, four in the fall. Before use, the inoculum was stored in the soil for not less than 9 months under natural conditions.

Table 1: Main characteristics of studied areas, *L. dispar* density and origin of *E. maimaiga*

<table>
<thead>
<tr>
<th>Locality</th>
<th>State Forest (Hunting) Enterprise</th>
<th>Geographical coordinates</th>
<th>Altitude, m a.s.l.</th>
<th>Tree species</th>
<th>Density of <em>L. dispar</em></th>
<th>Date of introduction</th>
<th>Origin of <em>E. maimaiga</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sadievo</td>
<td>Nova Zagora</td>
<td>42°31.783’N; 026°08.901’E</td>
<td>151</td>
<td>Q.r.</td>
<td>83</td>
<td>28.03.2008</td>
<td>Bulgaria</td>
</tr>
<tr>
<td>Assenovo</td>
<td>G. Oryahovitsa</td>
<td>43°17.695’N; 026°04.051’E</td>
<td>401</td>
<td>Q.c.</td>
<td>78</td>
<td>18.11.2009</td>
<td>USA</td>
</tr>
<tr>
<td>Slavyanovo</td>
<td>Popovo</td>
<td>43°17.090’N; 026°08.834’E</td>
<td>345</td>
<td>Q.c.</td>
<td>89</td>
<td>18.11.2009</td>
<td>USA</td>
</tr>
<tr>
<td>Ruets</td>
<td>Targovishte</td>
<td>43°12.119’N; 026°37.950’E</td>
<td>312</td>
<td>Q.c.; C.b.</td>
<td>76</td>
<td>18.11.2010</td>
<td>Bulgaria</td>
</tr>
<tr>
<td>Dalgach</td>
<td>Targovishte</td>
<td>43°12.966’N; 026°42.478’E</td>
<td>193</td>
<td>Q.ru.; T.p.</td>
<td>86</td>
<td>18.11.2010</td>
<td>Bulgaria</td>
</tr>
<tr>
<td>Solnik</td>
<td>S. Oryahovo</td>
<td>42°54.268’N; 027°44.296’E</td>
<td>202</td>
<td>Q.f.; Q.c.</td>
<td>183</td>
<td>05.04.2011</td>
<td>USA</td>
</tr>
</tbody>
</table>

\(a\) - C.b. – *Carpinus betulus* L.; Q.c. – *Quercus cerris* L.; Q.f. – *Quercus frainetto* Ten.; Q.r. – *Quercus robur* L.; Q.ru. – *Quercus rubra* L.; T.p. – *Tilia platyphyllos* Scop.  
\(b\) - egg masses per 100 trees

For the release of inoculum, within each experimental plot of 100 x 100 m, five circular sites were established - one central and four circles 50 m from the center to the magnetic north, south, east and west. Each circle contained at least five trees. The fungal introductions were conducted by mixing crushed infected larvae containing resting spores of *E. maimaiga* with soil, and dispersing the mixture around the base of 5 to 10 trees. The base of the tree was watered with 4-5 liters of water to achieve adequate humidity.

To monitor larval density, in each study site burlap bands were placed on 25 oak trees (including the treated trees) at a height of 1.3 m from the ground. Larvae of *L. dispar* were collected from the burlap bands 2-3 times per month from early May to late July and transported to the laboratory, where they were reared on fresh oak foliage in plastic boxes. The foliage was changed daily, dead gypsy moth larvae were placed in Petri dishes with moisturized filter paper at 20 °C for 5-7 days and then refrigerated at 5 °C until microscopic evaluation. Each cadaver was dissected individually and observed under light microscope at 125x magnification for the presence of conidia or azygospores of *E. maimaiga*.

To estimate the influence of the fungal infection on the density of the gypsy moth population, the number of egg masses on 100 trees in each study site was counted in the fall.
Results and discussion

Impact of *E. maimaiga* on *L. dispar* in Bulgaria

The introduction in Sadievo locality was conducted with dead gypsy moth larvae collected during the epizootic in 2005 in the village of Kremen. After the introduction, in the same year, *E. maimaiga* infected and killed 87.5% of the fifth and sixth instar *L. dispar* larvae. The reduction of egg masses was 96.4% and no egg masses were recorded two years later.

