8th EUROPEAN MEETING

“ENTOMOPATHOGENS AND INSECT PARASITIC NEMATODES: CURRENT RESEARCH AND PERSPECTIVES IN PEST BIOCONTROL”

at / à

Athens (Greece)
29 May – 2 June 2001

editor:
Bernard Papierok
Preface

This bulletin contains the proceedings of the 8th European meeting of the IOBC/WPRS Working Group “Insect Pathogens and Insect Parasitic Nematodes”. Entitled “Entomopathogens and Insect Parasitic Nematodes: Current Research and Perspectives in Pest Biocontrol”, this meeting was held in Athens, Greece, 29 May-2 June 2001, together with the meeting of the COST Action 842 “Biological Control of Pest Insects and Mites, with Special Reference to Entomophthorales”. On behalf of the Working Group and all participants, I take great pleasure in expressing my gratitude towards the Local Organizing Committee and especially its chairwoman, Dr Marie Anagnou-Veroniki, from the Benaki Phytopathological Institute, for the excellent organization of the meeting, which was scientifically and socially very fruitful.

More than 100 people from European countries but also from Canada, P.R. of China, Egypt, India, Israel, The Palestinian Authority, and Turkey, attended the meeting, which included oral presentations and poster sessions. Most of the contributions were devoted to entomopathogenic fungi and entomopathogenic nematodes, but there were contributions dedicated to bacteria, viruses or microsporidia also. Special events were organized: a full day workshop on the sampling of Entomophthorales, a professional meeting dealing with technical development, production, market development of nematodes, and an information session dedicated to the COST Action 850, which succeeded the previous COST Action devoted to entomopathogenic nematodes (COST Action 819), which ended on 31 May 2001.

The meeting in Athens was the first I was acting as the convener of the Working Group. Before ending this preface, I would like to thank the previous convener, Peter H. Smits, for the excellent job he did for 8 years for the cause of our Working Group and more generally for the cause of the enemies of pest insect and mites we are working with.

Bernard Papierok
Convener of the Working Group
Insect Pathogens and Insect Parasitic Nematodes
iv
Contents

Preface ...................................................................................................................................... iii
Contents ..................................................................................................................................... v
List of participants................................................................................................................... ix

Lecture and plenary presentations

Microbial control in Greece – Forty years history
  Christias Yamvrias .................................................................................................................. 1
Future needs in risk assessment of fungal Biological Control Agents
  Hermann Strasser .................................................................................................................. 5
New challenges for fungal bioinsecticides
  Jacques Fargues .................................................................................................................... 9

Entomopathogenic fungi

Fungus infection of the chicken mite *Dermanyssus gallinae*
  Tove Steenberg, Ole Kilpinen ............................................................................................... 23
Entomopathogenic fungi – Mycoinsecticides useful against lepidopteran pests in pulses
  Mukund Deshpande, Ajit Chandele, Pallavi Nahar, Ashok Hadapad, Ganesh Patil,
  Vandana Ghormade, Siegfried Keller, Urs Tuor .................................................................. 27
Impact of *Beauveria brongniartii* and *Metarhizium anisopliae* (Hyphomycetes) on
  *Lumbricus terrestris* (Oligochaeta, Lumbricidae)
  A. Hozzank, S. Keller, O. Daniel, Ch. Schweizer ................................................................. 31
Growth interactions between nematophagous and entomopathogenic fungi
  M.L. López-Serna, L.V. López-Llorea, J. Salinas .................................................................. 35
Persistent appearance of *Cordyceps gracilis* on Selatosomus-larvae near Siedlce
  (Poland)
  Ryszard Mietkiewski, Stanislaw Balazy .............................................................................. 39
Brassicaceous plants and insect pathogenic fungi
  Ingeborg Klingen, Ann Hajek1, Richard Meadow, J. Alan A. Renwick .............................. 43
A molecular method to differentiate between resting spores from different members of
  the *Entomophthora muscae*-complex
  Lene Thomsen, Annette Bruun Jensen ............................................................................ 45
Genetic variation among Latvian isolates of *Beauveria bassiana*
  Eriks Jankevics, Mara Kropa, Leide Grantina, Liga Jankevica ............................................ 51
Biological control of weevils (*Strophosoma spp.*) in Danish greenery plantations
  Jørgen Eilenberg, Charlotte Nielsen, Susanne Vestergaard, Susanne Harding,
  Amy Frølander, Anna Augustyniuk .................................................................................... 55
Occurrence of entomopathogenic fungi in soils from different parts of Spain
  E. A. A. Maranhão, C. Santiago-Alvarez ............................................................................ 59
On some little known epizootics in noxious and beneficial arthropod populations caused by entomophthoralean fungi
Stanislaw Balazy .............................................................................................................. 63

Investigations on entomophthoralean fungi in Latvia
Liga Jankevica, Zigrida Cudare ................................................................. 69

Survival of Erynia neoaphidis in aphid cadavers in a simulated winter environment
Tony Bonner, Simon N. Gray, Judith K. Pell ...................................................... 73

Investigations on the occurrence of entomopathogenic fungi and entomoparasitic nematodes in soils from Lower Austria
A. Hozzank, R. Wegensteiner, W. Waitzbauer, A. Burnell, Z. Mrácek, G. Zimmermann ................................................................................................................ 77

Results of preliminary investigations on the occurrence of Entomophthorales on aphids in Austria
Marek Barta, Mariana Stalmachová-Eliasová, Alexandra Hozzank, Ludovic Cagán, Rudolf Wegensteiner ................................................................. 81

Occurrence of entomopathogenic fungi in soils from Central Italy under different managements
Cezary Tkaczuk, Giancarlo Renella ........................................................................ 85

Interaction between entomopathogenic and saprophytic fungi
Zigrida Cudare, Mara Kropa ............................................................................... 91

Investigations on the virulence stability of Verticillium lecanii
Natalja Hetsch, Helga Sermann ........................................................................ 95

Influence of soil environment on growth and persistence of Beauveria brongniartii
Philip Kessler, Siegfried Keller ............................................................................... 99

Study on insecticidal, antifeedant and growth inhibitory properties of oosporein on selected pest organisms
Daniela Abendstein, Wolfgang Schweigkofler, Hermann Strasser ...................... 103

Effects of various species and isolates of entomopathogenic fungi on Alphitobius diaperinus (Coleoptera: Tenebrionidae)
Elzbieta Popowska-Nowak ............................................................................... 107

Occurrence of entomopathogenic fungi in soil from apple and plum orchards
Anna Sapieha-Waszkiewicz, Ryszard Mietkiewski, Barbara Marjanska-Cichon 113

Effect of temperature on conidia germination and vegetative growth of Metarhizium anisopliae
Hermann Strasser, Michaela Erschbamer ......................................................... 117

Distinction of the entomopathogenic fungal species Beauveria brongniartii and Beauveria bassiana by comparing their carbon utilization patterns
Barbara Pernfuss, Wolfgang Schweigkofler, Hermann Strasser .......................... 121

Fluorescence microscopic investigations on adhesion of spores of the entomopathogenic fungus Verticillium lecanii at larvae of Frankliniella occidentalis (Thysanoptera: Thripidae)
Ulrike Meyer, Helga Sermann ........................................................................ 125

Entomopathogenic nematodes

Danish surveys on insects naturally infected with entomopathogenic nematodes
Otto Nielsen, Holger Philipsen ........................................................................ 131

Studies on Steinernema feltiae related to control of sciarids
Solveig H. Salinas, Annette Sundby, Nina S. Johansen ........................................ 137
Host potential of insects from cruciferous crops to entomopathogenic nematodes and augmentation of nematodes through oil seed rape growing
Holger Philipsen, Otto Nielsen ................................................................. 141

Effect of entomopathogenic nematodes and insecticides on *Megaselia halterata* mortality
Agnieszka Sznyk-Basalyga, Andrzej Bednarek ............................................. 147

Impact of substrate conditions and application method on the efficacy of *Steinernema feltiae*
Arne Peters, Jenny Backes ............................................................... 151

Desiccation survival of *Heterorhabditis*: physiological and biochemical changes following dehydration
Liu Qi-zhi, Aharon Solomon, Itamar Glazer .................................................. 159

Application of entomopathogenic nematodes in controlling overwintering larvae of *Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Thaumetopoidea)
Oreste Triggiani, Eustachio Tarasco ......................................................... 165

Effect of *Beauveria bassiana* on the invasion and proliferation of the entomopathogenic nematode *Heterorhabditis indica* inside *Galleria mellonella* larvae
Omar M. Dar-Issa, Naim M. Iraki, Ralf-Udo Ehlers ....................................... 169

Effect of heat shock on the penetration and development of infective juveniles of two entomopathogenic nematodes strains *Heterorhabditis indica* and *H. bacteriophora* inside *Galleria mellonella* larvae
Basma Sandouka, Naim Iraki, Ralf-Udo Ehlers .............................................. 173

Molecular identification of eight isolates of entomopathogenic nematodes from West Bank and Gaza Strip, Palestine
Michael A. Sansour, Naim Iraki, Falah Younis, Sonja Hollmer, Ralf-Udo Ehlers ...... 177

Identification of bacterial symbionts of entomopathogenic nematodes by the use of universally primed-PCR (UP-PCR)
Otto Nielsen, Peter Stephensen Lübeck ....................................................... 181

Liquid culture production of biocontrol nematodes
Ralf-Udo Ehlers, Arne Peters .................................................................. 185

Integrated control of *Lycoriella solani* (Diptera: Sciaridae) with entomopathogenic nematodes and insecticides
Agnieszka Sznyk-Basalyga, Andrzej Bednarek .............................................. 189

Effects of Steinernematidae and Heterorhabditidae on the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)
Elzieta Pezowicz ....................................................................................... 193

The influence of cadmium ions on the behaviour of *Steinernema feltiae* Filipjev IJs in soil
Joanna Jarmul, Marta Kamionek .................................................................. 197

**Entomopathogenic bacteria**

Spinosad, a non-synthetic, naturally derived insect control agent
Richard Dutton, Costas Mavrotas, M. Miles, Petros Vergoulas ............................... 205

Screening *Bacillus thuringiensis* strains for activity against *Dociostaurus maroccanus* (Orthoptera: Acrididae)
Enrique Quesada-Moraga, Cándido Santiago-Alvarez ...................................... 209

Isolation and characterization of *Bacillus sphaericus* from soils in Greece
Sophia G. Aptsosglou, Stephanos I. Koliais ................................................... 215
Toxicity of wild *Bacillus thuringiensis* strains from Sardinia on *Lymantria dispar* (Lepidoptera: Lymantriidae) and comparison with commercial preparations

Emanuela Bazzoni, Marcello Verdinelli ................................................................. 219

Characterization and insecticidal activity of *Bacillus thuringiensis* strains isolated from hazelnut fields in Turkey

Remziye Nalcacioglu, Sabriye Dulger, Mustafa Yaman, Ali O. Belduz, Zihni Demirbag.................................................. 223

**Entomopathogenic viruses**

Abortive infection of the insect parovirus, *Mythimna loreyi* densovirus (MIDNV) in mammalian cells

Mohamed El-Far, Yi Li, Gilles Fédière, Said Abol-Ela, Max Bergoin, Peter Tijssen... 229

A nuclear polyhedrosis virus of the lackey moth, *Malacosoma neustria* (Lepidoptera: Lasiocampiidae) in Turkey

Mustafa Yaman, Remziye Nalcacioglu, Ali O. Belduz, Zihni Demirbag........................... 233

Record of densoviral diseases occurring among the noctuid populations in lucerne fields at El-Bahareya oasis in Egypt

Gilles Fédière, Mohamed A.K. El-Sheikh, Rabab El Mergawy, Moguib Salah, Maha Masri, Said Abol-Ela, Max Bergoin, Peter Tijssen ................................................. 237

Preliminary results on the biological activity of a Polish isolate of nucleopolyhedrovirus against *Panolis flammea* Schiff.

Iwona Skrzecz................................................................. 241

Persistence of the biological effect of codling moth granulovirus in the orchard – a preliminary field trial

Jutta Kienzle, Christof Schulz, Claus P.W. Zebitz, Juerg Huber ........................................ 245

Codling moth granulovirus – An efficient tool for codling moth control in IPM

Jutta Kienzle, Hubert Gernoth, Markus Litterst, Claus P.W. Zebitz, Juerg Huber ...... 249

**Miscellaneous**

Occurrence of microsporidia and other pathogens in associated living spruce bark beetles (Coleoptera: Scolytidae) in an Austrian forest

Bernhard Haidler, Rudolf Wegensteiner, Jaroslav Weiser.................................................. 257

Ultrastructure and temperature sensitivity of bacteriomes in *Prostephanus truncatus* (Horn)

R. G. Kleespies, C. Nansen, T. Adouhoun, A. M. Huger.................................................. 261

A new canestriniid mite (Acari, Astigmata, Canestriniidae) associated with *Blaps polychresta* Forsk. (Insecta, Coleoptera, Tenebrionidae)

Sh. M. O. El Bishlawy, S. F. M. Allam ........................................................................ 267

Utilization of essential oils and chemical substances alone or in combination against Varroa mite (*Varroa destructor*), a parasite of honeybees

S. F. M. Allam, M. F. Hassan, M. A. Risk, A. U. Zaki.................................................. 273
List of participants

ABD-ALLA, A.
Laboratoire de Pathologie Comparée CC101
Département de Microbiologie
Université Montpellier II
Place Eugène Bataillon
34095 Montpellier
France

ALEXANDRakis, Venizelos
NAGREF
Institute of Subtropical Plants and Olive Trees
Agrokipio
GR-73100 Chania
Greece

ALLAM, Sally
Department of Zoology and Agricultural Nematology
Faculty of Agriculture
Cairo University
Egypt
mshamseldean@hotmail.com

ANAGNOU-VERONIKI, Maria
Laboratory of Insect Pathology and Microbiology
Benaki Phytopathological Institute
8 Delta Str.
GR-14561 Kifissia, Athens
Greece
bpilibr@otenet.gr

ANDERMATT, Martin
Andermatt Biocontrol AG
Stahlevmatten 6
CH-6146 Grossdietwill
Switzerland
Andermatt@biocontrol.ch

APTOSOGLOY, Sophia
Laboratory of General Microbiology
Department of Biology
Aristotelian University of Thessanoliki
54006 Thessanoliki
Greece
Sapto@bio.auth.gr

BAIER, Urs
Hochschule Wædenswill
Abteilung Umweltbiotechnologie,
Grüntal, Posfach
CH-8820 Wædenswill
Switzerland

BALAZY, Stanislaw
Research Centre for Agricultural and Forest Environment, PAN
Bukowska 19
60-809 Poznan
Pologne
Balazy@man.poznan.pl

BERGOIN, Max
Laboratoire de Pathologie Comparée CC101
Département de Microbiologie
Université Montpellier II
Place Eugène Bataillon
34095 Montpellier
France
bergoin@ensam.inra.fr

BLUM, Bernard
I.B.M.A.
Postfach 252
CH-4009 BASEL
Switzerland
agrometrix.blum@bluewin.ch

BOEMARE, Noël
INRA, Laboratoire de Pathologie Comparée CC101
Département de Microbiologie
Université Montpellier II
Place Eugène Bataillon
34095 Montpellier
France
boemare@ensam.inra.fr

BOLLHALDER, Franz
Andermatt Biocontrol AG
Stahlevmatten 6
CH-6146 Grossdietwill
Switzerland
Sales@biocontrol.ch
FEDIERE, Gilles
IRD
Entomology laboratory/EC.IRD
26 Giza
12211 Cairo
Egypt
fediere@thewayout.net

GEORGIADOU, Afrodite
Laboratory of Insect Pathology and Microbiology
Benaki Phytopathological Institute
8 Delta Str.
GR-14561 Kiphissia, Athens
Greece
venus88@in.gr

GLAZER, Itamar
Agriculture Research Organization (ARO)
The Volcani Center
50-250 Bet Dagan
Israel
glazeri@netvision.net.il

GLOWACKA, Barbara
Forest Research Institute
Bitwy Warszawskiej 1920r. No. 3
00-973 Warszawa
Poland
b.glowacka@ibles.waw.pl

GRAMMATICAKIS, Nikos
Intrachem Hellas Ltd
31, Kiphissias Avenue
GR-11523 Athens
Greece

GRAY, Simon
Faculty of Science, Technology and Design
University of Luton
Park Square
Luton, Beds LU1 3JU
United Kingdom
simon.gray@luton.ac.uk

HASSANI, Mounir
Swiss Federal Institute of Technology
Institute of Microbiology
Schmelzbegsh 17
CH-8006 Zürich
Switzerland
hassani@micro.biol.ethz.ch

HOZZANK, Alexandra
Swiss Federal Research Station for Agroecology and Agriculture
Reckenholzstrasse 191
CH-8046 Zürich
Switzerland

HUBER, Jürg
Institute for Biological Control
Federal Biological Research Centre for Agriculture and Forestry (BBA)
Heinrichstrasse 243
D-64287 Darmstadt
Germany
J.Huber@bba.de

IRAKI, Naim
UNESCO Biotechnology Educational and Training Center for Palestinian Territory and Arab Countries
Bethlehem University
P.O. Box 9
Bethlehem, West Bank
Palestinian Authority
niraki@bethlehem.edu

JANKEVICA, Liga
Departement of Experimental Entomology
Institute of Biology
Miera Str. 3
LV-2169 Salaspils
Latvia
liga_jankevica@hotmail.com

KAMIONEK, Marta
Department of Zoology
Warsaw Agricultural University
Nowoursynowska 166
02-787 Warszawa
Poland

KESSLER, Philip
Swiss Federal Research Station for Agroecology and Agriculture
Reckenholzstrasse 191
CH-8046 Zürich
Switzerland
Philip.Kessler@fal.admin.ch
KLEESPIES, Regina G.
Institute for Biological Control
Federal Biological Research Centre for
Agriculture and Forestry (BBA)
Heinrichstrasse 243
D-64287 Darmstadt
Germany
R.Kleespies@bba.de

KLINGEN, Ingeborg
The Norwegian Crop Research Institute
Plant Protection Centre
Department of Entomology and Nematology
Høgskoleveien 7
N-1432 Ås
Norway
ingeborg.klingen@planteforsk.no

KONSTANTOPOULOU, Maria
Chemical Ecology & Natural Products
Laboratory NCSR
"Demokritos" Institute of Biology
P.O. Box 60228
GR-15310 Aghia Paraskevi Attikis
Greece
mkonstan@mail.demokritos.gr

KOUTSOBINAS, Dimitris
Elanco Hellas
Mesogion 335
GR-15231 Halandri Athina
Greece
elanco@acci.gr

LABROPOULOU, Vasiliki
Chemical Ecology & Natural Products
Laboratory NCSR
"Demokritos" Institute of Biology
P.O. Box 60228
GR-15310 Aghia Paraskevi Attikis
Greece
mkonstan@mail.demokritos.gr

LABUSCHAGNE, Louise
IPM Manager
Homegrown (Kenya) Ltd
P.O. Box 10222
Nairobi
Kenya
louise@timau.homegrown.co.ke

LARSEN, Brian
Borregaard Bioplant ApS
Helsingforsgade 27B
Aarhus N 8200
Denmark

LOPEZ-SERNA, M.L.
Department of Environmental Sciences and
Natural Resources
University of Alicante
03080 Alicante
Spain
mluisa@carn.ua.es

LYCOURESIS, Dyonysis
Agricultural University of Athens
Laboratory of Agricultural Zoology and
Entomology
Iera Odos 72
GR-11285 Votanikos, Athens
Greece

MAVROTAS, Costas
Dow Agrosciences Export SAS
Vouliamenis Avenue 85
GR-16674 Glyfada Athens
Greece
cmavrotas@dow.com

MAZOMENOS, Vassilios
Chemical Ecology & Natural Products
Laboratory NCSR
"Demokritos" Institute of Biology
P.O. Box 60228
GR-15310 Aghia Paraskevi Attikis
Greece
bmazom@mail.demokritos.gr

MEADOW, Richard
The Norwegian Crop Research Institute
Plant Protection Centre
Department of Entomology and Nematology
Høgskoleveien 7
N-1432 Ås
Norway
richard.meadow@planteforsk.no
MENTI, Hara
Municipality of Kiphissia
GR-14562 Kiphissia
Greece

MEYER, Ulrike
Humboldt University of Berlin
Department of Horticultural Sciences
Institute of Phytoecology
AG Applied Entomology
Dorstrasse 9
D-13051 Berlin
Germany
UMeyer73@aol.com

MICHELAKIS, Stelios
NAGREF
Institute of Subtropical and Olive Trees
Agrokipio
GR-73100 Chania
Greece

NAVON, Amos
Department of Entomology
Agriculture Research Organization (ARO)
The Volcani Center
50-250 Bet Dagan
Israel
navona@netvision.net.il

NIKAS, Haralambos
Elanco Hellas
Mesoghion 335
GR-15231 Halandri Athina
Greece
elanco@acci.gr

PAPIEROK, Bernard
Collection des Champignons
Institut Pasteur
25, rue du Dr Roux
75015 Paris
France
papierok@pasteur.fr

PELL, Judith
Entomology and Nematology Department
IACR-Rothamsted
Harpenden, Herts AL5 2JQ
United Kingdom
judith.pell@bbsrc.ac.uk

PETRERS, Arne
E-nema GmbH
Klausdorferstrasse 28-36
D-24223 Raisdorf
Germany
a.peters@e-nema.de

PEZOWICZ, Elzbieta
Department of Biology and Animal Environment
Warsaw Agricultural University
Nowoursynowska 166
02-787 Warszawa
Poland
pezowicz@alpha.sggw.waw.pl

PHILIPSEN, Holgen
Zoology Section, Department of Ecology
The Royal Veterinary and Agricultural University
Thorvaldsgsvej 40
DK-1871 Frederiksberg C
Denmark
hp@ecol.kvl.dk

PIGGOT, Simon
MicroBio
Unit 1
Harwood Industrial Estate
Harwood Road
Littlehampton BN17 7AN
United Kingdom
simon.microbio@dial.pipex.com
PITTARA, Irene
NAGREF
Ethalias 19 Halepa
GR-15125 Marousi
Greece
pubrel@nagref.gr

POPOWSKA-NOWAK Elzbieta
Institute of Ecology PAN
Dziekanów Lesny
05-092 Lomianki
Poland
ekolog@warman.com.pl

PROPHITOU, Dimitra
Aristotelian University of Thessanoliki
GR-54006 Thessanoliki
Greece

QI-ZHI, Liu
Department of Plant protection
Chinese Agricultural University
Beijing 100094
China
lqzwyz@public.cau.edu.cn

RAVENSBERG, Willem
Koppert Biological Systems
P.O. Box 155
2650 AD Berkel en Rodenrijs
The Netherlands
w.ravensberg@koppert.nl

RISHA, El-Sayed
Center of Virology
Faculty of Agriculture-IRD
Cairo University
Cairo
Egypt
saidlec@brainyl.ie-eg.com

RODITAKIS, Emmanouel
NAGREF
Plant Protection Institute Herakleon
P.O. 1802
GR-71110 Herakleon Crete
Greece

RODITAKIS, Nikos
NAGREF
Plant Protection Institute Herakleion
P.O. 1802
GR-71110 Herakleon Crete
Greece
roditak@chemistry.uoc.gr

POPOWSKA-NOWAK Elzbieta
Institute of Ecology PAN
Dziekanów Lesny
05-092 Lomianki
Poland
ekolog@warman.com.pl

SAHLEN, Ulrika
University of Umeå
Sofiehemsv. 55
S-90738 Umeå
Sweden

SALINAS, Solveig Haukeland
The Norwegian Crop Research Institute
Plant Protection Centre
Høgskoleveien 7
N-1432 Ås
Norway
solveig.haukeland@planteforsk.no

SAMISH, Michael
Kimron Veterinary Institute
P.O. Box 12
50-250 Bet Dagan
Israel
msami_vs@netvision.net.il

SANTIAGO-ALVAREZ, Cándido
Cátedra de Entomología Agrícola y Forestal,
Departamento de Ciencias y Recursos
Agrícolas y Forestales, E.T.S.I.A.M.
Universidad de Córdoba
Apartado 3048
14080 Córdoba
Spain
cr1saalc@uco.es

SAPIEHA-WASZKIEWICZ, Anna
Department of Plant Protection
University of Podlasie
ul. Prusa 14
08-110 Siedlce
Poland
mietkier@ap.siedlce.pl
SERMANN, Helga
Humboldt University of Berlin
Faculty of Agriculture and Horticulture
Phytomedicine / Applied Entomology
Dorfstrasse 9
D-13051 Berlin
Germany
helga.sermann@agrar.hu-berlin.de

SEWIFY, Gamal
Department of Agricultural Zoology
and Nematology
Faculty of Agriculture
Cairo University
Cairo
Egypt
arnaout@intouch.com

SHAMSELDEAN, Muhammad
Department of Agricultural Zoology
and Nematology
Faculty of Agriculture
Cairo University
El-Gamaa St.
Giza 12311
Egypt
mshamseldean@hotmail.com

SKRZECZ, Iwona
Forest Research Institute
Bitwy Warszawskiej 1920r. No. 3
00973 Warszawa
Poland
skrzeczi@ibles.waw.pl

STAROGIANNIS, George
Laboratory of Insect Pathology and
Microbiology
Benaki Phytopathological Institute
8 Delta Str.
GR-14561 Kiphissia, Athens
Greece
starojhon@hotmail.com

STATHAS, George
Laboratory of Biological Control
Benaki Phytopathological Institute
8 Delta Str.
GR-14561 Kiphissia, Athens
Greece
georgestathas@hotmail.com

STEENBERG, Tove
Danish Pest Infestation Laboratory
Skovbrynet 14
DK-2800 Lyngby
Denmark
t.steenberg@ssl.dk

STRASSER, Hermann
Institute of Microbiology
Leopold-Franzens-University of Innsbruck
Technikerstrasse 25
A-6020 Innsbruck
Austria
Hermann.Strasser@uibk.ac.at

STUBSGAARD, Lars
Borregaard Bioplant ApS
Helsingforsgade 27B
Aarhus N 8200
Denmark
borregard@bioplant.dk

SZNYK-BASALYGA, Agnieszka
Department of Biology and Animal
Environment
Warsaw Agricultural University
Nowoursynowska ul. 166
02787 Warszawa
Poland
sznyk@alpha.sggw.waw.pl

TARASCO, Eustachio
Dipartimento di Biologia e Chimica
Agro-Forstale ed Ambientale
Università di Bari
Via Amendola 165/A
70126 Bari
Italy
eustachio.tarasco@agr.uniba.it

THOMSEN, Lene
Department of Ecology
The Royal Veterinary and Agricultural
University
Thorvaldsensvej 40
DK-1871 Frederiksberg C
Denmark
Lene.Thomsen@ecol.kvl.dk
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution/Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEGENSTEINER, Rudolf</td>
<td>Institute for Forest Entomology, Forest Pathology and Forest Protection, Univ.-BOKU Vienna, Hasenauerstrasse 38, A-1190 Vienna, Austria, <a href="mailto:wegenst@ento.boku.ac.at">wegenst@ento.boku.ac.at</a></td>
</tr>
<tr>
<td>WILDEY, Kenneth</td>
<td>International Centre for Safe Commodity Storage, MAFF Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom, <a href="mailto:k.wildey@csl.gov.uk">k.wildey@csl.gov.uk</a></td>
</tr>
<tr>
<td>YAMVRIAS, Christias</td>
<td>Agricultural University of Athens, Laboratory of Agricultural Zoology and Entomology, Iera Odos 72, GR-11825 Votanikos, Athens, Greece</td>
</tr>
<tr>
<td>ZIMMERMANN, Gisbert</td>
<td>Institute for Biological Control, Federal Biological Research Centre for Agriculture and Forestry (BBA), Heinrichstrasse 243, D-64287 Darmstadt, Germany, <a href="mailto:G.Zimmermann@bba.de">G.Zimmermann@bba.de</a></td>
</tr>
</tbody>
</table>
Lecture and plenary presentations
Microbial control in Greece
Forty years history

Christias Yamvrias
Professor Emeritus
Agricultural University of Athens, Iera Odos 72, 11825, Votanikos, Athens, Greece

Research on insect pathology and microbial control of insect pests started in Greece in 1962. At that time, the Benaki Phytopathological Institute in Kiphissia, created a “Laboratory of Biological Control”, of which I became the head. At that time, I had just returned from France, where I had studied for four years the new “philosophie” of microbiological control and had obtained my Doctorate Diploma from the University of Paris. The name of the laboratory was later changed to “Laboratory of Insect Pathology and Microbiology”. And it was the only laboratory in Greece which performed such research.

The use of classical chemical control of insect pests was naturally the rule in Greece. Biological control methods, by using microorganisms, was something unknown. One of my primary goals was to convince scientists in plant protection of the value of insect pathogens as an alternative mean to chemicals. This was a difficult proposition; We needed energy to surpass all obstacles in order to develop these new ideas.

Unfortunately, due to insufficient financial resources we were unable to perform organized research work in the laboratory. Therefore, we decided to carry out experimental work in the field, and we planned to control lepidopterous larvae of insect pests using commercially available preparations of *Bacillus thuringiensis*. We were hoping for an opportunity to start biological control applications. Sampling methods, control methods, and application techniques were among our primary objectives in the field experiments.

This opportunity arisen from the President of the “Forest Plant Protection Society” in the region of Attiki. The President, a lady, questioned if somebody in the Benaki Phytopathological Institute knew the biological method to control processionary pine caterpillar (*Thaumetopoea pityocampa*) and to apply that method on damaged pine trees in a historical place near Athens (Kaissariani). She was aware of the fact that this method was safer for people visiting the area. So, I had the chance to perform my first application of biological control using a French preparation of *B. thuringiensis* named “Bactospéine”.

An essential aspect of that operation was that an officer, of the Forest Services of the Ministry of Agriculture, followed the entire process of the application. Interestingly, he became enthusiastic about the method and made all the necessary arrangements in order to use it in a large area of the country where pine trees grew. The method was to be applied for several years by the Forest Services. This first project was an important step for the introduction and development of microbiological methods in insect pest control in Greece.

Meanwhile, we were searching for a promising area to perform experiments with *B. thuringiensis*. It was essential to choose an economically important crop that was susceptible to insect attacks.

Since olive is an key cultivation in Greece, we decided to begin our studies with the olive moth (*Prays oleae*), the second most important olive insect enemy. Laboratory tests showed that the larvae of the insect were susceptible to *B. thuringiensis*. Only the larvae of the flower (or anthophagous) generation were susceptible to the bacteria because of their life habits,
crawling between open flowers. Usually, two to three insecticide treatments had been used by
the growers as an effective mean of preventing damage by the insect.

For our first trial, we selected an olive-grove in the island Cephalonia. During flowering
period, in May 1963, we sprayed olive trees with an aqueous solution of \textit{B. thuringiensis} WP
preparation, to control larvae of the anthophagous generation of the insect. In 1964, I
published, in the Annals of the Benaki Phytopathological Institute, the first paper dedicated to
the possibilities of controlling the olive moth using a \textit{B. thuringiensis} preparation.

Over several years, experiments were developed in controlling the olive moth. Results
showed that with one treatment against larvae of the flower generation of the moth, we could
have a significant decrease of olive moth population. Applying insecticides may destroy
beneficial insects at the beginning of their development. This biological control method is
now applied in all countries where olive trees are cultivated.

In 1989 I left the laboratory to take a new position at the Agricultural University of
Athens and I was replaced by Dr Maria Anagnou-Veroniki, who had joined the laboratory in
1977. During these years she prepared a Doctorate thesis on insect virology entitled “Studies
on viral infections of the olive fruit fly \textit{Bactrocera (Dacus) oleae} in Greece”.

Dr Anagnou-Veroniki had isolated from natural populations of \textit{Dacus oleae} a virus
which was classified in the Reovirus group. Artificial infections of virus-free flies, by
inoculation, using purified suspension of the virus, caused mortality in comparison to the
control flies. These virus particles were investigated in Dacus populations of various areas in
Greece. The investigation proved for the first time the significant role of the viruses on the
degeneration of the \textit{D. oleae} population. During this time, similar studies allowed to
recognize two other types of virus particles, morphologically different from the first one.
Since then, Dr Anagnou-Veroniki collaborated in all experiment projects, as well as on
diagnostic work of diseased insects, collected from different areas of Greece.

During the 40 year course of the laboratory, a large number of field trials were
performed. Their aim was to compare, not only for formulations of \textit{B. thuringiensis}, but also
for preparations of fungi or viruses, their efficacy against different insect species, having an
economically significant scale for Greek agriculture.

The development of the laboratory in a better situation created the conditions for research
work and the organized insectarium provided possibilities to rear insects, in order to perform
laboratory tests. In this way, we could study the mode of action of different entomopathogenic
microorganisms. In the early 70’s, commercial preparations of \textit{B. thuringiensis} were
available. Tests were performed by our team in order to examine the efficacy of those
formulations, from a practical point of view.

The main goal of all those research projects was to present a biological method for pest
control and to establish, if possible, an Integrated Pest Management system. The strategy for
such a system needed the collaboration of other specialists (e.g. on insect parasites and
predators). Such an IPM program was achieved for olive cultivation. This program covered
the three main insect pests: olive fruit fly, olive moth and black scale (\textit{Saissetia oleae}). For
\textit{Dacus} we used a mass trapping system, olive moth was treated with \textit{B. thuringiensis} and the
black scale was treated with white oils.

Together with olive cultivation, other Greek crops were given interest in the field of
biological control:
- Experimental control methods were performed against the codling moth (\textit{Cydia
  pomonella}), a serious orchard enemy, by using granulosis virus. Applications of a spray
  material of granulosis virus of codling moth (\textit{CpGV}) were made on apple orchards in
  1982-1983 at a location of Corinthos district. Sex pheromones were used as a monitoring
device for moth population. Results from the two year trial were encouraging. The
control of codling moth with viral preparation was equal to that of organophosphorous insecticides, used by the growers. This investigation was carried out within a framework of a Project of the European Community in which several European countries were involved.

For some years we studied the problem of applying an appropriate control method on cotton pink bollworm (*Platyedra gossypiiella*), which is the major cotton insect pest in Greece. It is susceptible to bacterial preparations of *B. thuringiensis*. By placing pheromone traps in the experimental areas, we monitored the moth presence, and consequently determined the time of application. Because of the particular biology of this insect species, we have tried different technical methods, to attract the new hatched larvae to the bacillus particles before entering into the cotton boll. As attractants, we used sugar or cotton seed-oil mixed with *B.t.* water solution in the tank. In some experiments we added low dosages of insecticides into the spray solutions of *B.t.* to enhance the efficacy. Eventhough the results were not very satisfactory, even if *B. thuringiensis* can, in a way, minimize the population of this pest.

Another effort was made to search for an efficient biocontrol method against the Colorado potato beetle (*Leptinotarsa decemlineata*) on potato crops. Field trials during the years 1988, 1989 and 1990 were undertaken at three different areas, using a *B. thuringiensis* sbsp. *tenebrionis* preparation. In these trials the efficacy of the product was tested by using various rates in relation to the various stages of the insect. Results showed a 96% reduction of the insect population. This method can be applied by growers as an effective and safe tool against this pest.

Laboratory tests were performed to compare the sensitivity of the potato tuber moth (*Phthorimaea operculella*) with *B.t.* formulations versus chemical insecticides (carbaryl, deltamethrin, triflumuron). Results showed that carbaryl from the insecticides used had good effectivity, but, less than that of the *B.t.* preparations.

During the same period (1992, 1993), another research program was to evaluate the effectiveness of two new strains of *B.t.* against the larvae of cotton bollworm (*Helioverpa (Heliothis) armigera*). This species of Noctuids was resistant to common preparations of *B. thuringiensis* (Bactospeine, Thuricide and Dipel) available at that time. The chemical cypermethrin was used as insecticide of reference. Results from field trials showed no difference, compared to the chemical.

The summer fruit tortrix (*Adoxophyes orana*) invaded Greece during the 1980’s. Field tests were created to study the effectiveness of a formulation containing as an active ingredient granulosis virus of *Adoxophyes orana* (*AoGV*) to control larvae of the insect. The chemical “lamba-cyhalothrine” was used as a reference. Results from the two years trial showed that there is no significant difference between viral and chemical insecticide.

Another area of interest was the symbiotic bacteria of *Dacus oleae*. This was a challenging subject for investigation. These symbiotic bacteria play a nutritional role for the olive fruit fly. If there would be a method for destroying those bacteria, it would be a good tool of control for this olive pest. However, their isolation from a head diverticulum of the adult insect and consequently their cultivation in vitro for identification deserved investigations. The research work has been made with the collaboration of two plant pathologists, Prof. Panagopoulos and Dr Psallidas of the Benaki Phytopathological Institute, and later on with an insect pathologist, Prof. Lüthy from Zürich. As a result from these investigations we could mention that those bacteria: a) differ from *Pseudomonas savastanoi*, which causes the olive knot, a serious disease of olives, and for which Petri wrote, in a paper in 1910, that they belonged to the same species, and b) those bacteria could be cultivated in artificial media.
In my lecture today, I tried to present a short history of the life of a laboratory specialized in insect pathology and microbial control of insect pests, that started in a period in which the development of those two new ideas had many difficulties to overcome. It is a simple history, but very important because we tried to implement new concepts in plant protection strategies in our country, at a time where the use of broad-spectrum insecticides by growers was the rule.

In Greece, the whole approach to pest control must be redeveloped using an Integrated Pest Management “philosophie”, that combines chemical, cultural and biological strategies to reduce harmful effects to the public health and the environment, and to give to the growers a profitable and productive farming, without disrupting the agro-ecosystem.
Future needs in risk assessment of fungal Biological Control Agents

Hermann Strasser
Institute of Microbiology, Leopold-Franzens University Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria

Abstract: This presentation contributes to the new EU policy on registration of biopesticides since the current Directive 91/414/EEC has been recently agreed by the EU and should be enacted at national level by May 2002. Despite this progress several key issues remain still unresolved and may impede exploitation of fungal biological control agents (BCAs). It is the author’s opinion that both the efficacy of the pathogen and data on the fate of inoculum and any major secreted metabolites are essential. To illustrate the future needs in risk assessment of fungal BCAs, methods and strategies are suggested which will help to standardise the risk assessment procedure.

Key words: Directive 91/414/EEC, fungal biocontrol agent, risk assessment, secondary metabolite

Introduction

There has been a sporadic debate about the criteria for registration of fungal BCAs and global harmonisation of registration procedures (OECD, 1998). Procedures for regulating biopesticides under the Directive 91/414/EEC are now being harmonized across Europe. One important area is to establish data requirements that take microbiological characteristics of BCAs into account. Currently, each applicant must still present sufficient data to fully assess the risks fungal BCAs pose to operators, consumers and the environment (Neale & Newton, 1999). Data on fungal toxins are often missing from registration documents (Strasser et al. 2000a).

It is a common opinion that both the efficacy of the pathogen and data on the fate of both inoculum and any major metabolites secreted are essential. These data will give some idea of the expected success of control and limitations of the product. In the general introduction for Annex II of the working document 91/414/EEC someone is instructed which information will be obtained by the characterisation and identification of a micro-organism. The interpretation of the “relevant aspects” which are necessary to complete the registration dossier are more or less defined in the different sections of Annex IIB and Annex IIIB. Nevertheless, on closer inspection it is still up to the national regulatory bodies to decide whether the safety data on a BCA are sufficient for a successful registration.

Scientists and industry are aware about fulfilling the rigid registration criteria defined in the EEC-directives. But there are still relevant guidelines missing how to control the efficacy of a BCA under standardized conditions, how to detect trace amounts of toxic metabolites in the production system, the final product and in the environment. Not to discuss whether and in which quantity do those metabolites enter the food chain and pose therefore risks to plants, animals and humans.

It is undisputed that the major hurdle in the registration, and subsequent commercialization, of fungal BCAs is risk assessment, particularly that of secreted fungal metabolites. Currently, little is known about the fate of these compounds in the environment even though this is one of the key questions asked by registration authorities. Studying the Biopesticide Manual (Copping, 1998) no information is available about fungal secondary
metabolites, although some of them are reported as toxigenous compounds in literature (Vey et al., 2001). With reference to the Biopesticide Manual data on mammalian toxicity for *Verticillium lecanii* and *Beauveria brongniartii* and environmental impact and non-target toxicity are published, respectively (Table 1). However, no information on metabolites is listed although both entomopathogens are known to excrete toxic metabolites and are already used for more than one decade as biological control agent.

Table 1. Extract from safety data of *Verticillium lecanii* and *Beauveria brongniartii*, two registered old active compounds (Copping, 1998).

<table>
<thead>
<tr>
<th>Biological insecticide</th>
<th>Mammalian toxicity</th>
<th>Environmental impact and non-target toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Verticillium lecanii</em></td>
<td>No skin and eye irritation</td>
<td>No allergic responses or health problems</td>
</tr>
<tr>
<td></td>
<td>No acute or chronic toxicity, Infectivity or hypersensitivity</td>
<td>Not pathogenic to non-target</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It has no adverse effects within the environment</td>
</tr>
<tr>
<td><em>Beauveria brongniartii</em></td>
<td>Acute oral LD50: rats &gt; 5 g/kg</td>
<td>Fish: trouts (30 days) 7.2 g/l</td>
</tr>
<tr>
<td></td>
<td>No toxicity, infectivity or pathogenicity from single dose of 1.1 x 10^9 cfu / kg (rats)</td>
<td>NOEL: 3 mg /l</td>
</tr>
<tr>
<td></td>
<td>Acute percutaneous LD50; rats &gt;2 g/kg; mildly irritant to skin (rabbit). considered to be non-toxic</td>
<td>Birds: dietary (5 days) ducks &gt; 4 g/kg</td>
</tr>
</tbody>
</table>

In section 2.8 of Annex IIB (“Information on the production of metabolites - especially toxins”), the following comment on toxins can be found: “Where a microbial species of the strain subject to the application is known to produce metabolites (especially toxins) known to have undesirable effects on human health and/or the environment and where these metabolites are produced in significant quantities during or after application, the nature and structure of this substance, its presence inside or outside the cell and its stability, its mode of action (including external and internal factors of the micro-organism necessary to action) as well as its effect on humans, animals or other non-target species shall be indicated. The conditions under which the micro-organism produces the metabolite(s) (especially toxin(s)) must be described”.

Regarding *B. brongniartii* the first attempt was made by our working group to provide data on the major toxic metabolites secreted by the production strains which form the active ingredient of Melocont®-Pilzgerste. Oosporein was the only major secondary metabolite produced by three commercial isolates of the fungus in submerged cultures and on sterilised barley kernels. None of the other toxins (bassianin, beauvericin and tenellin) normally produced by *Beauveria* species were detected on selected plants which were treated with Melocont®-Pilzgerste and inside the natural infected target host *Melolontha melolontha* (Strasser et al., 2000b).
Although *Metarhizium anisopliae* has been already registered in 1990 (Reinecke *et al*., 1990), neither information on destruxin accumulation in commercialised BCAs nor data on toxin enrichment in the environment were published (Table 2). In general no relevant data on this sensitive topic are available.

Table 2. *In vitro, in vivo* and *in situ* production of oosporein (*B. brongniartii*) and destruxin (*M. anisopliae*) by fungal entomopathogens (Amiri-Besheli *et al*., 1999; Strasser *et al*., 2000a).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Maximum amount of oosporein</th>
<th>Maximum amount of destruxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>300 mg/l</td>
<td>815 mg/l</td>
</tr>
<tr>
<td>Commercialised product (BCA)</td>
<td>3.2 mg/kg</td>
<td>?</td>
</tr>
<tr>
<td>Mycosed larvae</td>
<td>200 µg/larva</td>
<td>1 µg/larva</td>
</tr>
<tr>
<td>Plant biomass</td>
<td>n.d.</td>
<td>?</td>
</tr>
<tr>
<td>Potato tubers</td>
<td>n.d.</td>
<td>?</td>
</tr>
<tr>
<td>Soil (environment via BCA)</td>
<td>0.02 mg/m²</td>
<td>?</td>
</tr>
<tr>
<td>Soil (enrichment via larvae)</td>
<td>6.4 mg/m²</td>
<td>?</td>
</tr>
</tbody>
</table>

n.d. ….. not detected; ? ….. no data available from literature

**Future needs in risk assessment**

Because the lack of information regarding the efficacy of fungal pathogen and missing data on the fate of both inoculum and any major metabolites secreted, there are several innovation aspects discussed by many experts which should be investigated in future projects (Table 3).

Table 3. Key actions to be investigated in future projects.

<table>
<thead>
<tr>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>To identify the major metabolites secreted by fungal BCAs - i.e. those</td>
</tr>
<tr>
<td>most likely to enter the food chain.</td>
</tr>
<tr>
<td>To develop methodologies and tools (e.g. antibodies, species-specific</td>
</tr>
<tr>
<td>quantitative PCR, NASBA, species-specific microchips/microarrays) to</td>
</tr>
<tr>
<td>monitor metabolites and fungal BCAs in the environment.</td>
</tr>
<tr>
<td>To identify sensitive cell lines (i.e. biosensors) to detect selected</td>
</tr>
<tr>
<td>metabolites.</td>
</tr>
<tr>
<td>To determine the mode of action of metabolites to identify target sites</td>
</tr>
<tr>
<td>and potential risks (e.g. cytotoxicity assay).</td>
</tr>
<tr>
<td>To monitor major metabolites in the environment to see if they enter</td>
</tr>
<tr>
<td>the food chain.</td>
</tr>
</tbody>
</table>

It is the author’s opinion that these data will give some idea of the expected control and limitation of fungal products. The information will also help allay public fears regarding the safety of fungal BCAs.

Within the next few years we must screen different strains of mycoinsecticides, mycoparasites and mycoherbicides against their respective hosts to identify the most virulent or antagonistic isolates. The metabolite profile (type, quantity) and the regulation of selected
secondary metabolites must be determined to get a better understanding regarding stabilisation of virulence. Major metabolites, after purification and characterisation, should be screened against a range of cell lines (animal, plant and microbial) to identify sensitive biosensors. LD$_{50}$ for each compound must be determined. Assays and probes (biochemical, and molecular) will be developed in the future which could be used together with existing technologies to determine the mode of action of selected, major metabolites. Assays must be extended to plant and animal cell lines, young plants and various invertebrate pests. It is the aim of scientists to identify new compounds which are carcinogenic/mutagenic and cytotoxic.

A main key task in future will be the monitoring of metabolites in the environment. Samples should be taken from production systems, treated plants and soil. The metabolite profile, if any, will be compared with profiles obtained from controls. With the help of this information we can help to establish systems if metabolites of fungal BCAs really enter the food chain.

**Conclusion**

A major hurdle in the registration, and subsequent commercialisation, of fungal BCAs is risk assessment, particularly that of secreted fungal metabolites. Little is known about the range of metabolites produced and whether they enter the food chain so posing a risk to human and animal health. A RTD-EU project named “Risk assessment of fungal biocontrol agents (RAFBCA) is scheduled to work on these sensitive topics. This project will meet the major goals to monitor toxins of entomopathogenic fungi during production and post-application.

**Acknowledgements**

This presentation is supported by the EU-FAIR6-CT98-4105 research project BIPESCO.

**References**


New challenges for fungal bioinsecticides

Jacques Fargues
INRA - CBGP, Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet, 34980 Montferrier-sur-Lez, France

Abstract: Despite the importance of entomopathogenic fungi as natural regulators of many insect pest populations, only a few have been developed as bioinsecticides and used on an operational scale. The potential of these fungi for development as inundative control agents and, to a lesser extent, as classical control agents is promising. However, entomopathogenic fungi are often still considered as too dependent on environmental factors. Therefore one of the greatest challenges for improving fungi as microbial control agents is to identify and establish a hierarchy of pertinent environmental constraints and to develop ways to overcome them. Environmental conditions prevailing in the habitats of the targeted pest populations need to be analyzed so that well-adapted strains can be selected and application strategies optimized to suit their needs. To date, most of the isolate selection has focussed on the ability of pathogens to infect their target hosts. Few attempts have been made to select pathogens based on their tolerance to key factors such as UV radiation, temperature, and desiccation. A better understanding of the factors important in the initiation of fungal epizootics is necessary. A systematic approach must address the limiting environmental factors and the possibility of manipulating the environment to favor the pathogen (e.g. greenhouse climate). Modeling of beneficial pathosystems involving entomopathogenic fungi has to be improved and must take into account specific constraints of the targeted agroecosystem. The implementation of pathosystem and epizootiological models in agroecosystem models may be used to build operational models based on decision making in IPM crop protection. Recent advances in molecular biology and population genetics of entomopathogenic fungi provide promising perspectives for strain improvement, epidemiological tracking, and evaluation of genomic variations with a thorough understanding of their potential ecological consequences. A better understanding of the molecular basis of pathogenicity and virulence would permit the improvement of mycoinsecticide efficiency. Somatic hybridization (parasexual crossing) may be the most effective method for obtaining strains with a better environmental tolerance, as these traits are probably controlled polygenically. Population genetics will provide the scientific basis for the choice of either indigenous or exotic fungal isolates as the most suitable candidates for development as mycoinsecticides. In addition to the immediate goals of fungal genetics, phenotypic variation (nutritional requirements versus virulence) has been underestimated. Basic understanding of adaptative mechanisms and of the environmental determinism of pathosystem dynamics may be useful for optimizing progress in production, formulation and application techniques. Because of recent improvements and new perspectives, mycoinsecticides appear to be excellent candidates as components of IPM in an increasing number of agroecosystems.

Key words: microbial control, entomopathogenic fungi, IPM, climatic constraints, pathosystem dynamics, systems approach, modeling, molecular biology, population genetics, strain selection.

Introduction:
Current strategies for microbial control with entomopathogenic fungi

There is an increasing interest for developing the use entomopathogenic fungi as microbial control agents of invertebrate pests (Butt et al., 2001). The importance of fungi as natural regulators of many insect pest populations in natural and agricultural ecosystems has been
recognized for many years. Because of their percutaneous mode of infection, they do not need to be ingested. This makes the number of target hosts quite large, including plant and animal sucking insects, e.g. aphids, whiteflies, thrips, and triatomines and ticks. Moreover, they can infect non-feeding stages such as eggs or pupae (Ferron et al., 1991).

Four current strategies have been taken for pest control with pathogenic fungi: permanent introduction (Milner et al., 1982; Hajek et al., 1995), inoculative release (Ignoffo, 1981), inundative release (Ferron et al., 1991; Butt et al., 2001), and environmental manipulation (Helyer et al., 1992). The number of mycoinsecticides commercialized or pending registration has increased significantly within the last decade (Shah & Goettel, 1999; Butt & Copping, 2000). The development of these bioproducts has in part been driven by specific targeted markets, e.g. termites (Rath, 2000), and cockroaches (Mohan et al., 1999), and in part by the discovery of new isolates highly pathogenic for important groups of pests, e.g. locusts and grasshoppers (Lomer et al., 1997; Milner, 1997; Magalhaes et al., 2000).

The potential of these fungi for development as inundative control agents is promising, but often still under-estimated, because they are considered as too dependent on environmental factors. Therefore, one of the greatest challenges for improving fungi as microbial control agents is to identify and establish a hierarchy of pertinent environmental constraints and to develop ways to overcome them (Goettel & Fargues, 1996). All control strategies require an understanding of host-pathogen population dynamics and of its environmental determinism, in order to predict and manage the development of disease prevalence. In addition, the characterization of pertinent environmental constraints allowed us to select and manipulate fungal populations better-adapted to the targeted pest environments by using increasing knowledge in both genetical and phenotypical variability of these fungi. The purpose of this paper is to present an overview of some promising research areas for further development and implementation of fungal microbial control agents.

The systems approach

Because of the complexity of the interactions between pathogen populations and host populations, and a variety of environmental situations, the systems approach of insect mycosis have to be generalized. The basic components of the pathosystem consisted of the host populations, the pathogen populations and their environment, for which reason also called the disease triangle by plant pathologists (Robinson, 1976). The pathosystem analysis is essentially multi-disciplinary and basically holistic. It consists in analyzing the dynamics of the pathosystem, host-pathogen-environment, at each step of the dynamics of each population and each step of the dynamics of their interaction. It is clear that the pathosystem concept, including both pathosystem analysis and pathosystem management, unites these synthetic disciplines into one cohesive approach (Robinson, 1976). A crop pathosystem is a subunit of an agroecosystem and is defined on the basis of parasitism, which can involve wider boundaries in taking into account new constraints caused by the cultural system. Computer modeling is a particularly functional approach for understanding the pathosystem dynamics, because it is useful for artificially compartmentalizing a biological complex, and in doing so create simplified conceptual or physical representations (Carruthers & Smits, 1994). Mathematical models has only recently been applied to epizootiology of fungal diseases in insects (Kish & Allen, 1978; Brown & Nordin, 1982; Onstad & Carruthers, 1990; Hajek et al., 1993; Knudsen & Schotzko, 1999). There are two apparently paradoxical requirements in the purpose of building models. On one side, the discrete component approach is based on analytical experiments conducted to better understand the factors and processes involved at each step of the interactions. On the other side, by defining a model as a network of discrete
components that correspond closely with the pathosystem, it can be easily to relate both the structure of these models and the processes that regulate their dynamics (inputs, outputs and information links). Mechanistic modeling also provide a means of linking many different data into a larger system that can be evaluated over a wide area of interest where environmental constraints are also different (Hajek et al., 1993). Although incorporating spatial dynamics into disease models increase the complexity of the pathosystem, it provides a more realistic interpretation of the dynamics of the host plant-insect-pathogen interaction (Knudsen & Schotzko, 1999). As stated above, the pathosystem concepts is based on both analysis and management aspects, and that is why, pathosystem models based on detailed mechanistic experimental analyses, conducted under both controlled and semi-field conditions, have to be independently validated under natural conditions. In addition, according to the pathosystem-based strategy, it is imperative to take into account the other possible constraints relate to the other crop management practices. Elucidation of these relationships by means of simulation models lead to optimize the use of fungal microbial control agents in pest management strategies.

Environmental constraints in pest habitats: a base for modeling of pathosystem dynamics and management

The influence of climatic factors for aerial fungal diseases is well-known. Numerous studies underlined high humidity requirements for fungal infection in insects, but most of them are based on imprecise physical measure procedures and only vaguely represent the complexity of interactive underlying factors. Because of this lack of acute microclimatic investigations, pertinent climatic constraints and climatic requirements of fungi need to be reexamined.

A variety of environmental factors have been shown to cause dramatic effects on each compartment of the pathosystem (ie. each step of the disease). Salient parameters are solar radiation, temperature, water availability, precipitation, wind, and telluric factors. However, there are four difficulties in this approach. First, it is of fundamental importance to establish the "cause and effect" relation for identifying the pertinent explicative variable. Second, both space and time scales have to be investigated in taking into account constraints specific of the microbial population dynamics. Third, environmental parameters interact with each other in their impact on the pathosystem and, consequently, need experimental integration validations. Four, because of environmental heterogeneity and fluctuations, the hierarchy among the key-factors determining the dynamics of each pathosystem compartment change over space and time.

Several case-studies will allow us to illustrate the points enumerated above. When considering pathogen persistence in epigeal habitats, solar radiation is the most important factor of decay of fungal propagules. However, acute investigations showed that natural UVB radiation (295-320 nm) was the most pertinent explicative variable for modeling the detrimental effect of solar radiation outside (Smits et al., 1997). Under shaded/indirect light conditions, diffuse UVB may be capable of killing fungal inocula (Smits et al., 1996). The surfaces of leaves in the canopy are rarely distributed on an horizontal plane, but show a great variability of positions (plane slope and azimuthal orientation), causing highly significant changes in both direct and diffuse natural UVB irradiances. Thus, it is useful to combine biological models of pathogen persistence (Smits et al., 1997) with physical models of radiation penetration in plant canopy and canopy architecture (Sinoquet & Bonhomme, 1992; Mabrouk et al., 1997). Although UVA (320-400 nm) was also harmful for fungal propagules (Fargues et al., 1988, 1997c), this parameter did not appeared as an important constraints for mycoinsecticide use in greenhouse (Helyer et al., 1992).
Temperature conditions prevailing in arid locust habitats could severely limit fungal infection during the hottest periods. In South-East Morocco at 2000 m altitude, under diurnal air conditions of around 29 °C in June, the surface temperature of locust cuticle reached 45 °C for ca. 5 h per day and that of soil ca. 55 °C (J. Fargues, M. Rougier, P.H. Robert, J.P. Louveau & T. Ben Halima, unpublished data). Similar conditions prevailing in Sub-Saharan habitats of the Desert locust, may be unfavorable for isolates of *Metarhizium anisopliae* var. *acridum* in spite of their remarkable tolerance to the temperature constraints (35 °C) (Ouedraogo et al., 1997), and their capacity to infect locust hosts at low humidities (13 % RH%) (Fargues et al., 1997b). Moreover, fungal infection induced an alteration of the thermoregulating behavior in contaminated grasshoppers, preferentially seeking temperatures at 40-42 °C (Inglis et al., 1996; Blanford & Thomas, 2000) and fungal infection decreased as the amplitude of temperature increased (10-40 °C regime) (Inglis et al., 1999).

In humid tropical areas, the air moisture varied according to the insect habitats. When considering domestic and sylvatic habitats of *Rhodnius* spp. for vector biocontrol in Colombia, nocturnal relative humidity inside ranch houses did not reach 90 %, because of hygroscopic clay material-made walls, in contrast, air-saturated conditions prevailed in peridomestic palm trees (C. Luz, J. Fargues, M. Rougier & C. Romaña, unpublished data; Luz et al., 1994). High humidity appeared to be the most crucial climatic constraint, for example, when it reached 96 % RH for only 3-5 consecutive days, the high humidity duration per day required for total mortality in *Beauveria*-contaminated nymphs was 16 h per day (Fargues & Luz, 2000). Obviously, conditions prevailing in wild and peridomestic habitats appeared favorable to control vectors with *B. bassiana* according to both infection and recycling requirements (critical air humidity at ca. 96 % RH) (Fargues & Luz, 1998, 2000). Humidity constraints inside clay houses require that existing formulations be adapted, although fungus recycling was observed on nymph cadavers in palm-thatched roofs (Luz et al., 1994; C. Romaña, J. Fargues, P.H. Robert, unpublished data).

In most studies reported so far, high humidity conditions were considered as an absolute requirement for establishment of *Verticillium lecanii*-induced infection of the greenhouse whitefly (Ekboim, 1981; Osborne & Landa, 1992; Lacey et al., 1996). On chrysanthemum, fogging water over the crop at night for four consecutive nights of high humidity (≥ 95 % RH) per week provided good control of Homoptera by *V. lecanii* without provoking any adverse effect on the crop (Helyer et al. 1992). In contrast, fogging water over greenhouse tomato crops at night induces high risk of *Botrytis* epidemics (Elad & Shtienberg, 1995). Consequently, in Mediterranean greenhouse tomato crops a technique of confinement, by closing the ridge vents one hour earlier in the evening and one hour later in the morning, was tested. The night-humidity differential inside the tomato canopy between confined compartments and dry compartments reached 20 points, but, the efficiency of *B. bassiana* and *Paecilomyces fumosoroseus* mycoinsecticides was similar in dry greenhouse compartments and in humid compartments (J. Fargues, C. Vidal, N. Smits, M. Rougier, T. Boulard, M. Mermier, P. Nicot & B. Jeannequin, unpublished data). A similar high pathogenic activity was observed in small-scale field trials using *B. bassiana* and *P. fumosoroseus* against *Bemisia* whiteflies, under low-ambient-humidity conditions in Texas (Wraight et al., 2000). Boulard et al. (2001) demonstrated a significant boundary layer effect (with an air RH increase reaching 20 points at 5 mm from the tomato leaf under-surface during day time), showing a real disconnection between the air climate measurements, performed in the middle of the glasshouse or inside the plant canopy, and the microclimate prevailing in the habitat of the targeted insects. Environmental requirements for whitefly infection was then re-examined under controlled conditions on non excised leaves in regulated humidity sandwich cell systems (C. Vidal, J. Fargues, M. Rougier & N. Smits, unpublished data). Variation in
humidity-regulated air flow circulating over the under leaf surface in sandwich cells, would permit to model the laminar air flow characteristics (J.C. Roy, T. Boulard, J. Fargues, M. Rougier & N. Smits) and the boundary layer effect, obviously dependent in situ on both transpiration of the plant and the wind velocity (Boulard et al., 2001). Thus, the latter case-study provides definitive arguments for taking into account factors determining conditions of the boundary layer of the under leaf surface (Schuepp, 1993; Pachevpsky et al., 1999). The implication of these findings have to be integrated in the global approach of the modeling of the whole pathosystem.

From pathosystem to agroecosystem: implementation in the IPM context

As stated above, modeling of beneficial pathosystems involving entomopathogenic fungi has to be improved in taking into account specific constraints of the targeted agroecosystem. Moreover, in order to evaluate the efficacy of a fungus as a biocontrol agent, it is also necessary to consider that its effectiveness depends not just on its capacity to kill pests, but also on its capacity to reproduce on pests and thereby continue and compound its killing action (Thomas & Wood, 1997). The ecological approach includes, in addition of these, biotic factors relating to behavioral traits, as showed for aphids (Knudsen & Schotzko, 1999) and locusts (Magalhaes et al., 2000), for example. These efforts are thus aimed at determining how to better utilize entomopathogenic fungi as microbial control agents within an IPM framework. Finally, pathosystem models and epizootiological models have to be evaluated and validated under crop production conditions. Their implementation in agroecosystem models may used to build operational models based on decision making in IPM crop protection. It is clear that, in spite of their practical interest, these operational models on decision making in crop protection focused on the optimization of the pest control using entomopathogens and natural enemies (parasitoids and predators), are still now very limited (Van Lenteren, 1987; Bakker, 1995).

Climatic constraints as a base for pathogen selection

Characterization of pertinent environmental constraints in targeted pest habitats may be useful for optimizing selection of fungal isolates well-adapted to specific environments. To date, most of the isolate selection has focused on the ability of pathogens to infect their target hosts, i.e. by means of virulence and host specificity bioassays (Butt & Goettel, 2000). Knowledge of fungal growth according to temperature could be considered as a logical starting point for strain selection based on the tolerance to temperature constraints (Fargues et al., 1997a; Ouedraogo et al., 1997; Vidal et al., 1997). In many cases, the adaptability to temperature is related to the geoclimatic origin of the isolates. Thus, in P. fumosoroseus, Indian and Pakistani isolates are significantly more tolerant to upper temperature than European isolates (Vidal et al., 1997). UV light protectants in conidial formulations are used to counteract sunlight effects, but only limited protection is achieved. That is why it is interesting to look at a second possible strategy in selecting fungal isolates with increased resistance to irradiation. In Hyphomycetes, conidia of M. flavoviride isolates are the most UV-resistant and that of P. fumosoroseus the most UV-susceptible). Within each species, the difference in survival rates observed among isolates exposed to a given amount of UV light was 10 fold (Fargues et al., 1996).
The molecular biology and population genetics approaches

Recent advances in molecular biology and population genetics of entomopathogenic fungi provide promising perspectives for isolate identification, strain improvement, isolate grouping, epidemiological tracking, and for looking at genomic variations with a thorough understanding of their potential ecological (Couteaudier et al., 1998). On a mycoinsecticide registration viewpoint, they provide interesting information for strain labeling and for the risk assessment relate to gene exchanges.

Because of worldwide endemic occurrence of hyphomycetes well-known as potential microbial control agents, e.g. *Beauveria* spp. and *Metarhizium* spp., it is necessary to distinguish genotypes at the sub-specific level. Nowadays, useful molecular tools are being applied successfully to entomopathogenic fungi in order to analyze the consequences of intraspecific polymorphism. Genetic probes, such as mtDNA-based PCR primers (Typas et al., 1998), telomeric DNA-based PCR primers (Couteaudier & Viaud, 1997; Inglis et al., 1999), and specific microsatellite markers (Enkerli et al., 2001) provide interesting tools for identifying specific isolates, monitoring releases of biocontrol strains close to indigenous strains, and for investigating dynamics of clonal populations. RAPD-PCR and AFLP can be also used for monitoring the spread and fate of highly virulent isolates introduced as biopesticide in specific environments (Ozino et al., 1998; Boucias et al., 2000; Obornik et al., 2000; Bidochka, 2001). Bidochka et al. (1995) found clear differences between three *Entomophaga grylli* pathotypes based on pathotype-specific RAPD fragment patterns. The use of these cloned DNA probes showed evidence for infection preferences by *Entomophaga grylli* pathotypes in different life stages of grasshopper species (Bidochka et al., 1996). Interestingly, Bidochka (2001) reported, as a case-study in monitoring a fungal biocontrol release, the different aspects of this molecular approach.

There is no consensus about whether an indigenous isolate, originating from the target host, or an exotic fungus of different host and geographical origin, are likely to provide the most suitable candidate for development as a mycoinsecticide (Charnley, 1997). This debate, of practical importance, require basic information on the grouping effect of selection pressures relate to the insect host on fungal populations. Thus, the polymorphism within entomopathogenic hyphomycetes and the correlation between ribosomal haplotypes and some host insects have been recently established (Couteaudier et al., 1998). Using ITSs polymorphism within *Beauveria brongniartii*, Neuvéglise et al. (1994) distinguished one specific group, which contained all the isolates from *Hoplochelus marginalis* collected in Madagascar and La Réunion. Strains of this fungal species isolated from *Melolontha melolontha* clustered in a single 28S rDNA group I introns (Neuvéglise et al., 1997). A correlation between ribosomal haplotypes and host insects was also found within *B. bassiana* populations virulent to *Sitona* weevils and those virulent to *Ostrinia nubilalis* (Maurer et al., 1997; unpublished data reported by Couteaudier et al., 1998). However, speculations on host selection pressures appeared to be highly dependent on the variability of the fungus DNA markers used, and the fungus-insect pathosystem considered. Thus, various authors found no convincing evidence of host specific selection, in *P. fumosoroseus* (Tigano-Milani et al., 1995), *Paecilomyces farinosus* (Chew et al., 1997), and *Metarhizium* spp. (Curran et al., 1994), for example. Another approach to evaluate fungal population structure is the investigation of vegetative compatibility among fungal isolates. Couteaudier & Viaud (1997) analyzed vegetative compatible groups in *B. bassiana* and found a correlation between VCGs and RFLP patterns using a telomeric probe.

Because of recent advances in understanding of the molecular basis of pathogenicity and virulence (Clarkson et al., 1998), recombinant DNA techniques can be applied in strain
improvement programs (St Leger & Joshi, 1997). St Leger & Screen (2001) reported that their Expressed Sequence Tag (EST) project to identify the full range of genes expressed during the infection process by a wide-host-range strain and a narrow-host-range strains of *M. anisopliae*, has allowed them to identify thousands of genes expressed for pathogenicity (www.http:tegr.umd.edu). St Leger *et al.* (1996) developed the first genetically improved entomopathogenic fungus in increasing *M. anisopliae* virulence by overexpression of the gene encoding the regulated cuticle-degrading Pr1 protease by transformation of selected fungal strains. Improving virulence focused on the increasing of the speed with which isolates are able to infect and kill the host, is a realistic goal. In contrast, little is still now known on molecular basis of host specificity for enlarging fungal strain host ranges, in order to increase the potential market size. Fungal tolerance to environmental constraints appears to be governed by polygenic mechanisms too complex to be readily amenable to genetic manipulation (Charnley, 1997; St Leger & Screen, 2001).

In the absence of a sexual stage, natural mitotic recombinations, involved in parasexual exchanges between strains vegetatively compatible within clonal lineage, seem to occur inside co-infected insect hosts (Couteaudier & Viaud, 1997; Couteaudier *et al*., 1998). Combinations of desired genes can be achieved by anastomosis and somatic hybridization (parasexual crossing) (Messias & Azevedo, 1980; Bello & Paccola-Meirelles, 1998) or by means of protoplast fusion techniques (Viaud *et al*., 1998). However, St Leger & Screen (2001) underlined that the stability of transformed biotrophic fungi have to be improved. Exciting challenges over the prospects for genetic engineering in entomopathogenic hyphomycetes may be also tempered by an awareness of the necessary regulatory hurdles that will need to be climbed in order to get a transgenic fungal isolate approved for release (Charnley *et al*., 1997).

**The phenotypic variability approach**

If some environmental constraints may be partially overcome by both strain selection and manipulation based on genetical variability, there is a great phenotypic variability, which has been under-estimated and so under-utilized. This phenotypic variability is highly dependent on nutrition conditions and that which most physiological studies have been devoted to the propagule production. In fact, ecophysiological manipulations could be developed for improving strain virulence and environmental-stress tolerance. For example, one host-passage of a *M. anisopliae* isolate provided conidia hundredfold more virulent that in vitro-produced conidia (Fargues & Robert, 1983a). Submitted to accelerated biodegradation conditions in experimental soil, the persistence of the conidia collected on host-cadavers was threefold longer than that of in vitro-produced conidia (Fargues & Robert, 1991). Comparison of the influence of different nitrogen sources showed that one pepton provided an increase in virulence of in vitro-produced conidia, which was close to that induced by one host-passage (Fargues & Robert, 1983b). Because of the key importance of the quality of fungal propagules, the physiological approach for improving the ecological fitness of fungal microbial control agents might be developed intensively on fundamental bases (Magan, 2001).

**Conclusion**

Because of recent improvements, mycoinsecticides appear to be excellent candidates as components of IPM in an increasing number of agroecosystems. In conclusion, fungal pathogens offer a great potential for microbial control. They remain one of the greatest
untapped resources for pest management. New challenges for realizing their potential mainly consists of tackling environmental constraints for fungi. It is clear that fundamental understanding of adaptative mechanisms and pathosystem dynamics is still required, before the benefits of technological advances (production-formulation) and that of application strategies are realized.

Acknowledgements

The author is very grateful to Dr W. Meikle (USDA-ARS, European Biological Control Laboratory, Montpellier, France) for critical review of the manuscript and providing useful comments.

References


Shah, P.A. & Goettel, M. 1999: Directory of Microbial Control Products and Services (www.sipweb.org/directorymcp/directory.htm), Society for Invertebrate Pathology, Gainesville, Florida, USA.
Entomopathogenic fungi
Fungus infection of the chicken mite *Dermatomyssus gallinae*

Tove Steenberg, Ole Kilpinen
The Danish Pest Infestation Laboratory, Ministry of Food, Agriculture and Fisheries, Skovbrynet 14, DK-2800 Kgs. Lyngby, Denmark

**Abstract:** Although the blood-sucking mite *Dermatomyssus gallinae* is a major pest in the European poultry production, no information is available on the susceptibility of these mites to entomopathogens. Therefore, a survey of naturally occurring entomopathogens in mites from different parts of Europe was recently initiated. Furthermore, infection experiments with entomopathogenic fungi were carried out in the laboratory. Two isolates of each of the species *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* were tested in assays in which the mites were inoculated with high doses of conidia. All isolates were virulent, and some isolates caused high mortalities within the first 5 days of incubation. Further studies will include transmission and persistence experiments.

**Key words:** *Dermatomyssus gallinae*, chicken mite, entomopathogenic fungi, natural occurrence, isolate screening, *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*

**Introduction**

The chicken mite, *Dermatomyssus gallinae*, is prevalent in poultry systems throughout Europe. Heavy infestations may cause considerable economic losses and welfare problems in the poultry production. Besides the direct physiological effect chicken mites may also act as carrier of several important disease-causing agents, e.g. Salmonella (Zeman et al., 1982), spirochaetosis (Hungerford & Hart, 1937), and encephalitis (Durden et al., 1992). Some of these survive in the mite for several months, thus forming a potential source of reinfection of new flocks, as the mite can live without feeding for up to 9 months (Nordenfors et al, 1999). Chicken mites are also known to cause puritic dermatosis in humans (Baselga et al., 1997) and may create serious problems for workers in the poultry industry due to the nuisance of mites crawling on the skin.

Current attempts to control chicken mites in conventional poultry systems are mainly based on persistent synthetic insecticides. No efficient control methods are available in organic systems, where farmers must rely on thorough cleaning between flocks. Experience from conventional systems however has shown that control of chicken mites through cleaning alone is not effective. The enormous reproduction potential of the mites (Maurer & Baumgärtner 1992) implies that even small numbers of surviving mites can result in high population levels within the production cycle. Thus, there is a need for control methods which are applicable while the poultry is present in the houses, and this is a major constraint to many of the existing control measures.

Entomopathogenic fungi infect their hosts through the cuticle, and several species have wide host ranges. Therefore, entomopathogenic fungi may be expected to have a high control potential for blood-feeding mites like *D. gallinae*. We have recently initiated work aiming at selecting fungal pathogens for chicken mite control. This includes a survey of the natural occurrence of entomopathogens in chicken mite populations in different parts of Europe as
well as selection of fungal isolates suitable for application in the poultry house environment. This paper outlines the results obtained in a preliminary screening of fungal isolates against *Dermanyssus gallinae*.

**Materials and methods**

A total of six fungal isolates were tested against *D. gallinae*. Three of the isolates (one of each of the following species: *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus*) were included as they had previously shown high virulence to other pests such as flies and beetles (T. Steenberg, unpublished data). These three isolates originated from herbivorous beetles (*B. bassiana* and *M. anisopliae*) or from a cockroach (*P. fumosoroseus*). In addition, three other isolates of the species listed above, originating from a mite, a spider, and a tick respectively, were included in the test.

Adult female chicken mites were taken from a laboratory culture reared on caged poultry, and blood-fed 24 h prior to the inoculation. Groups of 10 chicken mites (10 replicates per isolate) were placed in petri dishes with sporulating fungal cultures for 2-3 min. This ensured maximum exposure to fungal spores. Control mites were left untreated. The mites were then transferred to glass tubes plugged with cotton wool, and incubated in plastic boxes at 25 °C/85 % RH for 16 days. Mortality was recorded at 1-3 day intervals.

**Results and discussion**

All six isolates were pathogenic to chicken mites (Fig. 1), so *D. gallinae* appears susceptible to members of this particular group of entomopathogens. The isolates originating from related hosts were not among the most virulent isolates, while the most virulent isolate was *B. bassiana* originally isolated from a beetle. This isolate caused 60 % mortality within 5 days of inoculation, and 90 % mortality after 16 days of incubation. Further screening of a wider range of fungal isolates will show whether even more virulent isolates can be selected.

![Mortality of *Dermanyssus gallinae* after inoculation with conidia of different entomopathogenic fungi.](image)

**Fig. 1.** Mortality of *Dermanyssus gallinae* after inoculation with conidia of different entomopathogenic fungi. Explanations: 0 – untreated control, 1 – *B. bassiana*, 2 – *P. fumosoroseus*, 3 – *M. anisopliae*, 4 – *M. anisopliae*, 5 – *P. fumosoroseus*, 6 – *B. bassiana*. 

0% 20% 40% 60% 80% 100% 0 5 10 15 Day Mortality
The ongoing survey of naturally occurring entomopathogens will show whether entomopathogenic fungi or other groups of entomopathogenic microorganisms are present in mite populations in poultry houses. Other studies will focus on the persistence of the selected fungi in the poultry house environment, and on transmission of the infection in mite populations.

References

Entomopathogenic fungi
Mycoinsecticides useful against lepidopteran pests in pulses

Mukund Deshpande, Ajit Chandele1, Pallavi Nahar, Ashok Hadapad, Ganesh Patil, Vandana Ghormade, Siegfried Keller2, Urs Tuor3
Biochemical Sciences Division, National Chemical Laboratory, Pune- 411 008, India
1 Department of Entomology, College of Agriculture, Pune- 411 005, India
2 Eidgenössische Forschungsanstalt für Agrarökologie und Landbau FAL, Postfach, CH-8046 Zürich - Reckenholz, Switzerland
3 Eidgenössische Technische Hochschule, Institut für Mikrobiologie, ETH Zentrum/LFV, Schmelzbergstr. 7, CH-8092 Zürich, Switzerland

Abstract: Under the biopesticides programme of the Indo-Swiss Collaboration in Biotechnology (ISCB), more than 50 different fungal strains have been isolated from the soil by dilution plate and Galleria baiting methods. A few strains (Nomuraea sp.) were isolated from naturally infected Spodoptera found on sugar beet. Using a bioassay with Helicoverpa armigera larvae, 4 promising strains (2 strains of Metarhizium sp., 1 strain of Nomuraea sp. and 1 strain of Beauveria sp.) were selected on the basis of % mortality (> 70 % in 7 days) for further large scale production and field trials. The biochemical characterization with regard to cuticle degrading enzymes, effect of different environmental conditions on the spore germination, and growth on solid substrates, such as beaten rice, sorghum, wheat and barley for maximum sporulation, were also studied. The preliminary trials for the control of Helicoverpa in the chick pea fields using spores of these 4 strains were promising.

Key words: biocontrol, entomopathogenic fungi, lepidopteran pest control, pulses

Introduction

Control of insect pests, particularly by their natural enemies comprising parasites, predators and pathogens in agro-ecosystems is a continuous process. Fungi constitute a large group of more than 500 species which can parasitize insects. Most of the taxonomic groups contain entomopathogenic genera, such as Metarhizium, Beauveria, Verticillium, Nomuraea, Entomophthora, Neozygites, to name a few (Deshpande, 1999). Fungal biocontrol agents are promising because they act without ingestion, can be mass-produced very easily and are quite host specific.

Among the lepidopteran pests, the gram pod borer, Helicoverpa armigera Hübner (Lepidoptera: Noctuidae), is a polyphagous pest which attacks more than 180 host plants (Reed & Pawar, 1981). The present investigations deal with isolation and identification of entomopathogenic hyphomycetous strains for the effective control of Helicoverpa on pulses.

Material and methods

All the hyphomycetous cultures (56 strains) were isolated using the single spore isolation method. They were maintained by sub-culturing every 15 days on PDA (potato dextrose agar) and/ or SMYP (Sabouraud malt yeast extract peptone agar) at 28 °C, as well as by keeping agar plugs in 10 % glycerol at -20 °C. Strains were isolated from soil, either by soil dilution
plating or *Galleria* bait method, as described by Goettel & Inglis (1997), or from naturally infected insects (infected *Spodoptera* larvae found in sugar beet fields).

**Isolation of entomopathogenic hyphomycetes from soil by soil dilution plating method**

A sieved soil (20 g) mixed with 100 ml of 0.185 % tetra sodium pyrophosphate was incubated on a shaker at 28 °C for 3 h, and allowed to settle for 15 sec. The suspension (0.1 ml) was spread on semi-selective medium (glucose, 2 %; peptone, 1 %; agar, 1.8 %) with antibiotics such as tetracyclin (0.005 %), streptomycin (0.06 %), cycloheximide (0.005 %) and dodine (0.01 % v/v) added after autoclaving. The plates were incubated at 28 °C for 10-15 days in the dark. The colonies developed were plated on PDA and/or SMYP. Identification was carried out using slide culture technique on the same media.

**Isolation using the Galleria bait method**

A 60 g sample of soil was filled in a plastic vial (4.2 x 6.5 cm) and 4 *Galleria mellonella* (greater wax moth) larvae were added and incubated in the dark at 25 °C. For the first 5 days, the tubes were turned up side down daily to keep the larvae moving in the soil. After 14 days, the larvae were examined and attributed to one of the 4 following categories: Healthy, Infected with fungus, Infected with other pathogens, or Dead due to other factors. The hyphomycetous fungi were isolated from infected larvae on selective medium as described above.

**Spore germination studies**

The spore germination of 4 selected strains suspended in 0.1 % Tween 80, was studied on 3 different media, i.e. PDA, SMYP agar and chitin agar (chitin, 0.5 %; agar, 2 %) at 28 °C for 48 h.

**Insect bioassay**

The insect bioassay was performed using the dipping method (Goettel & Inglis, 1997), with *Helicoverpa armigera* larvae as targets. Conidia (at the concentration of 1.5 x 10⁶) were suspended in 30 ml of water containing 0.1 % Tween 80.

**Estimation of enzyme activities**

The chitin modifying enzyme activities, viz. chitinase and chitosanase were estimated using acid swollen chitin and acid swollen chitosan, respectively as described earlier (Patil *et al.*, 2000). The chitin deacetylase (CDA) activity was estimated using ethylene glycol chitin as the substrate. Deacetylation was visualized using 3-methyl-2-benzothiazoline (MBTH) as described by Kauss & Bauch (1988). The enzyme unit was defined as the amount of enzyme required to produce 1 µmole of product per min.

**Results and discussion**

**Field survey and collection of soil samples**

In the western-central region of India, Maharashtra State, pulses, sugarcane, vegetables are being cultivated in two different seasons. Heavy infestation of lepidopteran pests has been observed occasionally. Therefore, field surveys for the isolation of entomopathogenic fungi from various parts of Pune and Ahmednagar districts were conducted in September 2000. In each location five soil samples were collected for the soil dilution plating as well as for the *Galleria* bait method.

**Isolation of entomopathogenic Hyphomycetes**

A total of 56 entomopathogenic hyphomycetous strains were isolated. Among them, 22 strains of *Metarhizium* sp. and 8 strains of *Paecilomyces* were isolated from soil by dilution plate method, while 7 strains of *Beauveria* sp. and 4 strains of *Paecilomyces* sp. were isolated
using the *Galleria* bait method. Furthermore, 15 strains of *Nomuraea* sp. were isolated from *Spodoptera* larvae found infected in a sugar beet field.

**Selection of the most promising strains**

Among the 56 isolates, only 4 strains, 2 of *Metarhizium* sp. (M1311 and M34412), 1 of *Beauveria* sp. (B3301) and 1 of *Nomuraea* sp. (N812), showed >70 % mortality within 7 days against *Helicoperva armigera* larvae, irrespective of the isolation method. Furthermore, *Nomuraea* isolates also showed high % mortality (>80 %) with *Spodoptera* as test insect. Among the majority of the remaining isolates, 50-70 % mortality was recorded within 7 days after treatment. However, some isolates caused <50 % mortality. In addition to the % mortality, the fungal growth on the insect host was considered as one of the parameters for the selection of the strains for further experiments (data not shown).

**Solid state fermentation of the identified isolates**

Among the tested substrates, sorghum supported maximum sporulation in the case of *Metarhizium* sp. and *Beauveria* sp. strains. In the case of *Nomuraea* sp., the maximum sporulation was observed on barley (Table 1).

Table 1. Solid state fermentations for maximum sporulation of entomopathogenic fungi (values given as conidia/ g substrate).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>M1311 <em>Metarhizium</em> sp.</th>
<th>M34412 <em>Metarhizium</em> sp.</th>
<th>N812 <em>Nomuraea</em> sp.</th>
<th>B3301 <em>Beauveria</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beaten rice</td>
<td>1.1 x 10^6</td>
<td>3.2 x 10^5</td>
<td>3.1 x 10^4</td>
<td>4.5 x 10^5</td>
</tr>
<tr>
<td>Cooked rice</td>
<td>7.2 x 10^5</td>
<td>1.1 x 10^6</td>
<td>6.2 x 10^4</td>
<td>4.1 x 10^5</td>
</tr>
<tr>
<td>Sorghum</td>
<td>1.5 x 10^7</td>
<td>1.6 x 10^7</td>
<td>0.4 x 10^5</td>
<td>5.4 x 10^5</td>
</tr>
<tr>
<td>Corn</td>
<td>9.8 x 10^5</td>
<td>6.2 x 10^5</td>
<td>1.3 x 10^5</td>
<td>3.6 x 10^4</td>
</tr>
<tr>
<td>Barley</td>
<td>3.7 x 10^6</td>
<td>0.72 x 10^6</td>
<td>3.7 x 10^5</td>
<td>3.3 x 10^5</td>
</tr>
</tbody>
</table>

Table 2. Germination of spores of the selected strains on different media.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Medium used</th>
<th>% spore germination</th>
<th>Time required for germination (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1311</td>
<td>PDA</td>
<td>54.8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>SMYP</td>
<td>75.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Chitin agar</td>
<td>82.2</td>
<td>18</td>
</tr>
<tr>
<td>M34412</td>
<td>PDA</td>
<td>90.1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>SMYP</td>
<td>91.2</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Chitin agar</td>
<td>89.0</td>
<td>18</td>
</tr>
<tr>
<td>B3301</td>
<td>PDA</td>
<td>84.0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>SMYP</td>
<td>72.0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Chitin agar</td>
<td>72.0</td>
<td>15</td>
</tr>
<tr>
<td>N812</td>
<td>PDA</td>
<td>83.8</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>SMYP</td>
<td>56.1</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Chitin agar</td>
<td>65.9</td>
<td>48</td>
</tr>
</tbody>
</table>
**Spore germination studies of the identified strains**

In case of the *Metarhizium* sp. strains, the maximum spore germination (82.2 % and 91.2 %) was recorded with chitin agar and SMYP, respectively after 18 h. For *Beauveria* sp. and *Nomuraea* sp, the maximum spore germination (>80 %) was observed on PDA after 15 and 36 h, respectively (Table 2).

**Extracellular enzyme activities of the identified isolates**

The main structural component of the cuticle is chitin, a β-1,4-linked polymer of N-acetylglucosamine. The enzymatic hydrolysis of chitin facilitates the entry of the fungal pathogen into the insect host. The constitutive production of chitin metabolising enzymes, viz. chitinase, chitosanase and chitin deacetylase, was studied in all the four strains. Interestingly, *Metarhizium* and *Nomuraea* isolates constitutively produced high levels of extracellular chitin deacetylase and chitosanase activities suggesting their involvement in the initial penetration of the insect cuticle (data not shown).

**Acknowledgements**

The work presented is part of the Indo-Swiss Collaboration in Biotechnology ISCB (Project ISCB-BP1). We thank the Department of Biotechnology, New Delhi, India and the Swiss Development Corporation, Switzerland for their financial support.

**References**


Impact of Beauveria brongniartii and Metarhizium anisopliae (Hyphomycetes) on Lumbricus terrestris (Oligochaeta, Lumbricidae)

A. Hozzank, S. Keller, O. Daniel, Ch. Schweizer
Federal Research Station for Agroecology and Agriculture, Reckenholzstr. 191, 8046 Zürich, Switzerland

Abstract: Two different studies were performed to investigate the impact of the entomopathogenic fungus B. brongniartii on earthworms. First the impact of the entomopathogenic fungus B. brongniartii on natural populations of earthworms was studied. Worms were collected in plots treated with the commercial product of B. brongniartii and in plots without treatment. Species of the following genera were found: Lumbricus, Nicodrilus, Allolobophora and Octolasion. No differences were found in species diversity, abundance and biomass in plots treated with B. brongniartii in comparison with untreated plots.

For the second investigation worms were kept in plastic boxes and subjected two the following treatments: 1. no additives, 2. fresh barley grains, 3. barley grains with B. brongniartii (commercial product) and 4. barley grains with M. anisopliae. The bioassay lasted two months. Additional food was added only at the beginning of the second month. After one month mortality and weight changes of L. terrestris, the position of grains and spore density in the test boxes were checked. After a further month mortality was checked again, as well as spore densities in the soil and in the faeces. Only three L. terrestris died in total irrespective of their treatment. None of them showed any signs of fungal infection.