Introductions of *E. maimaiga* in Assenovo and Slavyanovo area were conducted with an inoculum from the USA. As a result of the introduction epizootics occurred in both experimental sites in 2010. Mortality of young larvae reached 44-55%, whereas mortality of the late instar larvae was 95-98%; no defoliations were observed in the stands. The reduction of the egg masses was 55.1-81.8%, and 100% a year later. Interestingly, in 2010, gypsy moth epizootics caused by *E. maimaiga* were recorded not only in Assenovo and Slavyanovo, but also in many other areas in the adjacent forests of SFE Gorna Oryahovitsa and State Hunting Enterprise (SHE) Popovo.

Introductions of *E. maimaiga* in Ruets and Dalgach in the region of SFE Targovishte were conducted with a mixed inoculum from the epizootic near Slavyanovo (collected in the summer 2010) and Sofia (collected in 2005 near Kremen, stored in soil substrate for about 5 years). In May and June 2011 frequent and heavy rainfall occurred in the region of Targovishte. We suppose that this favoured the establishment of *E. maimaiga* and resulted in an epizootic that killed almost all middle and late instar larvae of the pest. Defoliation in the experimental sites was not observed and no egg masses at all of *L. dispar* were recorded. Furthermore, conidial infections were registered in larvae in many areas near Targovishte. We believe that this caused the rapid suppression of the outbreak in the oak forests of Northeastern Bulgaria, where the strongest gypsy moth infestations in Bulgaria had been reported in the past.

For the introduction in Solnik, inoculum from the USA was released in April 2011. In the late spring of 2011, 80.4% mortality of the late instar gypsy moth larvae was registered in the release site. The reduction of gypsy moth egg masses was 77.6% in 2011 and 86.3% in 2012.

In 2011, on the Black Sea coast in the region of SHE Nessebar (30-50 km from Solnik) strong defoliation of oak forests by *L. dispar* was registered. In 2012, however, gypsy moth in this region as well as all over central Black Sea coast was suppressed by the pathogen. As seen in other areas, adjacent to release plots (e. g. Popovo, see above), we believe that this demonstrates the self-disseminating capacity of the fungus. It is well known that *E. maimaiga* can spread more than 100 km in one season (Elkinton et al., 1991).

Infestations of Bulgarian forests by *L. dispar*

Figure 1 presents data of the forest area infested by different gradations of *L. dispar* in a period of 60 years. Before the first introduction of *E. maimaiga* in Bulgaria in 1999, 492 to 1,028 thousand ha of forests were affected by the pest each decade, and annual defoliation reached from 150,000 to 370,000 ha. After the introduction of *E. maimaiga*, no large-scale pest calamities were observed and the gypsy moth’s annual infestation area did not exceed 25,000 ha, only 2-5% of the infestation levels observed before the introduction.
From 2010 to 2013, gypsy moth is undergoing another outbreak, but only 71,000 ha of forest were severely affected. By extrapolation we estimate that by the end of the current gradation (expected for 2017/18), the total forest area affected by gypsy moth will not exceed 150,000 ha, corresponding to only 15-30% of the infestation levels observed during gradations before the establishment of *E. maimaiga*.

The decrease of *L. dispar* damages to the forest after a one-time introduction of *E. maimaiga* shows that the pathogen effectively reduces and regulates the pest density. After the introduction of *E. maimaiga* in Bulgaria, chemical control of gypsy moth was used in very small areas only, dismissing the previous practice of large-scale use of microbial and chemical insecticides. *E. maimaiga* is expanding its range in the Balkan countries (Georgiev et al., 2012) and in the near future it is expected to spread into other areas of Southeast Europe. The high virulence and species specificity of *E. maimaiga* and its ability to reduce *L. dispar* density characterize the fungus as an effective, economical and environmentally safe biological control option for *L. dispar*.

**Acknowledgements**

The study was supported by National Science Fund of Bulgaria, Project DO-02/282/2008.

**References**