Key words: biological control, earthworms, entomopathogenic fungi, Lumbricus, Beauveria, Metarhizium, bioassay, infection

Introduction

Every year the larvae of Melolontha melolontha L. (Coleoptera: Scarabaeidae) cause severe damage in agricultural areas. Since the early nineties this pest is controlled with the entomopathogenic fungus B. brongniartii. The commercial product consists of barley grains colonised with this fungus. So far there are no investigations about the effect of B. brongniartii on earthworms. Fungi as well as earthworms play an important role in soil community. Especially earthworms are believed to be irreplaceable in agricultural soils because they assist drainage, aeration and turnover of organic material. On the other hand they are important vectors of microbial propagules since they live in the upper part of the soil and transport a large amount of soil through their bodies. Earthworms feed preferentially on fungi associated with plant remains (Piearce, 1978; Moody, 1995; Schönholzer, 1999). Previous work showed that number of spores in the soil increased after the passage through the gut of earthworms (Tiwari & Mishra, 1992).

Two questions should be answered by the following study: 1. Is there an influence of the application of B. brongniartii grains on earthworm populations in the field; 2. Do B. brongniartii and M. anisopliae have the ability to infect L. terrestris in bioassays?
Material and methods

The field investigations were undertaken at two different sites in the Swiss midland (Thurgau and Matten). Each of these sites has been treated a year before with a commercial product of *B. brongniartii* (Beauveria Schweizer, a registered product of E. Schweizer Samen AG, Thun). Worms were collected by hand without using any chemicals in two sampling holes (50x50x35) per plot. At both sites four untreated plots and four treated plots with fungal densities of 3800 CFU/g soil (Thurgau) and 260 CFU/g soil (Matten) were checked, resulting in eight samples per treatment. Worms were transported in plastic boxes with soil. In the laboratory they were killed in hot water (60 °C) and stored in formaldehyde (4 %) for three weeks to get a constant weight. After three weeks they had been identified using morphological features.

Bioassays were done with juveniles of *L. terrestris* obtained from a meadow near the Swiss Federal Research Station for Farm Management and Agricultural Engineering at Tänikon. The plots were treated with formalin (10 litres of 10 % formalin per square meter), which forced the worms to come to the surface. Worms were washed twice with fresh water, placed in plastic boxes with soil and stored at 10 °C for two weeks. Individuals weighing 0,5-3,0 g were placed individually in 1000 ml test boxes. They contained 550 g native sieved soil slightly compressed by centrifugation to achieve a water tension of 100 cm and a sedimentation density of 1. The boxes with the worms were stored under experimental conditions to get them adapted to soil humidity. After a week they were randomly divided into 6 groups with 20 *L. terrestris* each and subjected to the following treatments: BU: no additives, BK: 10 fresh barley grains, B: 10 barley grains with *B. brongniartii* (commercial product), MU: no additives, MK: 10 fresh barley grains, M: 10 barley grains with *M. anisopliae*. The bioassay lasted two month. Additional food was added only at the beginning of the second month. After a month mortality and weight change of *L. terrestris*, the position of the grains and the fungus densities in the test boxes was checked. After a further month mortality was checked again, as well as the fungus densities in the soil and in the excrements. From treatments B and BK 10 g faeces and 10 g soil each were taken and shaked for 3 hours. Soil suspensions were diluted up to $10^{-4}$ if necessary and plated on selective medium, two replicates were done. The data were statistically analyzed either with a paired t-test or with an analysis of variance (ANOVA).

Results and discussion

Field investigations

Specimens of the following genera of earthworms were found: *Lumbricus, Nicodrilus, Allolobophora* and *Octolasion*. No differences were found in species diversity, abundance and biomass at sites treated with *B. brongniartii* in comparison with untreated sites (Fig. 1). However, the two sites differed in species composition and density, which is attributed to different geographical regions and soil type.

Bioassays

Three *L. terrestris* died, two of them in the first month in treatment BK (pure barley grains), and one in the second month in treatment M (barley grains with *M. anisopliae*). None of them showed any signs of fungal infection. The weights at the beginning of the bioassay and a month later as well as the weight changes are given in Table 1. In the *Beauveria* treatment the weight change was largest in the absence of either grains, in the *Metarhizium* treatment it was largest in presence of fungus colonized grains and in absence of grains.
Lumbricus sp.

L. rubellus

L. terrestris

Nicodrilus sp.

N. longus

N. nocturnus

N. caliginosus

Allolobophora

A. chlorotica

ch.

A. icterica

A. rosea

Octolasion sp.

O. tyrtaeum

lact.

species

mean number

HF-Ma

F-TG

U-TG

U-Ma

Fig. 1. Mean number of individuals. HF-MA, F-TG: treated sites, U-TG, U-MA: untreated.

Table 1. Weights of *L. terrestris* at the beginning of the bioassay, after one month and the weight difference. Different letters within the same frame refer to statistical differences (ANOVA).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight (g): mean (standard deviation) and statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>start</td>
</tr>
<tr>
<td><strong>Beauveria</strong></td>
<td></td>
</tr>
<tr>
<td>BU</td>
<td>2.35 (0.97) a</td>
</tr>
<tr>
<td>BK</td>
<td>1.83 (0.64) a</td>
</tr>
<tr>
<td>B</td>
<td>2.01 (0.98) a</td>
</tr>
<tr>
<td><strong>Metarhizium</strong></td>
<td></td>
</tr>
<tr>
<td>MU</td>
<td>2.22 (0.77) a</td>
</tr>
<tr>
<td>MK</td>
<td>2.42 (1.08) a</td>
</tr>
<tr>
<td>M</td>
<td>2.33 (1.07) a</td>
</tr>
</tbody>
</table>

The weight loss in the *Metarhizium* treatment is attributed to the barley grains, which were completely colonized with the fungus, thus leaving no food for the earthworms.

In the treatment with pure barley grains a single colony of *B. brongniartii* was present in both soil and faeces. 7.8 colonies per Petri dish of *M. anisopliae* were found in the soil corresponding to 1019 CFU/g soil (Table 2). The faeces contained 3.1 times less, namely 2.5 colonies per Petri dish corresponding to 323 CFU/g. The treatment with commercial *B. brongniartii* resulted in 6.9x10^3 colonies per Petri dish corresponding to 9.1x10^5 CFU/g soil. The fungus concentration in the faeces was 2.9 times higher and reached 19.8x10^3 colonies per Petri dish corresponding to 25.8x10^5 CFU/g. At the same dilution (10^3), three colonies of *M. anisopliae* were found in the Petri dish with soil suspension and none in the faeces suspension.

The results of the treatment B (grains with *B. brongniartii*) demonstrated that *L. terrestris* ingest fungal material together with their food. The concentration in the faeces was about three times higher than that of the surrounding soil (Table 2). Tiwari & Mishra (1992) also found an increased mean number of fungal propagules in the cast of earthworms. This enrichment within digestive tract of earthworms may be due to a selective feeding on *Beauveria* spores. The activity of the earthworms also led to a distribution of fungus material. Nearly a million CFU/g were found in the surrounding soil. Thorpe *et al.* (1996) showed in
their investigation that the transport of bacteria only occurred in the presence of *L. terrestris*. Therefore these animals can to be seen as vectors to disseminate fungal material.

Table 2. Survival of *L. terrestris* and mean number of colonies of *B. brongniartii* and *M. anisopliae* in soil and faeces of earthworms. (treatments: 1, pure barley grains; 2, grains with *B. brongniartii*; 3, grains with *M. anisopliae*)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% living <em>L. terrestris</em></th>
<th>Mean number of colonies per Petri dish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>B. brongniartii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>6910</td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>—</td>
</tr>
</tbody>
</table>

Neither in control plots, where *B. brongniartii* was applied at dosages used for control purposes, nor in bioassays, where dosages reached about 10-100 fold increased concentrations (H. Strasser, 1999; P. Kessler, pers. comm.), earthworms were not harmed. We can conclude that the application of *B. brongniartii* for white grub control has no negative effects on earthworms. On the contrary especially *L. terrestris* is able to disseminate fungal material. It even may be responsible for the distribution of fungal material from the inoculation points to the surrounding soil and therefore able to increase the possibilities for white grubs to come in contact with this biocontrol agent. It is also possible that the faeces protect the fungal material from inactivation by biotic and abiotic influences.

Acknowledgement

The authors thank all colleagues of the Federal Research Station for Agroecology and Agriculture, Zürich, for their support in the development of techniques concerning the bioassays.

References

Growth interactions between nematophagous and entomopathogenic fungi

M. L. López-Serna, L.V. López-Llorca, J. Salinas
Departamento de Ciencias Ambientales y Recursos Naturales, Universidad de Alicante, Ap. Correos 99, 03080 Alicante, Spain

Abstract: Dual cultures of both entomopathogenic (EF) and nematophagous (NF) fungi were performed on growth media of increasing nutrient content. Growth inhibition of both EF and NF increased with the nutrient content of the media used. Evidence of an antagonistic role of diffusible metabolites was found. Changes in hyphal morphology upon colony merging were assessed under poor nutrient conditions. Microscopical studies of the zone of contact of fungal colonies showed no signs of direct mycoparasitism. Our results suggest that growth interference between nematophagous and entomopathogenic fungi occurs in vitro only under high nutrient conditions.

Key words: Entomopathogenic fungi, nematophagous fungi, fungal metabolites, mycoparasitism

Introduction

One of the objectives in the development of biological control is to enhance the population of an antagonist when it is already present in the environment, or to introduce it if it was absent. However, this new situation must be achieved with minimum disturbance. The aim of this study is justified by the fact that most of the nematophagous or entomopathogenic fungi are facultative pathogens, and they spend their non-infective period in the soil. The effects of the application of biological control agents (BCAs) on non-target organisms such as plant pathogens (Benhamou & Brodeur, 2000) or entomopathogenic nematodes (Kaya & Koppenhofer, 1996) has been reported. Compatibility between entomopathogenic fungi and insect predators or parasitoids has been documented (Danfa et al., 1999). On the contrary, no information is available on the effects of entomopathogenic fungi on nematophagous fungi (Bourne & Kerry, 1999), and vice versa.

Material and methods

Fungal isolates and culture conditions
Five isolates of entomopathogenic fungi, one of the following species (Paecilomyces fumosoroseus, Metarhizium anisopliae, P. farinosus, Beauveria bassiana and Verticillium lecanii) were grown on Corn Meal Agar (Oxoid) at 25 °C in the dark. The nematophagous fungi used were nine isolates of Verticillium chlamydosporium (75, 64, 144, 76, 149, 52, 65, 84 and 123) and a Paecilomyces lilacinus isolate, which were grown as entomopathogens.

Colony interactions and growth patterns
Tests were carried out in triplicate in Petri dishes containing 1 % w/v Water Agar (WA, low nutrient conditions), Corn Meal Agar (CMA, medium nutrient conditions) and Sabouraud Dextrose Agar (SDA, high nutrient conditions). Inoculated dishes were incubated at 25 °C in the dark. The inoculum consisted of discs (4.5 mm diameter) taken from the growing margin of fungal cultures. Interactions between nematophagous (NF) and entomopathogenic fungi
(EF) were assessed as described under each nutrient conditions. Plates were inoculated with the two fungi simultaneously, at a distance of 4.5 cm. Plates with EF or NF growing alone (for differences on growth patterns) were included as controls. Radius colonies were measured every 2-3 days. Zones where fungal growth was abnormal, defined by changes in pigmentation and growth pattern compared to controls, were scored when colonies merged.

**Hyphal interactions**

Hyphal interactions between EF and NF were observed under low nutrient conditions using a cellophane overlay technique. Colony interaction experiments were carried out as described above but with the agar surface covered with a sterile cellophane membrane. In the day of contact of fungal colonies, a cellophane square (0.5 cm²) was removed from the contact zone and placed on a microscope slide. Signs of coiling, adpressed growth, vacuolation, granulation, formation of appressoria and penetration in hyphae of the two fungi were scored.

**Effects of fungal metabolites on growth: fungal metabolites test**

The effect of fungal metabolites on growth was tested according to Horvath et al. (1995). Mycelial plugs of a given fungus were placed on SDA with conidia (100,000) of the fungus to be tested on. These tests were carried out in triplicate, including as controls plates with the same fungus growing in the medium and over it. Haloes of inhibition or activation of the germination and growth of conidia, as well as diameter of the mycelium of the colony, were measured 4 days after inoculation. Halo/colony diameter was calculated using the formula of Bidochka & Khachatourians (1990).

**Results**

**Colony interactions and growth patterns for EF**

NF did not cause significant changes on growth rate of EF when growing on WA. When close to contact, fungus growth became slower, and sometimes it almost stopped. No morphological changes in EF colonies were found.

Changes in growth patterns were detected on CMA when colonies were near to contact. Sometimes, growth almost stopped. *P. lilacinus* was the NF that induced the highest inhibitor. Colony lysis was apparent for *P. fumosoroseus, V. lecanii* and *B. bassiana*. This latter entomopathogenic fungus was very sensitive to most *V. chlamydosporium* strains.

NF retarded and almost stopped growth of EF on SDA (Fig. 1). Pigment production was found for all EF, except *V. lecanii*. Moreover, *B. bassiana* formed a halo surrounding the colony, that increased upon contact with NF colonies.

**Colony interactions and growth patterns for NF**

EF did not cause clear changes on growth rate of NF on WA. In some cases, when colonies were close to merging with EF colonies, a growth rate reduction was found on NF. Growth of NF was never arrested by EF. Furthermore, no changes in colony morphology were observed in this medium.

Changes in growth of NF were found on CMA, as for WA. When the colonies were about to merge, their growth rate decreased. Hyphal lysis was observed, upon colony merging.

On SDA, twelve days after inoculation, NF growth was arrested. In some cases, they were unable to merge with the EF colony. NF decreased their growth towards the EF colony and sometimes produced a brown pigment. Strain 123 (*V. chlamydosporium*) produced the highest amount of pigment when growing with *B. bassiana*. When growing with *B. bassiana* too, strains 144 and 52 (*V. chlamydosporium*) got lysed when touched by the halo formed by the entomopathogen.
**Hyphal interactions**

No signs of direct mycoparasitism were found for most EF-NF interactions. However, granulation and vacuolation were frequent, for most combinations. Adpressed growth was more frequent on the plates of interactions than on the control ones, excepting for combinations including *B. bassiana*. Appressoria-like structures were found in some combinations including *M. anisopliae*, *P. fumosoroseus* or *P. farinosus*. No signs of penetration and coiling structures were found.

**Fungal metabolites tests**

Preliminary studies were carried out with *M. anisopliae* vs. *V. chlamydosporium* 64, and *M. anisopliae* vs. *V. chlamydosporium* 123.

*M. anisopliae* had a strong effect against the two NF tested. When inoculated over *V. chlamydosporium* 64, *M. anisopliae* colony was smaller than that of the control, but not when plated with *V. chlamydosporium* 123. This kind of results was not found for nematophagous fungi.

**Conclusion**

Analysis of NF-EF interactions revealed growth reduction or arrest when the colonies were close to merging. This could be due to competition for nutrient space. Similar results were observed when a fungus was plated with itself (data not shown). These effects were higher on CMA than on WA, and were maximum on SDA. On SDA, production of metabolites was assumed because of the great morphological changes observed. In WA, no metabolites seemed to be secreted on the medium, but maybe on the contact zone. This could be the cause of the vacuolation and granulation of the hyphae. The absence of coiling and penetration structures between EF and NF, when growing in the poorest conditions, indicates no ecological adaptation to this way of life. Adpressed growth can be a chemotropical response to the metabolites excreted in the contact zone (Chet *et al.*, 1997). Experiments to ascertain interactions between NF-EF interactions in soil should be carried out. These studies will determine the effect of the environment on the physiology of the inoculum. This in turn will determine the biocontrol potential of these antagonists.
Acknowledgments

This work was financially supported by the FAIR5-PL99-3444 project from the European Community. M.L. López-Serna was financially supported by grants from the University of Alicante (Spain).

References


Bourne, J.M. & Kerry, B.R. 1999: Effect of the host plant on the efficacy of *Verticillium chlamydosporium* as a biological control agent of root-knot nematodes at different nematode densities and fungal application rates. Soil Biology and Biochemistry 31: 75-84.


Persistent appearance of *Cordyceps gracilis* on *Selatosomus*-larvae near Siedlce (Poland)

Ryszard Mietkiewski, Stanislaw Balazy
Academy of Podlasie, Department of Plant Protection, Prusa street 14, 08-110 Siedlce, Poland
Research Centre for Agricultural and Forest Environment, Polish Academy of Sciences, Bukowska street 19, 60-809 Poznan, Poland

**Abstract:** The occurrence of the entomopathogenic fungus *Cordyceps gracilis* on larvae of *Selatosomus aeneus* was checked during 20-year period in the suburban area near Siedlce (Poland). Despite considerable land use changes and increased human interference the disease persisted at relatively high level. The fungus was not found infecting either other insect species occurring in soil or *Galleria mellonella* and *Tenebrio molitor* larvae used as bait insects.

**Key words:** entomopathogenic fungi, *Cordyceps gracilis*, *Paraisaria dubia*, host specificity, pathogen’s survival.

**Introduction**

The fungi of the genus *Cordyceps* have been not frequently recorded in European countries, except *C. militaris* which occurs rather regularly on caterpillars and lepidopteran pupae in forests. Another species of worldwide distribution, *C. gracilis* Montaigne et Durieu, seems to be not rare in Europe, though the number of its records is much more limited. This fungus, as well as its anamorph, *Isaria dubia* Delacroix (1893), were originally recognized as pathogenic for caterpillars, mostly of the genus *Hepialus* (Petch, 1933 ; Favre, 1942) and in most of European collections, both ascogenous and conidial sporulation occurred together. Mains (1957, 1958, 1959), who revised North and South American collections, found the perfect stage of this fungus on caterpillars, wireworms and unidentifie d hosts, but never associated with conidial sporulation. Similar observations were reported later from Soviet Union by Koval (1984).

The anamorph of the fungus were discovered in the vicinity of Siedlce (Poland) about 20 years ago on wireworms and pupae of *Selatosomus aeneus* (Coleoptera: Elateridae) and misidentified by S. Balazy as *Isaria nipponica* Kobayasi (Mietkiewski & Balazy, 1982). One year later, Samson & Brady (1983) published detailed descriptions of the conidial sporulation structures connected with *C. gracilis* ascogenous stromata on *Hepialus* spp. caterpillars under the new generic name *Paraisaria dubia* (Delacroix) Samson et Brady. The fungus appeared identical with those occurring on *Selatosomus* wireworms in Poland. Later on, in 1993, some dead specimens of this host were found with both conidial sporulation and ascigenous stromata, which allowed to rectify its identification and confirm the work by Samson & Brady (1983). The research carried out in the Polish locality contributed to better know *in situ* the development of the fungus and its persistence in suburban area despite considerable changes in land use and human interference.
Materials and methods

The site conditions and *S. aeneus* larvae sampling method have been described by Mietkiewski & Balazy (1982). However, during the last 20 years, the extensively utilised fields with some parts of fallows were changed in part into allotment gardens and in part settled or used as children playground. All the area became intensively frequented during the whole vegetation period. The occurrence of the pathogen and infection level were checked in 1980s at irregular time intervals, whereas starting from 1993, regular spring and late autumn samples were taken from 10 holes 25x25 cm and 30 cm deep. After sifting the soil all dead and alive wireworms were selected and identified. The fungal pathogen was isolated by the same methods as described by Mietkiewski & Balazy (1982). Additionally, in late autumn 1998 and in spring 2000, the soil samples were taken to determine the fungal pathogens using *Galleria mellonella* L. and *Tenebrio molitor* L. larvae as bait-insects.

Results and discussion

Because of land use changes there were very limited possibilities to adequately check the click-beetle population in private possessions, but the general recognition showed only their sporadical occurrence in cultivated gardens. In not utilized parcels, however, covered by spontaneous vegetation, the density of *S. aeneus* larvae was generally lower than in 1980/81 (respectively 21.9 and 61.8 indiv./m²), but the mean mortality percent caused by the fungus was higher (34 % and 53 % respectively) (Table 1).

<table>
<thead>
<tr>
<th>Date</th>
<th>Numbers of collected elaterid larvae</th>
<th>Selatosomus aeneus</th>
<th>Agriotes ustulatus</th>
<th>Athous sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>alive</td>
<td>dead</td>
<td>alive</td>
<td></td>
</tr>
<tr>
<td>1993 (21.XII)</td>
<td>10</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1995 (12.IV )</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1996 (11.XII)</td>
<td>7</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1997 (7.V)</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1997 (15.X)</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>1999 (31.III)</td>
<td>5</td>
<td>9</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>2000 (12.IV )</td>
<td>4</td>
<td>11</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>51</td>
<td>35</td>
<td>4</td>
</tr>
</tbody>
</table>

* Cordyceps sporulation on one specimen.

Table 1. Mortality of wireworms caused by *Cordyceps gracilis* in soil on the studied locality (vicinity of Siedlce).

These results seemed to indicate that the pathogen was less sensitive to the human interference in the suburban zone than its host. Despite the occurrence of other click-beetle larvae (*Agriotes ustulatus* Schall. and *Athous* sp.) and almost continuous occurrence of single caterpillars of Noctuidae (mostly of the Agrotinae subfamily), some predaceous beetle larvae and great number of *Phyllopertha horticola* L. white grubs, only fungal infections of...
S. aeneus caused invariably by C. gracilis were noted. Independently of the collection period, conidial sporulation of the fungus occurred on synnemata in soil and developed more abundantly in humid chambers. The Cordyceps stromata were found only once under natural conditions in late autumn 1993 on few specimens (one in the check-hole). As results of the use of the bait-insect method, only typical polyphagous components of entomopathogenic fungi were obtained, with dominance of Metarhizium anisopliae (Metschnikoff) Sorokin (Table 2). This fungus was shown to be predominantly isolated from soil at relatively high temperatures (Tkaczuk et al., 2000).

Table 2. Mortality of bait-insect larvae at different temperatures on the soil from the studied locality (vicinity of Siedlce) in late autumn and early spring (in %).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galleria mellonella</td>
<td>Tenebrio molitor</td>
</tr>
<tr>
<td></td>
<td>14 °C 24 °C</td>
<td>14 °C 24 °C</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>14 °C 24 °C</td>
<td>Galleria</td>
</tr>
<tr>
<td></td>
<td>14 °C 24 °C</td>
<td>Tenebrio</td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>14 °C 24 °C</td>
<td>molitor</td>
</tr>
<tr>
<td>14 °C 24 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>48.4 10.4</td>
<td>21.0 1.0</td>
</tr>
<tr>
<td>Metarhizium anisopliae</td>
<td>23.6 54.6</td>
<td>15.0 83.0</td>
</tr>
<tr>
<td>Paecilomyces farinosus</td>
<td>10.2 2.1</td>
<td>14.0 2.9</td>
</tr>
<tr>
<td>P. fumosoroseus</td>
<td>5.9 1.3</td>
<td>17.0 5.9</td>
</tr>
<tr>
<td>Common entomopathog.</td>
<td>88.1 66.3</td>
<td>67.0 86.0</td>
</tr>
<tr>
<td>Cylindrocarpon sp.</td>
<td></td>
<td>20.0</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Mucor sp.</td>
<td>3.0 1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Sterile mycelia</td>
<td>1.5 3.9</td>
<td>8.0 3.0</td>
</tr>
<tr>
<td>Other mycoses</td>
<td>4.5 3.9</td>
<td>11.0 5.0</td>
</tr>
<tr>
<td>Total mycoses</td>
<td>92.6 67.2</td>
<td>78.0 91.0</td>
</tr>
<tr>
<td>Nematodes</td>
<td>11.7 42.3</td>
<td>44.8 8.0</td>
</tr>
<tr>
<td>Other causes</td>
<td>7.4 18.1</td>
<td>22.0 8.0</td>
</tr>
<tr>
<td>Total mortality</td>
<td>100.0 100.0</td>
<td>100.0 100.0</td>
</tr>
</tbody>
</table>

Apart caterpillars of some Hepialus species and larvae of S. aeneus (this contribution), the real host range of C. gracilis has been rather poorly recognized. In monographs of Cordyceps by Kobayasi (1941) and Koval (1984) only lepidopteran larvae (in the latter also pupae) have been mentioned. In afore quoted papers Mains has specified only the family of Elateridae within order rank taxa. Dörfelt & Conrad (1978) have given some more detailed data on site conditions from one of South German localities, quoting as hosts of C. gracilis caterpillars of Geometridae, Lasiocampidae (probably Macrothylacia rubi L.) and Noctuidae.

According to the data of the literature and the results of this research, one can conclude that C. gracilis is able to infect soil inhabiting caterpillars of a relatively wide species scope, and some strictly selected click-beetle larvae. Such phenomena have been known in some other Cordyceps species, as for instance in C. entomorrhiza (Dicks.) Link ex Fr. (Balazy, 1986), which is narrowly oligophagous in relation to the ground beetles of the subfamily Carabinae, but also infects the predaceous bug Himacerus apterus Fabr. (Heteroptera: Nabidae). Such species may serve as good material for studying factors which determine host-pathogen specificity.
References


Brassicaceous plants and insect pathogenic fungi

Ingeborg Klingen, Ann Hajek¹, Richard Meadow, J. Alan A. Renwick²

The Norwegian Crop Research Institute, Plant Protection Centre, Department of Entomology and Nematology, Høgskoleveien 7, N-1432 Ås, Norway

¹ Department of Entomology, Cornell University, Ithaca, New York 14853-0901 U.S.A.
² Boyce Thompson Institute, Ithaca, New York 14853, U.S.A.

Brassicaceous plants are attacked by a wide range of pest insects, and insect pathogenic fungi are being explored for control of these pests. Isothiocyanates, produced by brassicaceous plants upon mechanical damage, infection or pest insect attack, might potentially cause a problem when using insect pathogenic fungi as control agents against soil dwelling *Brassica* pest insects.

The aim of this study was to investigate the potential interactions between brassicaceous plant roots, the isothiocyanates they produce, and insect pathogenic fungi. First, the *in vitro* toxicity of 2-phenylethyl isothiocyanate to *Metarhizium anisopliae* was confirmed, further the effect of 2-phenylethyl isothiocyanate on *Tolypocladium cylindrosporum* was tested for the first time. Since rutabaga might produce high levels of the fungitoxic 2-phenylethyl isothiocyanate upon wounding, we also investigated the effect of exposure to grated rutabaga roots on the virulence of *M. anisopliae* using *Galleria mellonella* as a model insect. To also utilize a more realistic system, we investigated whether presence and abundance of *M. anisopliae* and *T. cylindrosporum* were affected in soil if brassicaceous plants were growing. Our studies demonstrated that isothiocyanates can inhibit insect pathogenic fungi in petri dish experiments, but when using a more realistic fungus/plant/soil assay no fungal inhibition was found.

Details on this contribution will be found in the publication originated from this work:

A molecular method to differentiate between resting spores from different members of the *Entomophthora muscae*-complex

**Lene Thomsen, Annette Bruun Jensen**

*Department of Ecology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*

**Abstract:** *Entomophthora*-specific primers were constructed for nested amplification of the ITS II region. The two successive amplifications and the more sensitive and specific primers used in the nested PCR, enabled us to overcome problems with poor DNA quality from whole resting spore bearing fly cadavers. The nested primers amplified fragments of all the tested species belonging the genus *Entomophthora* and irregularly from the genera *Entomophaga* and *Eryniopsis*. No bands were amplified from species belonging to the genera *Pandora* and *Conidiobolus*. This method allowed a genetic analysis of resting spores naturally present in different fly host species collected in the field. Comparing the nested PCR-RFLP profiles of the different resting spores with profiles of known *Entomophthora* species, it was possible to assign the resting spores to species level.

**Key words:** Entomophthorales, *Entomophthora muscae*-complex, resting spores, Diptera, nested PCR-RFLP, ITS II, genetic variation, *in vivo*

**Introduction**

The fungus *Entomophthora muscae sensu lato* (Entomophthorales: Zygomycota) is a common pathogen on adults from several families of Diptera, Cyclorrhapha, such as Anthomyiidae, Muscidae, Fanniidae, Psilidae, and Scathophagidae (Eilenberg, 2000). The actual use of *E. muscae sensu lato* for practical biological control purposes is, however, still hampered by our lack of knowledge on important aspects of the fungal life cycle.

Keller (1984) recognized several different types within *E. muscae* species complex differentiated by conidial morphology and pathobiological characters. At present four separate species have been described. *E. schizophorae*, *E. syrphi*, *E. muscae sensu stricto*, *E. scatophagae* from *Scathophaga stercoraria*, but with a conidia morphology similar to *E. muscae sensu stricto*. A fifth yet undescribed species *E. muscae* “group B” was identified by Keller (1984).

Resting spores of *E. muscae s. l.* are frequently found in agriculturally important fly species from Anthomyiidae (Thomsen & Eilenberg, 2000), but often without exact determination of the conidial morphological type. It is, however, known that both *E. muscae s. str.* originating from *Delia radicum* as well as *E. muscae “group B”* originating from *Botanophila fugax* are able to form resting spores (Thomsen et al., 2001). Resting spores are also known for *E. syrphi* whereas to our knowledge, resting spores of *E. scatophagae* have never been observed. The overwintering strategy of *E. schizophorae* is unclear and resting spores of this species have never been certified *in vivo*, although the fungus has the ability to form resting spores *in vitro* (Eilenberg et al., 1990).

Molecular characteristics of isolates have previously been used to differentiate between species and/or isolates within Entomophthorales. Most studies have used the unspecific primed RAPD profiles (Hodge et al., 1995; Nielsen et al., 2001). However, as we wanted to use *in vivo* material possibly containing non-fungal DNA, a PCR method employing fungus
specific primers was envisaged. Jensen and Eilenberg (2001), using Entomophthorales-specific primers, found that the non-coding ITS II region of the nuclear rDNA was especially variable in the genus *Entomophthora*. The use of *in vivo* material for DNA extraction can result in co-extraction of components inhibiting DNA polymerisation, resulting in low amounts of PCR products for RFLP (Goller *et al*., 1998). The use of a two-step PCR with nested primers increases both the sensitivity and the specificity of the PCR significantly, and can overcome problems with relative impure DNA extractions (Goller *et al*., 1998). *Entomophthora*–specific primers were designed for nested amplification of the ITS II and by use of a nested PCR-RFLP approach, the presence of resting spores from the different species in the *E. muscae* complex in different fly hosts collected from the field was analysed.

**Material and methods**

**Isolates**

35 isolates from the *E. muscae* complex from different fly-hosts were represented: *E. schizophorae* from *Musca domestica* (1), *E. syrphi* from *Melanostoma* spp. (3) and *Platycheirus peltatus* (2), *E. scatophagae* from *S. stercoraria* (5), *E. muscae* *s. str.* from *D. radicum* (6), *E. muscae* *s. str.* from *M. domestica* (1), *E. muscae* (group B; sensu Keller (1984)) from *B. fugax* (2), and resting spores from *M. mellinum* (1), *P. peltatus* (1), *S. stercoraria* (3), *D. radicum* (9) and *B. fugax* (1). In order to test the specificity of the nested primers, one isolate from each of the following species were used; *Entomophthora chromaphidis*, *Entomophthora culicis*, *Entomophthora planchoniana*, *Entomophthora thripidum*, *Entomophaga maimaiga*, *Eryniopsis ptychopterae*, *Pandora neoaphidis* (= *Erynia neoaphidis*) and *Conidiobolus obscurus*. The material was either *in vitro* isolates grown in GLEN medium, or *in vivo* material sporulating with conidia or containing resting spores. *In vivo* material was freshly frozen, kept in 70 % alcohol, or dry (only resting spores).

**DNA extraction and amplification**

The total DNA was extracted from the samples as described by Jensen & Eilenberg (2001), except that cadavers with resting spores were carefully crushed with a micro pestle in a 1.5 ml eppendorf tubes after addition of the extraction buffer. Amplification of the ITS II was done by nested PCR. Nested PCR consists of two successive PCRs using two primer pairs (see Fig. 1). The first PCR used the primers nu-5.8S-5′ (Jensen & Eilenberg, 2001) and ITS 4 (White *et al*., 1990) with total DNA as template. The second PCR used the newly designed *Entomophthora* specific nested primers constructed on the basis of the sequences of ITS II from *E. schizophorae*, *E. muscae* *s. str.* (one isolate from *M. domestica* and one isolate from *D. radicum*), *E. scatophagae*, and *E. syrphi* (A.B. Jensen, L. Thomsen & J. Eilenberg, unpublished data) as well as the sequence of the ribosomal repeat for *Entomophaga aulicae* and *Penicillium clavigerum* with GenBank accession numbers U35394 and L14533 respectively. The sequences of the nested primers were: ITS II 20-5′ (5′-ACA GGA GGT TTG TTT GTT TG-3′) and ITS II 1500-3′ (5′-CTT GCT TGA TTT GAA ATG WAG-3′).

The PCR conditions were initial denaturation for 5 min at 96 °C followed by 35 cycles with denaturation for 1 min at 96 °C, annealing for 1 min at 62 °C (ITS II) or 58 °C (nested-ITS II), extension for 1 min at 72 °C and a final extension for 10 min at 71 °C.

**RFLP**

Each nested amplification was cut with the restriction enzymes: *Alu I*, *Dde I*, *Dra I*, *Hae III*, *Hha I*, *Rsa I*, *Sau IIIa*, and *Taq I* (New England Biolabs) according to the manufacturers instructions using 5 µl PCR product. The fragments were separated on a 1.5 % agarose gel and visualized with ethidium bromide. For each enzyme the length of the different fragments
was used as a character, and then all the fragment lengths were scored as present or absent for each isolate.

**A) First PCR**

Primer nu-5.8S-5’

126 bp

5.8 s

ITS II

LSU

Primer ITS 4

60 bp

**B) Second PCR**

Primer ITS II 20-5’

31 bp

5.8 s

ITS II

LSU

Primer ITS II 1500-3’

22 bp

Amplicon from first PCR

Fig. 1. The principle of nested PCR. A) The first PCR is performed on total DNA with the primers: nu-5.8S-5’ positioned in the 5.8S coding region and ITS 4 positioned in the LSU coding region. B) The second PCR is performed with the diluted amplicon from the first PCR as template. The newly constructed, nested primers (ITS II 20-5’ and ITS II 1500-3’) anneals within the first PCR product. The arrows represent primer position and direction. The number of base pairs from the start of the primer to the end of the coding region is also indicated.

**Results and discussion**

**DNA amplification**

The new nested primers amplified distinct bands for all the tested *Entomophthora* species including the resting spore isolates, and as expected from the position of the primers, approximately 135 bp shorter than the ITS II (Fig. 2, lanes 1-7), except for *E. culicis* where two bands were amplified using the nested primers (Fig. 2, lane 8). More inconsistently, weaker bands were amplified from one species each of *Entomaphaga* and *Eryniopsis*, two other multi-nucleate genera from Entomophthoraceae (Fig. 2, lanes 9-10). The nested primers never amplified bands from the uni-nucleate entomophthoraceous species (*P. neoaphidis = E. neoaphidis*) or the ancylistaceous species (*C. obscurus*) even the first amplification resulted in distinct bands (Fig. 2, lanes 11-12). Based on these results we conclude that the new primers are specific for the genus *Entomophtora*, but given the right conditions amplification might also occur for other multi-nucleate taxa from Entomophthoraceae. The specificity and sensitivity of these nested primers make the use of this approach ideal for *in vivo* material where the DNA quality might not be optimal.

**PCR-RFLP**

Within the *E. muscae* complex considerable variation of the nested-ITS II was seen using the PCR-RFLP. Polymorphism was seen with all the tested enzymes.

The RFLP profiles generally showed that each host was correlated with one fungal genotype, at least at the host genera level and apparently without any interaction between the different fungal isolates and host species. There was almost no genetic variation between isolates originating from the same host species. This is accordance with studies on members from the *Entomophaga grylli* complex (Bidochka et al., 1995), but in contrast to a study on the genotypic variation in *P. neoaphidis* where the groupings were more correlated to geographic origin of the isolates than the host species (Nielsen et al., 2001). None of the resting spore isolates could be assigned to *E. schizophorae*.

All together the nested PCR-RFLP approach allowed us to assign resting spores from the *Entomophthora muscae* complex to the species level. Furthermore, this method could be a valuable tool for more detailed studies analysing the host-pathogen specificity and possible interactions in the *E. muscae* complex using *in vivo* material, irrespective of whether conidia or resting spores are formed in the cadavers.

**Acknowledgements**

We thank Anne Grundschober, Ann Hajek, Richard Humber, Vibeke Kalsbeek, Ingeborg Klingen and Charlotte Nielsen for providing isolates, and Verner Michelsen and Stig Andersen for the identification of the flies. Kirsten Ploug and Mette Vingaard are thanked for skilled technical assistance. The Carlsberg Foundation and the Ministry of Food, Agriculture and Fisheries supported this study.

**References**


Genetic variation among Latvian isolates of *Beauveria bassiana*

Eriks Jankevics, Mara Kropa\(^1\), Lelde Grantina\(^1\), Liga Jankevica\(^1\)

LU Biomedical Research and Study Center, Ratsupites str.1, Riga, LV 1067, Latvia

\(^1\) Department of Experimental Entomology, Institute of Biology, University of Latvia, Miera str. 3, Salaspils, LV 2169, Latvia

**Abstract:** Entomopathogenic fungi of the genus *Beauveria* are the most commonly and frequently natural occurring agents causing pest insect diseases in Latvia. Our purpose was to search for different *Beauveria bassiana* isolates and to compare their characteristics. Various isolates were studied with respect to colony morphology, growth intensity, sporulation, biological activity and molecular characteristics. We used *B. bassiana*-specific primers and PCR based technique that amplified fragment of *B. bassiana* DNA. Sequence analysis was performed for PCR products. Results revealed the existence of DNA sequence differences which could be used for isolate identification.

**Key words:** *Beauveria bassiana*, entomopathogenic fungi, characterization, PCR differentiation

**Introduction**

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin has been extensively used for the control of many important pests of various crops. This species is the most commonly and frequently natural occurring agent causing pest insect diseases in Latvia. We recorded epizootics caused by *B. bassiana* in populations of *Galeruca tanaceti* L., *Leptinotarsa decemlineata* Say and *Pieris brassicae* L. The development of this pathogen as a microbial control agent requires development of highly specific and sensitive fungal detection systems. Such systems are necessary for controlling quality of products and monitoring the presence of entomopathogenic fungi in the environment. They should allow the identification and differentiation of naturally existing as well as genetically engineered and novel strains (Hegedus & Khachatourians, 1996). For this purpose different molecular genetic methods have been suggested. Techniques which rely on DNA probes to create restriction fragment length polymorphisms (RFLP) fingerprints have been used to distinguish between individual isolates (Hegedus & Khachatourians, 1996; Castrillo *et al*., 1999).

A study was conducted to assess phenotypic and genetic variation within different Latvian populations of *B. bassiana*. Its aim was to compare characteristics of isolates in order to found DNA sequences differences to be used for identification.

**Material and methods**

**Isolates**

Four isolates originated from different sources (Table 1) were selected from the collection of bio-agents preserved in the Institute of Biology, University of Latvia. Cultures were maintained on Malt agar or Sabouraud dextrose agar (SDA).

**Spore characteristics**

Three hundred conidia of each isolate, from 10-day-old cultures on SDA, were examined for conidial shape and size, using a light microscope fitted with a micrometer.
Table 1. *Beauveria bassiana* isolates: original host, collection sites and year of isolation.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source of isolation and collection site</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Monospore isolate of strain R (ex <em>Galeruca tanaceti</em>, Riga, 1965)</td>
<td>1999</td>
</tr>
<tr>
<td>M30</td>
<td>Monospore isolate of strain R (ex <em>G. tanaceti</em>, Riga, 1965)</td>
<td>1999</td>
</tr>
<tr>
<td>L2-S</td>
<td>Soil, Riga district, Saulkalne</td>
<td>1999</td>
</tr>
<tr>
<td>L4-R</td>
<td><em>Leptinotarsa decemlineata</em>, Riga district, Ropali</td>
<td>2000</td>
</tr>
</tbody>
</table>

**Macroscopic morphology of colonies and colony growth rate**

Round pieces (4 mm Ø) of cultures were punched into a centre of six 90 mm Malt agar plates and incubated at 26 °C. The diameter of the colonies were measured every 24 h for 10 days. Colour and type aspect were recorded for 10-days old colonies.

**Bioassay**

To test the relative pathogenicity, fourth instar larvae of *L. decemlineata* (laboratory culture kindly provided by Dr. R. Kleespies, BBA, Institute for Biological control, Darmstadt, Germany) were inoculated by contact with a suspension containing 1.10^7 conidia/ml. Experimental and control insects were kept individually in sterilized 120 ml glass flasks with potato top as food supply. They were observed daily to determine the mortality. Ten insects per replica and four replicas were used for each isolate.

**DNA isolation and PCR**

Total genomic DNA was isolated from fungal spores or cultures as described by Pfeifer & Khachatourians (1993). DNA concentration was determined by spectrophotometry. The *Beauveria*-specific PCR primers: P1 (5’AAGCTTCGACATGGTCTG) and P3 (3’GGAGGTGGTGAGGTTCTGTT) (Hegedus & Khachatourians, 1996) were used to amplify fragment of *B. bassiana* DNA. The PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer). 1 µg of the DNA samples was used in PCR mix. The PCR reaction mixture contained the following components: fungal DNA – 1 µl; 10x PCR buffer – 5 µl; MgCl2 – 5 µl; BSA (1 mg/ml) – 4µl; dNTP (10 mM) – 1 µl; Primer1 (10 pM/µl) – 2 µl; Primer2 (10 pM/µl) – 2 µl; Taq polymerase 5 U/µl (MBI Fermentas) – 1 µl; water till 50 µl.

The reaction parameters were: initial denaturation for 3 min at 94 °C, followed by 30 cycles of 95 °C. – 30 sec, second step 55 °C – 30 sec, third step 72 °C – 1 min. The final step of the reaction was followed by extension by at 72 °C for 7 min. At the end of the reaction the PCR products were separated by electrophoresis on 1 % agarose gel, stained with ethidium bromide and visualised under short wave UV light. Sensitivity was increased using a double amplification procedure where additional Taq polymerase was added after 25 cycles followed by an additional cycles as described above.

**DNA sequencing and comparison of isolates**

PCR fragments were purified from 1 % agarose gel by Jetsorb kit (Genomed) and sequenced. Sequence analysis was performed by ABI PRISM 310 Genetic Analyser (Perkin Elmer) using BigDye Terminator Mix according to the manual. Data were analysed using the program DNASIS for Windows 2.1 (Hitachi).

**Results and discussion**

After 10 days growth on SDA, colour of colonies showed wide variations between white and yellow according to isolates. Other variable features were observed. 2 types of conidial shape were found among conidia from 10 days-old cultures on SDA: globose and ellipsoidal. Their
size was 2.6 – 3.7 µm. Isolates had very few blastospores. Isolates M1 and M30 exhibited similar total conidial productions (1.5-2.109 conidia/cm²). Isolates L2-S and L4-R produced less amount of conidia, 7.8.10^8 and 5.9.10^8 respectively. Colony growth rate of isolates differs significantly (Table 2). Isolate M30 on Malt agar grows faster than other isolates. Isolates obtained from G. tanaceti and L. decemlineata had high virulence on larvae of the Colorado potato beetle, L. decemlineata (Table 2).

Table 2. Comparison of conidial size, colony growth on Malt agar and virulence of *Beauveria bassiana* isolates*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Culture</th>
<th>Conidial size, µm</th>
<th>Colony growth, mm/day</th>
<th>Mortality % <em>L. decemlineata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Monosporic</td>
<td>2.8 - 3.7</td>
<td>2.30 ±0.15 c</td>
<td>86.0 ± 4.6 a</td>
</tr>
<tr>
<td>M30</td>
<td>Monosporic</td>
<td>2.9 - 3.5</td>
<td>3.31 ± 0.21 a</td>
<td>90.8 ± 3.4 a</td>
</tr>
<tr>
<td>L2-S</td>
<td>Multisporic</td>
<td>2.7 - 3.6</td>
<td>2.88 ± 0.16 b</td>
<td>79.4 ± 4.4 b</td>
</tr>
<tr>
<td>L4-R</td>
<td>Multisporic</td>
<td>2.6 - 3.7</td>
<td>2.86 ± 0.08 b</td>
<td>95.8 ± 3.6 a</td>
</tr>
</tbody>
</table>

*Means with the same letter are not significantly different (P>0.05), mortality of *L. decemlineata* larvae in control (no inoculation) – 0%.

Concerning the molecular study, *B. bassiana* isolates showed characteristic PCR amplification. Sequence analysis of PCR products revealed some differences among isolates (Fig. 1). After comparison of sequences of isolates M1 and M30, which exhibited an homology of 97.7 %, it was found that there are nucleotide exchange in position 431 and two insertions: isolate M1 insertion in position 483, isolate M30 insertion in position 503. After comparison of isolate L2-S from soil (DNA homology of 93.9 %), we found 8 nucleotide exchanges, 1 insertion in position 271 and deletions in position 397 and 594. Sequences of the Latvian material were compared with sequence published by Hegedus & Khachatourians in 1996 (94.3 % homology).

In conclusion, Latvian populations of *B. bassiana* appeared to be not homogenous according to source of isolation and localities. They exhibited differences as regards colony morphology, growth rates, sporulation, virulence and DNA sequence. This could be used for identification of isolates.

References


Fig. 1. Alignment of the *B. bassiana* DNA sequences. Mismatched nucleotides are framed (H & K – published sequence (Hegedus & Khachatourians, 1996), L2-S, M1, M2 – sequences of Latvian isolates).
Biological control of weevils (*Strophosoma* spp.) in Danish greenery plantations

Jørgen Eilenberg, Charlotte Nielsen, Susanne Vestergaard, Susanne Harding, 
Amy Frølander, Anna Augustyniuk

Department of Ecology, The Royal Veterinary and Agricultural University, 
Thorvaldsensvej 40, 1871 Frb.C., Denmark;

1 Department of Agrocenology, Institute of Ecology PAS, Dziekanow Lesny, 
05-092 Lomianki, Poland

**Abstract:** In Danish greenery plantations, *Strophosoma melanogrammum* and *S. capitatum* cause needle damage, especially in *Abies procera*. No chemical treatment of these weevils is allowed in forestry, so biological control provides a potential alternative method. We studied the laboratory mortality and field effects of several insect pathogenic fungi and the field effect of *Metarhizium anisopliae*. The preliminary data prove that *M. anisopliae* is capable of killing both species of weevil in the laboratory. A field release experiment performed in order to control ovipositing females during spring 2001 has so far proved that it is possible to obtain high fungus prevalences in the treated plots (up to 90.2 % infected *S. melanogrammum*).

**Key words:** *Abies*, Curculionidae, entomopathogenic fungi, *Metarhizium anisopliae*, control

**Introduction**

Greenery from *Abies procera* and *A. nordmanniana* is an economically important forest product in Denmark. No exact total estimate of the cultivated areas exists, but for *A. procera* the area is at least 8.500 ha, while for *A. nordmanniana* it is at least 21.500 ha (Kaj Østergaard, personal communication). The market demands very high quality, the greenery must be well-shaped, the needle colour must be nicely green, and no damage from feeding of insect pests should be present. The greenery has, due to the high crop quality, a high export value, more than 100 millions USD per year.

The production of greenery was earlier based on the use of chemical pesticides. Now, the chemical pesticides are to be phased out. In state forestry, chemical pesticides will be completely banned from year 2003 (The Danish Environmental Protection Agency, 1998). For privately owned forests, there is similarly a political pressure from the state authorities to phase out chemical pesticides. Furthermore, the few chemical pesticides still allowed in private forests do not include products for control of weevils in greenery plantations.

Two species from the genus *Strophosoma* (Coleoptera: Curculionidae) are important pest species in greenery production in Denmark: *S. melanogrammum* and *S. capitatum* (Harding, 1993 ; Kirkeby-Thomsen & Ravn, 1997). The damage is due to the feeding by the adult weevils on the needles. Many aspects of their life-cycle are similar: adults overwinter in the soil and are active in the spring and again in the autumn. Later instar larvae are found in the soil around the trees. The completion of the life-cycle is assumed to last two years.

The growers are now in a difficult situation: these pest insects cause economically significant damage on the decorative greenery, and since no chemical pesticides to control them are allowed, there is a strong demand need for alternatives.
Two ongoing projects in Denmark (the EU project BIPESCO and a project granted by the Danish Ministry of Environment and Energy) aim to develop biological control of weevils by using insect pathogenic fungi. Specifically, the projects aim to:

- assess the natural occurrence of entomopathogens in the soil and on *Strophosoma* spp.
- perform bio-assays to assess the virulence of selected isolates
- release *Metarhizium anisopliae* and other fungi in a greenery plantation
- evaluate the effect on target and non-target insects
- study the dispersal of the fungus after release.

**Materials and methods**

A 0.3 ha greenery plantation in Bidstrup (Zealand, Denmark) of 15 year old *A. procera* culture was used for field experiments in 2001 and for collection of insects for bio-assays. Sampling of weevils and soil from the plot before treatment assessed the natural occurrence of insect pathogenic fungi in the plot. All soil samples were taken with a cylindrical soil sampler at three depths (0-5; 5-15 and 15-25 cm) and baited with *Galleria* and *Tenebrio* larvae.

Beetles for bio-assays were collected during autumn 2000 and used after checking for natural infection. Beetles were stored at 5 °C until used. Four isolates of hyphomycetous fungi were selected (see Table 1). Cultures of fungi were grown on SDA, and conidia were harvested after 12-14 days of growth at 23 °C. The viability of conidia was tested after incubation at 23 °C for 24 hours.

Two bioassays procedures were compared: (1) Dipping of insects: insects were dipped for 10 sec in a suspension of $10^7$ conidia per ml in 0.05 % Triton X-100. Weevils were carefully transferred individually to plastic cups (30 ml) containing 5 ml of 3% water agar and a small twig of *A. procera*; (2) Dipping of twigs: small twigs of *A. procera* were dipped for 10 sec in a suspension of $10^7$ conidia per ml. Twigs were placed in 5 ml 3 % water agar in a 30 ml plastic cups. One weevil was then carefully transferred to each cup. In both experiments the plastic cups were sealed with polyvinyl chloride (PVC) ‘cling-film’, a semi-permeable membrane that ensured humid conditions without condensation. The cups were incubated at 20 °C with a 16:8 h photoperiod. The control treatment consisted of either weevils or twigs treated with 0.05 % Triton X-100 only. Three times 10 weevils were used per treatment and the mortality of the test insects was recorded every second day for three weeks. Median lethal time (LT$_{50}$) was estimated in Mathematica vers. 4.0 (Wolfram Research, Inc.), by the method described by Throne *et al.* (1995).

The *M. anisopliae* BIPESCO 5 strain was applied on 25.04.2001 and 09.05.2001 in two plots each covering around 125 m$^2$, by spraying suspensions of $4.6 \times 10^{13}$ and $1 \times 10^{14}$ conidia per ha, respectively. Both plots were thus treated twice. Two control plots were treated with 0.05 % Triton X-100. Weevils were sampled from emergence cages once a week, and incubated individually in 30 ml plastic cups with *A. procera* in 3 % water agar. The mortality was recorded daily for four weeks, and fungal infections were diagnosed.

**Results and discussion**

Both *Beauveria bassiana* and *Paecilomyces* spp. were isolated from the soil in the greenery plantation. Incubated *Strophosoma* spp. showed that *B. bassiana* and *Paecilomyces* spp. occurred naturally. By contrast, *M. anisopliae* was recorded neither in the plantation soil, nor on the weevils.

Preliminary data from the bio-assays are shown in Table 1. As shown, dipping the insects resulted in a more rapid kill than dipping the twigs in a conidia suspension. Dipping the twigs
is, however, more comparable to the field situation. Among the tested isolates, *M. anisopliae* BIPESCO 5 proved to be one of the most promising.

Table 1  Comparison of LT$_{50}$ values of *Strophosoma melanogrammum* for four isolates, by dipping insect or dipping twigs.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Dipping insects LT$_{50}$ in days</th>
<th>Dipping twigs LT$_{50}$ in days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Metarhizium anisopliae</em> BIPESCO 5</td>
<td>18.1</td>
<td>22.1</td>
</tr>
<tr>
<td><em>M. anisopliae</em> YF1/1-05/00</td>
<td>17.4</td>
<td>34.5</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em> p.Chot.</td>
<td>20.4</td>
<td>43.0</td>
</tr>
<tr>
<td><em>Paecilomyces fumosoroseus</em> S-1/3-06/00</td>
<td>27.0</td>
<td>33.7</td>
</tr>
</tbody>
</table>

Table 2. Field infection of adult *Strophosoma melanogrammum* and *S. capitatum* from emergence cages after treatment of the plantation with *Metarhizium anisopliae*.

<table>
<thead>
<tr>
<th>Date</th>
<th><em>Strophosoma melanogrammum</em></th>
<th><em>Strophosoma capitatum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Infected/ No. Incubated</td>
<td>Prevalence (%)</td>
</tr>
<tr>
<td>19.04.2001</td>
<td>0/2</td>
<td>0</td>
</tr>
<tr>
<td>25.04.2001</td>
<td>0/13</td>
<td>0</td>
</tr>
<tr>
<td>02.05.2001</td>
<td>29/55</td>
<td>52.7</td>
</tr>
<tr>
<td>09.05.2001</td>
<td>46/51</td>
<td>90.2</td>
</tr>
<tr>
<td>16.05.2001</td>
<td>11/16</td>
<td>68.8</td>
</tr>
<tr>
<td>23.05.2001</td>
<td>1/5</td>
<td>20.0</td>
</tr>
<tr>
<td>30.05.2001</td>
<td>1/3</td>
<td>33.3</td>
</tr>
<tr>
<td>12.06.2001</td>
<td>2/4</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Prevalence data for adult *S. melanogrammum* and *S. capitatum* from the 2001 field experiment are shown in Table 2. For *S. melanogrammum* the highest prevalence (90.2 %) was found on May 9, a result of the first release of the fungus and the highest prevalence for *S. capitatum* was obtained on May 16 (78.1 %). In the untreated control plots, 82 individuals of *S. melanogrammum* were sampled and incubated, but only one was found to be infected by *M. anisopliae* while no infected individuals of *S. capitatum* were found. The infected individual was captured from one of the emerging traps on the borderline of an untreated plot.

The experiments were promising since we actually managed to initiate infections in the treated plots. The effect on the population development is, however, still unexplored, since we have not been able so far to monitor eggs or larvae in the field. The effect of the *M. anisopliae* treatment on population level will thus not be monitored before eggs laid in spring 2001 reach the adult stage (in autumn 2002). However, based on these preliminary results we conclude that *M. anisopliae* has a potential for future biological control of *Strophosoma* spp. Our future work aims to improve the timing and method for field application as well as to develop methods to monitor eggs and larvae.
References

Occurrence of entomopathogenic fungi in soils from different parts of Spain

E. A. A. Maranhão, C. Santiago-Alvarez
Departamento de Ciencias y Recursos Agrícolas y Forestales. E.T.S.I.A.M., Universidad de Córdoba. Apartado 3048, 14080 – Córdoba, Spain

Abstract: The natural occurrence of entomopathogenic fungi in soils from Andalucía, Extremadura and Canary Islands, Spain, was studied using the Galleria mellonella bait method. Entomopathogenic fungi have been isolated from 81.25 % of the soil samples. Incubation temperatures were 10, 15, 20, 25 and 30 ºC. The most abundant species were Beauveria bassiana (89.7 % of samples) and Metarhizium anisopliae (43.5 %), while both Paecilomyces sp. and Verticillium lecanii were isolated only once (2.5 %). B. bassiana was isolated from soils samples kept at 10, 15, 20, 25 or 30 ºC; similarly, M. anisopliae was isolated at 20, 25 or 30 ºC. On the other hand, 33 % of soil samples yielded B. bassiana as well as M. anisopliae and 2.5 % yielded B. bassiana and either Paecilomyces sp. or V. lecanii. Neither the percent of organic matter nor the pH have any relationship with the occurrence of B. bassiana and M. anisopliae in the soil.

Key words: Beauveria bassiana, Metarhizium anisopliae, “Galleria bait method”.

Introduction

The soil habitat contains a rich fauna of invertebrates, including insects, as well as a high diversity of microorganisms. Among these, some entomopathogenic fungi have great potential as biocontrol agents. Their occurrence and distribution depend on many environmental factors such as temperature, humidity, soil type, etc. (Rath et al., 1992; Vanninen, 1995; Mietkiewski & Tkaczuk, 1998; Tkaczuk, et al. 2000). Furthermore, the presence of other soil born microorganisms, responsible of the fungistatic effect, and even plant exudates, can affect their survivorship in the soil (Walstad et al., 1970).

The “Galleria bait” method proposed by Zimmermann (1986) allows the isolation of entomopathogenic fungi from soil and afford insights on their biodiversity. This method also allows the isolation of more virulent strains in regard to the selective media. On the other hand, the incubation temperature of soil samples baited with insects can be used as a selective factor for isolation of entomopathogenic fungi (Tkaczuk et al., 2000).

The aim of this contribution was to study the diversity and frequency of entomopathogenic fungi in soils from different parts of Spain.

Materials and methods

Soil samples were taken from different biotopes of Andalucia, Extremadura and Canary Islands, Spain. The samples were collected at 10-20 cm depths, then transferred to plastic Petri dishes baited with 10 last instar larvae of G. mellonella (Zimmermann, 1986). Dishes were incubated at different temperatures (10, 15, 20, 25 and 30 ºC) during 7 days. After this period, dead insect without visible mycelium on the surface, were disinfected in 1% sodium hypochloride during 3-4 min, rinsed twice in sterile water and incubated at 25 ºC in moist chambers. Dead insects with visible mycelium on the surface were rinsed only in sterile water. The alive larvae were observed every 3 days until the 21st day.
Fungi were identified using light microscope and pure cultures were obtained on Saboraud-Dextrose-Agar medium (SDAY) supplemented with yeast extract (2 %) (pH adjusted to 6.5). The isolated strains were stored at 4 ºC on Malt-Agar.

For each soil sample, texture, pH and content of organic matter were recorded.

Results and discussion

Entomopathogenic fungi were isolated from 39 out of 48 soil samples (81.25 %), from Andalucía, Extremadura and Canary Islands. Among the positive samples, the frequency of occurrence of the fungal species was: B. bassiana, 89.7 %; M. anisopliae, 43.5 %; both Paecilomyces sp and Verticillium lecanii, 2.5 % (Fig. 1). On the other hand, the simultaneous presence of the two former species was observed in 33 % of the soil samples while 2.5 % yielded B. bassiana and either Paecilomyces sp. or V. lecanii. Opportunistic fungi like Fusarium sp., Aspergillus sp., Rhizopus sp., as well as sterile mycelium, were also observed on several dead larvae.

Fig. 1. Frequency of occurrence of entomopathogenic fungi in soils from Andalucía, Extremadura and Canary Islands.

Overall, in this study, B. bassiana dominated in almost all biotopes, except in soils from peninsular sea coast, which no fungus was isolated from, and in the sandy-loam soils from grasslands of Canary Islands where M. anisopliae was the dominant species. These results suggest that B. bassiana is favoured by soil and climatic conditions of Andalucia and Extremadura. This situation is similar to the results obtained by Tarasco et al. (1997) in Southern Italy and in the UK by Chandler et al. (1998) but differed from those observed in other regions of Northern Europe. In Polish soils, M. anisopliae and P. fumosoroseus were the dominant species (Mietkiewski et al., 1995, 1996). These species also appear more abundant than B. bassiana in Germany (Kleespies et al., 1989). Similary, in soils from temperate and near-northern habitats in Canada, M. anisopliae was the more abundant species (Bidochka et al., 1998).

The pH of the samples ranged between 4.94 and 8.73 ; organic matter between 0.36 and 18.18 %, but neither the pH nor the organic matter have any relationship with the occurrence of fungi in the soil, agreeing with the results obtained by Tarasco et al. (1997) and Bidochka et al. (1998).

In relation to incubation temperature, in soils from Andalucía and Extremadura, B. bassiana infected larvae at temperatures ranging from 10 to 30 ºC, but the highest
percentage of infection was at 15, 20 and 25 °C. *M. anisopliae* doesn’t cause infection at 10 °C and maximum mortality was observed at 25 and 30 °C (Fig. 2). These results confirm both the capacity of *B. bassiana* to grow in a wide temperature range (Fargues *et al*., 1997) and the greatest temperature requirements of *M. anisopliae* (Tkaczuk *et al*., 2000). In soils from Canary Islands, *M. anisopliae* caused infection on *Galleria* larvae at 18, 25 and 28 °C, with a maximum mortality at the highest temperature. By contrast *B. bassiana* caused more infection at 18 °C (Fig. 3). The dominance of *M. anisopliae* in such conditions confirms the results of Tkaczuk & Mietkiewski (1996) and Kleespies *et al.* (1989).

![Fig. 2. Mortality of *Galleria mellonella* larvae baited in soils from Andalucia and Extremadura at different incubation temperatures.](image1)

![Fig. 3. Mortality at different temperatures of incubation of *Galleria mellonella* larvae baited in soils from grasslands in Canary Islands.](image2)

The various Spain isolates of *B. bassiana* or *M. anisopliae* are currently under study to determine wheter they correspond to different strains or to a single strain with broad thermal activity, host specificity, etc.
Acknowledgements

E.A.A. Maranhão was given a scholarship from CNPQ, Brazil.

References


On some little known epizootics in noxious and beneficial arthropod populations caused by entomophthoralean fungi

Stanislaw Balazy
Research Centre for Agricultural and Forest Environment, Polish Academy of Sciences,
Bukowska 19 street, 60-809 Poznan, Poland

Abstract: Some hitherto unknown or poorly recognized examples of high mortality caused by entomophthoralean fungi in folio-, sapro- and entomophagous insect populations in Europe, mostly in Poland, are briefly described. The sharpest mortality peaks were observed in the case of infections occurring in short periods, by species of the genera Entomophaga and Zoophthora and by Conidiobolus gustafssonii. These pathogens all appear to be mono- or narrowly oligophagous, similarly as species of Pandora. Several Erynia species affect heavily adult dipteran populations in moist sites moderately shaded by arboreal vegetation or lake side bushes. They persist from the beginning of May to the end of October, whereas diseases caused by Entomophthora culicis usually increase in mid summer. Batkoa apiculata, known as a polyphagous entomopathogen, affects many insects, predominantly saprophagous and entomophagous species, but spectacular mortality peaks have never been observed. Several-day rainy periods stimulate mortality increase whereas longlasting cool and wet weather restricts epizootic strength.

Key words: Mycoses, Entomophthorales, epizootics, foliophageus insects, predacious arthropods

Introduction

More or less detailed data on epizootics caused by fungous diseases in arthropod populations are often reported, but they mostly concern economically important pest insects or mites. The term “epizootic” has often been used in an unequivocal sense, as referring to an unusually high number of disease cases or “high mortality level”, despite weak relation to real hosts’ population density, and irrespective of their distribution patterns in habitats, pathogens’ selectivity regarding host species or its development stage, and other possible inaccuracies (Fuxa & Tanada, 1997). Prevailing descriptions of spectacularly strong host population declines caused by pathogenic fungi concern mostly medically important insects (Steinkraus, 1990), or serious pest outbreaks in agriculture and forestry (Pell et al., 2001). Mycoses in so called “indifferent” or beneficial arthropod species have been usually treated as occasional ones, of no or little significance for biocenotic regulation processes. This led to some arbitrarily anthropocentric overestimation of this group of entomopathogens as almost univocally favourable, which was expressed for instance by Batko (1974) as “the foes of our enemies”.

Investigations on entomopathogenic fungi in different natural and semi-natural ecosystems and in agriculturally managed areas led the author to regularly observe cases of high or very high host mortality, suggesting local or extensive epizootics often caused by little known entomophthoralean species. The aim of this contribution was to present significant examples of such phenomenons.
Material and methods

In the early 1970s, at the start of his investigations on the Entomophthorales, the author who considered mostly protected by law ecosystems of the Wielkopolski National Park (WNP), tried to establish a list of local species and to recognize differences between particular biotopes. For these purposes, careful searches were carried out in different stands, particularly intensive in the forest floor vegetation and underbush up to the height of about 3 m. Apart from careful gathering of all infected arthropod specimens, the cases of extraordinarily high mortality were treated with special attention in order to establish extent areas and quantitative characteristics of affected populations. Such observations were continued in subsequent years and steadily extended on the surroundings of WNP, including agricultural areas and other national parks. Since 1981 the investigations in rural areas have prevailed and a unified concept of quantitative estimations on the series of square 2 x 2 m research plots have been explored as a standard. In 1990s grasslands and wetlands were included into the research programme realized in Poland and in some limited areas of Germany (Southern Bavaria) and France (Picardie).

Results and discussion

For the most part well described epizootics are those observed during host outbreaks or in gregarious populations of some arthropods of agricultural importance, such aphids or spider mites for instance. In addition to these already studied situations, the following few noteworthy examples, related to various entomophthoralean species, deserve to be briefly described.

Entomophaga tenthredinis (Thaxter) Batko
This species has been regularly observed in WNP and in normally managed cereal crops and meadows on Dolerus-like larvae. However, mortality attaining 70-98 % (as counted on small plots directly on plants) appeared regularly in a number of clearances in an old oak-horn beam stand (Galio silvatici - Carpinetum) at the turn of June-July during the period 1974-1980, on small tenthredinid larvae unlike Dolerus species, feeding in forest floor vegetation. The fungus did not infect Pteronidea ribesi larvae despite their abundant occurrence on wild gooseberry bushes in surrounding stands. Since the early 1980s, the disease cases have become increasingly rare in this area. Earlier epizootic appearance of this species was reported by Fresenius (1858) in Tenthredo larvae on black alder and by Zimmermann & Huger (1984) in Dolerus sp. from winter barley crops and a meadow.

Entomophaga diprionis Balazy
E. diprionis was found in Diprion simile cocoons collected during sawflies outbreaks in Scots pine stands of Nowogród, Gniewkowo and Solec Kujawski forest districts (Central Poland), in October-December 1974 and 1975 (materials obtained from the Forest Research Institute in Warsaw). In some samples 40 to 75 % of larvae were mummified. Apart from single cases of Beauveria bassiana (Balsamo) Vuillemin and chalcidoid or ichneumonoid parasitoids, the majority of them was affected by the Entomophthorale, formerly identified by the author as E. tenthredinis (Swiezynska & Górnas, 1976; Balazy, 1993). Probably the epizootics in Wisconsin (USA) described by Klein & Coppel (1973) concern the same pathogen. Unfortunately, field studies dedicated to this species have not been carried out so far.

Zoophthora bialowiezensis Balazy
Numerous cases of mycoses caused by this species in foliophagous geometrid caterpillars (mostly Cheimatobia and Hibernia species) were noted at the end of May 1975 in Bialowieza
National Park (BNP). Pretty regular checking in 1980s showed at the turn of May-June of 1981, 1982, 1985 and 1986, a mortality rate estimated as higher than 90 % in all deciduous or mixed stands of BNP (Polish part), and after these peaks no damages from geometrids were noted for 3-5 years. Occasional collections in 1990s yielded single infected specimens. However, they have never been done during the geometrid outbreaks.

**Zoophthora arginis** Balazy

Epizootics caused by this fungus, discovered by Mrs Professor Dr. Z. Machowicz-Stefaniak, appeared regularly at the end of larval development of *Arge berberidis* feeding on ornamental *Berberis* spp. In a municipal park in Lublin, during the observations period 1975-1981, estimated mortality rate reached 80-90 %, but the larval populations of the following generation were restored invariably at the high level, causing almost total defoliation of invaded shrubs. Observations were not continued after 1981, but this pathogen was recorded in new localities in the periphery of Poznan city (Dr. M. Tomalak) and in Oberheim near Laufen (Bavaria, Germany – S. Balazy). However, in numerous parks or hedges where berberry shrubs were even strongly invaded by *A. berberidis*, no larvae were found infected by this pathogen.

It is much more difficult to determine the mortality rate in populations of hosts species which do not tend to aggregate. High mortality cases are quite frequent in herbivorous, saprophagous and predacious host populations, but they seldom concern important noxious species. The following may serve as particularly noteworthy examples.

**Conidiobolus gustafssonii** Balazy

This species occurred on *Ectobius lapponicus* in dry Scots pine forests of Central Pomerania (Northern Poland). On the basis of direct counting of alive specimens proportion to cadavers attached to forest floor vegetation, the mortality was roughly estimated within the limits of 20-75 % on patches disseminated on a total area of about 10.000 ha. Mortality peaks appeared only once yearly, between 15 June – 5 July. Despite relatively high mortality no remarkable fluctuations of host population were noticed. Single infected individuals were reported from several other localities of Poland.

**Entomophaga batkoi** (Balazy) Humber

The fungus was found on *Oligolophus tridens* (Arachnoidea: Opiliones), in deciduous and mixed forests of WPN. The first infected individuals appeared in the last days of July and the mortality increased to reach 70 % or more by the end of September (2-7 cadavers/1 m²). The mycosis grew every next year on separated patches of the areas from 0,2 to about 5 ha. Disease development led to extreme (sometimes total) decline of harvest spider number on affected plots, but some relatively big areas remained unaffected.

**Batkoa apiculata** (Thaxter) Batko

This species differs from the majority of other entomophthoralean members by its conspicuously polyphagous character, although the representatives of Auchenorrhyncha and adults of Diptera are preferred. Moreover, the fungus is tolerant of cool or even cold weather, quickly reacting to a habitat humidity augmentation by the increase of its prevalence, especially during late summer, and it persists active till the autumnal ground-frost period. Despite its common frequency in the majority of wooden and shrubby associations, strongly eruptive epizootics have not been observed even in dense insect populations. As a matter of fact, the total numbers of infected hosts and the longlasting activity of the fungus, including periods unfavourable for other pathogens, could indicate a rather very important role among natural enemies, though unnoxious and beneficial forms prevail among its hosts. High mortality cases were particularly frequent in reserve deciduous and mixed forests, but during longlasting summer droughts, single infected individuals may be found only in moist sites.
**Pandora echinospora** (Thaxter) Humber

Extraordinarily strong epizootic on lauxaniid flies appeared in WNP deciduous and mixed forests in August to October 1976, 1977 and 1980, on an area of about 4000 ha. 3 to over 500 host cadavers were found per m² in forest floor and undergrowth vegetation, according to sampled patches. The fungus produced almost exclusively resting spores (*Tarichium richteri*) and only few cases of conidial sporulation occurred each year in the early phase of disease appearance (turn of July/August). Very deep decline of host population was observed till the mid-80s but single infected individuals were found every year, with noticeable increase in 1990.

**Pandora lipai** Balazy, Eilenberg et Papierok

Every year the observed ratios of infected cadavers to alive specimens of *Rhagonycha lignosa* adults were as high as 30-50 % in deciduous forests of WNP and BNP from the last ten days of May to mid June. The disease appeared in small plots (0,1-0,5 ha) scattered in the stands, though the host population seems to be rather regularly distributed. Only single cases of infected *R. lignosa*, *Cantharis rustica*, *C. livida* and seldom some other host cantharid-species were noticed out of these reserve stands.

**Zoophthora aphrophorae** (Rostrup) comb. nov.

This species was overlooked in Balazy’s (1993) review, where strains infecting mirid bugs *Dicyphus pallidus*, *Stenodema* spp., and *Lygus* sp., and occasionally found on plant hoppers were described under the name *Zoophthora miridis*. Further investigations, especially those in Bavarian, French and Polish grasslands, showed a simultaneous and rather common occurrence of this fungus on representatives of both orders. Furthermore, cultures originating from these different host do not show differences in morphology and development. Although this fungus commonly appeared in most meadows and rushes, epizootics have been observed only, but continuously since 1977, on *D. pallidus* in several stands of oak-horn beam forest undergrowth in WPN. The cadavers were found on the underside of *Stachys silvatica* leaves, often up to 12 individuals on one plant, and the mortality estimated on the basis of alive/dead individuals ranged from 70 to 95 %. Periodically, 2-3 years of very low *D. pallidus* population density were observed. The pathogen survived mostly on few individuals of *Stenodema* sp. or plant hoppers, seldom on other bugs of the family Miridae.

**Data on further entomophthoralean species**

Conspicuously increased mortalities caused by different entomophthoralean species in populations of many other insects of hardly estimable density were often observed in various habitats during and after short-periodical rainy weather in all late spring and summer seasons. It usually, but not always, coincided with heavy epizootics on widely studied important pests such as aphids, weevils, anthomyid flies and others. The following events can be mentioned as especially typical and frequent, keeping in mind that all the examples presented in this contribution did not exhaust the list of ascertained cases of noteworthy entomophthoralean fungi effects on arthropod populations:

- mycoses in adults of many dipteran species caused by *Pandora muscivora* in deciduous forests, affecting individuals belonging to at least 10 families including big rhagionid and therevid species;
- *Zoophthora opomyzae* Balazy on adult opomyzid flies and at least three *Entomophthora* species on empidids. Infected specimens occurred singly and in groups of about 5-30, appearing often regularly for many subsequent days on the same plants in different afforestations and their surrounding, as well as lake-side rushes in Poland and Bavaria;
- *Zoophthora giardii* Balazy, which only infected in the same stand the grasshopper *Meconema thalassinum*;
- *Furia sciarae* (Olive) Humber affecting dense population of nematocerous gnats mostly of the family Sciaridae in deciduous fresh and moist stands;
- *Tarichium rhagonycharum* Balazy on *R. lignosa*. This fungus appeared normally dispersed in single cadavers but in one case (28 May 1991 in WPN), a number of groups of several dozen individuals were found on linden beech and hazel in a reserve stand underwood. Similar groups of small ichneumonids infected by *Zoophthora ichneumonis* Balazy occurred in BNP (found on 27 July 1995), but during a longlasting drought period, as the only cases of entomophthoroses on forest undergrowth leaves.

However, the most regular situation is represented by the entomophthorosis of adult dipterans in relation with extremely moist habitats in forest and wetlands shaded by trees and arborescent bushes. In WNP about twelve species of Entomophthorales form teams infecting these insects continuously from the beginning of May till the end of October, usually with two sharp peaks at the turns of May-June and June-July and one rather smooth in September-October. The first disease cases appear at the beginning of May and are caused by *Erynia variabilis* (Thaxter) Remaudière et Hennebert on small Psychodidae; this pathogen also ends mycoses appearance in the last days of October on flies of the same family. Subsequent dominant species are *E. ovispora* (Nowakowski) Remaudière et Hennebert and *E. conica* (Nowakowski) Remaudière et Hennebert, whereas the early summer peak is caused mostly by *Entomophthora culicis* (A. Braun) Fresenius. In mid-summer *Erynia sepulchralis* (Thaxter) Remaudière et Hennebert appears as the subdominant pathogen, whereas species as *Entomophaga kansana* (Hutchinson) Batko, *Eryniopsis longispora* (Balazy) Humber, *Erynia aquatica* (Anderson et Ringo) Remaudière et Hennebert, *E. curvispora* (Nowakowski) Remaudière et Hennebert behave locally as non-significant components.

The microclimatic or temporal weather conditions generally favourable for the development of fungi often enhance effects of entomopathogens treated as rare or only associated with strongly epizootic species. Such cases were ascertained in a mountain wet site in south Germany, where *Zoophthora phalloides* Batko ranked with *Pandora neoaphidis* (Remaudière et Hennebert) Humber (= *Erynia neoaphidis* Remaudière et Hennebert) as mortality factors of *Microphium carnosum* on nettle, and in a Polish riparian rush where *Zoophthora occidentalis* occurred as the only mortality agent in aphid colonies on bramble leaves. Longlasting observations in forest floor vegetation of WNP showed frequent cases of the complex occurrence of *Entomophthora planchoniana* Cornu, *P. neoaphidis* and *Verticillium lecanii* (Zimmermann) Viégas as principal pathogens, with *Conidiobolus obscurus* (Hall et Dunn) Remaudière et Keller, *Z. phalloides* and *Hirsutella aphidis* Petch as accessory ones on the aphids *Capitophorus similis*, *Cryptomyzus ribis* and *Urololeucon tussilaginis* after wet summer periods and in autumn.

To conclude, fairly extensive author’s recognitions in different ecosystems seem to show that entomopathogenic fungi as a whole affect beneficial entomaphagous as well as phyto- and saprophagous arthropods, among them some species of great significance for plant protection, matter cycling and soil forming processes (Balazy, 2000).

It is the author’s opinion that the often similar observed situations justify the necessity of restoration refuge habitats of arthropods and their pathogens in countryside, in order to increase the potential of biocenotic regulation in agricultural and forest ecosystems. Such activity corresponds well with the guidelines for the integration of agriculture with biodiversity preservation recommended by the Council of Europe.
References

Investigations on entomophthoralean fungi in Latvia

Liga Jankevica, Zigrida Cudare
Department of Experimental Entomology, Institute of Biology, University of Latvia, Miera street 3, Salaspils, LV 2169, Latvia

Abstract: Fermentative activity and biochemical composition (content of amino acids and fatty acids) of Latvian strains of entomophthoralean fungi were determined. Amount of amino acids depended on nutrient conditions. Strains of Conidiobolus obscurus, C. thromboides and Basidiobolus ranarum exhibited high virulence on different aphid species, spider mites and flies. The Conidiobolus strains caused 95 to 98% mortality in the aphid Aphis gossypii within 48 h. B. ranarum caused 100% mortality in the mite Tetranychus urticae from 12 to 24 h following exposure to the fungus. Some strains were tested in greenhouse conditions against aphids and spider mites. Fungal strains that remained able to germinate after the process of dehydration were selected.

Key words: Conidiobolus obscurus, C. thromboides, Basidiobolus ranarum, biochemistry, virulence, aphids, pest control

Introduction

The order Entomophthorales (Zygomycetes) are an important group of entomopathogenic fungi. They cause lethal infections and can regulate insect and mite populations through epizootics. This order consists of more than 220 arthropod pathogenic species spread out in 3 families and 11 genera (Balazy, 1993). Bio-preparations based on Entomophthorales may be considered for the following reasons: 1) these fungi have a high host specificity, 2) they are well adapted to environmental conditions, 3) they have a tendency to cause epizootics (Keller, 1998).

For many years, the Institute of Biology located in Salaspils has been working on problems involved in the development of microbiological methods for plant protection in Latvia. Research on entomophthoralean fungi was performed since 1969. Observations on natural epizootics and collecting of diseased insects were done regularly in central and western part of Latvia. These investigations showed that epizootics are caused by Conidiobolus obscurus, C. thromboides, C. coronatus, Basidiobolus ranarum, Erynia neoaphidis, Entomophthora muscae, Zoophthora radicans, Entomophthora sp. (Jegina et al., 1977; Petrova et al., 1997; Cudare, 1998). Target pests are aphids, spider mites, flies and thrips. The collection of entomopathogenous microorganisms (including Entomophthorales) was created in the Institute of Biology in 1993.

The aim of studies was to extend knowledge of Entomophthorales and to clarify the biological properties and their role in regulation of pest populations. Attention was paid to investigation of qualitative properties of isolates. Some procedures were done, namely: 1) the determination of morphological properties of fungal strains; 2) the testing of host range and virulence; 3) the determination of optimal growth conditions. The surface structure of spores was investigated by electron microscope. The main objective was to prepare the ground for the field application of entomophthoralean mycoinsecticides by increasing the knowledge particularly in the following areas: biochemical composition of strains, virulence and viability under standard and non-standard conditions, viability of spores during drying process.
Material and methods

Fungal cultures and cultivation
Local strains of entomophthoralean fungi: *C. obscurus* E-68 from *Aphis pomi* (isolation by J. Cinovskis & K. Jegina); *C. thromboides* from *Neomyzus circumflexus* (by K. Jegina); *C. obscurus* 79 from soil and *B. ranarum* from *Myzus persicae* (by Z. Cudare) were used in investigations.

For biochemical research, the cultures were grown in glucose/pepton medium (40 g glucose, 20 g peptone, 1.36 g KH₂PO₄, 1 g yeast extract in 1 l water), soya–starch media and Malt extract. The cultures were grown in 750 ml retorts (100 ml medium in each), on shaker (180 rpm) at 24 °C. Cytochemical investigations were according to the methods described by Sumner & Sumner (1969).

For laboratory tests and field trials cultures were grown in Petri dishes and plates (23 x 32 cm) on Malt agar for 3 weeks at 22 – 24 °C. Cultures were dried (at 32-35 °C, with forced aeration) and pulverized two weeks before application. Structure of dried mycelium and resting spores were investigated by electron microscope.

Determination of biochemical composition and fermentative activity
Biochemical composition of cultures was determined in collaboration by the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. Fatty acid composition was determined using standard substance *C₂₂H₄₆* (Suglanovich et al., 1982). The content of amino acids was determined following the method of Beker & Vasiljeva (1972). Fermentative activity was determined according to Rukhlyayeva & Polygalina (1981), using a FEK- 56 PM.

Determination of pathogenicity and efficiency
Suspensions of fungal cultures (titre: 5.2.10⁵ resting spores; 0.1.10⁵ conidias) were spread on cucumber leaves infested with *Aphis gossypii* and on bean leaves infested with *Tetranychus urticae*. The treatments were kept under conditions: 24 °C, 60-70 % relative air humidity and 16 h light. Mortality in aphids was recorded every 6 h after application.

*C. obscurus* suspensions made from fresh and dry cultures (titre : 7.10⁵ resting spores) were spread on cucumbers against *A. gossypii* and *N. circumflexus* in greenhouses located in Marupe (350 m²). The control plants were spread with 1% milk powder suspension. Backpack air-blast sprayer was used. Titres of spores and conidia suspensions were estimated using a haemocytometer. Mortality was recorded after 24 and 48 h, by inspecting 15 plants. Part of infected insects was inspected microscopically.

Results and discussion

In previous studies Jegina (1988) and Cudare (1989) showed morphological characteristics of various strains (morphology of colonies, spore size, shape, surface structure, sporulation, growth rate). Biochemical composition (content of lipids, fatty acids and amino acids) of dried cultures was compared. Structural and biochemical changes have been elucidated in spores and mycelium during growing on different media. On rich nutrient media or in the pest insect the intensive development of polysaccharides and lipids are characteristic for all cultures. On glucose/peptone medium, the percentage of proteins in the dry biomass of *C. obscurus* ranged from 30 to 33 %. 17 amino acids were determined in the biomass of *C. obscurus* and *B. ranarum* isolates (Table 1) after cultivation on glucose/peptone medium. The amount of amino acids is higher when cultures grew on Malt extract. The dry biomass of *C. obscurus* cultures after cultivation on glucose/peptone medium contained lipids at concentrations 20.5 to 35.5 %, *C. thromboides* 24 % and *B. ranarum* 7.4 to 22.0 % respectively. Mono- and poly-
unsaturated fatty acids were characteristic for tested cultures. The experimental data showed that the amount of lipids in biomass differs in developmental stages of fungi (Cudare, 1990). Lipids of C. obscurus 79 contained 19 fatty acids: C_{12:0}, C_{14:0}, C_{14:1}, C_{15:0}, C_{16:0}, C_{16:1}, C_{16:2}, C_{17:0}, C_{18:0}, C_{18:1}, C_{18:2}, C_{18:3}, C_{20:0}, C_{20:1}, C_{20:2}, C_{20:4}, C_{21:0}, C_{22:0} and C_{24:0}. The fatty acids C_{20:4} and C_{12:0} were the most variable among the C. obscurus isolates (Cudare, 1990). Significant differences were observed in fatty acid composition. It was determined that lipids of C. obscurus are richer in fatty acids than those of B. ranarum. The results showed that amount and composition of fatty acids depend on the fungal species and nutrient conditions. The experimental data confirmed that fungi can accumulate neutral lipids even growing on unfavourable medium such as peptone/glucose medium (Cudare, 1990). Cytochemical investigations showed that neutral lipids are accumulated in fungal mycelia and spores. Polysaccharides and lipid accumulated in optimal growth conditions could allow spores to survive after dehydration and were important in the process of reactivation of spores.

Table 1. Contents of amino acids in biomass of the 2 isolates C. obscurus 79 and B. ranarum.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount in biomass, g/100 g</th>
<th>Amino acid</th>
<th>Amount in biomass, g/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. obscurus</td>
<td>B. ranarum</td>
<td>C. obscurus</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.15</td>
<td>0.02</td>
<td>Alanine</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.06</td>
<td>0.003</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.12</td>
<td>0.02</td>
<td>Valine</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.29</td>
<td>0.04</td>
<td>Methionine</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.18</td>
<td>0.05</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Serine</td>
<td>0.15</td>
<td>0.04</td>
<td>Leucine</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.39</td>
<td>0.06</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Proline</td>
<td>0.12</td>
<td>0.01</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.15</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Fermentative activity of cultures significantly depends on used media and stage of culture development. Highest proteolytic activity was found during intensive formation of biomass: C. thromboïdes – 22.13 units/g; C. obscurus E-68 – 11.77 units/g; C. obscurus 79 – 14.83 units/g B. ranarum – 8.24 units/g. Investigations showed that the proteolytic activity of C. obscurus 79 isolate increased when the culture were grown on soya-starch media (22.25 units/g). Addition of glucose to nutrient media (10 g/l) decreased proteolytic activity for 7 units.

Latvian isolates exhibited high pathogenicity and broad host spectrum. For example, C. obscurus E-68 can infect 15 aphid species as well as Tetranychus cinnabarinus (Cudare, 1998). After 48 h mortality caused by C. obscurus E-68 in A. gossypii was 95.7 ± 2.1 %, by C. thromboïdes 95.8 ± 2.9 %, by B. ranarum 91.4 ± 3.3 % (mortality in control 3.9 ± 2.2 %). After 48 h mortality caused by C. obscurus E-68 in T. urticae was 97.6 ± 1.7 %, by C. thromboïdes 97.6 ± 2.4 % (mortality in control 4.6 ± 2.2%). B. ranarum caused 100 % mortality of T. urticae after 24 h.

C. obscurus E-68 had high efficiency against A. gossypii and N. circumflexus on cucumbers. Mortality of aphids after 48 h ranged from 66 to 86 %. Mortality of aphids is higher when the relative humidity and temperature are higher (Fig. 1). No significant differences were found between the efficiency of suspensions made from fresh and dry cultures, which confirmed that C. obscurus E-68 spores remained able to germinate after dehydration at 32-35 °C.
In the process of dehydration the size of *C. obscurus* and *C. thromboides* spores decreases, surface structure and form vary. Matured spores were stable and remained viable under dehydration.

![Mortality, % (corrected after ABBOT)](image)

Relative humidity and temperature during the trial: *52-72%, 18-27 °C; ** 69-73 %, 20-27 °C

**Figure 1. Efficiency of *C. obscurus* E-68 against *Aphis gossypii* and *Neomyzus circumflexus* on cucumbers (field trial in greenhouse).**

**References**


Survival of *Erynia neoaphidis* in aphid cadavers in a simulated winter environment

Tony Bonner, Simon N. Gray, Judith K. Pell
Faculty of Science, Technology and Design, University of Luton, Park Square, Luton, Beds, LU1 3JU, UK; 1 Plant and Invertebrate Ecology Division, IACR-Rothamsted, Harpenden, Herts, AL5 2JQ, UK

Abstract: A possible mechanism for the winter survival of *Erynia neoaphidis*, which does not appear to produce resting spores, was tested. Dried, *E. neoaphidis* mycosed pea aphid (*Acyrthosiphon pisum*) cadavers were stored in a temperature regime alternating (12 h/12 h) between 8 °C and -1 °C at a range of relative humidities for 24 weeks. No conidia were discharged by cadavers incubated under optimal conditions for sporulation (18 °C and 100 % RH) after storage at 90 or 100 % RH for 6 weeks or more. Cadavers stored at 50 or 20 % RH for up to 24 weeks discharged conidia when incubated under optimal conditions for sporulation, and the infectivity of these conidia against *A. pisum* was not significantly reduced compared to conidia discharged by freshly prepared cadavers. The results support the hypothesis that *E. neoaphidis* can over-winter within an aphid cadaver at low RH.

Key words: *Erynia neoaphidis*; winter survival; relative humidity; aphid cadaver; conidia.

Introduction

The entomophthorarlean fungus *Erynia neoaphidis* Remaudière et Hennebert (=*Pandora neoaphidis* (Remaudière et Hennebert) Humber) is pathogenic to a wide range of aphid species, frequently causing epizootics in the field when environmental conditions are favourable (e.g. Wilding, 1975). It therefore has potential as a biological control agent of aphids. However, natural epizootics often occur too late in the development of the aphid population to prevent crop damage. Results from field trials where applications of this fungus have been made to control aphid populations have been inconsistent (e.g. Wilding et al., 1990; Latteur & Godefroid, 1983). Reliable and effective exploitation relies on an improved understanding of the life cycle of *E. neoaphidis*, particularly mechanisms of persistence in the environment.

Currently it is not known how *E. neoaphidis* overwinters in the absence of aphid hosts. Other entomophthorarlean species produce asexual (azygospore) or sexual (zygospore) thick-walled resting spores which survive through the winter but these have not been observed for *E. neoaphidis* (Balazy, 1993). Wilding (1973) demonstrated that dried mycosed cadavers of *Acyrthosiphum pisum* which contained hyphal bodies survived many months at 4 °C and 20 % RH in the laboratory. Feng *et al.* (1992) gained similar results for mycosed cadavers stored from November to March both at 4 °C in a refrigerator and when suspended from shrubs 0.5 m from the ground in the field, but found that the hyphal bodies did not survive when cadavers were stored buried in polypropylene containers plugged with cotton wool in the soil.

The aim of this study was to test the hypothesis that *E. neoaphidis* is able to survive simulated winter conditions when stored in the form of hyphal bodies within an aphid cadaver at low relative humidity (RH).
Materials and methods

Production of dried mycosed cadavers

_Erynia neoaphidis_, reference X4, from the IACR-Rothamsted culture collection, was maintained by repeated _in vivo_ passage through the pea aphid _A. pisum_ using the methods of Wilding (1973). Dead infected aphids were collected as soon as they died, dessicated at 20 % RH and stored at 4 °C for a maximum of 4 weeks before use.

Storage of mycosed cadavers under simulated winter conditions at different relative humidities

A single mycosed aphid cadaver was placed in each of 80 wells of four sterile polystyrene 96-well microtitre plates (TPP, Switzerland). Each plate was maintained at a different relative humidity; 100 %, 90 %, 50 % or 20 %, achieved by the addition of aqueous glycerol solutions to the remaining 16 wells (0 % w/v, 27.5 % w/v, 73 % w/v and 90.05 % w/v respectively; Johnson, 1940). A 2 mm air gap was maintained between the lid and base of each plate by means of a cork spacer, allowing the atmosphere above all wells to equilibrate to the required relative humidity. The plates were sealed using laboratory sealing film (Whatman, Maidstone, UK) and incubated in an alternating temperature regime of 8 °C for 12 h followed by -1 °C for 12 h. These conditions simulated the mean day and night temperatures for the period of November-March in the southern UK (data from the Meteorological Office, Bracknell, UK). As the dimensions of the wells were too small to allow insertion of a humidity monitor, the ability of the experimental system to maintain the required humidity was tested by adding the appropriate glycerol solutions to larger polystyrene sample tubes with a similar ratio of air space:volume to the 96-well plates. The RH was confirmed using a Tinytalk® II relative humidity data logger (RS Components Ltd, Corby, UK).

Determination of rates of discharge and infectivity of conidia from stored cadavers

Ten cadavers were sampled from each of the plates after 3, 6, 9, 12, 15, 21 and 24 weeks’ storage and pre-incubated for 1 h at 100 % RH and 18 °C to encourage sporulation. Conidia discharged over the following 168 h were collected, under optimal conditions for sporulation, in a sporulation monitor as described by Pell _et al._ (1998) and the number of conidia quantified microscopically every 5 h. Electronic images of the conidia at each time interval were captured using a JVC TK1280E colour CCD camera connected to a Videologic DVA4000 video adapter mounted in a Macintosh Quadra 650 computer and the numbers and dimensions of conidia were determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). A further ten cadavers were sampled from each treatment after 18 weeks and, where conidia were discharged, their infectivity against _A. pisum_ was tested using the bioassay system of Morgan _et al._ (1995).

Results and discussion

Large numbers of conidia were discharged from cadavers which had been stored for three weeks at 90 and 100 % RH, but such cadavers failed to discharge conidia after 6 or more weeks storage. Cadavers stored at 50 % RH discharged fewer conidia in total after 3 weeks storage than those stored at higher RH, although the peak rates of discharge of conidia after 3 weeks storage did not differ significantly between these treatments (Fig. 1). In contrast to those stored at higher RH, cadavers that had been stored at 50 % RH for the full 24 weeks still discharged conidia on incubation, although the peak rate and number of conidia discharged decreased significantly (_P_<0.05, t-test) as a result of storage (Fig. 2). The rate of discharge of conidia by cadavers that had been stored at 20 % RH was initially the lowest of all treatments, but only decreased slightly over the 24 week storage period and was greater than that for any
other treatment after 9 or more weeks storage (Fig. 1). Infectivity of conidia discharged from cadavers stored for 24 weeks was not significantly different to conidia discharged from freshly prepared cadavers.

Fig. 1. Peak rate of discharge of *E. neoaphidis* conidia from aphid cadavers previously stored under simulated winter conditions at 20 % (●), 50 % (■) and 90 % (○) RH. Error bars = standard errors of the mean (which are less than the size of the symbols at 90 % RH), n=10.

Fig. 2. Discharge of *E. neoaphidis* conidia from aphid cadavers previously stored under simulated winter conditions at 50 % RH for 3 (●), 9 (■) and 24 (○) weeks. Arrow indicates peak rate of discharge after 3 weeks storage. Error bars = standard errors of the means, n=10.

Desiccation on silica gel is commonly used as a method for the preservation of microbial cultures (Grivell & Jackson, 1969). Metabolism is suspended and the cultures retain their viability. It is likely that the metabolism of *E. neoaphidis* biomass within aphid cadavers is
also slowed at low RH, allowing it to retain viability for longer. In addition, Hallsworth & Magan (1995) showed that conidia of deuteromycete entomopathogenic fungi contained elevated concentrations of polyols when produced at low water activity ($A_w$). If such polyols were produced by *E. neoaphidis* within cadavers at low RH (and hence low $A_w$), they might have a cryoprotective effect thus enhancing survival under winter conditions. The results support the hypothesis that *E. neoaphidis* can over-winter within an aphid cadaver at low RH. There is potential that crop management regimes could be optimised to favour long term survival of this entomogenous fungus during winter.

**Acknowledgements**

JKP was supported by the Ministry of Agriculture Fisheries and Food (MAFF) of the UK. IACR-Rothamsted receives grant aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK.

**References**


Investigations on the occurrence of entomopathogenic fungi and entomoparasitic nematodes in soils from Lower Austria

A. Hozzank, R. Wegensteiner, W. Waitzbauer\textsuperscript{1}, A. Burnell\textsuperscript{2}, Z. Mrácek\textsuperscript{3}, G. Zimmermann\textsuperscript{4}

Institute of Forest Entomology, Forest Pathology and Forest Protection, Univ.-BOKU, Hasenauerstrasse 38, 1090 Vienna, Austria
\textsuperscript{1} Inst. for Ecology and Nature Conservation, Vienna University, Althanstrasse 14, 1090 Vienna, Austria;
\textsuperscript{2} Department of Biology, National University of Ireland (NUI), Maynooth, Co. Kildare, Ireland;
\textsuperscript{3} Laboratory of Insect Pathology, Inst. of Entomology, Czech Academy of Sciences, Branisovská 31, 37005 Ceské Budejovice, Czech Republic;
\textsuperscript{4} Federal Biological Research Centre, Institute for Biological Control, Heinrichstrasse 243, 64287 Darmstadt, Germany

Abstract: The occurrence of entomopathogenic fungi and entomoparasitic nematodes in soils from Lower Austria was studied using larvae of \textit{Galleria mellonella} and \textit{Tenebrio molitor} as bait insects. Soil samples were taken from forest stands, conventionally and organic treated fields, vineyards, orchard, hedgerows and fallow in 1998 and 1999. Entomopathogenic fungi were isolated from a major part of the soil samples (84\%). The two most common species of fungi were \textit{Beauveria bassiana} (74\%) and \textit{Metarhizium anisopliae} (29\%). \textit{Paecilomyces fumosoroseus} was isolated only from 3\% of the samples. Entomoparasitic nematodes were found in 25\% of the samples only.

Key words: entomopathogenic fungi, entomoparasitic nematodes, occurrence, survey, organic farming

Introduction

Entomopathogenic fungi and entomoparasitic nematodes in soil can be important factors in the regulation of pest insect populations. Studies in this field have been published by authors from several countries, however little work has been done on the occurrence of these two antagonistic groups in Austria. Until now only one study reported about the occurrence of fungi and nematodes in soil in Austria with special focus on forest sites (Wegensteiner \textit{et al.}, 1998). The aim of this investigation was to compare the distribution and diversity of entomopathogenic fungi and entomoparasitic nematodes in organic treated field sites, with conventionally treated sites and with sites without cultivation as well as with forest sites.

Material and methods

Sampling sites

All the sampling sites were located in the north-eastern part of Lower Austria: seven sites near Großmugl (district Korneuburg), five sites near Göllersdorf (district Hollabrunn) and five sites near Groß Enzersdorf (district Gänserndorf). The cultivated sites differed in their cultivation (conventional: using herbicides, insecticides and chemical fertiliser; organic: using
no herbicides, no insecticides and only special fertiliser), method of tillage and soil type (Tables 1 and 2).

Table 1. Number of sampling sites, type of site, cultivation and treatment of different sampling sites.

<table>
<thead>
<tr>
<th>Number</th>
<th>Site</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>forest sites</td>
<td>no treatment</td>
</tr>
<tr>
<td>2</td>
<td>hedgerows</td>
<td>no treatment or organic</td>
</tr>
<tr>
<td>2</td>
<td>fallows</td>
<td>no treatment (except cutting)</td>
</tr>
<tr>
<td>1</td>
<td>orchard</td>
<td>conventionally treated</td>
</tr>
<tr>
<td>1</td>
<td>vineyard</td>
<td>organic treated</td>
</tr>
<tr>
<td>1</td>
<td>vineyard</td>
<td>conventionally treated</td>
</tr>
<tr>
<td>4</td>
<td>field sites</td>
<td>organic treated</td>
</tr>
<tr>
<td>4</td>
<td>field sites</td>
<td>conventionally treated</td>
</tr>
</tbody>
</table>

**Sampling and incubation**

Soil samples were taken to a depth of about 30 cm, in 1998 and 1999. Fungi and nematodes were isolated using the *Galleria* bait method (Bedding & Akhurst 1975; Zimmermann, 1986), with *Galleria mellonella* and *Tenebrio molitor* as bait insects. 200-250 cm$^3$ soil was filled into honey glasses; in each glass four larvae of *G. mellonella* or *T. molitor* were added. Glasses were controlled at day 3, 7, 10, 14, 17 and 21. Samples were incubated at room temperature (20 ± 2 °C) in a moist chamber.

**Identification**

Fungi and nematodes were identified by morphological features. In addition nematodes were also identified by molecular techniques (RFLP).

**Results and discussion**

Entomopathogenic fungi were isolated from a major part of all of the soil sub-samples (84 %). The most common species was *Beauveria bassiana* which was found in soil samples from all sampling sites. *Metarhizium anisopliae* was found in samples of eight sampling sites, *Paecilomyces fumosoroseus* was only isolated from one locality only, from the hedgerow. In the orchard and in the hedgerow (no treatment), significantly more larvae infected with entomopathogenic fungi occurred in comparison with other sites (p<0.001). At least two fungal species were found in all organically grown fields (Table 2). In contrast to the results of a former study (Wegensteiner et al., 1998), only *B. bassiana* occurred in the samples of the two forest sites (Table 2).

Entomopathogenic nematodes were found only in 25 % of the samples only. 17 isolates of entomoparasitic nematodes were identified: two *Steinernema* species: *S. feltiae* and one not conclusively identified species (*S. affinis / intermedium*) (Table 2). Interestingly, differences in occurrence of entomoparasitic nematodes were found at short distances: *S. feltiae* was found in soil samples from an oak forest, whereas no entomopathogenic nematodes could be isolated from soil of a birch stand (next to the oak stand).

The occurrence of entomopathogenic fungi and entomoparasitic nematodes is influenced by a number of biotic and abiotic factors (Kleespies et al., 1989; Vänninen et al., 1989), as well as by the method used for isolation and incubation (Mietkiewski et al., 1994). We also
found more samples with entomopathogenic fungi in samples from clay in comparison with samples from sandy soils. In addition, organic farming may provide a more suitable habitat for entomopathogenic fungi and entomoparasitic nematodes than other forms of agricultural tillage; as it is shown in table 2, there could be an influence of cultivation on the occurrence of entomopathogenic fungi and entomoparasitic nematodes. *P. fumosoroseus* and *S. intermedium / affinis* were found in the organic treated plots only. Sturhan (1996) showed that 97.2 % of *Steinernema* species were found in the upper layer of the field. In organic farming only the upper 30-40 cm were ploughed, which could explain the presence of *S. intermedium / affinis* in the plots we studied. The occurrence of three different entomopathogenic fungal species, especially *P. fumosoroseus*, can be correlated with the fact that no fungicides have been used in the organic treated sites (Table 2).

Table 2. Relation between treatment and occurrence of entomopathogenic fungi and entomoparasitic nematodes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Site</th>
<th>Soil type</th>
<th>Fungus species</th>
<th>Nematode species</th>
</tr>
</thead>
<tbody>
<tr>
<td>forest</td>
<td>sandy chernozem, brown earth</td>
<td><em>B. bassiana</em></td>
<td><em>S. feltiae</em></td>
<td></td>
</tr>
<tr>
<td>fallow</td>
<td>calcareous colluvium</td>
<td><em>B. bassiana</em></td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>hedges</td>
<td>calcareous colluvium</td>
<td><em>B. bassiana</em></td>
<td><em>M. anisopliae</em></td>
<td></td>
</tr>
<tr>
<td>field</td>
<td>calcareous grey flood plain soil</td>
<td><em>B. bassiana</em></td>
<td><em>S. feltiae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. anisopliae</em></td>
<td><em>S. affinis / intermedium</em></td>
<td></td>
</tr>
<tr>
<td>vineyard</td>
<td>calcareous colluvium</td>
<td><em>B. bassiana</em></td>
<td><em>S. feltiae</em></td>
<td></td>
</tr>
<tr>
<td>hedges</td>
<td>calcareous grey flood plain soil</td>
<td><em>B. bassiana</em></td>
<td><em>S. affinis / intermedium</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. anisopliae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>field</td>
<td>calcareous colluvium, calcareous chernozem</td>
<td><em>B. bassiana</em></td>
<td><em>S. feltiae</em></td>
<td></td>
</tr>
<tr>
<td>vineyard</td>
<td>calcareous soil consisting of loamy-silt tertiary material</td>
<td><em>B. bassiana</em></td>
<td><em>M. anisopliae</em></td>
<td></td>
</tr>
</tbody>
</table>

Even if the results of this study cannot be representative neither for soils from Austria nor for effects of organic farming versus conventional farming, they indicate that there might be some influences concerning treatment. Furthermore, it was shown that hedgerows and division into small woods most probably are important for persistence of entomopathogenic fungi and for entomoparasitic nematodes in agro ecosystems.

**Acknowledgements**

We want to thank all the farmers who allowed us to make observations in their fields and the Federal Government of Lower Austria for financial support.
References


Results of preliminary investigations on the occurrence of Entomophthorales on aphids in Austria

Marek Barta, Mariana Stalmachová-Eliasová, Alexandra Hozzank1, Ludovic Cagán, Rudolf Wegensteiner1
Institute of Plant Protection, University of Nitra, Slovakia
1 Institute of Forest Entomology, Univ.-BOKU-Vienna, Austria

Abstract: Until now, no data were available on the occurrence of Entomophthorales in aphids populations in Austria. Aphids were collected at different localities in Vienna, Lower Austria and Styria in June and October 2000. During these two sampling periods four entomophthoralean species were found on various aphid species living on herbaceous plants or trees: Erynia neoaphidis, Conidiobulus obscurus, Entomophthora planchoniana and Neozygites fresenii. No Entomophthorales were observed on Dreyfusia nordmannianae.

Key words: Entomophthorales, aphids, Erynia neoaphidis, Conidiobulus obscurus, Entomophthora planchoniana, Neozygites fresenii

Introduction

Beside predators and parasitoids, fungi are the most important limiting factors for aphid populations (Suter & Keller, 1977). Numerous studies have been done investigating the occurrence of Entomophthorales in populations of these insects (e.g. Shands et al., 1963; Rabasse & Robert, 1975; Wilding & Perry, 1980; Remaudière et al., 1981; Hatting et al., 1999). As no studies have been performed in Austria yet, a preliminary approach was carried out to get first information about the diversity of entomophthoralean fungi in this country.

Material and methods

Aphids were collected at different localities in June and October 2000: in Vienna (Federal Research Station), in a few sites in Lower Austria and Styria. Dead or moribund aphids were collected and stored separately in vials. Fungal infections were identified in the laboratory mainly using morphological features of primary spores (Keller, 1987, 1991).

Results and discussion

The first sampling, which started in mid June, brought evidence of Erynia neoaphidis, Conidiobulus obscurus and Entomophthora planchoniana on Metopolophium dirhodum. No Entomophthorales were found on Dreyfusia nordmannianae, collected at different sites in the foothills of the Alps and in the Alps from heavily infested fir trees (Abies alba). Inspection of dead D. nordmannianae showed only presence of hyphomycete fungi of the genera Cladosporium, Alternaria and Fusarium.

Furthermore, laboratory infection experiments were fulfilled to test the susceptibility of D. nordmannianae to strains of Erynia neoaphidis maintained in vitro. Three strains were tested: sk(NR)1 and sk(NR)92, both originating from Slovakia, and GR42 from Greece (kindly provided by Bernard Papierok); no aphid died from infection by the fungus.
A second sampling series was performed through October, which allowed to find several entomophthoralean fungi on different aphid species (Table 1).

Table 1. List of infected aphid species collected in Austria, with corresponding host plants, and the involved entomophthoralean species (October 2000).

<table>
<thead>
<tr>
<th>Aphid species</th>
<th>Host plant species</th>
<th>Fungus species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhopalosiphum padi</em> L.</td>
<td><em>Padus avium</em> Mill.</td>
<td>Neozygites fresenii</td>
</tr>
<tr>
<td></td>
<td><em>Zea mays</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Rhopalosiphum padi</em> L.</td>
<td><em>Hordeum sativum</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Sitobion avenae</em> F.</td>
<td><em>Zea mays</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Capitophorus elaeagni</em> del Guercio</td>
<td><em>Elaeagnus</em> sp.</td>
<td>Neozygites fresenii</td>
</tr>
<tr>
<td></td>
<td><em>Hippophae rhamnoides</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Myzus persicae</em> Sulzer</td>
<td><em>Persicae vulgaris</em> Mill.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td></td>
<td><em>Sinapis alba</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td></td>
<td><em>Daucus carota</em></td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td></td>
<td><em>Brassica napus</em></td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Anoecia corni</em> Fab.</td>
<td><em>Cornus sanguinea</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Dysaphis crataegi</em> Kalt.</td>
<td><em>Crataegus</em> sp.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Aphis fabae</em> Scop.</td>
<td><em>Euonymus europaeus</em> L.</td>
<td>Neozygites fresenii</td>
</tr>
<tr>
<td></td>
<td><em>Euonymus europaeus</em> L.</td>
<td>Neozygites fresenii</td>
</tr>
<tr>
<td><em>Aphis nasturtii</em> Kalt.</td>
<td><em>Rhamnus catharticus</em> L.</td>
<td>Neozygites fresenii</td>
</tr>
<tr>
<td><em>Macrosiphum euphorbiae</em> Thomas</td>
<td><em>Rosa canina</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entomophthora planchoniana</td>
</tr>
<tr>
<td><em>Aphis pomi</em> de Geer</td>
<td><em>Malus domestica</em> Borkh.</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Brevicoryne brassicae</em> L.</td>
<td><em>Brassica oleracea</em> var. capitata</td>
<td>Erynia neoaphidis</td>
</tr>
<tr>
<td><em>Brachycorynella asparagi</em> Mordv.</td>
<td><em>Asparagus officinalis</em> L.</td>
<td>Erynia neoaphidis</td>
</tr>
</tbody>
</table>

Acknowledgements

We want to thank the Austrian Academic Service for financial support within the scientific co-operation Austria-Slovakia (Project 29s19).
References


Occurrence of entomopathogenic fungi in soils from Central Italy under different managements

Cezary Tkaczuk, Giancarlo Renella
Department of Plant Protection, University of Podlasie, ul. Prusa 14, 08-110 Siedlce, Poland
Department of Soil Science and Plant Nutrition, University of Florence,
P. le delle Cascine, 28, 50144- Firenze, Italy

Abstract: The spectrum and frequency of occurrence of entomopathogenic fungi in the soil under different managements (woodland, grassland, lucerne and wheat) in Central Italy was studied. Fungi were isolated from soil by means of *G. mellonella* larvae as baits. Three entomopathogenic fungal species: *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* were isolated. *B. bassiana* was detected from all investigated soils and was the only fungal species isolated from woodland soil, infecting up to 77 % of bait insects. In the soil from other types of management, *B. bassiana* occurred in lower frequency, infecting from 2.2 to 14.3 % of larvae. *Paecilomyces fumosoroseus* was isolated from all soils with the exception of the woodland whereas *M. anisopliae* occurred only once in the soil from wheat field. The frequency of the occurrence of entomogenous fungal species in the soil under different managements, measured by % of infected *G. mellonella* larvae, was as follows: 77.3 % woodland, 27.5 % wheat field, 16.5 % lucerne and 9.3 % grassland.

Key words: entomopathogenic fungi, isolation, soil, insect bait method

Introduction

Fungi infecting insects and other arthropods play a relevant role in controlling their populations in nature and have potential as biocontrol agents for use in microbial pest control. Several of the most common entomogenous fungal species have cosmopolitan distribution and often decimate insect population in spectacular epizootics. Pedosphere is the main reservoir for these fungi (Ignoffo *et al.*, 1978; Ferron, 1981).

Zimmermann (1986) proposed the “insect bait method” for the isolation of entomogenous fungi from soil, which allows investigations on spectrum composition and frequency of these insect pathogens in soil habitat as compared to the selective medium method. The “insect bait method” has been widely used for isolating entomopathogenic fungi from soils in Germany (Kleespies *et al.*, 1989), Finland (Vänninen *et al.*, 1989; Vänninen, 1995), Denmark (Steenberg, 1995), UK (Chandler *et al.*, 1997; Mietkiewski *et al.*, 1997), Poland (Mietkiewski *et al.*, 1991, 1995b; Tkaczuk & Mietkiewski, 1996) and Norway (Klingen, 2000). In our knowledge, little is known about occurrence of these fungi in the soils in the Mediterranean area and how the land management influences the occurrence of entomopathogenic fungi in Italian soils (Tarasco *et al.*, 1997).

The aim of this study was to determine the spectrum and frequency of entomopathogenic fungi in soils from Central Italy under four different managements.

Material and methods

Soils were sampled in October 2000 from the Vicarello experimental fields located in Tuscany, Central Italy. The soil type is Vertic Xerochrept (USDA, 1992) under four different
managements: woodland, grassland, lucerne and wheat. The first three soils were sampled at 10 cm depth while the wheat soil was sampled at 23 cm, all by means of a Dutch auger. All soils were air dried for 2 days at 20 °C and sieved in order to remove stones and other debris. About 250 ml of soil were placed into a plastic box and ten *Galleria mellonella* larvae were added. Ten boxes were prepared per treatment and all were incubated at 21 °C for 25 days.

The larval mortality was monitored first at day 6 and then every 3 days afterwards until the end of incubation time. Boxes were turned and agitated every day to ensure that the larvae come into maximum contact with the soil. Dead larvae were surface sterilized in 1 % sodium hypochloride for 30 sec, rinsed three times in sterile distilled water and placed in humid conditions to encourage fungal outgrowth and sporulation. Factors causing mortality were identified as far as possible. Preliminary identification of fungi was confirmed by slide preparation.

**Results**

Three entomopathogenic fungal species: *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* were isolated from the sampled soils (Tab. 1). *Beauveria bassiana* was detected from all the studied soils and was the only fungal species isolated on *Galleria mellonella* larvae from woodland soil, infecting up to 77 % of bait insects.

<table>
<thead>
<tr>
<th>Factor of mortality</th>
<th>Type of management</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lucerne field</td>
</tr>
<tr>
<td><strong>Entomopathogenic fungi</strong></td>
<td></td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>7.2</td>
</tr>
<tr>
<td><em>Metarhizium anisopliae</em></td>
<td>–</td>
</tr>
<tr>
<td><em>Paecilomyces fumosoroseus</em></td>
<td>9.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>16.5</td>
</tr>
<tr>
<td><strong>Fungi of unproved entomo-pathogenic abilities</strong></td>
<td></td>
</tr>
<tr>
<td><em>Cylindrocarpon sp.</em></td>
<td>1.0</td>
</tr>
<tr>
<td><em>Fusarium sp.</em></td>
<td>14.4</td>
</tr>
<tr>
<td><em>Gliocladium sp.</em></td>
<td>7.2</td>
</tr>
<tr>
<td><em>Mucor sp.</em></td>
<td>14.4</td>
</tr>
<tr>
<td><em>Aspergillus sp.</em></td>
<td>9.3</td>
</tr>
<tr>
<td>Non-sporulated mycelium</td>
<td>8.3</td>
</tr>
<tr>
<td><strong>Other factors</strong></td>
<td></td>
</tr>
<tr>
<td>Nematodes</td>
<td>–</td>
</tr>
<tr>
<td>Undefined causes</td>
<td>8.3</td>
</tr>
</tbody>
</table>

In soils under other type of management *B. bassiana* occurred in lower infecting frequencies: 14.3, 7.2 and 2.1 % in wheat, lucerne and grassland soils, respectively.
In the soil taken from wheat and lucerne fields, and also from grassland, besides *B. bassiana* *Paecilomyces fumosoroseus* was found, infecting 12.2, 9.3 and 7.2 % of *G. mellonella* larvae respectively.

The fungus *Metarhizium anisopliae* occurred only once in the soil from wheat field, but with a very low infectivity (below 1 %).

The total frequency of infection by entomogenous fungal species in soils under different managements, as measured by % of infected *G. mellonella* larvae, was as follows: 77.3 % woodland, 27.5 % wheat field, 16.5 % lucerne and 9.3 % grassland.

Apart from specific entomopathogenic fungal species, opportunistic or saprophytic fungi like *Aspergillus* sp., *Fusarium* sp. and *Gliocladium* sp. were also observed on the dead larvae.

**Discussion**

The occurrence of entomopathogenic fungi in Italian soils has been investigated mostly through observations on cadavers or insects with visible symptoms of disease (Ozino, 1989; Nanni et al., 1988; Triggiani, 1986; Triggiani & Lipa, 1989). In our knowledge, this is the first study reporting the presence and the infectivity of entomopathogenic fungi in soils from Central Italy under four different management using the “insect bait method”. Previously, Tarasco et al. (1997), when studying the occurrence of entomogenous fungi in Southern Italian soils using larvae of *G. mellonella* as “bait insect”, reported that among three isolated fungal species (i.e. *Beauveria bassiana*, *Paecilomyces lilacinus* and *Metarhizium anisopliae*), *B. bassiana* was the most widespread entomopathogenic fungal species, especially in uncultivated area. Our results agree with these previous findings. In fact, in our study, *B. bassiana* infected much more *G. mellonella* larvae in the forest soil than in agricultural cultivated fields. These data also agree with the results obtained in Finnish soils by Vänninen (1995). Kleespies et al. (1989), when investigating the spectrum of entomopathogenic fungi in the soils under different crop systems in Germany, confirmed the high frequency of *B. bassiana* in the forest soil. Forest litter and soil appeared to be the best habitat for this fungus in Poland (Tkaczuk & Mietkiewski, 1998; Bajan et al., 1995).

Another interesting finding of the present study was the isolation of *Paecilomyces fumosoroseus* from Italian soils using the “insect bait method”. This species was found in all the investigated soils with the exception of the forest soil. *P. fumosoroseus* is a rather common species occurring in intensively cultivated agricultural soils from North-Eastern Europe regions (Bajan, 1973; Kleespies et al., 1989; Mietkiewski et al., 1991; Tkaczuk & Mietkiewski, 1996; Mietkiewski et al., 1995b). The fungus appeared more rarely isolated from the soil in Finland (Vänninen, 1995) and Denmark (Steenberg, 1995).

Surprisingly, the relatively thermophilic fungus *Metarhizium anisopliae* was isolated in one case only in our experiment. Tarasco et al. (1997) reported a similar result from Southern Italy. The situation in Italian soils appeared therefore different from the situation observed in Northern and Central Europe, where *M. anisopliae* seems to be common species isolated from soil: Germany (Kleespies et al., 1989), Finland (Vänninen et al., 1989; Vänninen, 1995), Denmark (Steenberg, 1995) and Poland (Mietkiewski et al., 1991/92, 1995).

**Acknowledgements**

The authors wish to thank Dr Pagliai, Dr Bazzoffi and Dr Pellegrino from the Institute for Soil Study and Protection, for the access to the study area and details about the soil and the experiments.
References


Interaction between entomopathogenic and saprophytic fungi

Zigrida Čudare, Mara Kropa
Institute of Biology, University of Latvia, Miera Street 3, Salaspils, 2169 Latvia

Abstract: Interactions between Latvian strains of entomopathogenic (Conidiobolus obscurus, Conidiobolus thromboides, Beauveria bassiana and Metarhizium anisopliae) and saprophytic fungi (Trichoderma spp. and Penicillium spinulosum) were studied. The experiments did not show any antagonistic phenomenons between species of entomophthoralean fungi. Trichoderma spp., which is antagonistic to pathogens of plant diseases, inhibited the growth of C. obscurus and B. bassiana. Territorial antagonisms between some fungi were observed.

Key words: entomopathogenic fungi, saprophytic fungi, interaction

Introduction

Using pathogens against pest plants, or pathogenic fungi against pest insects or plant diseases, is considered as environmentally friendly. In such a strategy, a good knowledge about interrelations between these microorganisms is of great importance. Especially, it is necessary to elucidate interactions between the different fungal species, which were most frequently mentioned either as responsible for epizootics in insect populations, as plant protection agents, or common in soil samples. Previous investigations described interactions between B. bassiana and other fungi: 5 Penicillium species, Aspergillus flavus and Trichoderma viride (Bajan, 1978).

In Latvia, the entomophthoralean fungi, Conidiobolus obscurus and C. thromboides, are known to reduce pests in biocenosis, causing epizootics mostly in populations of flies and aphids (Jegina et al., 1977). Strains isolated from insects or soil exhibited high virulence and broad host spectrum.

Promising agents in pest insect biological method are the entomopathogenic fungi Beauveria bassiana (Tsiboulskaia, 1972) and Metarhizium anisopliae (Jegina, 1972).

Saprophytic fungi of the genus Trichoderma are antagonistic to phytopathogenic fungi (Botrytis, Fusarium) and are used in the production of bio-preparations in Latvia.

Furthermore, the saprophytic fungus Penicillium spinulosum can infect insects. All the above-mentioned fungi are parts of the system: soil-plant-plant pathogen-insect-insect pathogen-antagonist, which deserves to be investigated.

The aim of the present work was to study interactions between these fungi, since not infrequently the methods of plant protection which are recommended for the reduction of pathogens involve introduction into the soil, of biological preparations containing entomopathogenic fungi or the fungal antagonist Trichoderma.

Material and methods

Fungal cultures
Local strains of entomopathogenic fungi used in investigations were: Conidiobolus obscurus 79, isolated from soil; C. obscurus 68, from Aphis pomi de Geer; C. thromboides, from Neomyzus circumflexus; Metarhizium anisopliae, from Selatosomus aeneus L.; Beauveria
bassiana, from Galeruca tanaceti L. and their monospore isolates. Tested saprophytic fungi were Penicillium spinulosum and Trichoderma spp.

**Procedure for experiments**
Fungal cultures were grown in Petri dishes (diameter 10 cm) at 22-24 °C in glucose-malt extract-agar medium. Before inoculation fungal cultures were kept in the above-mentioned medium for 5 days. Then 4 x 4 mm pieces of 2 different cultures were inoculated on to Petri dish (Fig. 1). Dishes with inoculated 2 pieces of the same species were used as control. The experiment was repeated 5 times. The average diameter of culture colonies on the 5th day after inoculation was used as indicator growth intensity.

In some cases fungal cultures were grown for 10 days to observe the way the released spores developed.

![Figure 1. Schematic representation of the procedure.](image)

**Results and discussion**

**Interaction between entomopathogenic fungi**
During the growth in Petri dishes the culture colonies of various entomophthoralean strains or monospore isolates were not clearly delimited from one another.

The strain *C. obscurus* 79, isolated from soil, grew more intensively than *C. obscurus* 68 and *C. thromboides*, both isolated from aphids. On the 5th day of growth, the size of *C. obscurus* 79 colonies were 9.9 x 5.6 and *C. obscurus* 68, 6.1 x 4.5 cm; *C. obscurus* 79, 9.7 x 5.4 and *C. thromboides*, 8.0 x 4.3 cm; *C. obscurus* 68, 8.4 x 3.9 and *C. thromboides*, 8.2 x 4.0 cm. In comparison, average size of colonies in control were for *C. obscurus* 79, 8.1 x 4.1, for *C. obscurus* 68, 7.8 x 3.8, and for *C. thromboides* 8.5 x 5.0 cm.

Antagonistic zones between cultures were not observed. All the entomophthoralean cultures had their characteristic morphological features. Colonies of *Conidiobolus* isolates merged. After 3 days conidia and resting spores formation was observed in all colonies. Light layers of conidia were observed for intensively sporulated cultures.

In experiments involving either *C. obscurus* or *C. thromboides* and Beauveria bassiana, the entomophthoralean cultures grew faster than those of *B. bassiana*. In all experiments, colonies of entomophthoralean fungi covered the medium, thus impeding the expansion of *B. bassiana* colonies. On the 5th day of growth, the average size of *C. obscurus* 79 colony was 4.7 x 5.7, and *B. bassiana* colony 1.6 x 2.4 cm only; for *C. obscurus* 68 colony 7.8 x 5.75 cm, and *B. bassiana* 2.8 x 3.3 cm; for *C. thromboides* – 6.0 x 8.0 cm and *B. bassiana* – 2.8 x 3.3 cm. In control, average size of *B. bassiana* colonies was 8.3 x 4.3 cm. Colonies of entomophthoralean fungi had their characteristic morphological features. Colonies of *B. bassiana* were smooth. Since, beginning with the 3rd day growth, *C. obscurus* intensively
sporulated, the growth of *B. bassiana* colonies was impeded also by the mycelium formed from disseminated conidia of the Entomophthora. The colonies of *B. bassiana* were clearly delimited from *C. obscurus* culture.

Unexpectedly, we observed that after 10 days of culture, there were cases when *B. bassiana* intensively sporulated and the spores trapped on *C. obscurus* culture, where they germinated. Indeed, in such situations, *B. bassiana* grew over entomophthoralean mycelia. Examining samples of these sites under microscope, hyphae of both fungi can be seen. Using for inoculation *B. bassiana* monospore cultures, with markedly different growth intensity, it has been observed that these differences survived also in interaction with entomophthoralean fungi. The intensively growing monosporal cultures form larger colonies.

*M. anisopliae* can also impede the growth of entomophthoralean colonies because its vegetative growth is more expansive. At the time when the average size of *M. anisopliae* colonies was 8.8 x 5.7 cm, the size of the colonies of *C. obscurus* 79, *C. obscurus* 68 and *C. thromboide* was 7.6 x 4.1, 7.0 x 3.4 and 4.2 x 2.9 cm, respectively. In control average size of *M. anisopliae* was 8.5 x 5.1 cm. In some places around the colonies of entomophthoralean fungi an antagonistic zone can be seen. *M. anisopliae* developed an elevation along the borderline with the *C. obscurus*. Territorial antagonism of *M. anisopliae* appeared regarding *B. bassiana*.

The mutual interactions between different species of entomophthoralean fungi did not display any antagonistic phenomenons. There was no growth inhibition between the studied species. By contrast, the cultures of *C. obscurus* and *C. thromboide* grew faster than those of *B. bassiana*, the development of which was impeded. Unlike this Hyphomycete, *Metarhizium anisopliae* was showed to be able to impede the growth of the studied entomophthoralean species because of its more rapid vegetative growth. Territorial antagonism between fungi can be observed.

**Interaction between entomopathogenic and saprophytic fungi**

The cultures of *Trichoderma* spp. usually actively sporulated in the place of inoculation. The mycelium arising from the germinated spores is an additional factor that ensures the fact that cultures of these fungi covered the medium in Petri dishes, thus inhibiting the growth of the entomopathogenic fungal species *C. obscurus* and *B. bassiana*. Moreover, a clearly pronounced territorial antagonism of the fungus appeared. Around the colonies of *C. obscurus* and *C. thromboide*, a light, circular zone emerged. For instance: on the 5th day of growth, the average size of the colony of *C. obscurus* 79 was 4.1 x 3.4 cm but that of fungus *Trichoderma* spp. – 9.0 x 5.5 cm. Colonies of *Trichoderma* grew faster than those of *B. bassiana*: the size of the colonies was 9.0 x 7.2 cm for *Trichoderma* spp. and 5.2 x 3.7 cm for *B. bassiana*. These results agree with the data published by Bajan (1978), which showed that the growth of *B. bassiana* was impeded by *T. viride* on each of the used media. The two fungi differed in their expansiveness and formed a clear antagonistic zone. It has been established that *Trichoderma* inhibits the multiplication of nitrogen fixing bacteria (Pavlovitcha, 1978).

The cultures of entomopathogenic fungus *M. anisopliae* and saprophytic fungus *Trichoderma* spp. did not show territorial antagonism (*M. anisopliae*: 5.6 x 4.7; *Trichoderma* spp.: 6.3 x 5.0 cm).

Between the entomopathogenic species *C. obscurus*, *C. thromboide* or *B. bassiana*, and the saprophytic fungus *P. spinulosum*, there is a pronounced antagonism. The colonies of *P. spinulosum* and those of *C. obscurus* or *B. bassiana* were separated by a light zone where the growth of cultures was totally inhibited. Growth was approximatively the same for all species: *C. obscurus* 79: 7.8 x 3.8 cm; *B. bassiana*: 7.9 x 4.0 cm; *P. spinulosum*: 5.2 x 4.1, 4.9 x 3.7 cm. The growth intensity was more intensive for *M. anisopliae* than *P. spinulosum*. Size of *M. anisopliae* colonies achieved approximately 7.9 x 5.7 cm.
It seemed that some of these fungi act antagonistically on nutrient media against test-cultures. Remaining of influence of these cultures in plants/soil system, should deserve further investigation.

The interactions between the microflora and fauna of the soil are numerous and greatly influenced by the physicochemical factors of the habitat. The saprophytic phase is essential for the completion of the developmental cycle of entomopathogenous fungi (Keller & Zimmermann, 1989). One could suppose that saprophitical capacity could define interactions between entomopathogenic and saprophytic fungi.

As a conclusion, the present investigations allowed us to suppose that it is not advisable to introduce into the soil the entomopathogenic fungi *C. obscurus*, *C. thromboides* and *B. bassiana* simultaneously with the fungus *Trichoderma*, which is antagonistic to plant pathogens.

References

Bajan, C. 1978: Interactions between selected species of entomopathogenic and saprophytic fungi. Polish Ecological Studies. 4, 2: 5-54.


Investigations on the virulence stability of *Verticillium lecanii*

Natalja Hetsch, Helga Sermann  
*Humboldt-University of Berlin, Institute of Horticultural Sciences, Department of Phytomedicine, Lentzeallee 55-57, 14195 Berlin, Germany*

**Abstract:** Four strains of the entomopathogenic fungus *Verticillium lecanii* from different origins were studied as regards a possible influence of time of culture and passage on a host on the virulence. Differences were observed between the origin strains and the re-isolates as regards their virulence against the thrips *Frankliniella occidentalis*. One Bulgarian strain and 2 Siberian strains have been positively influenced in their virulence by host passage, thus exhibiting a limited virulence stability. By contrast, the minor negative change of virulence in a strain isolated in Germany resulting from host passage, could be considered as an expression of a stabilized virulence.

**Key words:** *Verticillium lecanii*, virulence stability, reisolates

**Introduction**

The fungus *Verticillium lecanii* (Zimmermann) Viegas is a widespread entomopathogen, whose morphological and physiological characteristics vary according to the origin of strains. Strains isolated under different conditions exhibited differences not only in their ability to react to the environmental conditions, but also in their virulence stability. This is important to be considered for the practical application. We therefore examined the virulence stability of four *V. lecanii* strains from three different geographical origins.

**Materials and methods**

Blastospores have been used in infection experiments. They were produced in shake culture on malt extract medium (1 % yeast extract, 3 % malt) following a standardized production method (Hirte *et al.*, 1989).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Year</th>
<th>Original host</th>
<th>Geograph. location</th>
<th>Further development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vb3/50</td>
<td>1978</td>
<td><em>Trialeurodes vaporariorum</em></td>
<td>Bulgaria</td>
<td>50x passed through <em>T. vaporariorum</em>. After 10 passages, 1x agar medium; since 1990, maintained on malt extract agar.</td>
</tr>
<tr>
<td>Vs</td>
<td>Approx. 1985</td>
<td><em>Trialeurodes vaporariorum</em></td>
<td>Siberia</td>
<td>10 years, on Czapek-Dox complete medium only</td>
</tr>
<tr>
<td>Vn</td>
<td>1997</td>
<td><em>Tetranychus urticae</em></td>
<td>Siberia</td>
<td>At first, passed through original insect host repeatedly. Then cultivated on Czapek-Dox complete medium and malt extract agar</td>
</tr>
<tr>
<td>V24</td>
<td>1989</td>
<td><em>Myzus persicae</em></td>
<td>Germany</td>
<td>Since isolation, no host-passage. Maintained on malt extract agar</td>
</tr>
</tbody>
</table>
The larvae I of the western flower thrips *Frankliniella occidentalis* were used as test insects. The original *V. lecanii* isolates have been compared to the reisolates after a 5 times host passage. The virulence was recorded as a degree of the pathogenicity (mortality, in %) against the test insect. The passages on the larvae were carried out at 20 °C and 80 % relative humidity. The mortality of *F. occidentalis* was tested with a standard Petri dish test after a direct application of 5.5 ml of the blastospore-suspension (concentration: $10^6$ spores/ml) on detached leaves of *Phaseolus vulgaris* in Petri dishes at 20 °C and 95 % relative humidity (Hirte et al., 1989).

**Results**

Original strains exhibited high differences in their virulence against larvae I of *F. occidentalis* (Fig. 1).

![Fig. 1. Pathogenicity of blastospores of 4 original Verticillium lecanii strains to larvae I of Frankliniella occidentalis at 20 °C.](image)

The highest mortality was observed for the strain V24, whereas the lowest virulence was noticed for the strain Vb3/50. The mortality of larvae differed by more than 50% at day 7 after infection. Up to the last day of notation, this difference came down to 25%. The strain Vs reached a high degree of virulence also, while the strain Vn turned out to be virulent on a moderate degree.

Considering the virulence after passages through host insect, virulence of the strains Vb3/50, Vs and Vn increased highly following five passages through *F. occidentalis*. Moreover, the virulence reached a standardized level, without significant differences (Fig. 2).

The original strain Vb3/50 (from Bulgaria), which was not considered as virulent enough before the reisolation, showed the strongest increase of virulence after the passage, while both the Siberian strains (Vs and Vn) showed only a moderate increase. The fast increase of the mortality is noticeable in these three strains during the 3-5 days after inoculation (Fig. 2).

Strain V24 exhibited an unexpected divergent virulence development after passages through the western flower thrips. The strain which showed a stable virulence after 10-years of maintenance on malt extract agar without host passage, caused a weaker final mortality after 5 reisolations. Within the first days of post infection, the mortality of the passaged strain raised faster, but decreased significantly in the second phase. Thereby the reisolate of strain V24 has not reached its original virulence during the performed passage test.
Fig. 2. Pathogenicity of blastospores of reisolates of 4 strains of *Verticillium lecanii* (after 5 passages through the host insect) to larvae *Frankliniella occidentalis* at 20°C.

**Discussion**

From the literature it is clear that virulence of *V. lecanii* strains are among other dependant on the manner of cultivation.

In 1990 Wick reported that a long-lasting culture of isolates on malt agar led to a significantly reduced virulence already after three months of culture. According to Jackson *et al.* (1985), the original host can also play an important role for the stability of virulence. In addition, Hirte *et al.* (1989) reported on an induced host adaptation at which high virulent strains have been gained. Even a unique passage through the corresponding host led to an increase of the virulence. However, all the authors pointed out that the stability of the virulence is family-specific.

By the adaptation of strains to a medium optimally weighted out, they became domesticated by long-lasting cultivation without passages through the hosts in laboratory. With the exception of strain V24, the studied strains obviously developed a decrease of virulence during the cultivation period, which could be compensated after a 5 times passage through the host.

All the examined strains have a different geographic origin and were isolated originally from different hosts, none of them, however, from a thrips. While the virulence of the three strains Vb3/50, Vs and Vn increased by passages through *F. occidentalis*, the strain V24 kept a constantly high virulence against the thrips without passages for a period of almost 10 years. The increase of the mortality within the first days and the almost identical progress of mortality of the three strains could be explained for several reasons.

So, the percentage of fast germinating spores could increase and thereby cause a faster infection. Moreover, changes in enzymatic and toxic capabilities could also occur. In any case, the data presented here deserve further investigations.

**References**


Wick, M. 1990. Untersuchungen zur biologischen Bekämpfung von *Myzus persicae* (Sulz.) durch den entomopathogenen Pilz *Verticillium lecanii* (Zimm.) Viegas und die parasitoide Blattlausschlupfwespe *Aphidius matricariae* Hal. (Homoptera; Aphididae; Hyphomycetales; Moniliaceae; Hymenoptera; Aphididae). Humboldt-Universität zu Berlin, Institut für Gartenbauwissenschaften, Dissertation.
Influence of soil environment on growth and persistence of *Beauveria brongniartii*

Philip Kessler, Siegfried Keller
Swiss Federal Research Station for Agroecology and Agriculture, FAL Reckenholz, Reckenholzstrasse 191, 8046 Zürich, Switzerland

**Abstract:** The objective of this study was to examine the effects of abiotic and biotic soil factors on growth and persistence of *B. brongniartii* on barley kernels in the soil environment. Field trials in different regions of Switzerland have been conducted applying fungus kernels in different soil types and at different seasons. For assessing the changes of the population density of *B. brongniartii*, the fungus has been reisolated from soil samples regularly taken from trial sites using an isolation method on selective medium and the *Galleria* bait method. First results indicated that the applications in spring and summer were successful at the most sites, whereas treatments at the end of the vegetation period were not. Multiple regression analysis revealed dependence of fungus growth on the number of hours in the optimal temperature range (20-25 °C), as well as clay and organic material content in the soil during the first three months following the application. After this period the *B. brongniartii* population usually reached its maximum and began to decrease.

**Key words:** fungal growth, inoculum, microbiological activity, soil temperature, humidity

**Introduction**

*Beauveria brongniartii* is an entomopathogenic fungus which has been used for several years in Switzerland to control the larvae of the European cockchafer (*Melolontha melolontha*). Since 1991, a product based on barley kernels colonised with the fungus is commercially available. Over 360 hectares of grasslands and orchards have been treated so far with this product. A majority of the farmers was satisfied with the treatment. Reasons for insufficient success are attributed to factors such as dry soils or inappropriate application handling (Keller, 2000; Matzke, 2000). Application of the biocontrol agent is achieved by tilling barley kernels overgrown with fungal mycelium into the soil. Subsequently the fungus grows and sporulates on the kernels and builds up the inoculum. For an efficient control of the host, a large amount of conidia has to be present in the soil and must remain viable under a wide range of environmental conditions. The objective of our study is to examine the effects of abiotic and biotic soil factors on growth and survival of *B. brongniartii* in the soil.

**Methods**

Field trials have been conducted in different regions of Switzerland (Table 1). All sites were meadows with the exception of VS3 and VS4 which were orchards. The study site has been divided in several plots of 20x20 m² for different treatments including four replications of each. At VS1 the plot size was only 20x15 m² and at VS3 and VS4 the plots correspond to the tracks between the tree rows (4x40 m², 4x20 m² respectively).

For the application we used fungus material that has been grown on sterilised barley kernels (product available from Eric Schweizer Samen AG, Thun, Switzerland). 40-50 kg/ha of the fungus kernels were tilled into the soil at a depth of 5-10 cm using an adapted tilling
machine. At each site different plots were treated once with the fungus kernels at different seasons in 1999. At each site four control plots were not treated.

Soil samples were taken from a depth of 5 to 18-20 using a cylindrical soil sampler with an inner diameter of 5.5 cm. For each sampling period 10 samples were collected per plot. The samples were taken before the application, 1 month and 3 months after the application and stored in plastic bags at 4 °C no longer than 6 months. To assess the fungal density in the soil, the Galleria Bait Method (GBM) was applied to each soil sample and colony forming units (CFU) per gram dry soil on selective medium were determined for each plot (mixture of 10 soil samples) (Zimmermann, 1986; Strasser et al., 1996).

Soil factors such as pH, content of clay, sand, organic material and salt have been analysed by Eric Schweizer Samen AG, Thun, Switzerland. Catalase activity has been measured to assess microbiological activity in the soil (Beck, 1971). Soil temperature was measured every hour either by sensors that have been placed directly at the study sites in a depth of 10 cm (HOTDOG DT1, ELPRO-BUCHS AG, CH-9471 Buchs, Switzerland) or by neighbouring meteorological station of the SMI (Swiss Meteorological Institute). Daily rainfall has been measured by the SMI stations.

Table 1: Mean of soil parameters of all plots at all trial sites.

<table>
<thead>
<tr>
<th>Region</th>
<th>Site</th>
<th>Clay %</th>
<th>Sand %</th>
<th>Organic %</th>
<th>pH</th>
<th>Salt Si</th>
<th>Catalase O2/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE Switzerland</td>
<td>TG1</td>
<td>19.7</td>
<td>40.4</td>
<td>3.8</td>
<td>7.0</td>
<td>239.4</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>TG2</td>
<td>11.0</td>
<td>54.5</td>
<td>2.8</td>
<td>7.6</td>
<td>251.3</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>TG3</td>
<td>12.7</td>
<td>56.6</td>
<td>3.4</td>
<td>6.7</td>
<td>242.8</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>TG4</td>
<td>42.5</td>
<td>20.5</td>
<td>8.1</td>
<td>6.3</td>
<td>259.9</td>
<td>6.08</td>
</tr>
<tr>
<td>Central Switzerland</td>
<td>UR</td>
<td>29.0</td>
<td>31.7</td>
<td>9.0</td>
<td>6.4</td>
<td>194.0</td>
<td>4.30</td>
</tr>
<tr>
<td>South Switzerland</td>
<td>VS1</td>
<td>14.8</td>
<td>22.2</td>
<td>2.1</td>
<td>7.7</td>
<td>625.8</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
<td>VS3</td>
<td>3.4</td>
<td>57.5</td>
<td>1.5</td>
<td>7.5</td>
<td>217.1</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>VS4</td>
<td>32.2</td>
<td>23.0</td>
<td>4.9</td>
<td>7.3</td>
<td>226.4</td>
<td>3.20</td>
</tr>
</tbody>
</table>

Results and discussion

**Growth of B. brongniartii**

Data obtained with the GBM showed increased *Galleria* mortality due to *B. brongniartii* in soil samples 3 months after spring and summer application, whereas soil samples from autumn treatments showed no or only slight mortality increase. Similar results were obtained from analyses with selective medium (Table 2). Multiple regression analysis revealed a model explaining variance of fungal growth ($R^2=0.77$, $F=16.01$, $p<0.001$) including the variables optimal temperature ($b=0.52$, $p<0.05$), clay content ($b=1.15$, $p<0.05$) and catalase activity ($b=-1.47$, $p<0.05$).

In order to build up the inoculum, the fungus has to grow and sporulate in the soil. Walstadt et al. (1967) indicated that muscardine fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* are unable to sporulate at temperatures below 10 °C. Colony growth rate of *B. bassiana* measured on *Spodoptera* cadavers in the soil decreased as temperature dropped from 28 to 13 °C (Studdert & Kaya, 1990). The temperature for optimal growth of *B. brongniartii* ranges from 22 to 25 °C. The fungus is still able to grow and sporulate at a temperature at 10 °C, but it takes more time, and sporulation seems to be unlikely at temperatures below 5 °C (Aregger-Zavadil, 1992). The temperature range in the soil after
spring and summer treatment is much more favourable for fungal growth compared to the temperature ranges after autumn treatment which corresponds to the findings mentioned above.

Table 2: Increase of the presence (%) and density (CFU/gr dry soil) of *B. brongniartii* during the first 3 after the application with fungus kernels at different seasons. Climatic conditions (optimal growth temperature at 10 cm and rainfall) are indicated for this period.

<table>
<thead>
<tr>
<th>Site</th>
<th>Application period</th>
<th>GBM (before application)</th>
<th>GBM (after application)</th>
<th>Selective Medium (before application)</th>
<th>Selective Medium (after application)</th>
<th>Increase n hours</th>
<th>Temp. 20-25°C</th>
<th>Rainfall mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG 1</td>
<td>spring</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>74</td>
<td>+74</td>
<td>774</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>5</td>
<td>17.5</td>
<td>0</td>
<td>48</td>
<td>+48</td>
<td>285</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>5</td>
<td>12.5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>2.5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>270</td>
</tr>
<tr>
<td>TG 2</td>
<td>spring</td>
<td>22.5</td>
<td>47.5</td>
<td>808</td>
<td>3685</td>
<td>+2878</td>
<td>559</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>32.5</td>
<td>35</td>
<td>281</td>
<td>282</td>
<td>+1</td>
<td>568</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>40</td>
<td>30</td>
<td>275</td>
<td>199</td>
<td>-75</td>
<td>568</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>25a</td>
<td>22.5b</td>
<td>420a</td>
<td>292b</td>
<td>-128</td>
<td>270</td>
<td>280</td>
</tr>
<tr>
<td>TG 3</td>
<td>spring</td>
<td>5</td>
<td>37.5</td>
<td>209</td>
<td>148</td>
<td>-61</td>
<td>735</td>
<td>428</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>39</td>
<td>+33</td>
<td>442</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>12.5</td>
<td>7.5</td>
<td>140</td>
<td>0</td>
<td>-140</td>
<td>190</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>7.5a</td>
<td>0b</td>
<td>10a</td>
<td>86b</td>
<td>+76</td>
<td>270</td>
<td>280</td>
</tr>
<tr>
<td>TG 4</td>
<td>spring</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>580</td>
<td>407</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>0</td>
<td>27.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>175</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0a</td>
<td>0b</td>
<td>0a</td>
<td>0b</td>
<td>0</td>
<td>0</td>
<td>282</td>
</tr>
<tr>
<td>UR</td>
<td>summer</td>
<td>7.5a</td>
<td>42.5</td>
<td>8</td>
<td>2254</td>
<td>+2245</td>
<td>c</td>
<td>362</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>0</td>
<td>10</td>
<td>8</td>
<td>81</td>
<td>+73</td>
<td>c</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>5a</td>
<td>7.5b</td>
<td>29a</td>
<td>68b</td>
<td>39</td>
<td>c</td>
<td>196</td>
</tr>
<tr>
<td>VS1</td>
<td>summer</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>4892</td>
<td>+4892</td>
<td>843</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>+12</td>
<td>0</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0a</td>
<td>0b</td>
<td>0a</td>
<td>0b</td>
<td>0</td>
<td>0</td>
<td>149</td>
</tr>
<tr>
<td>VS3</td>
<td>spring</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>1305</td>
<td>+1305</td>
<td>483</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>+3</td>
<td>0</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0a</td>
<td>20b</td>
<td>0a</td>
<td>9b</td>
<td>+9</td>
<td>0</td>
<td>147</td>
</tr>
<tr>
<td>VS4</td>
<td>spring</td>
<td>0</td>
<td>50</td>
<td>33</td>
<td>4409</td>
<td>+4376</td>
<td>822</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>autumn</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>50</td>
<td>+47</td>
<td>0</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0a</td>
<td>0b</td>
<td>0a</td>
<td>0b</td>
<td>0</td>
<td>0</td>
<td>147</td>
</tr>
</tbody>
</table>

*a*: value spring 1999; *b*: value autumn 1999; *c*: no data available

Humidity is known to be a crucial factor for growth and sporulation of muscardine fungi (Walstadt et al., 1970; Fargues & Luz, 1998; Aregger-Zavadil, 1992). As the relative humidity remains at values of 99% in the soil even under dry conditions and can drop under 95% only in the very upper layers (Griffin, 1963), we conclude that the relative humidity was not a critical factor in our field trials. Nevertheless clay content is a significant variable in our regression model. Soils with higher clay content are known to have better water capacity due to smaller pore sizes, which could explain the positive effect on the development of *Beauveria.*
Catalase activity, which is highly correlated with organic content, is often associated with microbiological activity in the soil. Studdert & Kaya (1990) measured a reduced growth rate of B. bassiana in organic soils, which they attributed to a inhibitory effect of microbiological activity. This corresponds to our model, where catalase has a negative effect on CFU production of B. brongniartii.

**Persistence of B. brongniartii**

Three months after application the B. brongniartii population usually reached its maximum and began to decrease. This study is still ongoing.

**Conclusions**

It is crucial to apply the fungus kernels in spring and summertime for a successful treatment, when the climatic conditions favour the growth and the sporulation of B. brongniartii on the kernels in the soil.

The application in soil with higher content of organic material can result in reduced CFU of B. brongniartii, probably due to inhibition by higher microbiological activity in the soil.

The influence of factors such as temperature, soil moisture, pH, and biotic environment on the persistence of B. brongniartii in soil will be studied in more details.

**Acknowledgements**

The work is part of the EU-project BIPESCO (FAIR6-CT98-4105) and supported by grants from the Swiss Federal Office for Education and Science. We are grateful to Dr H.R. Oberholzer for measuring the catalase activities.

**References**


Matzke, H. 2000: Field application of Beauveria brongniartii, established on peeled barley kernels (remarks to a field demonstration). IOBC wprs Bull. 23 (8): 87-92.


Study on insecticidal, antifeedant and growth inhibitory properties of oosporein on selected pest organisms

Daniela Abendstein, Wolfgang Schweigkofler¹, Hermann Strasser
University of Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria
¹ Research Center Laimburg, 39040 Auer (BZ), Italy

Abstract: The entomopathogenic fungus Beauveria brongniartii, the highly effective control agent against the cockchafer Melolontha melolontha, produces the secondary metabolite oosporein. For registration purposes data on the toxicity of secondary metabolites synthesised by the fungus are required. Although toxicity of oosporein on bacteria and fungi with varying extents were reported, little is known about its properties on insects. This study shows that oosporein has no insecticidal, antifeedant or growth inhibitory effects, when ingested by lepidopterous larvae of Capua reticulana, Cydia pomonella (both Tortricidae) and Mamestra brassicae (Noctuidae).

Key words: Beauveria brongniartii, non-target pest insects, oosporein, risk-assessment

Introduction

The use of biological control agents (BCAs) on the basis of microorganisms for the control of soil dwelling insect pests is in progress and has already reduced inputs of harmful synthetic chemical pesticides (Butt et al., 1999). Entomopathogenic fungi used in the biological pest control (BPC) like Beauveria, Fusarium, Tolypocladium or Metarhizium are known to synthesize chemical diverse secondary metabolites (Khachatourians, 1996). Some of these metabolites are considered to be important pathogenicity determinants in the host-pathogen relationship, for others varying toxicity has been described. There is concern that the use of entomopathogenic fungi in the BPC may lead to an increase of toxic microbial metabolites in the environment. Therefore, for registration of a fungal BCA in Europe the Directive 91/414/EEC demands data on the potential risk arising from the applied organism and its metabolites on the environment (Strasser et al., 2000).

Beauveria brongniartii, used for the control of the cockchafer (Melolontha melolontha, Scarabaidae), excretes the red dibenzoquinone oosporein. Because of its antibiotic effect on gram-positive bacteria, oosporein is believed to play a major role in the colonisation of infected insects by B. brongniartii. Studies concerning the effect of B. brongniartii on non-target soil dwelling animals like the earthworm Lumbricus terrestris (Hozzank et al., 2003) and different Carabidae species (Traugott et al., 2000) were performed. Effects of oosporein were till now studied on Galleria mellonella, Plutella xylostella and Phaedon cochlearia larvae, only (Abendstein & Strasser, 2000; Abendstein et al., 2001).

Therefore, the aim of this ongoing study is to investigate insecticidal, antifeedant and growth inhibitory effects of oosporein on the non-target soil dwelling pest organisms Mamestra brassicae (Noctuidae), Cydia pomonella and Capua reticulana (both Tortricidae).
Materials and methods

**Maintenance of insect cultures**

*C. reticulana, C. pomonella* and *M. brassicae* were reared at 22 °C, 70 % RH and a 18:6 h light:dark photoperiod. Larvae (L) were fed with “Capua-medium” for *C. reticulana* and with “Cydia-medium” for *M. brassicae* and *C. pomonella*, respectively. In the toxicity tests L2 to L3 larvae of *C. reticulana* (weight 0.003 g), L2 to L4 larvae of *C. pomonella* (weight 0.02 g) and L3 larvae of *M. brassicae* (weight 0.14 g) were used.

The “Cydia-medium” was composed as follows: 50 g corn meal, 50 g brewers yeast, 60 g wheat germ, 45 g apple pulp, 5 g ascorbic acid, 1.5 g hydroxybenzoate, 1.5 g benzoic acid, 2.7 ml formaldehyde (37 %), 25 g agar and 850 ml water. Constituents of the “Capua-medium” were: 235.35 g soaked beans, 35.31 g brewers yeast, 3.53 g ascorbic acid, 2.21 g 4-hydroxy-benzoic acid methyl ester, 1.1 g sorbic acid, 2.14 ml formaldehyde (37 %), 14.12 g agar and 700 ml water.

**Bioassay test-systems**

Pure oosporein was dissolved in acetone:methanol (1:1) in the concentration of 6.5 mg/ml. Oosporein solution and solvent solution (control), respectively, were dropped on pieces of rearing food (food weight for *C. reticulana* 0.25 g, for *C. pomonella* 0.35 g and for *M. brassicae* 1.40 g). The bioassays were carried out in plastic boxes (Ø 9.8 cm) at the rearing conditions of insects mentioned above. Mortality was estimated after 1, 7 and 14 days, the development of the insects was observed until complete metarmorphosis. Twenty larvae were tested for each insect species and test system. The bioassays were repeated twice.

In the choice bioassay, test organisms were offered a choice of two pieces of food enriched with 0.325 mg (*M. brassicae*) and 0.195 mg (*C. reticulana* and *C. pomonella*) oosporein, respectively, and two pieces of food treated with distilled water. As a control larvae were fed with food treated with solvent solution.

In the no-choice test, either one piece of oosporein enriched food or one piece of food treated with solvent solution was offered to the larvae. *M. brassicae* were fed with 0.46 mg oosporein/larva.

Mortality of insects fed with treated and untreated food were compared by means of the Fisher statistical Test and the test of parallelism ($\chi^2$ Yates correction).

**Results**

In both test systems, choice as well as no-choice bioassay, oosporein showed no acute toxicity to *C. reticulana, C. pomonella* and *M. brassicae*. There were no significant differences (P > 0.05) in the survival of larvae fed with oosporein treated food and fed with untreated food at any type of insect species, respectively (Fig. 1). Mortality rate ranged from 25 to 20 %. Dead larvae, that had consumed oosporein, did not show any abnormal discolouring nor melanisation spots.

Oosporein had no antifeeding effect. There were no preferences of *C. reticulana, C. pomonella* and *M. brassicae* in the consumption of treated and untreated food in the choice bioassay. In the no-choice assay *M. brassicae* consumed completely the offered food, and 7 days after application fresh untreated food had to be offered again. *M. brassicae* larvae had eaten 0.46 mg pure oosporein, corresponding to approximately 3.3 mg/g living weight.

No growth inhibitory effect of oosporein was observed to *C. reticulana, C. pomonella* and *M. brassicae*. In the no-choice bioassay treated and untreated *M. brassicae* larvae showed the same development into pupae and imagos (Fig. 2; P > 0.05). After 60 days incubation...
larvae that consumed oosporein developed to 70 % to imagos and to 20 % to pupae. Larvae
that had no contact with oosporein developed to 65 % to imagos and 20 % to pupae.

![Graph](image)

Fig. 1. Survival [%] of *C. reticulana*, *C. pomonella* and *M. brassicae* in a choice bioassay
after 14 days (n = 20). The food was enriched with 0.39 mg oosporein (*C. pomonella*
and *C. reticulana*) and 0.65 mg (*M. brassicae*), respectively.

![Graph](image)

Fig. 2. Stages of development [%] of *M. brassicae* in a no-choice bioassay during a 60 days
incubation at 22°C and a feeding with ■ oosporein (0.46 mg) enriched food and
non-treated food, n = 20.

**Discussion**

Chemical and toxicological features of oosporein were reviewed by Strasser et al. (2000).
Besides a non-specific ATPase inhibitory activity (i.e. oosporein inhibits the activity of the
erthrocyte membrane ATPase), the pigment also caused alterations in erythrocyte
morphology and promoted varying degrees of cell lysis (Jeffs & Khachatourians, 1997). It showed antibiotic effects on gram-positive, but had little effect on gram-negative bacteria. The fungicidal activity was moderate.

Up to now scarce information is available whether oosporein is a "bioactive compound" showing insecticidal properties on pest organisms. Therefore, three typical insect pests from fruit plantations were selected to investigate the behaviour of the oosporein. The investigated insect species *C. reticulana, C. pomonella* and *M. brassicae* were not susceptible to oosporein. Oosporein showed neither repellent, antifeedant nor toxic effects and did not alter metamorphosis. Similar observations were made when the toxicity of oosporein was tested in feeding bioassays on *G. mellonella* (Abendstein *et al.*, 2001), *P. xylostella* and *P. cochleariae* (Abendstein & Strasser, 2000).

It could be demonstrated that oosporein, although fed to larvae in high doses, showed no significant negative effect. In the case of *M. brassicae* the larvae consumed 3.3 mg/g living weight. For comparison, toxicity studies of oosporein in mice and hamsters indicated a LD50 value of 0.5 mg/kg body weight, when injected intraperitoneally (Wainwright *et al.*, 1986).

On the basis of these results it can be suggested that oosporein poses no marked risk to insect populations in soil. For a general assumption, however, further studies on more soil dwelling insect species are needed.

**Acknowledgements**

This project is funded by the Austrian Science Fund (FWF 12791-BIO) and will be finalized with assistance of the European RTD programme FAIR6-CT98-4105. The authors are indebted to Dr Tariq Butt, University of Wales Swansea, for his scientific support.

**References**


Effects of various species and isolates of entomopathogenic fungi on 
*Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

Elzbieta Popowska-Nowak  
*Institute of Ecology Polish Academy of Science, Department of Agrocenology,  
Dziekanów Lezny, 05-092, Lomianki, Poland*

**Abstract:** Effects of 43 isolates from 5 species of entomopathogenic fungi of the class Deuteromycetes: *Beauveria bassiana* (15 isolates), *Metarhizium anisopliae* (9 isolates), *Paecilomyces farinosus* (6 isolates), *P. fumosoroseus* (8 isolates) and *Verticillium lecanii* (5 isolates) on mortality of larvae and adults of the lesser mealworm *Alphitobius diaperinus*, a pest of broiler houses, has been studied under laboratory conditions. The aim was to select for further studies the isolates most pathogenic towards the insect. Beetles and larvae were sprayed with a spore suspension at a concentration of $10^8$/ml. Adults appeared quite resistant to fungi. The greatest mortality (40 %) was observed when using an isolate of *M. anisopliae*. By contrast, larvae were more sensitive to the fungal infection. All isolates from *B. bassiana*, *M. anisopliae* and *P. farinosus* produced 100% mortality of larvae. The isolates of the two other species, *P. fumosoroseus* and *V. lecanii*, usually caused a lower mortality.

**Key words:** entomopathogenic fungi, *Alphitobius diaperinus*, adults, larvae, biological control

**Introduction**

For many years, the lesser mealworm *Alphitobius diaperinus* (Panzer) was noted as a pest of stored grain and products (Gould, 1948). In the 1950s, it was discovered in poultry houses in the USA (Gould & Mouses, 1951). Recently, it appeared as one of the most common beetles in poultry houses in the USA (Axtell & Arends, 1990; Rueda & Axtell, 1997).

At the end of the 1970s and beginning of the 1980s, the occurrence of this insect was observed in hen-cotes and poultry houses in Poland. In recent years it became increasingly frequent.

This beetle is threatening as a potential vector of such pathogenic agents as fungi (mainly *Aspergillus*), bacteria (*Escherichia*, *Salmonella*, *Bacillus*, *Streptococcus*), viruses (e.g. causing Marek's and Newcastle diseases), and parasites (*Eimeria*, or larval nematodes) (Steelman, 1996). Moreover, these insects destroy polyurethane thermal isolation of poultry houses by boring tunnels when they move and pupate (Voughan *et al.*, 1984; Steelman, 1996).

The purpose of this study was to select under laboratory conditions, isolates of different fungal species highly pathogenic to larvae and adult lesser mealworms.

**Material and methods**

Ten beetles or 3-week-old larvae were placed on Petri dishes with filter paper on the bottom, in three replicates. A spore suspension with a concentration of $10^8$/ml in 0.01 % Tween 20 solution was prepared using three-week cultures of fungi. The concentration of the suspension was determined using a hematocytometer. The beetles or larvae in each dish were infected with 1 ml of spore suspension from one of the 43 selected isolates of 5 species of pathogenic fungi. In the control, insects were sprayed with 1 ml of 0.01 % Tween 20 solution. Dead
beetles were counted for 21 days and removed to separate dishes. The following fungal species were used: *B. bassiana* (15 isolates), *M. anisopliae* (9 isolates), *P. farinosus* (6 isolates), *P. fumosoroseus* (8 isolates) and *V. lecanii* (5 isolates). Most isolates were taken from the collection of the Department of Agroecology of the Institute of Ecology PAS. A few strains originated from insects (Table 1). However, most strains were isolated from the forest soil or litter, cultivated soil, poultry litter or from the soil taken in places of temporary deposition of poultry litter near poultry houses.

Table 1. List of studied strains which were isolated from insects (isolate labelling from the Department of Agroecology of the Institute of Ecology PAS Culture Collection).

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Isolate labelling</th>
<th>Original host</th>
<th>Year of culture collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bassiana</em></td>
<td>4</td>
<td><em>Galeruca tanaceti</em> (beetle)</td>
<td>1971</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td><em>Ostrinia nubilalis</em> (caterpillar)</td>
<td>1978</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td><em>Leptinotarsa decemlineata</em> (beetle)</td>
<td>1982</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td><em>Coccinella</em> (beetle)</td>
<td>1983</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td><em>Cicadodea</em> (bug)</td>
<td>1983</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td><em>Leptinotarsa decemlineata</em> (beetle)</td>
<td>1985</td>
</tr>
<tr>
<td></td>
<td>Ka</td>
<td><em>Coccinella</em> (beetle)</td>
<td>1992</td>
</tr>
<tr>
<td><em>M. anisopliae</em></td>
<td>BP</td>
<td><em>Melolontha melolontha</em> (grub)</td>
<td>1997</td>
</tr>
</tbody>
</table>

The significance of differences in the mortality of adult beetles and larvae was tested by LSD test at P=0.05 on arcsin\sqrt{x} transformed data. LT50 were calculated by using a probit analysis with POLO program.

**Results and discussion**

The highest mortality in beetles was observed after treatment with spore suspensions of three isolates of *M. anisopliae*: M, 11, and 3K (Fig. 1). Isolate M was responsible for 40 % mortality in adults. For the remaining 6 isolates, the number of dead beetles did not differ from the control (Fig. 1).

Only five *B. bassiana* isolates caused adult mortality that differed from the control, and the mortality ranged from 16 to 20 % (Fig. 2).

Isolates of the three remaining fungal species had no significant effect on the mortality of adult lesser mealworms.

Given these results, only the isolates M, 11, and 3K of *M. anisopliae* were chosen for further testing the fungal infection of adults.

Larvae of *Alphitobius diaperinus* appeared more susceptible to infection by the tested fungi. Spores of all isolates of *B. bassiana*, *M. anisopliae*, and *P. farinosus* achieved 100 % mortality. In the case of the remaining two fungal species, *P. fumosoroseus* and *V. lecanii*, only several isolates produced 100 % mortality of larvae. Differences were also observed in the lethal time (LT50 differed even for various isolates of the same fungal species). Data are shown for six isolates of *B. bassiana*, 22, 33, 34, 85, 1K, and 3K (Table 2), and five isolates of *M. anisopliae*, BPd, B4, 9,1K, and 2K (Table 3), respectively. Indeed, these isolates were selected for further tests.
For the isolates of the three remaining fungi (P. farinosus, P. fumosoroseus and V. lecanii), LT50 ranged from about 5 to 11 days, thus they were not used in further experiments.

Table 2. Values of LT50 for 6 isolates of B. bassiana bioassayed upon larvae of A. diaperinus (1isolates from naturally infected insects, see Table 1, 2isolate from forest litter, 3isolates from poultry litter, *the values followed by the same letter are not significantly different).

<table>
<thead>
<tr>
<th>Isolates</th>
<th>LT50 ± confidence limits at 95 %</th>
<th>Slope ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 1</td>
<td>4.321 (3.307-4.722)ab*</td>
<td>9.706±2.015</td>
</tr>
<tr>
<td>33 1</td>
<td>3.943 (3.204-4.413)ab</td>
<td>7.818±1.672</td>
</tr>
<tr>
<td>34 1</td>
<td>3.700 (3.071-4.137)a</td>
<td>7.397±1.444</td>
</tr>
<tr>
<td>85 2</td>
<td>3.944 (3.183-4.352)ab</td>
<td>9.974±2.570</td>
</tr>
<tr>
<td>1K 3</td>
<td>4.414 (3.221-4.955)b</td>
<td>8.279±2.365</td>
</tr>
<tr>
<td>3K 3</td>
<td>4.065 (3.310-5.558)ab</td>
<td>7.244±1.540</td>
</tr>
</tbody>
</table>

Table 3. Values of LT50 for 5 isolates of M. anisopliae bioassayed upon larvae of A. diaperinus (1isolate from forest litter, 2isolate from cultivated soil, 3isolate from naturally infected insect, see Table 1, 4isolates from the soil in the place of poultry litter deposition, *the values followed by the same letter are not significantly different).

<table>
<thead>
<tr>
<th>Isolates</th>
<th>LT50 ± confidence limits at 95 %</th>
<th>Slope ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 1</td>
<td>3.951 (2.836-4.454)a*</td>
<td>9.095±2.831</td>
</tr>
<tr>
<td>B4 2</td>
<td>4.319 (3.239-4.851)ab</td>
<td>8.679±2.354</td>
</tr>
<tr>
<td>Bpd 3</td>
<td>3.719 (2.933-4.221)a</td>
<td>7.450±1.844</td>
</tr>
<tr>
<td>1K 4</td>
<td>3.624 (2.788-4.240)a</td>
<td>5.285±1.016</td>
</tr>
<tr>
<td>2K 4</td>
<td>4.711 (3.443-5.332)b</td>
<td>7.916±2.123</td>
</tr>
</tbody>
</table>

The pathogenicity of fungi to the lesser mealworm was studied in the USA and Denmark. The experiments conducted in the USA concerned isolates of B. bassiana obtained from naturally-infected domestic flies and poultry-houses infested with lesser mealworms (Steinkraus et al., 1991, Crawford et al., 1998). In Denmark, the effects of different fungal species and isolates on the mortality rate of Alphitobius diaperinus larvae and adults were tested, including those originating from naturally infected insects (Steenberg et al., 2001). These studies, like the present one, showed that adult were more resistant than larvae to fungal infections. Nonetheless, when strongly pathogenic isolates were applied under laboratory conditions, the mortality in adult beetles reached even 92 % (Steenberg et al., 2001).

Unfortunately, no strain originated from naturally infected target insects was tested in this study. However, some of the studied isolates originated from the habitat where they could be in contact with the lesser mealworm. Further investigations should be centred upon searching for isolates with higher activity on the adult stage.
110

Fig. 1. Mortality of adult lesser mealworms 21 days after infection with a suspension of spore isolates of *Metarhizium anisopliae*.

* mortality significantly different from the control
1 isolate from cultivated soil,
2 isolate from forest soil
3 isolate from the soil in the place of poultry litter deposition

Fig. 2. Mortality of adult lesser mealworms 21 days after infection with a suspension with a suspension of spore isolates of *Beauveria bassiana*.

* mortality significantly different from the control
1 isolates from naturally infected insects (see Table 1),
2 isolates from forest soil,
3 isolate from poultry litter
Acknowledgements

This study was supported by the National Committee of Scientific Investigations (KBN), Grant KBN 6PO4G 02116.

References

Occurrence of entomopathogenic fungi in soil from apple and plum orchards

Anna Sapieha-Waszkiewicz, Ryszard Mietkiewski, Barbara Marjanska-Cichon
University of Podlasie, Siedlce, Poland

Abstract: The aim of this investigation was to estimate the influence of pesticide applications on spectrum and occurrence of entomopathogenic fungi in soil taken from apple and plum orchards in which chemical control of pests diseases and weeds has been used. Soil samples in both orchards were collected from sward between rows and from herbicides fallow under trees. For comparison soil samples were taken also from arable field adjacent to the orchards. Soil samples were baited with *Galleria mellonella* larvae at 22 °C. Five species of entomopathogenic fungi were isolated. *Meta-rhizium anisopliae* was the dominant species isolated from all soil samples. *Beauveria bassiana* was dominated in soil under sward in both kinds of orchards. *Paecilomyces fumosoroseus* occurred less frequently than *M. anisopliae* and *B. bassiana*. The highest mortality of *G. mellonella* larvae caused by this species was observed in samples from arable soil. *M. flavoviride* and *P. farinosus* were rare. There was not significant differences in occurrence of fungi between autumn and spring, however more abundant infections were observed in autumn.

Key words: orchards soil, *Galleria* bait method, *Beauveria, Metarhizium, Paecilomyces*

Introduction

Orchards in Poland, in which a traditional plant protection programme is applied, are among cultivated area where the level of chemical pesticide applications is the highest. Consumption of pesticides in commercial orchards, given in active ingredients, reached 30 kg per hectare (Makosz, 1992), whereas average consumption of pesticides in all agricultural crops amounted to only 1,4 to 1,6 kg active ingredient (Pruszynski, 1989). Besides their advantages, pesticides have a negative influence on environment, thereby on soil which is a reservoir of entomopathogenic fungi which limit pests populations. Usual pests in orchards are often infected by entomopathogenic fungi (Niezborala, 1978; Jaworska, 1979; Mietkiewski, 1981; Mietkiewski et al., 1992a). Pesticides use may have therefore an impact on the natural occurrence, infectivity and population dynamics of these fungi in soil.

In Polish agricultural soils receiving low pesticide applications, occurrence of entomopathogenic fungi was evaluated already. *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces fumosoroseus* appeared to be the major species.

The aim of this study was to determine the spectrum and abundance of entomopathogenic fungi in soil samples taken from sward between rows and from herbicides fallow under trees in apple orchard and plum orchard. For comparison, soil samples were taken from arable field adjacent to the investigated orchards.

Material and methods

In the autumn (September 1999) and in spring (May 2000) samples of soil (slightly loamy sand) were taken from an apple orchard, a plum orchard and for comparison, from adjacent fields on which winter wheat and oat were cultivated. Soil samples in orchard were collected from sward beetween rows and from herbicides fallow under trees.
The soil from each stand was air dried for 2 days at 20±2 °C and sieved in order to remove stones and other debris. Soil was placed in plastic Petri dishes and 10 fifth – instar *Galleria mellonella* larvae were added to each of 10 Petri dishes per treatment. Dishes with soil and larvae were incubated at 22 °C. Dishes were turned and agitated every day to ensure that the larvae came in to maximum contact with the soil. First observation of larval mortality was monitored after 7 days and next ones every 5 days.

Dead larvae were surface sterilised in 1 % sodium hypochlorite for 30 s, rinsed three times in sterile distilled water and placed in moisture chamber to encourage fungal outgrowth and sporulation. If external fungal growth was already visible, larvae were only rinsed in sterile distilled water in order to remove soil before being placed into humid conditions. Identification was confirmed by slide preparations. Analyses were done according to the following statistical pattern:

\[ Y_{ijk} = m + a_i + b_j + c_k + a_i b_j + a_i c_k + b_j c_k + e_{ijk} \]

where \( m \): the mean of population, \( a_i \): the effect of \( i \)-th fungus species, \( b_j \): the effect of \( j \)-th soil, \( c_k \): the effect of \( k \)-th season, \( a_i b_j \), \( a_i c_k \), \( b_j c_k \): the interaction effect, and \( e_{ijk} \): the experimental error.

**Results and discussion**

Five entomopathogenic fungi, i.e. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Metarhizium flavoviride* (Gams et Rozsypal), *Paecilomyces farinosus* (Holm ex Gray) Brown et Smith, *Paecilomyces fumosoroseus* (Wize) Brown et Smith, were isolated from orchard and adjacent arable field soils. *M. anisopliae* was the dominant species isolated from all soil samples (Table 1). Several studies have demonstrated domination of this fungus in soils from Poland (Mietkiewski et al., 1988, 1991, 1996), from Germany (Kleespies et al., 1989), from Japan (Yaginuma, 1990). Only in English soils *M. anisopliae* occurred less frequently than *B. bassiana* (Mietkiewski et al., 1997; Chandler et al., 1998). In our study, *B. bassiana* and *P. fumosoroseus* occurred less frequently while *P. farinosus* and *M. flavoviride* occurred sporadically (Table 1).

There were significant differences in the occurrence of fungi between different stands. *M. anisopliae* was dominant in soil taken from herbicides fallow, infecting approx. 29 % larvae in apple orchard and approx. 21,5 % larvae in plum orchard, as well as in arable soil (approx. 27,5 % in average). By contrast, *B. bassiana* was dominant in soil taken from sward in both kinds of orchards (Table 1). *P. fumosoroseus* occurred less frequently than *M. anisopliae* and *B. bassiana*. The highest mortality (approx. 15 % of *G. mellonella* larvae) caused by this species was observed in samples from arable soil. In soil from orchards mortality of larvae was lower and did not seem to depend on stand (Table 1). Similar results were obtained by Mietkiewski et al. (1992b) in a study where entomopathogenic fungi were detected in orchard soils using *Tribolium destructor* as bait insect. At that time *P. fumosoroseus* was not isolated. Absence of the fungus could be result from the use of a different bait insect.

Generally speaking, there were not significant differences in occurrence of fungi between autumn and spring. However more abundant infections were observed in autumn (Table 1). Mietkiewski et al. (1994, 1996) observed similar results in soils from mid-forest meadows in Sudety mountains and from hops plantations.
Table 1. Mortality (%) of *Galleria mellonella* larvae after 30 days of contact with soil taken from apple orchard, plum orchard and adjacent wheat or oats field in autumn and spring.

<table>
<thead>
<tr>
<th>Factor of mortality</th>
<th>Autumn</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apple orchard</td>
<td>Plum orchard</td>
<td>Wheat field</td>
<td>Apple orchard</td>
<td>Plum orchard</td>
<td>Wheat field</td>
<td>Oats field</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Herbicides fallow</td>
<td>Sward</td>
<td>Herbicides fallow</td>
<td>Sward</td>
<td>Herbicides fallow</td>
<td>Sward</td>
<td>Herbicides fallow</td>
<td>Sward</td>
<td></td>
</tr>
<tr>
<td>Entomopathogenic fungi</td>
<td><strong>Beauveria bassiana</strong></td>
<td>13.3</td>
<td>28.0</td>
<td>6.6</td>
<td>21.3</td>
<td>10.7</td>
<td>8.0</td>
<td>27.0</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td><strong>Metarhizium anisopliae</strong></td>
<td>29.3</td>
<td>13.3</td>
<td>24.0</td>
<td>17.3</td>
<td>25.3</td>
<td>28.0</td>
<td>16.0</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td><strong>Paecilomyces fumosoroseus</strong></td>
<td>9.3</td>
<td>6.7</td>
<td>12.0</td>
<td>8.0</td>
<td>14.7</td>
<td>1.0</td>
<td>4.0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td><strong>Paecilomyces farinosus</strong></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td><strong>Metarhizium flavoviride</strong></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.9</td>
<td>48.0</td>
<td>42.6</td>
<td>49.3</td>
<td>53.4</td>
<td>38.0</td>
<td>47.0</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.0</td>
<td>16.0</td>
<td>14.7</td>
<td>51.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungi of unproved entomopathogenic abilities</td>
<td><strong>Aspergillus sp.</strong></td>
<td>13.3</td>
<td>10.8</td>
<td>9.3</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>0.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td><strong>Penicillium sp.</strong></td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>0.0</td>
<td>6.7</td>
<td>3.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><strong>Gliocladium sp.</strong></td>
<td>1.3</td>
<td>1.3</td>
<td>5.3</td>
<td>0.0</td>
<td>1.3</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><strong>Mucor sp.</strong></td>
<td>8.0</td>
<td>14.7</td>
<td>13.3</td>
<td>14.7</td>
<td>9.3</td>
<td>15.0</td>
<td>12.0</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td><strong>Fusarium sp.</strong></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.0</td>
<td>7.0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td><strong>Non-sporulating mycelium</strong></td>
<td>10.7</td>
<td>13.3</td>
<td>9.3</td>
<td>16.0</td>
<td>9.3</td>
<td>18.0</td>
<td>10.0</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.6</td>
<td>41.4</td>
<td>38.5</td>
<td>34.7</td>
<td>30.6</td>
<td>45.0</td>
<td>34.0</td>
<td>48.0</td>
</tr>
<tr>
<td>Others</td>
<td><strong>Nematodes</strong></td>
<td>4.0</td>
<td>5.3</td>
<td>9.4</td>
<td>8.0</td>
<td>8.0</td>
<td>15.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><strong>Undefined causes</strong></td>
<td>9.3</td>
<td>5.3</td>
<td>9.4</td>
<td>8.0</td>
<td>8.0</td>
<td>2.0</td>
<td>18.0</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.3</td>
<td>10.6</td>
<td>18.8</td>
<td>16.0</td>
<td>16.0</td>
<td>17.0</td>
<td>19.0</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Acknowledgements

This research was founded by the State Committee for Scientific Research (KBN) through the Research Project № 5 P06C 00119.

References


Effect of temperature on conidia germination and vegetative growth of *Metarhizium anisopliae*

Hermann Strasser, Michaela Erschbamer

*Institute of Microbiology, Leopold-Franzens University Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria*

**Abstract:** Nine *Metarhizium anisopliae* var. *anisopliae* strains were subjected to seven different temperatures between 10 and 37 °C, in order to study the effects of temperature on germination of airborne conidia and the fungal growth behaviour on Sabouraud Dextrose Agar (SDA) plates. The loss of ability to germinate at temperatures higher than 30 °C was determined. Additionally, conidia were exposed to 50 °C for 60 min to simulate a freeze-drying process for downstream processing. These investigations are of prime interest regarding production of formulated conidia and their registration as a safe highly virulent biocontrol agent.

**Key words:** *Phyllopertha horticola*, Scarabaeidae, entomopathogen, *Metarhizium anisopliae*, temperature, fungal growth

**Introduction**

*Metarhizium anisopliae* var. *anisopliae* is a promising antagonist to control the garden chafer *Phyllopertha horticola* (L.) (Scarabaeidae), which is a major pest in Europe (Strasser, 2000). Attributes such as pathogenicity, mass production, formulation and persistance in soil, respectively, were already well characterized for *Metarhizium* production strains (Zimmermann, 1982; Reinecke et al., 1990). Nevertheless, besides these factors, information on the resistance of conidia to various degrees of temperatures are still missing (McCommon & Rath, 1994). Therefore, the objective of the present investigation was to estimate the thermal tolerances of nine *M. anisopliae* var. *anisopliae* isolates, which are potential *P. horticola* control agents. Besides the vegetative growth of the fungi, the influence of temperature on the germination of airborne conidia as well as the conidiation of the mycelium was determined.

**Materials and methods**

**Fungal isolates and maintenance**

Nine single-spore isolates of *Metarhizium anisopliae* var. *anisopliae*, strain KVL275, V245, V208, IMBST9601, IMBST9602, IMBST9609, 9631, FAL376, FAL538 were used in this study. The isolates, provided from BIPESCO-partners, were maintained on Sabouraud-Dextrose Agar (SDA medium, Merck) at 25 °C and 80 % relative humidity (RH).

**Effect of temperature on fungal growth and conidia germination**

Conidia suspensions (50 to 100 conidia per 50 µl Tween 80; 0.5 % v/v) were plated on SDA and incubated at 10, 20, 25, 30, 33, 35 and 37 °C and >80% RH for 21 days (n=4).

Additionally, conidia suspensions diluted with Tween 80 (0.5 % v/v) and dry harvested airborne conidia were incubated in vials at 20, 36, 45 and 60 °C for 5, 10, 30 and 60 min. Then a sterile SDA solution was inoculated with these conidia/conidia suspensions and cultivated for 24 h at 25 °C and 200 rpm (n=3). Conidia germination was assessed microscopically (per treatment, n=40).
Table 1: Effects of different temperatures on vegetative growth of nine *M. anisopliae* isolates cultivated on SD-agar for 21 days: (xxx) good growth, (xx) weak growth, (+++) sporulation, (++) weak sporulation, (-) no growth/ no sporulation.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>CFU (SD)</th>
<th>Temperature [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>KVL 275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>284 (±81)</td>
<td></td>
</tr>
<tr>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>156 (±33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65 (±21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>V245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>133 (±30)</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>134 (±37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>104 (±50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95 (±32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42 (±20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>V208</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>102 (±18)</td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>89 (±44)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59 (±19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 (±39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>IMBST96 01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>44 (±8)</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>41 (±10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 (±9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27 (±17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>IMBST96 02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>186 (±28)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 (±47)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>112 (±24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>132 (±12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (±5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>IMBST96 09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>68 (±20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>47 (±19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44 (±35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (±15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>9631</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>149 (±27)</td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>106 (±42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>121 (±54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37 (±48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>FAL376</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>389 (±22)</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>175 (±62)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>121 (±54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37 (±48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td>FAL538</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xx</td>
<td>212 (±33)</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>128 (±16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>69 (±24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 (±8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (±0)</td>
<td></td>
</tr>
</tbody>
</table>
Results and discussion

Optimal growth was observed for all *M. anisopliae* var. *anisopliae* strains at 20 to 30 °C. No isolate grew at temperatures higher than 35 °C. Except strain IMBST9602 and IMBST9609, all strains were able to grow at 10 °C, but only strain KVL275, V245 and IMBST 9631 showed conidiation (Table 1).

According to the presented data all tested isolates belong to the meso-thermoactive group. The optimal germination temperature range for the tested *M. anisopliae* var. *anisopliae* isolates was 22-30 °C. This indicates, that the most rapid germination and growth of the fungus, which probably results in the fastest death of the insects, is likely to occur at 25 °C for all tested strains. This is important, because the range of temperatures over which the fungus has a high germination rate should be matched to those occurring in the environment in which it will be applied (McCammon & Rath, 1994).

![Germination (%) of *M. anisopliae* strain IMBST 9609 after treating the conidia at different temperatures and incubation time, respectively: (a) 20 °C, (b) 36 °C, (c) 45 °C and (d) 60 °C (n=3).](image-url)
The morphology of *Metarhizium* isolates was temperature dependent, especially at temperatures higher than 30 °C. At temperatures up to 25 °C the isolates showed a green to olivaceous-buff colonies. An increase led to a decolouring of the conidia (i.e. brownish, yellowish or white pigmentation). The harvested conidia from each temperature regime, which were again incubated at 20 °C showed with one exception (strain FAL376) efficient vegetative growth and the conidiophores produced ripe airborne conidia (Fig 1.). At high effect of high temperatures (> 40°C) the conidia were effectively damaged. At temperatures of 45 and 60 °C germination was significantly reduced after 10 min. After 60 min-treatment, only one isolate showed still a minor germination activity. No negative effects were observed on the viability of conidia when treated and incubated at 20 and 36 °C.

The viability and stability of conidia is influenced by different drying techniques (Hong *et al*., 2000). Kiewenick (personal communication) demonstrated that rapidly drying with warm air has a negative effect on the viability of *M. anisopliae* strains V245 and V208 conidia. Latest investigations showed that stability of *M. anisopliae* conidia is greatly influenced by the moisture content of the conidia. Hong *et al*. (1997) have developed a model to quantify relations between conidia longevity and environment. However, they still validate the parameters because there is a variation in the sensitivity of longevity to temperature and equilibrium relative humidity. For ongoing research more attention will be paid to conidial production and formulation methods in order to maximize the effectiveness of selected, entomopathogenic *M. anisopliae* isolates.

**Acknowledgements**

This work is part of the ongoing EU-FAIR6-CT98-4105 research project BIPESCO.

**References**


Distinction of the entomopathogenic fungal species

Beauveria brongniartii and Beauveria bassiana
by comparing their carbon utilization patterns

Barbara Pernfuss, Wolfgang Schweigkofler1, Hermann Strasser
Institute of Microbiology, Leopold-Franzens University Innsbruck, Technikerstraße 25,
6020 Innsbruck, Austria
1 Research Centre for Agriculture and Forestry Laimburg, 39040 Auer (BZ), Italy

Abstract: Several strains of entomopathogenic Beauveria spp. were tested for their carbon utilization profile using the BIOLOG™ microtiter plate procedure. Monospore cultures of five Beauveria brongniartii- and three B. bassiana-strains were used to inoculate BIOLOG SFP- and SFN-microtiter plates. With this two plate systems the metabolization of 130 carbon sources was investigated. The results were transcribed into a four-character-code and the generated matrix was used as an input file for a Neighbour Joining Analysis. Furthermore, the BIOLOG data were statistically analysed by student's t-test. The methodology presented here allows to distinguish B. brongniartii and B. bassiana with high reliability, based on physiological carbon utilization tests. By comparing the absolute carbon utilisation profiles with student's t-test, the species B. brongniartii could be distinguished from B. bassiana with a probability of more than 95%. The bootstrap analysis of 130 carbon sources grouped into four categories resulted in a separate clustering of these two Beauveria species.

Key words: Beauveria brongniartii, Beauveria bassiana, carbon utilization, chemical taxonomy

Introduction

As nomenclature and taxonomy of the genus Beauveria are still controversial, and specialists for the morphological characterization are rare, various attempts have been made to find additional methods for a reliable distinction of species within this genus.

Physiological studies as well as genetic and immunological techniques were tested for their suitability to distinct Beauveria spp. Nevertheless, the entomopathogenic species B. bassiana and B. brongniartii are still subjects for investigation (Todorova et al., 1998).

In the course of the EU funded project BIPESCO (FAIR-CT98-4105), several strains of entomopathogenic Beauveria spp. were physiologically characterized by their carbon utilization patterns. These patterns were used to test whether they are suited to distinct B. brongniartii and B. bassiana.

Material and methods

Beauveria spp. strains
Five highly virulent isolates of B. brongniartii IMBST 95031 (No. 1), IMBST 95041 (No. 2), FAL 166 (No. 3), FAL 546 (No. 4) and IMBST 97011 (No. 12), were used in this study. The three B. brongniartii strains IMBST 95031, IMBST 95041 and IMBST 97011 were isolated from pasture soil infested with Melolontha melolontha (Scarabaeidae) in Kramsach (Tyrol), Austria. FAL 166 and FAL 546 are commercial single spore isolates obtained from FAL-Reckenholz, Zürich, Switzerland.
Furthermore, three *B. bassiana* strains - CH I (No. 9), FAL 257 (No. 10) and FAL 342 (No. 11) - were generously put at our disposal by FAL-Reckenholz.

**Cultivation**

All isolates were cultivated on Sabourand-2-Glucose agar (S2G) or Chitin-peptone agar (CiP) at 25 °C until spores could be harvested (20 to 50 days). S2G broth was purchased (Merck 1.08339) and 18 g l⁻¹ agar was added. Chitin-peptone agar was composed of 10 g l⁻¹ chitin (Sigma C-7170), 5 g l⁻¹ peptone (Merck 7214) and 18 g l⁻¹ agar (pH 5.6).

The isolates were numbered (see above) and decimal numbers designate how often the strains were subcultured. When fungi were grown on CiP, strains are marked with stars (*). Isolates without stars were grown on S2G agar.

**Carbon utilization patterns**

Airborne conidia were used to inoculate BIOLOG SFP- and SFN microtiter plates. Following Dobranic & Zak (1999) the tetrazolium dye MTT – Dimethylthiazolyl-diphenyl-tetrazolium bromide was used as a redox indicator. With the named two plate systems the metabolization of 130 carbon sources was investigated. All experiments were done twice.

Data analysis was performed by calculating the average of relative carbon utilization, whereby the absorption [OD₅₉₀ nm] of the reference well without a carbon source was subtracted from the values measured for each well containing a defined carbon source. The second highest absorbency for each strain and experiment, respectively was set to 100 %. These data were grouped into four categories, which are defined as follows : < 10 % (group 1), < 40 % (group 2), < 70 % (group 3) and > 70 % carbon utilization (group 4).

The data of absolute utilization of the different carbon sources (OD₅₉₀ minus OD of the reference well) were statistically analysed by student's t-test.

**Neighbour Joining Analysis**

On the basis of the results on the carbon utilization by the 22 cultures of *Beauveria brongniartii* and *B. bassiana*, a four character matrix was constructed, ready to use for phylogenetic computer programs. Basically, the four-character code for carbon utilization was transcribed into a nucleotide code (carbon utilization < 10 % A, carbon utilization < 40 % T, carbon utilization < 70 % C, carbon utilization > 70 % G).

The generated matrix was used as the input file for a Neighbour Joining Analysis (Saitou & Nei, 1987). A statistical bootstrap analysis of the resulting topology was done with 1000 replicates.

The program “TreeView” (Page, 1998) was used for the graphical representation of the resulting treefile as a phylogram and as a slanted cladogram, respectively.

**Results and discussion**

The 22 cultures were grouped into three clusters (Fig. 1) : i) a *B. brongniartii* cluster with the Swiss isolates No. 3.2 and No. 4.2, ii) a cluster with all other *B. brongniartii* isolates (Austria) and iii) a cluster with *B. bassiana* strains (Switzerland). Isolates belonging to the same strain which differ in the number of subculturing do cluster together (i.e. No. 1.2 and No. 1.4 in cluster 2 ; No. 10.2 and No. 10.4 in cluster 3). The same situation could be observed with isolates of the same strain subcultured on different media (i.e. No. 12.2 and No. 12.2* in cluster 2).

By comparing the absolute carbon utilization profiles with students t-test, the species *B. brongniartii* could be distinguished from *B. bassiana* with a probability of more than 95 %. The Neighbour Joining Analysis of 130 carbon sources grouped into four categories resulted in a separate clustering of *B. brongniartii* ecotypes and *B. bassiana*. From a taxonomic point
of view it is questionable whether the Swiss strains (cluster 1) and the Austrian strains (cluster 2) of *B. brongniartii* are conspecific.

The methodology presented here allows to distinguish *B. brongniartii* from different geographic regions and *B. bassiana* with high reliability, based on physiological carbon utilization tests.

---

**Fig. 1.** Slanted cladogram (left) and phylogram (right) of *B. brongniartii* and *B. bassiana* strains. Matrix generated by using 130 carbon utilization values, grouped into four categories.

---

**References**


Fluorescence microscopic investigations on adhesion of spores of the entomopathogenic fungus *Verticillium lecanii* at larvae of *Frankliniella occidentalis* (Thysanoptera: Thripidae)

Ulrike Meyer, Helga Sermann
Humboldt-University of Berlin, Institute of Horticultural Sciences, Department of Phytomedicine (WG Applied Entomology), Lentzeallee 55-57, 14195 Berlin, Germany

Abstract: *Frankliniella occidentalis* is an important pest in horticultural systems. Until now no satisfactory control methods are available and there are investigations in order to develop biological control methods. Adhesion processes of spores of the entomopathogenic fungus *Verticillium lecanii* to the insect cuticle were investigated using fluorescence-microscopic procedures. Results showed a high number of spores per insect after low-dose application, the efficacy of an indirect application, a better adhesion to dead than to living larvae and a better adhesion of conidia compared to blastospores. In addition the loss of spores after 24 h and the localization on the insect have been recorded.

Key words: *Frankliniella occidentalis*, *Verticillium lecanii*, biological control, adhesion processes

Introduction

*Frankliniella occidentalis* is an important pest in horticultural systems. To control its populations is a problem which has not been solved satisfactorily until now. Trials to establish a biological control method with help of entomopathogenic fungi such as the deuteromycete *Verticillium lecanii* (Zimmermann) Viégas have been performed.

In relation to these trials we analysed the adhesion-processes of spores at the host insect cuticle using fluorescence-microscopic procedures.

Material and methods

*F. occidentalis* was reared on bush-beans (*Phaseolus vulgaris* 'Marona'). Larvae at stage L II were used in the experiments.

Blastospores and conidia of the fungus (*V. lecanii* strain V24 K2) were produced according to the method of Hirte et al. (1989). The inoculum has been stained by the fluorescent-dye Calcofluor white M2R (SIGMA).

Experimental infections were performed using the "Petri dish test" standard method (Wolff, 1999). Per variant 50 larvae (10 per Petri dish) have been sprayed with a spore suspension (amount: 5.5 mls per dish) at concentrations 2-3x10^5, 10^6 and 10^7 spores/ml using a potter precision laboratory spray tower (Burkard Manufacturing Co. Ltd.). Either conidia or blastospores were used. Besides this direct application procedure, indirect application procedure was performed.

To observe spores on the insect cuticle, insects were examined either immediately after application or after different periods of time. Insects were killed by ethyl acetate prior to removing from the support.

The attached spores (dorsal, dorsal and ventral) have been counted under a fluorescence-microscope (BX40 FS by Olympus) with objectives Plan 10x/0.25 Ph1 and Plan 20x/0.40 Ph1.
(excitation filter: 355-425 nm, barrier filter: 460 nm), and their localization on the insect has been recorded.

**Results**

A set of results was gained. They are presented in 6 topics.

**Topic 1**

Higher was the spore concentration of the applied suspension, higher was the number of spores attached to insects. Similar trends were observed whether insects were living or dead. (Table 1).

**Table 1:** Number of spores/larva (L II) of *F. occidentalis* and proportion of larvae without spores after direct leaf application (27.5 ml/variant) with conidial suspension of *V. lecanii* (V24 K2) [spore concentrations: $3 \times 10^7$, $3 \times 10^6$ and $3 \times 10^5$ sp./ml; addition of Calcofluor white M2R]; variant a) larvae killed before application, variant b) larvae killed immediately after application (50 insects/variant).

<table>
<thead>
<tr>
<th>applied spore concentration</th>
<th>application on dead insects</th>
<th>application on living insects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>spores/larva (total)</td>
<td>larvae without spores (%)</td>
</tr>
<tr>
<td>$3 \times 10^7$ spores/ml</td>
<td>33.3 a*</td>
<td>0</td>
</tr>
<tr>
<td>$3 \times 10^6$ spores/ml</td>
<td>3.08 b</td>
<td>8</td>
</tr>
<tr>
<td>$3 \times 10^5$ spores/ml</td>
<td>0.66 c</td>
<td>46</td>
</tr>
</tbody>
</table>

*Different letters show significance.*

**Topic 2**

After indirect application the highest number of spores on insects was noticed between 0 and 3h (ventral > dorsal) following application.

In the case of low spore-concentrations ($3 \times 10^5$ spores/ml), more spores were observed on the larvae after indirect application than after direct application (Fig. 1).

**Topic 3**

The spores adhere better to dead larvae than to living ones (Fig. 2).

**Topic 4**

Conidia adhere stronger to both dead and living larvae than blastospores (Fig. 2).

**Topic 5**

The loss of conidia (i.e. the proportion of conidia which did not remain on insects) reached 29.6 % after 24 h (Table 2).

**Topic 6**

Whatever situation, the highest number of spores was observed on the abdomen of the larvae. Spore numbers on the thorax and the extremities were lower.

**Discussion**

The fact that higher number of spores were recorded on dead insects than on living ones seems to concern larvae only. Indeed, Wolff *et al.* (1999) observed a not significant higher number of spores on adult insects after application on the living adults.
Fig. 1: Number of spores of *V. lecanii* (strain V24 K2) per larva (L II) of *F. occidentalis* after direct and indirect leaf application (27.5 ml/variant) with a conidial suspension of [spore concentrations: $3 \times 10^7$, $3 \times 10^6$ and $3 \times 10^5$ sp./ml; addition of Calcofluor white M2R].

![Number of spores/insect](image)

Fig. 2: Number of spores of *V. lecanii* (strain V24 K2) per larva (L II) of *F. occidentalis*, according to the type of spores (conidia or blastospores), after direct leaf application (27.5 ml/variant) with a spore suspension of *V. lecanii* (V24 K2) [spore concentration: $3 \times 10^7$ spores/ml; addition of Calcofluor white M2R]; variant a) larvae killed before application, variant b) larvae killed immediately after application (50 insects/variant).

![Number of spores/insect](image)

The better adhesion of conidia compared to blastospores is probably due to their mucilaginous coating. Such a result was noticed in the case of adults already (Wolff *et al.*, 1999), but the difference between adults and larvae were smaller.

The loss of spores after a period of walking on the leaf is caused by moving and cleaning of insects. Interestingly, the loss could reach 40% and even more in the case of the adults, which are more active (Wolff *et al.*, 1999).

In direct application many factors can lead to a loss of spores, such as moving, hiding or cleaning activities of insects at a certain moment. After indirect application, in relation to the nearly symmetrical infection potential on the leaf, contact between insects and fungal inoculum could be favoured, allowing to balance a possible loss of spores.
All the presented results seemed to indicate a promising possibility of using *V. lecanii* for the control of *F. occidentalis.*

Table 2: Loss of conidia of *V. lecanii* (strain V24 K2) on larva (L II) of *F. occidentalis* and proportion of larvae without spores after direct leaf application (27.5 ml/variant) with a conidial suspension [spore concentration: $3 \times 10^6$ spores/ml; addition of Calcofluor white M2R]; variant a) recording immediately after application, variant b) recording 24 h after application (50 insects/variant).

<table>
<thead>
<tr>
<th></th>
<th>spores/larva</th>
<th>larvae without spores (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>immediately after application</td>
<td>2.84 a*</td>
<td>2 %</td>
</tr>
<tr>
<td>24h after application</td>
<td>2.0 b</td>
<td>20 %</td>
</tr>
<tr>
<td>total loss</td>
<td>0.84</td>
<td>–</td>
</tr>
<tr>
<td>loss in percent</td>
<td>29.6</td>
<td>–</td>
</tr>
</tbody>
</table>

*Different letters show significance.

Acknowledgements

The authors wish to thank the Deutsche Bundesstiftung Umwelt for supporting this project.

References


Entomopathogenic nematodes
Danish surveys on insects  
naturally infected with entomopathogenic nematodes

Otto Nielsen, Holger Philipsen  
Zoology Section, Department of Ecology, The Royal Veterinary and Agricultural University,  
Thornvaldsenvej 40, 1871 Frederiksberg C, Denmark

Abstract: More than 6000 insects were sampled in a two year period in order to display infections by entomopathogenic nematodes. The highest number of isolates were obtained from *Phyllopertha horticola* larvae (50 isolates), *Delia radicum* larvae and puparia (4 isolates), *Cantharis* spp. pupae and adult (4 isolates), *Hepialus lupulinus* larvae (3 isolates) *Agriotes lineatus* larvae (1 isolate), ceculionid larvae (1 isolate) and *Pterostichus nigrita* larvae (1 isolate). The main focus of the study was put onto the cabbage root fly, *D. radicum*. Approximately 2500 larvae and 1500 puparia were sampled but only two larvae (0.08 %) and two puparia (0.13 %) yielded isolates. The infected *D. radicum* larvae and the infected *A. lineatus* larva were found at plants that approximately three months earlier had been inoculated with an indigenous isolate of *S. feltiae*. It was concluded that the low records of isolates from *D. radicum* probably were due to a combination of low abundance of entomopathogenic nematodes, low susceptibility of the insects and a very low turnover rate of infected individuals. In contrast, epizootic-like conditions occurred in regard to the *P. horticola* larvae collected at a golf course.

Key words: Insect survey, Denmark, natural infection, natural host, *Delia radicum*, *Sitona* spp., *Bibionis* spp., *Hepialus lupulinus*, *Agriotes lineatus*, *Phyllopertha horticola*, *Pterostichus nigrita*, *Cantharis* spp., entomopathogenic nematodes, *Steinernema affine*, *S. intermedium*, *S. feltiae*, cabbage, cultivated soil

Introduction

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are commonly found by the *Galleria* bait method (Bedding & Akhurst, 1975) in which *Galleria mellonella*, *Tenebrio molitor* or other susceptible larvae are placed in soil samples. If infective stages of entomopathogenic nematodes occur in the soil, these might infect the bait larvae and isolates are obtained. Alternatively, isolates may be obtained through the incubation of field collected insects. The advantage of the latter method is that information is gained about the infectivity of the nematodes towards the insects sampled under natural conditions. In addition, this method allows to possibly obtain isolates that are very specific to the particular host in which they were found, and as such, perhaps would not have been detected by the *Galleria* method. A list of insects naturally infected by entomopathogenic nematodes with reference to the original discovery has been given by Peters (1996). Some of the references are unpublished or it is a matter of personal communications, which reflects the sporadic discovery of natural infected insects. A large proportion of nematode species have never been found in hosts under natural conditions but have been obtained by baiting, whereas several examples of natural hosts are given for *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* (Peters, 1996 and references herein).

The first record of insects naturally infected by entomopathogenic nematodes in Denmark was given by Bovien (1937) who isolated *S. feltiae* and *S. affine* from bibionid
larvae. The association of *Bibio* spp. and *S. affine* in Denmark was rediscovered subsequently by Poinar & Lindhardt (1971).

The main focus of the present work was to search for insects infected by entomopathogenic nematodes in cultivated areas in Denmark, with the aim of gaining information on the possible influence of the nematodes on insect populations. Special attention was given to the cabbage root fly, *Delia radicum* (Diptera: Anthomyiidae).

**Materials and methods**

**Soil and insect samples**

Soil samples were taken in relation to plants, including the root and the surrounding soil. Each sample was approximately 15 cm in diameter down to a depth of approximately 15 cm. The soil was kept in plastic bags and either processed on the same day or placed at 5 °C until insect extraction as described below. The highest proportion of samples were taken in organically grown cabbage fields to recover *D. radicum* larvae and pupae. A minor proportion of the larvae (< 1 %) were collected from plants which have been inoculated with *Steinernema feltiae* (DK1) approximately three months earlier. Additional sampling was carried out in pea and alfalfa fields to obtain larvae of the clover weevils, *Sitona* spp. Along with the sampling of *D. radicum* and *Sitona* spp., a range of other insects where obtained including larvae of wireworms, diptera, weevils and predatory beetles.

**Extraction of insects from soil samples**

The insects were extracted by sieving or flotation. In addition, larger sized insects were also sampled directly by hand. Sieving was performed by placing a soil sample on a sieve (mesh=600 µm; diameter=50 cm) from where the soil was flushed away by water. Larger pieces of plants and stones were removed by hand and the remaining material was transferred to a tray with water from which the insects were picked by a pipette. Flotation was performed by suspending the soil sample in water in a bucket (10 l) and decanting it through the same sieve as used above. The soil was suspended and decanted repeatedly until only stones and sandy parts of the soil was left in the bucket. The main difference between the sieving and the flotation method was that most soil was retained in the bucket by flotation and thus reduced flushing on the sieve. Samples, where primarily the last instar larvae or puparia of *D. radicum* were of interest, were processed on a sieve with a larger mesh (1500 µm).

**Incubation of insects**

Alive larvae were allowed to dry on paper tissue and then placed individually in small cups (35 ml) with a lid and a piece of moist filter paper to prevent dehydration of the insects. The larvae were examined at two to four days intervals for at least two weeks or until death. Larvae that pupated during this period were assumed uninfected. Dead larvae were placed individually on water traps and examined two to three times a week for up to three weeks for nematode infection. All incubations were at room temperature (18-25 °C). Water traps were constructed by placing a piece of filter paper (approximately 3x3 cm) on a small plastic lid in a Petri dish (5 cm) with water covering the bottom.

Overwintering *D. radicum* puparia were placed directly without incubation on water trap. These water traps were larger (10-cm-petri dishes). They were firstly placed at room temperature for two weeks and observed regularly. They were then stored at 5 °C for six months and examined approximately every three weeks during this period.

**Identification of entomopathogenic nematodes**

Isolates of entomopathogenic nematodes that were obtained from incubation of insects were cultured and identified to species on the basis of infective juvenile morphology together with
PCR-RFLP according to Reid et al. (1997). When several isolates originated from the same site, a representative proportion was cultured and identified by PCR. The restriction enzymes \textit{Alul}, \textit{Ddel}, \textit{HhaI} and \textit{HinfI} were used.

**Results and discussion**  

More than 6000 insects were sampled in the surveys from 1997 to 1999. The Figure 1 gives an overview of the results in terms of numbers of collected insects and number of infected individuals. Most of the sampled insects were either \textit{D. radicum} larvae (~2500) or puparia (~1500) from organic farms. Despite the high number of \textit{D. radicum}, only two last instar larvae (0.08 \% of total larvae) and two winter puparia (0.13 \% of total puparia) yielded nematode isolates. The nematodes from the infected \textit{D. radicum} puparia emerged after few weeks of incubation on water traps and storage for the winter did not yield additional isolates. Further, infected individuals were obtained from the following insects: \textit{Hepialus lupulinus} larvae, \textit{Phyllopertha horticola} larvae, an unidentified curculionid larva, \textit{Bibio} spp. larvae, a \textit{Agriotis lineatus} larva, a \textit{Pterostichus nigrita} larva and \textit{Cantharis} spp. pupae and one adult.

![Fig. 1. Total number of insects sampled and number of infected individuals in relation to insects species. Details on the infected insects are given in Table 1.](image)

The nematode isolates were all identified as steinernematids (Table 1). \textit{Steinernema feltiae} was the most frequent species and was isolated from the curculionid larva, \textit{D. radicum} larvae and puparia, \textit{Bibio} spp. larvae, a \textit{Agriotis lineatus} larva, \textit{Hepialus lupulinus} larvae and \textit{P. horticola} larvae. \textit{S. feltiae} (DK1) had been inoculated approximately three months earlier to the specific plants from where the infected \textit{D. radicum} larvae and the infected \textit{Agriotis lineatus} larva were sampled. The three isolates obtained could thus be reisolates of
DK1. *S. affine* was isolated from a *Cantharis* sp. adult, *P. horticola* larvae and a *P. nigrita* larva. *S. intermedium* (or C1) was isolated from *Cantharis* spp. pupae. *S. intermedium* and C1 are morphologically similar but the isolate examined by PCR had a banding pattern identical to C1 (data not shown). All infected insects were either collected by hand directly in the field or found by the flotation technique (no isolates obtained by the sieving method).

Table 1. Infections of collected insects by steinernematid nematodes (Denmark, 1997-1999).

<table>
<thead>
<tr>
<th>Nematode species</th>
<th>Insect order or family</th>
<th>Insect species</th>
<th>Stage(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Steinernema feltiae</em></td>
<td>Curculionidae</td>
<td>Not identified</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td><em>Delia radicum</em></td>
<td>L*, P</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td><em>Bibio</em> sp.</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Elateridae</td>
<td><em>Agriotes lineatus</em></td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Hepialidae</td>
<td><em>Hepialus lupulinus</em></td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Scarabaeidae</td>
<td><em>Phyllopertha horticola</em></td>
<td>L</td>
</tr>
<tr>
<td><em>Steinernema affine</em></td>
<td>Cantharidae</td>
<td><em>Cantharis</em> sp.</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Scarabaeidae</td>
<td><em>Phyllopertha horticola</em></td>
<td>L</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pterostichus nigrita</em></td>
<td>L</td>
</tr>
<tr>
<td><em>Steinernema intermedium</em></td>
<td>Cantharidae</td>
<td><em>Cantharis</em> sp.</td>
<td>P</td>
</tr>
</tbody>
</table>

*S. feltiae* has been supplied to the plants approximatively three months prior to collection of infected insects.

The present work demonstrates that infected insects can be found in systematic surveys. The insects were either collected by hand or extracted by the use of water (flotation or sieving). Among infected insects which were mainly hand collected (data not shown), *P. horticola* yielded the highest number of nematode isolates. This species has previously been found infected by nematodes under field conditions and Peters (1996) referred to unpublished reports where up to 80% of the population was infected. *P. horticola* larvae is relatively easy to collect by hand in the field in autumn where they are active just below the soil surface. Furthermore, the lower temperatures at this time of the year reduces the turnover rates of cadavers which enhances the probability of obtaining infected individuals. These conditions were similar for the *H. lupulinus* larvae, and thus the probability of finding infected larvae can be expected to be relatively high if infective juveniles of entomopathogenic nematodes are present in the soil simultaneously. A related species, *H. californicus* has been observed infected by *H. hepialus* in California (Stock et al., 1996).

Bibionid larvae are slightly smaller than *P. horticola* and *H. hepialus* larvae, but they still have a size that makes hand collection possible. *Bibio* spp. overwinter as larvae in the soil, which extends the period where individuals can be collected. The common species, *Bibio hortulanus*, can be found in “nests” and thus high numbers may be easy to collect. The association of this insect with steinernematids has previously been reported (Bovien, 1937; Poinar & Lindhardt, 1971) and bibionid larvae could be a frequent host for these nematodes.

Also the infected *Cantharis* spp. were hand collected and even though a high proportion was infected, the total number of individuals collected is to low to allow generalization about susceptibility of this insect. Infection of Cantaridae by entomopathogenic nematodes is, to our knowledge, reported here for the first time.
The remaining nematode isolates were obtained from insects extracted from soil samples using the flotation method. The main focus was on *D. radicum*. This insect is relatively small and according to our experience, this method allowed the optimal sampling in regard to the purpose of this study. Relative low numbers of *D. radicum* individuals were infected. In addition to low susceptibility of the insects, a low turn over rates of the cadavers could explain such a situation. Observations in the laboratory have shown that turn over rates are less than one week for this species. Another explanation could be the absence of or low numbers of entomopathogenic nematodes in the sampled fields. This is supported by the results of parallel baiting in the specific fields (data not shown).

When extracting *D. radicum*, a range of further insects were found. Among these, one *Agriotes lineatus* larva and one *Pterostichus nigrita* larva were found infected by entomopathogenic nematodes. *A. lineatus* has previously been found infected by *S. carpocapsae* (Poinar & Veremchuk, 1970). However, infection of wireworms with *S. feltiae* is, to our knowledge, observed for the first time in this study. *Agriots* spp. larvae spend three years in the soil and are as such potential hosts for entomopathogenic nematodes. However they are regarded as relatively resistant to infections due to physical barriers (Eidt & Thurston, 1995). Infection of the carabid, *Pterostichus nigrita* under field conditions is also here reported for the first time. However, laboratory experiments with different carabid beetles have demonstrated that carabids, and among these *P. vulgaris*, were infected by *S. feltiae* and *H. bacteriophora* (Jaworska, 1989). Laboratory experiments with other carabid species also showed that some species are susceptible to entomopathogenic nematodes (Bathon, 1996; Georgis et al., 1991; Jaworska, 1989) and it is as such not unlikely that infections of carabid beetles are possible under field conditions. Attempts to directly measure the impact of entomopathogenic nematodes on predatory beetles under fields conditions requires extensive work. Indeed, the results so far gave inconsistent results (Koch & Bathon, 1993) or could not display any negative impact (Ropek & Jaworska, 1994).

**Acknowledgements**

We wish to thank Jan Martin at our institute for his help by the collection and identification of insects. The technical assistance of Pernille Frandsen is highly appreciated. The work was financially supported by the Danish Ministry of Agriculture (Contract no. BIO 96-KVL-1).

**References**


Studies on *Steinernema feltiae* related to control of sciarids

**Solveig H. Salinas, Annette Sundby, Nina S. Johansen**

*The Norwegian Crop Research Institute, Department of Entomology & Nematology, Hogskoleveien 7, 1432 Aas, Norway*

**Abstract:** The entomopathogenic nematode *Steinernema feltiae* is used to control sciarid flies (*Bradysia* spp.) by many Norwegian growers of ornamentals in glasshouses. The growers occasionally complain of poor effect of *S. feltiae* when glasshouse temperatures approach 30 °C or more. Survival of *S. feltiae* infective juveniles at 24, 30 and 35 °C was tested in a controlled experiment at the Norwegian Crop Research Institute (NCRI). At ambient temperatures of 30 and 35 °C the survival of *S. feltiae* infective juveniles appeared to be not negatively affected.

**Key words:** *Steinernema feltiae*, Sciaridae, poinsettia, biological control

**Introduction**

Sciarid flies (*Bradysia* spp.) are pests of young ornamental plants and the larvae damage roots, stems and leaves of seedlings and cuttings. The larvae also feed on organic debris and fungi. In Norway the main sciarid pest is *Bradysia paupera*. Biological control of sciarids is necessary because there has been an increase in insecticide resistance, and growers are increasingly using various biological methods for control of other pests, where the use of insecticides is undesirable (Gripwall, 1994; Gouge & Hague, 1995). The nematode *Steinernema feltiae* (Filipjev) is the species most commonly used for control of sciarids.

Many norwegian growers are satisfied with *S. feltiae* as a biological control agent for sciarids, however there has been concern that poor control is achieved when temperatures approach 30 °C or more in the glasshouses. This paper reports on the survival of the infective stages of *S. feltiae* in poinsettia cuttings at different temperatures over time.

**Materials and methods**

Poinsettia cuttings (cv. Lilo) were planted in Jiffy-7 38 mm peat pellet-pack-trays and were incubated for two weeks at 24 °C, 80-95 % RH (relative humidity) for rooting. The plants were not infested with sciarid flies.

After 13 days 150 plants/pellets were treated with *S. feltiae* (Nemasys) at a rate of 1 million per m², which was equivalent to approx. 1150 per plant. Nematodes were applied using a pipette (Finnpipette Digital 1 - 5 ml, Labsystems) in 1 ml tap water. Immediately after treatment, 30 plants were sampled to count the number of extracted nematodes per plant/pellet, and to establish the extraction efficacy. Nematodes were extracted using a modified Baermann funnel technique (Hooper, 1986). The remaining plants were incubated in equal numbers at 24, 30 and 35 °C (RH 80-90 %). The plants were watered once a day, care was taken not to water the plants too much so that the nematodes would be washed out of the peat pellets.

At days 4, 10 and 21 after the nematode treatment, 10 plants per temperature were randomly taken and nematodes were extracted. The number of nematodes were counted for each plant/pellet and average numbers per day/temperature were compared.
Results and discussion

In Figure 1 the average number of nematodes extracted indicate that there is little difference in survival of *S. feltiae* infective juveniles between the tested temperatures. Surprisingly survival appears to be better at 35 °C after 4 and 10 days based on the number of nematodes extracted. The extraction efficacy was estimated at 54 %, consistant with results from similar work (Hass *et al.*, 1999).

The variation in the extraction efficacy may in part explain the apparent higher number of nematodes at 35 °C. However it was noted during the course of the experiment, that the peat pellets in the incubators at 30 and 35 °C tended to dry out quickly, which may have resulted in an increased survival of the infective juveniles compared to the situation at 24 °C where the pellets were constantly moist. In this experiment the infectivity of the infective juveniles was not tested. Haukeland (1993) has shown that at temperatures above 30 °C *S. feltiae* is not able to reproduce or cause mortality to *Galleria mellonella*.

In conclusion it appears that at ambient temperatures of 30 and 35 °C the survival of *S. feltiae* infective juveniles is not negatively affected. Overwatering at higher temperatures may have a more detrimental effect on nematode survival.

![Fig. 1. Survival of Steinernema feltiae in peat pellets (with Poinsettia cuttings) at different temperatures (°C). Figures represent the number of nematodes extracted (using a modified Baerman funnel), bars represent confidence intervals at the 95 % level.](image)

Acknowledgements

We would like to thank the Poinsettia growers association for supplying Poinsettia cuttings and the nematode laboratory at NCRI for extraction of nematodes.
Table 1. Percentage of *Steinernema feltiae* that have survived at different temperatures in peat pellets with *Poinsettia* cuttings. The percentage for each day/temperature has been calculated from the average number of nematodes extracted at day 0.

<table>
<thead>
<tr>
<th>Days after application</th>
<th>% <em>S. feltiae</em> infective juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 °C</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>21</td>
<td>9</td>
</tr>
</tbody>
</table>

References


Haukeland, S. 1993: Isolation and observations of steinernematid nematodes (Steinernematidae: Rhabditida) from Norway and Reading, UK. PhD thesis, University of Reading, UK.

Host potential of insects from cruciferous crops to entomopathogenic nematodes and augmentation of nematodes through oil seed rape growing

Holger Philipsen, Otto Nielsen
Zoology Section, Department of Ecology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

Abstract: Larvae of pollen beetles (Meligethes spp.), the brassica pod midge (Dasyneura brassicae), the cabbage seed weevil (Ceutorhynchus assimilis), and the cabbage moth (Mamestra brassicae) were collected in the field and exposed to entomopathogenic nematodes in the laboratory. Pollen beetle larvae, seed weevil larvae and cabbage moth larvae were all relatively easily infected while infections of pod midge larvae were observed only twice. The number of nematodes produced in the cadavers was positively related to the size of the insects. The recovery of D. brassicae and Meligethes spp. was further studied after exposure to entomopathogenic nematodes during the pupating process. The recovery of D. brassicae was almost unaffected by the nematodes whereas recovery of Meligethes spp. decreased with increasing number of nematodes. The susceptibility of oil seed rape insects to entomopathogenic nematodes was indirectly demonstrated under field conditions. Soil grown with oil seed rape for three years was compared to soil grown with grain using baiting with Tenebrio molitor. While entomopathogenic nematodes were almost absent in soil grown with grain, almost all samples were positive for nematodes in soil grown with oil seed rape.

Key words: Host potential, soil-pupating insects, entomopathogenic nematodes, Ceutorhynchus assimilis, Dasyneura brassicae, Mamestra brassicae, Meligethes spp.

Introduction

Entomopathogenic nematodes of the genera Steinernema and Heterorhabditis occur in the soil as non-feeding infective juveniles. Since they are obligate parasites of insects, they depend on the presence of these for completion of their life cycle. This work focused on the host potential of soil-pupating insects living in oil seed rape, ranging from tiny gall midges to large noctuid larvae.

The main attention was paid to the pollen beetle (Meligethes spp.), and the brassica pod midge, Dasyneura brassicae. These species are in most years very numerous in oil seed rape and even though the insects are relatively small, the sum of biomass passing through the soil may be of significance to a nematode population. The larvae of the pollen beetle feed on pollen of oil seed rape plants until they reach a size of approximately 4 mm. Feeding by the adults may cause damage to the plants, depending on the developmental stage of the plant, as open flowers are not damaged. Dasyneura brassicae larvae live within the pods and may cause severe damage due to the destruction of the pod walls. The larvae enter the soil for pupating when they are approximately 2 mm long.

Additional studied insects were the cabbage seed weevil, Ceutorhynchus assimilis, and the cabbage moth, Mamestra brassicae. The cabbage seed weevil larvae live within the pods of oil seed rape and reach a size of approximately 5 mm. The cabbage moth is a relatively large insect (approximately 40 mm) and has as such a great potential for propagation of nematodes. The cabbage seed weevil and the cabbage moth are normally of minor importance as pest insects.
Little information is so far available about the interactions of these insects and entomopathogenic nematodes. Likewise information about soil-pupating insects are in general rare, but several experiments have been made especially in regard to saw flies (Battisti, 1994; Georgis & Hague, 1988; Mrácek & Spitzer, 1983). Most of the insects taken into account in the present work are relatively small. The knowledge about the success of nematode infections in small insects is limited. As far as the authors are aware, the smallest insects studied are *Frankliniella occidentalis* (Tomalak, 1994), sciarid flies (Gouge & Hague, 1995), second instar *Otiortynchus sulcatus* larvae (Kakouli-Duarte & Hague, 1999) and first and second instars *Delia radicum* larvae (Bracken, 1990).

The aim of this work was therefore to study the host potential of these soil-pupating insects to entomopathogenic nematodes. This included the estimation of mortality and infection rates and nematode producing capacity according to species. Furthermore the effect of oil seed rape growing on entomopathogenic propagation was studied under field conditions.

**Materials and methods**

**Sampling of larvae**

Pollen beetle larvae, cabbage stem weevil larvae and pod midge larvae were sampled by collection of oil seed rape plants in June and July. The plants were laid in heaps of approximately 30-40 plants on large pieces of plastic. After a period of time, larvae could be collected from the plastic surface after lifting the plants. Cabbage moth larvae were sampled directly in the field. For all species, only the most active larvae with maximum sizes were used.

**Mortality rates, infection rates and nematode production in insects**

The larval stage of the insects were exposed individually or in groups to different levels (0-200 nematodes per larva) of entomopathogenic nematodes (*Heterorhabditis* and *Steinernema* isolates). The tests were carried out at room temperature (20-24 °C) in either Petri dishes (10 cm-diameter) lined with filter paper or in small cups (height-diameter = 42-30mm), jars (height-diameter = 9.5-6.5cm) or boxes (height-width-length = 6-17-22 cm) with soil (heat sterilised sandy loam with a water content of 11 %). Petri dishes were kept separately in plastic bags to avoid evaporation, while cups, jars and boxes were closed with lids. Larvae that died during the experiments were placed individually on water traps and emerging nematodes were counted.

**Recovery of pod midges and pollen beetles after exposure to nematodes**

The larval stages of the insects were placed individually in cups with 10 ml soil (see above) and various numbers of nematodes. The cups were closed with lids and left at room temperature (20-24 °C) for approximately three weeks after which they were stored at 5 °C until extraction of the insects from the soil. Cups with pod midges had been supplied with a piece of sticky material on the inside of the lid to catch emerging adults. The soil from each individual cup from where no adults were visible was then rinsed on a sieve and poured into a tray with water.

**The effect of oil seed rape growing on entomopathogenic nematode propagation**

These experiments were carried out at the experimental site Højbakkegård. An area of 114×36 m was grown with oil seed rape for three subsequent years. In the autumn of the last year 64 soil samples were taken. Each of these samples consisted of 40-50 soil cores (1×10 cm) sampled within an area of 2×2 m. In addition, another 64 similar samples were taken on both side of the area grown with oil seed rape in an area that had been grown with grain. The
samples were taken to the laboratory and a sub sample from each sample was baited with six *Tenebrio molitor* larvae in a 10 cm-Petri dish for a period of one week. Dead larvae were placed on water traps to reveal infections with entomopathogenic nematodes.

**Results and discussion**

An overview of the results from the host potential experiments is given in Table 1. The highest mortality rates were observed with seed weevil larvae (93 %) followed by cabbage moth larvae (81%) and pollen beetle larvae (56 %). The pod midge larvae were apparently unsusceptible to entomopathogenic nematode with mortality rates of 0-2 %. Also infection rates for the pod midges were low (0-1 %, only two infected individuals observed) while infection rates reached 81 % for the cabbage moth larvae, 75 % for the seed weevil larvae and 40 % for the pollen beetles respectively. The production of nematodes in the cadavers was related to host size. A maximum of 2675 (mean around 1300) was observed in the seed weevil and a maximum of 2150 (mean around 1000) in the pollen beetle. The maximum production in the cabbage moth was 95.750 (mean of 47.000).

Table 1. Mortality rates, infection rates and nematodes produced per larva for different insect species collected from oil seed rape (*M. brassicae* was collected on cabbage). The numbers are minimum and maximum numbers obtained in different experimental designs (Petri dishes with filter paper or small containers with soil) with different isolates and numbers of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*).

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Mortality rates (%)</th>
<th>Infection rates (%)</th>
<th>Nematodes per larva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pod midge (<em>D. brassicae</em>)</td>
<td>0-2</td>
<td>0-1</td>
<td>2-71</td>
</tr>
<tr>
<td>Seed weevil (<em>C. assimilis</em>)</td>
<td>55-93</td>
<td>22-75</td>
<td>150-2.675</td>
</tr>
<tr>
<td>Pollen beetles (<em>Meligethes spp.</em></td>
<td>16-56</td>
<td>0-40</td>
<td>100-2.150</td>
</tr>
<tr>
<td>Noctuids (data for <em>M. brassicae</em>)</td>
<td>81</td>
<td>81</td>
<td>4.800-95.750</td>
</tr>
</tbody>
</table>

The recovery experiments were based on experiments in small cups with moist soil and different numbers of nematodes. The recovery of the pod midges was more or less unaffected by the presence of nematodes. The recovery of the control larvae was about 80 % while the minimum recovery of exposed larvae was about 60 % (see left part of Fig. 1). The recovery of pollen beetles was, however, dramatically affected (see right part of Fig. 1). The lowest exposure level reduced the recovery from around 75 % in the control to around 50 % and less than 10 % of the insects were recovered at the highest exposure level.

Pollen beetles are often very numerous in oil seed rape and their high susceptibility to entomopathogenic nematodes could have an effect on nematode augmentation. This is indicated by the results obtained from the baiting in soil grown with oil seed rape or grain (Fig. 2). Entomopathogenic nematodes were almost absent in the area grown with grain while almost all samples were positive in the area grown with oil seed rape. The overall conclusion of the present work is that several insect species from oil seed rape are potential host for entomopathogenic nematodes and may contribute to nematode augmentation in the field.
Fig. 1. Recovery of pod midge (left) or pollen beetle (right) pupae or adults after exposure to different levels of *S. feltiae* during the pupation process.

Fig. 2. Effect of oil seed rape growing on the entomopathogenic nematode propagation in the field as estimated by baiting with *Tenebrio molitor*. The two middle sections of the field have been grown with oil seed rape for three years prior to baiting while the surrounding area was grown with grain. A bar represents that one or more bait larvae was infected by nematodes. The total size of the experimental area was 114×72 m.

**Acknowledgements**

We wish to thank e-nema, Kiel (Germany) and Reitzel, Copenhagen (Denmark) for providing nematodes. The technical assistance of Pernille Frandsen is highly appreciated. The work was financially supported by the Danish Ministry of Agriculture (Contract no. BIO 96-KVL-1).
References


Kakouli-Duarte, T. & Hague, N.G.M. 1999: Infection, development, and reproduction of the entomopathogenic nematode Steinernema arenarium (Nematoda: Steinernematidae) in the black vine weevil Otiorhynchus sulcatus (Coleoptera: Curculionidae). Nematology 1: 149-156.


Effect of entomopathogenic nematodes and insecticides on *Megaselia halterata* mortality

**Agnieszka Sznyk-Basalyga, Andrzej Bednarek**  
*Agricultural University, Department of Animal Biology, Nowoursynowska str. 166, 02-787 Warsaw, Poland*

**Abstract:** *Steinernema feltiae* is known to successfully control sciarid populations in mushroom cultivation. In contrast, this nematode appeared unable to insufficiently control populations of phorid flies. In our investigation we focused on the possible influence of low doses of insecticides applied into substrate, as a factor stimulating infection by nematodes, against *Megaselia halterata* (Phoridae). The following commercial biopreparations based on *Steinernema feltiae* were used: OVINEMA (Poland), NEMAPLUS (Germany) and ENTONEM (The Netherlands). The insecticide used was trigard (active ingredients: cyromazine), at a concentration ten times lower than the recommended dose. The results showed that combination of invective juveniles and low doses of insecticide cause higher mortality of *M. halterata* larvae compared to individual factor treatment, suggesting that integrated pest control is a prospective tool for control of phorid pest in mushroom cultivation.

**Key words:** *Megaselia halterata*, Phoridae, Diptera, entomopathogenic nematodes, *Steinernema feltiae*, cyromazine

**Introduction**

The phorid fly *Megaselia halterata* is one of the major pests in cultivated mushrooms in Poland (Dmoch, 1984). These Diptera are common in environments such as decaying plant material, animal excrement and rotten wood infested with fungi. In the cultivated mushroom fields flies attack freshly pasteurized mushroom compost. Larvae feed on mushroom mycelium. The mushroom production is affected when larval densities exceed 100 larvae per 30 g of compost (Hussey, 1961). Phorid flies are also vectors of pathogenic microbials, nematodes and pest mites (Binns *et al*., 1979). This is often underestimated by Polish growers because they do not directly associate fly with diseases. Furthermore adult flies both annoy the pickers and may cause allergies. Currently, hygiene measures and chemical methods could be use only for the control of adults. There are not sufficient methods to control phorid population in mushroom cultivations. Scheepmaker *et al*. (1998) tested entomopathogenic nematodes for the control of phorid larvae but with insufficient results. Other investigations showed that the combine application of different factors could be more sufficient than a selected one. For instance, Bednarek *et al*. (1998) found that simultaneous treatments with entomogenous fungi and nematodes together with a chemical insecticide treatment at low concentration were more efficient than individual factor for control of grubs. The aim of this study was to investigate the influence of the combine application of the entomopathogenic nematode *Steinernema feltiae* and the insecticide trigard at a concentration ten times lower than the recommended one on the mortality of *M. halterata* larvae. This insecticide is commonly used in Poland to control larvae from the family *Sciaridae* in mushroom cultivation.
Material and methods

Three commercial strains of *Steinernema feltiae* were used: OVINEMA (Oviplant Ltd., Poland), NEMAPLUS (E-nema GmbH, Germany) and ENTOM (Koppert B.V., The Netherlands). Nematodes IJs were applied directly after delivery from producers. Before each test viability of nematodes was checked.

The insecticide trigard 5 WP (active ingredient: cyromazine) was supplied by Novartis Crop Protection AG, Switzerland. The concentration of 3 g/l/m² is recommended for use in the professional pest control measures.

Phorid flies were collected by exhauster from a mushroom growing cellar near Warsaw and then used immediately in the test.

Plastic pots (diameter 11x16 cm) were filled each with 100 g of compost. This substrate was treated with 8800 IJs (app. 50 x 10⁶ IJs/100m²) of the appropriate nematode strain or trigard’s solution at 3g/l (i.e. the recommended dose) or 0.3g/l. In separate pots trigard (0.3g/l) and nematode IJs were treated simultaneously. As a control, pots with substrate were treated with sterile water only. Afterwards 30 adult flies were put into each pot. Pots, covered with a lid, were then kept in a chamber at a temperature of 24 °C. To catch new generation of *M. halterata* flies yellow sticky traps were placed to the cover of pots five days after the beginning of the test. The number of emerging fly adults was counted after the next 25 days. The test comprised six replicates for each factor.

The effectiveness of the control measure was evaluated based on the percentage of fly emergence. Results were analyzed using ANOVA.

Results and discussion

Application of different nematode strains of *S. feltiae* caused a reduction of fly emergence from 49 % (ENTONEM) to 28 % (OVINEMA) (Figs 1, 2 and 3). By applying trigard at the recommended concentration (3 g/l) a reduction of fly emergence approx. 87 % was obtained. This reduction was app. 56.5 % when trigard was used at 0.3 g/l. A combine treatment of nematode IJs and trigard (0.3 g/l) caused a reduction of fly emergence approx. 85.3 % in

![Control 100%](image)

**Fig. 1.** Effect of trigard (at 3 and 0.3 g/l respectively) and biopreparation OVINEMA, either alone or in combination, on the emergence of phorid flies in mushroom cultivation.
Fig. 2. Effect of trigard (at 3 and 0.3 g/l respectively) and biopreparation NEMAPLUS, either alone or in combination, on the emergence of phorid flies in mushroom cultivation.

Fig. 3. Effect of trigard (at 3 and 0.3 g/l respectively) and biopreparation ENTONEM, either alone or in combination, on the emergence of phorid flies in mushroom cultivation.

average. This reduction seemed to depend on the nematode strain: 88 % (ENTONEM), 86 % (OVINEMA) and 82 % (NEMATOP). The results showed that \textit{S. feltiae} strain ENTONEM was the most effective for the control of \textit{M. halterata} larvae, both in treatment alone (reduced fly emergence by 49 %) and in combine treatment (88 %). Moreover the results showed that combine treatment with nematode IJs and low concentration of trigard caused similar level of phorid emergence than trigard alone applied at
the recommended concentration. On the other side the treatment with nematode IJs alone, irrespective from *S. feltiae* strain, caused significant lower reduction of fly population in comparison to the combine treatment. It confirms the results gained by other investigators (Bednarek et al., 1998).

It seems that the efficiency of chemical or biological methods for control of phorid flies could depend on the place of the application. In our investigation the insecticide and nematode IJs were applied directly into the compost. This can increase the reduction of fly emergence, because phorid larvae occur in the compost contrary to sciarid larvae which occur in the casing. When nematodes and insecticide were applied on the casing they did not achieve as high phorid reduction as in our study (Scheepmaker et al., 1997).

**References**


Dmoch, J. 1984: Fauna of mushroom in Poland. SGGW-AR.


Impact of substrate conditions and application method on the efficacy of *Steinernema feltiae*

**Arne Peters, Jenny Backes**
*E-nema GmbH, Klausdorferstrasse 28-36, 24223 Raisdorf, Germany*

**Abstract:** The effect of the moisture on three potting soils used in greenhouses on the infectivity of *Steinernema feltiae* (nemaplus®) was investigated. Substrate 1 (Potgrond) was composed of 85 % black peat and 15 % white peat (weight by weight). Substrate 2 (seed) was composed of 50 % black peat, 30 % white peat and 20 % ground coconut-shell fibres. Substrate 3 (Steck) contained 75 % white peat and 25 % Perlite (grain size 1-4 mm). The infectivity of *Steinernema feltiae* in these substrates was tested using mealworm larvae (*Tenebrio molitor*). The two factors substrate type and water content (w/w) both had significant effects on nematode efficacy and factor interaction was statistically significant. Neither the water content nor the water potential was sufficient to predict nematode infectivity across the 3 types of substrate. Nematode infectivity was lower in substrate 3 and was significantly decreased in substrates 1 and 2 when 25 % Perlite was added. In another experiment, the nematodes were either incorporated into or spray applied on top of the substrate where the mealworms were included. In substrates 1 and 2, nematode infectivity against mealworms was statistically better when mixed into the substrate. In substrate 3, infectivity was lower and no difference was noted between the two application methods. In order to avoid settling of nematodes during application within aqueous suspensions carboxymethyl-cellulose (CMC) was added. A concentration of 0.4 % CMC reduced nematode settling speed from 0.58 to 0.02 cm/min.

**Key words:** *Steinernema feltiae*, *Tenebrio molitor*, potting soil, spray-adjuvants, application, Perlite, Dosatron

**Introduction**

The use of *Steinernema feltiae* to control sciarid flies in greenhouses is a well established biocontrol method and efficient nematode strains are widely available. Nevertheless, there are still a number of occasions where nematodes fail revealing notable gaps in our understanding of the system. This work addresses two important non-biotic factors: the composition and moisture of the substrate and the application techniques for entomopathogenic nematodes in the greenhouse.

**Material and methods**

*Assessing effect of substrate composition and moisture*

Three commonly used substrates were selected (Table 1) and adjusted to a water content of 40 to 80 % (w/w). Water contents were measured with an heated balance (Sartorius) where water was evaporated at 105 °C. 200 ml of the substrate was then filled into plastic containers and 20 mealworms were added to each container. Four hundred dauer juveniles of *S. feltiae* (nemaplus®) were added and the containers were incubated for 7 days at 20-22 °C before insect mortality was assessed. Three replicates were done per substrate-moisture combination and an additional control without adding nematodes. Mortality values were corrected according to Abbott’s formula.
Table 1. Composition of substrates investigated in the study. They were kindly provided by Klasmann-Deilmann, Germany.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Components and proportion in % by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black peat</td>
</tr>
<tr>
<td>Potgrond</td>
<td>85 %</td>
</tr>
<tr>
<td>Seed</td>
<td>50 %</td>
</tr>
<tr>
<td>Steck</td>
<td>–</td>
</tr>
</tbody>
</table>

The water potential (i.e. the suction force the soil exerts on a water column via a porous ceramic body in hPa) of the substrates at different moistures was measured at 20 °C using tensiometers. The containers were sealed to avoid water evaporation and the tensiometers were left for at least one week before the readings were taken since values drifted notably in the first few days. Numbers in Fig. 1 represent the readings when no further change in the water potential was recorded.

![Fig. 1. Water potential of three potting substrates at different water contents measured at equilibrium state.](image)

Comparing nematode incorporation and spray application
Two methods of nematode application were compared on the different substrates at 70 % moisture. Nematodes were either incorporated into the substrate by adding 2 ml to the substrate and gently mixing the substrate before filling it into the plastic container to a height of 8 cm. In comparison, the substrate was first filled into the container and nematodes were sprayed onto the substrate in 0.2 l/m² of water using a pneumatic spray can. The application rate was 50 nematodes/cm² in both treatments. Each container contained 20 mealworms and and mortality was assessed after 7 days incubation at 20 °C. One untreated control with 20 mealworms in the substrate was included.
Assessing effect of Perlite

Only substrate 3 contained Perlite. To investigate the effect of this commonly used substrate additive, nematode efficacy was measured in substrates 1 and 2 with and without adding 25 % Perlite according to the methodology described in experiment 1. Water content was adjusted to 70 %.

Testing sedimentation retardents

The possibility to slow down sedimentation of *S. feltiae* dauer juveniles in aqueous suspensions by increasing the viscosity was investigated. Fifty million nematodes in a conventional clay-powder formulation were dispersed in 5 litre tap water in a rectangular vessel (25 cm x 15 cm x 20 cm height). Carboxymethylcellulose (CMC MK40; Henkel, Germany) was added at 0.1, 0.2, 0.3, 0.4 and 0.5 % (w/w) and stirred gently. After 15 min the suspension was stirred vigorously and a sample of approx. 3 ml was taken at 3 cm depth. The suspension was left without stirring and further samples at 3 cm depth were taken at fixed time intervals. The syringes for drawing the samples were placed before starting the experiment to avoid agitating the suspension by the sampling process. Hundred µl of the sample was diluted with 4.9 ml water and the concentration of nematodes in this dilution counted in 1 ml using an inverted microscope. The settling speed (in cm/min) was estimated by dividing 3 cm by the time (min) when no more nematodes were found in the sample.

The effect of adding CMC on the distribution of spray-applied nematodes over time was tested using a Dosatron™ Di16 (Dosatron International, France). This device mixes up to 1.6 % of a concentrate into tap water. It is driven by the tap water pressure. A conventional 25 million package of *S. feltiae* was mixed into 750 ml of water, 0.4 % CMC was added and stirred. After a 10 min soaking period the suspension was stirred vigourously and spraying
started. A suspension without CMC was used as a control. The nematode concentrate was sucked from the bottom of the vessel into the Dosatron where it is continuously mixed with tap water and fed to the application device, a simple strikler. Samples were taken from the sprinkler at fixed time intervals and the nematode numbers were counted in 500 µl of the sample.

Results

Effect of substrate composition and moisture

The proportion of mealworms killed by *S. feltiae* at different substrate-moisture combinations is shown in Fig. 2. There is a marked decline in nematodes caused mortality at lower moistures levels. This decline, however, occurs at different thresholds in the various substrates which is reflected by the statistically significant interaction of the factors "substrate" and "water content" moisture (ANOVA, see Fig. 2). In the "Potgrond" the decline occurred sharply at 40 % water content, whereas a more continuous decline was observed in "Seed". In "Steck" there is a notable decline from 80 to 70 %, virtually no change down to 50 % and another sharp decline in nematode infection at 40 % water content. The water potential measured in the different substrates (Fig. 1) at different water contents did not explain the interaction between "substrate" and "water content" shown in Fig. 2. For instance, the water potential decreased continuously with declining water content in the "Potgrond" substrate. In contrast, nematode infection only decreased at the lowest level tested (40 % water content).
The water potential in "Steck" was only slightly decreasing with declining water content while there is a marked drop in nematode infection from 80 to 70 % water content.

**Nematode incorporation versus spray application**
In the substrates "Potgrond" and "Seed", the proportion of nematode infected mealworms was significantly higher if nematodes were incorporated into the substrate than if they were spray applied on top of the substrate (Fig. 3). No difference was observed in "Steck" substrate and nematode infection was lower in this substrate, which corroborates the findings shown in Fig. 2. Mortality in the untreated control was zero.

**Effect of Perlite**
Since nematode infectivity was consistently lower in the "Steck" substrate compared to the substrates that did not contain Perlite (see Table 1), the effect of adding Perlite to the other substrates was tested. The addition of Perlite decreased nematode infectivity significantly (Fig. 4).

![Graph](image)

Fig. 4. Proportion of killed mealworms (*Tenebrio molitor*) 7 days after application of 20 nematodes per insect. The substrates were tested with and without adding 25% Perlite (1-4mm grain size). The substrate ‘Steck’ contains 25% Perlite originally and consequently was not tested without. Twenty mealworms tested per container in 4 replicates.

**Effect of sedimentation retardents**
Sedimentation of *S. feltiae* dauer juveniles was decreased significantly by adding CMC to the nematode suspension (Fig. 5). The settling speed in the pure aqueous suspension was 0.58 cm/min. Adding 0.1 % CMC decreased the settling speed to 0.1 cm/min. A concentration of up to 1 % CMC is still sufficiently fluid to be applied with a Dosatron (data not shown). When nematodes were applied with a Dosatron without adding CMC, there was a sharp decline in
Fig. 5. Sedimentation of *Steinernema feltiae* dauer juveniles in aqueous suspensions with different concentrations of carboxymethylcellulose (CMC). The estimated settling speed in cm/min is given on the figures.

Fig. 6. Concentrations of *Steinernema feltiae* dauer juveniles at different times of spray application using a Dosatron. Carboxymethylcellulose (CMC) was added to the nematode concentrate (0.4 %) and compared to a pure aqueous suspension.
nematode numbers to the end of the application. Since the Dosatron sucks the liquid from the bottom of the container, the upper layer deprived from nematodes is taken in at the end of application. By adding 0.4 % CMC a sufficient number of nematodes remained in the upper layer to allow an even application of nematodes up to the end of application (Fig. 6). The drop in nematode concentration at 12 min was due to air being sucked into the Dosatron.

Discussion

The impact of potting substrates and water content on nematode infectivity is a neglected area in entomopathogenic nematode research. This study showed that both are significant factors which can explain the variability of nematode performance in practice.

Very few papers on entomopathogenic nematode research give information on the water potential (Koppenhöfer et al., 1995, 1997). It was expected that the water potential (in hPa) could explain the differences between the substrates with regard to the effect of the water content (in %) on nematode infectivity. This was not the case, but it shall be noted that the kinetics of changes in the water potential was not recorded. The time for nematode infection (7 days) and measuring water potential (at least 2 weeks) was different. Accordingly, the water potential might change with time with the change in substrate structure due to gravity forces. There was no decrease in nematode infectivity at high moisture levels. This was unexpected since the negative impact of oversaturated soil on nematode movement is well known (Ishibashi & Kondo, 1990). Probably the critical level was not reached in this study. In fact the 90 % levels were omitted from the study because the substrate started to float in water.

The impact of Perlite granules on nematode infectivity was serious. The basis for this negative impact has yet not be elucidated. Unfortunately, Perlite is widely used in cutting propagation where sciarids also are a major problem. The only recommendation for a good nematode efficacy in these substrates is keeping the substrate moisture constantly on a high level.

The incorporation of nematodes in the substrate has been successful with the slug nematode (Phasmarhabditis hermaphrodita) on arable crops (Wilson et al., 1999). It has not been tried in greenhouses, so far. Despite promising results in this study, there are two factors which may compensate for the advantage of having the nematodes in the substrate. Since the nematodes have to be incorporated before potting, there is a time gap of at least 1 week between nematode application and the occurrence of the first sciarid larvae. Nematode numbers can drop significantly in such a period of time (Ishibashi & Kondo, 1986). Second, sprayed nematodes would concentrate in the upper substrate layer, where most sciarid larvae are living. Experiments with sciarid larvae are currently carried out to find out, whether nematode incorporation is advantageous in practice.

The use of sedimentation retardants offer a solution for many types of spray application. Carboxymethylcellulose was used in this study but there is a wide range of thickeners which could be used as well (Burges, 1999). The method can open new forms of application. The application of nematodes with irrigation systems including drip irrigation hoses that supply the plants via a hole on top of the main hose can now be used without the restrictions in application time faced before (Conner et al., 1998).

Acknowledgements

We would like to express our thanks to the staff of the Botanical Garden of the University of Kiel for providing the Dosatron.
References


Desiccation survival of *Heterorhabditis*: physiological and biochemical changes following dehydration

Liu Qi-zhi, Aharon Solomon\(^1\), Itamar Glazer\(^2\)

*Department of Plant Protection, China Agricultural University, Beijing 100094, China*

\(^1\) *Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208 USA*

\(^2\) *Department of Nematology, ARO, The Volcani Center, Bet Dagan 50250, Israel*

**Abstract:** Heterorhabditid nematodes are known to be very poor anhydrobiotes and there is a considerable variation in desiccation tolerance among different heterorhabditid isolates. In the present study we attempted to elucidate the causes for the variation among heterorhabditid strains in regard to desiccation tolerance, using a comparative approach. Exposure to 97 % RH for 72 h caused reduction of 10-25 % in desiccation survival of all the nematode strains. After additional exposure to 85 % RH for 24 h there was no further viability reduction in steinernematids, whereas, drastically reduced to 30-50 % in heterorhabditid strains. Nematode initial water contents were similar. After 72 h at 97 % RH the water contents of all strains were reduced by 20 to 25 % w/w. Further exposure to 85 % RH for 24 h, the heterorhabditis lost additional 22-24 %, the steinernematids however did not lose any significant amount of water. The concentration of trehalose in the hydrated heterorhabditid strains was lower than that of the steinernematids. Following 72 h at 97 % RH trehalose levels were not increased significantly in the hydrated heterorhabditid strains, however it was increased in steinernematids. The levels of glycogen have been decreased significantly in all tested nematodes.

**Key words:** Anhydrobiosis, nematode desiccation toleration, water loss, glycogen, trehalose

**Introduction**

Heterorhabditid nematodes are known to be highly susceptible to desiccation (Womersley, 1990). Under various desiccation regimes IJ survived for minutes (O’Leary & Burnell, 1997) or hours (Surrey & Wharton, 1995). Recently we demonstrated (Liu & Glazer, 2000) that the IJs of *H. bacteriophora* are capable of surviving in RH ≥ 93 % up to 18 days. We further showed that under controlled desiccation regime there is a considerable variation in desiccation tolerance among different heterorhabditids isolated from the soil of temperate, sub-tropical, semi-arid or arid regions in Israel (Glazer et al., 1991).

In the present study we attempted to elucidate the causes for the variation among heterorhabditid strains in regard to desiccation tolerance, using a comparative approach. We determined the relations between desiccation tolerance and water content, changes in trehalose and glycogen among the nematode strains that displayed poor moderate or good desiccation tolerance ability. The heterorhabditid strains tested here were selected from 13 strains that were tested previously (Liu & Glazer, 2000).

**Materials and methods**

**Nematode culture**

The method of nematode culture was described by Liu & Glazer (2000). The *Heterorhabditis bacteriophora* HP88 was obtained from USA. *Heterorhabditis* strains, HIS-28, HIS-33 and
SIS15 were isolated from the soil of arid region in Israel and SCB-L1 from China. Both steinernematid strains belong to *S. feltiae*.

**Desiccation regime**
Anhydrobiosis was induced by exposure of the nematodes to 97% RH for 72 h as described in Liu & Glazer (2000). Different RH levels were generated by enclosing the nematodes in desiccator as described by Liu & Glazer (2000).

**Water content measurements**
Water content (WC) was determined by gravimetric method. Nematode clumps were obtained from water suspensions (Control) and from the exposure to 97, 93 or 85% RH for 0, 4, 8, 12, 24 and 72 h. The fresh weight of the clumps was determined on an analytical balance after exposure, then kept frozen in liquid nitrogen until use. The WC was calculated as percent of the dry weight getting from the sample lyophilised in a Cherist L-1 lyophilizer (Germany) at –56°C for 24 h. Each WC value is the average of three replicates.

**Glycogen and trehalose determination**
Glycogen and Trehalose were extracted from lyophilised frozen clumps of fresh hydrated nematodes (Control), dehydrated clumps of IJs after been exposed to 97% RH for 72 h. The determination method was referred to Solomon (1999).

**Statistical analysis**
An arcsine of square root transformation was used on percentage survival data. General Linear Model (GLM) Procedure of SAS (1990) was used for analysis of variance.

**Results and discussion**
Substantial differences in nematode survival were observed between steinernematids and heterorhabditid strains tested strains. After 72 h exposure to 97% RH viability was reduced by 10-25% in all the nematode strains with no significant differences (P> 0.001). After additional exposure to 85% RH no further significant reduction was recorded in SIS-15 and SCB-L1 strains. Whereas, survival of the heterorhabditid strains was drastically reduced (Fig 1).

![Fig. 1. Effect of different desiccation regimes on survival of entomopathogenic nematode strains (HP88= a strain of *Heterorhabditis bacteriophora*; HIS28 and HIS33= strains of *Heterorhabditis* spp.; SIS15 and SCB-L1= strains of *Steinernema feltiae*). Bars indicate SEM.](image)

Similarly to previous studies (Liu & Glazer, 2000; Womersley, 1990), the steinernematids showed better capability than heterorhabditids. However, despite overall poor
survival in heterorhabditids, distinct differences were observed in HIS-28 strain, showed the highest survival rate in 85 % RH and HIS-33 strain, exhibited the poorest survival rate. These two strains were both recently isolated from the coastal plain of Israel. The HP88 strain has been reared in the laboratory in the past 12 years. The differences in desiccation survival levels cannot be attributed to the lab conditions or to typical climatic conditions.

The initial WC levels for all strains tested was similar, ranging between 61 to 67 % w/w. After 72 h at 97 % RH the WC of all strains was reduced by 20 to 25 % w/w. After additional exposure to 85% RH the heterorhabditid strains lost additional 22-24 % of their WC, whereas the steinernematids did not lose any significant amount of water (Fig. 2).

Water loss rate was considered to be a major factor contributing to the survival level of different nematodes (Womersley, 1998). More specifically to heterorhabditids (O’Leary & Burnell, 1997). However, the present study showed that different water loss can explain the differences in survival inter-genus but not intra-genus. There was no difference in water content among the heterorhabditid strains but they have differential survival ability (Fig. 1).

![Graph showing water content of various nematodes strains](image)

Fig. 2. Water content of various nematodes strains (HP88= a strain of Heterorhabditis bacteriophora; HIS28 and HIS33= strains of Heterorhabditis spp.; SIS15 and SCB-L1= Strains of Steinernema feltiae) following different desiccation regimes. Bars indicate SEM.

The trehalose concentration was significantly ($P< 0.001$) lower in heterorhabditid strains tested here than that of the steinernematids (Fig. 3), ranging from 0.17 to 0.23 µg/mg protein. Following 72 h at 97 % h no significant increase was measured with most nematodes tested here with the exception of steinernematids. The Trehalose level in SIS-15 strain increased 2.5 fold (Fig. 3). Under the same desiccation condition the level of glycogen has been decreased significantly in all nematodes tested (Fig. 4). While 2-2.5 folds decrease in glycogen concentrations were recorded among the heterorhabditid strains, only 20 and 30 % reduction was measured in the steinernematids.

It has been shown in various studies with true anhydrobiotic nematodes that desiccation is accompanied by a decline in concentrations of lipids and glycogen (Crowe & Madin, 1975, Womersley et al., 1998). Accumulation of trehalose and glycerol has been recorded (Crowe & Madin, 1975; Womersley & Smith, 1981). In the present study a decrease in glycogen concentration was recorded among the different heterorhabditid strains following dehydration (Fig. 4). However, the concentrations of trehalose remained the same (Fig. 3).
Fig. 3 Trehalose levels in fresh and dehydrated (72 h exposure to 97 % RH) IJ of various nematode strains (HP88= a strain of *Heterorhabditis bacteriophora*; HIS28 and HIS33= strains of *Heterorhabditis* spp.; SIS15 and SCB-L1= Strains of *Steinernema feltiae*). Bars indicate error.

Fig. 4. Glycogen levels in fresh and dehydrated (72 h exposure to 97 % RH) infective juveniles of various entomopathogenic nematodes strains (HP88= a strain of *Heterorhabditis bacteriophora*; HIS28 and HIS33= strains of *Heterorhabditis* spp.; SIS15 and SCB-L1= Strains of *Steinernema feltiae*). Bars indicate SEM.

We propose two possible explanations to these findings: 1) another metabolite, other than trehalose, produces and plays an important role in desiccation tolerance of heterorhabditids, 2) heterorhabditids just don’t have the physiological mechanism enabling to survive to low RH conditions. We previously suggested that heterorhabditid might escape desiccation stress by moving to deeper and moister layers of soil (Liu & Glazer, 2000). This response requires high energy level, which can be obtained from glycogen.

**Acknowledgements**

Thanks are due to Mrs. L. Salame, to “US-Israel Binational Agriculture Research and Development Fund” (US-2858-97R), to the German Ministry of Science Research Grant 116/3-1 and to the International Foundation for Science (IFS) Grant No. D/2107-2F.
References


Application of entomopathogenic nematodes in controlling overwintering larvae of *Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Thaumetopoeidae)

Oreste Triggiani, Eustachio Tarasco
Dipartimento di Biologia e Chimica Agroforestale ed Ambientale, Sez. Entomologia e Zoologia, Facoltà di Agraria, Università degli Studi di Bari, Via Amendola 165/A 70126 Bari, Italy

Abstract: In January 2001, gel suspensions of IJs of *Steinernema feltiae*, *S. carpocapsae* and *Heterorhabditis bacteriophora* were injected in *Thaumetopoea pityocampa* nests in order to control overwintering larval populations. The best results were obtained with *S. feltiae* which was able to limit the winter populations of the lepidoptera under the threshold of 50 %. The nematode completed its life cycle and also reproduced itself in cadavers of the insect.

Key words: *Pinus halepensis*, gel suspensions, *Steinernema feltiae*, *S. carpocapsae*, *Heterorhabditis bacteriophora*, nests, *Phryxe caudata*

Introduction

Due to its larvae which feed on the needles, *Thaumetopoea pityocampa* (Pine processionary) is a destructive enemy of pine trees, present throughout the mediterranean area. Moreover, the last instar have tufts of long nettling hairs that can cause an allergy to humans and warm-blooded animals.

In July the female lays around two or three needles a large group of eggs covered with silver hairs from the abdomen.

The larvae hatch in September and start to build a silk nest, which is made thicker and more compact before winter. They spend the cold season inside. At the end of February-beginning of March, the larvae leave the pine trees in procession to pupate in clearings under the soil.

In January 2000 attempts were performed in a *Pinus halepensis* reforestation, to control overwintering larval populations by injecting in nests gel suspensions with two indigenous strains of *Steinernema feltiae* (Nematoda, Steinernematidae).

The results were promising and encouraging. Further trials were therefore performed in January 2001 in the same reforestation and they are reported in the present contribution. Three species of entomopathogenic nematodes suspended in two different gels were injected with the aim of increasing larval mortality and confirming the poor influence of nematodes on the larval endoparasite *Phryxe caudata*.

Material and methods

Native *Steinernema feltiae* (ItS-C12), *S. carpocapsae* and *Heterorhabditis bacteriophora* isolated from Southern Italian soil were tested during January and February 2001.

The field temperature during the experiment was 7.5 °C, it reached 14.5 °C at noon. In the nests the temperature was up to 8-9 °C higher.
The IJs nematodes were suspended in an acrylic polymer (Idrosorb SR 2002 (Nigem®) and a silicon gel (Compex gel®) in order to slow down the water evaporation and to prolong nematodes life (Triggiani & Tarasco, 2000a & b).

Of to 210 nests (30 nests per strain and per gel and 30 as control) were selected before the experiment.

Three ml of each gel containing 300,000 IJs of a given nematode species were injected in a single nest with a syringe equipped with a special canula (Triggiani & Tarasco, 2000a & b) while 3 ml of the same two gels, without nematodes, were forced in the nests as control.

Ten nests were collected per nematode strain and per gel, and for the control as well. They were examined in laboratory at the intervals of 14, 21 and 28 days after the treatment.

All the dead caterpillars found in nests were dissected for nematode and endoparasite presence. The stage of nematodes was noted.

Results and discussion

Effect on caterpillars

No *T. pityocampa* larvae were found killed by nematodes in the control and only a very low number were parasitized by *P. caudata*.

The three nematode species were able to parasitize the larvae in the nests during January and February, even if there were differences in the level of parasitism.

The suspension of nematodes in Idrosorb showed a different activity towards the overwintering larvae: *H. bacteriophora* acted poorly during the period of experimentation never reaching 4 % of larva mortality. *S. carpocapsae* had a slow but constantly increasing mortality in the course of the weeks. After four weeks 9 % of the caterpillars were killed.

*H. bacteriophora* in Compex gave better results after 3 weeks, with 9 % mortality while the higher larval mortality by *S. carpocapsae* was evident after 4 weeks (3.4 %).

The best results were gained with the suspension of *S. feltiae* in Idrosorb, since larval mortality gradually increased from 28 % in the second week, up to 54 % in the fourth week.

The same nematode species in Compex gave good results also, which were not statistically different from those obtained with Idrosorb (45 % of caterpillars parasitized after 4 weeks).

After 3 weeks, almost all the larvae of *T. pityocampa* killed by *S. feltiae* both in Idrosorb and in Compex contained adults of the nematode while the other species had not reached the adult stage.

After 4 weeks, the number of *S. feltiae* adults decreased and the percentage of juveniles increased.

Effect of the nematodes on *Phryxe caudata*

The pupae of the endoparasite collected in the nest during the time of the experiment gave rise to adults and no pupa was proved to be parasitized by nematodes. A very low percentage of young larvae of *P. caudata* were found dead in the caterpillars but no nematodes were recovered from them.

Discussion

Our results point out 1) the capability of the nematodes suspended in gels to not percolate from the nest, 2) the absence of a negative effect of the gels on the IJs of the tested entomopathogenic nematodes.

The best results were obtained with *S. feltiae*, which appeared able to contain the winter populations of *T. pityocampa* under the threshold of 50 % either suspended in Compex or
Idrosorb. Furthermore, whatever the type of gel, *S. feltiae* proved its capability to complete its life cycle and reproduce in cadavers of the caterpillars.

With regard to *Phryxe caudata*, because no parasites were found infected with the nematodes, we assume that the few dead young larvae found in the caterpillars died by feeding on the internal tissue of *T. pityocampa* infected with the symbiotic bacteria released by the nematodes (G.O. Poinar Jr, personal communication).

References


Effect of *Beauveria bassiana* on the invasion and proliferation of the entomopathogenic nematode *Heterorhabditis indica* inside *Galleria mellonella* larvae

Omar M. Dar-Issa, Naim M. Iraki, Ralf-Udo Ehlers

UNESCO Biotechnology Educational and Training Center (UNESCO BETCEN) for Palestinian Territory and Arab Countries. Bethlehem University, P.O. Box 9, Bethlehem, Palestinian Authority;

Dept. for Biotechnology and Biological Control, Institute for Phytopathology, University Kiel, 24223 Raisdorf, Germany

Abstract: In this work, we studied the effect of insect-infection with the fungus *Beauveria bassiana*, on the invasion and proliferation of the insect-pathogenic nematode *Heterorhabditis indica*. Both of these pathogen strains were isolated from Palestine. Results showed that when *Galleria* larvae were infected with nematode Infective Juveniles (IJs) only, the mean percent penetration was 7.37. However, when the larvae were infected with *B. bassiana* for 24, 48, 72, or 96 h before nematode application, the mean percent penetration was significantly reduced to 1.72, 1.40, 1.70, and 0.00 respectively. On the other hand, simultaneous application of both pathogens did not cause any significant decrease in nematode penetration. The total nematode production inside *B. bassiana*-infected *Galleria* was dramatically reduced compared to the control. When the larvae were infected with nematodes only (control), the number of nematodes collected, after two weeks from insect death, was 106x10^3 nematodes/larva (14380 nematode/IJ). However, when the Galleria larvae were simultaneously infected with both agents, the total number of nematodes produced was restricted to 35 x 10^3 nematodes/larva (5866 nematode/IJ). A more dramatic effect was observed when larvae were infected with *B. bassiana* for 24, 48, 72, or 96 hours before nematode application; 2.16 x 10^3 nematodes/larva (1256 nematode/IJ) in the case of 24 h preinfection period, while the other preinfection regimes caused a total inhibition of nematode production. Increasing the number of IJs inside preinfected larvae by injection reduced the inhibitory effect of the fungus on the development of IJs to hermaphrodites. The results indicate the occurrence of antagonistic interactions between the two pathogens inside the infected insect. Hence, combining these two biocontrol agents in a short-term biocontrol program may reduce the sustainability of the nematode.

Key words: *Heterorhabditis indica*, *Beauveria bassiana*, *Galleria mellonella*, penetration, infective juvenile development

Introduction

Biocontrol agents, such as nematodes, and fungi have been successfully applied to reduce the use of chemical pesticides. Their use involves, in some cases, a dual application of two agents. The applied pathogens may interact during their application resulting in an increased or a decreased efficiency of the biocontrol process. Similar consequence may result from interactions between the applied agent and the naturally occurring organisms (Timper & Kaya, 1989). The nature of such interactions and their effect on the sustainability of each biocontrol agent has not been studied adequately (Barbercheck & Kaya, 1991). Most of the research conducted on the use of combined biocontrol agents focused mainly on their effect on the mortality of the target insect, yet little studies dealt with the reproduction capacity of the applied biocontrol agent. The aim of this work was to study the effect of the fungus...
Beauveria bassiana on the penetration, and proliferation of the entomopathogenic nematode Heterorhabditis indica inside Galleria mellonella larvae.

Materials and methods

IJs of Heterorhabditis indica
Two-weeks old IJs were obtained from a monoxenic culture maintained as described by Lunau et al. (1993).

Beauveria bassiana spores
Spores were harvested from B. bassiana culture as described by Zhang & Watson (1997), and stored at 4 °C till use.

Infecting Galleria larvae with B. bassiana and nematodes
Appropriate volume of the pathogen suspension was mixed with sand to form a 10 % moistened infecting medium. Last instar Galleria larvae were exposed to 1.16 x 10^7 B. bassiana spores or 100 IJs in wells of a multi-well plate (one larva per well). After each exposure larvae were washed 3 times with sterile distilled water.

Determination of IJs penetration by pepsin digestion
Infected larvae were dissected and incubated in pepsin solution for two hours at 37 °C at continuous rotary shaking (120 rpm). The pepsin solution contained 8.0 g/l pepsin (Sigma), 23 g/l NaCl, and 940 ml distilled water. The pH was adjusted to 2.0 with HCl. The number of IJs penetrated into the insect was then counted under the microscope.

Determination of IJs recovery to hermaphrodites, and total production of nematodes
At the end of the infection period, larvae were taken from sand, washed and transferred to a Petri dish lined with wet filter paper and left for 72 h at 25 °C. Hermaphrodites were monitored in the dissected larvae under the microscope. For determining the total production of nematodes, each infected larva was washed and placed on a small white trap in the dark at 25 °C for two weeks. At the end of this period, the IJs and the adults in the cadaver were determined under the microscope.

Injection of IJs into Galleria larvae
IJs suspended in twenty µl of sterile Ringer solution were injected into the haemolymph of last instar Galleria larvae. The number of injected IJs ranged from 27-46 per larva.

Statistical analysis
The results presented in Table 1 are means of two independent experiments. The collected data were statistically analyzed by paired samples t-test using the SPSS 9.0 software. Means were tested for significant difference at P value of 0.05.

Results and discussion

Effect of B. bassiana on the penetration of H. indica IJs into G. mellonella larvae
A non-significant effect of B. bassiana on penetration was observed when the fungal pathogen was applied simultaneously with the IJs (6.0 compared to 7.37 % in control). However, preinfecting the Galleria larvae with B. bassiana for periods of 24 h and longer caused a dramatic decrease in the penetration of the nematodes (1.72 % compared to 7.37 % in the control). Furthermore, preinfection for a period of 96 h resulted in a total inhibition of IJs penetration (Table 1). Similar effect of B. bassiana was reported by Barbercheck & Kaya (1991). Also, a decreased penetration of IJs into a host that had already been infected with the same nematode was reported by several workers (e.g. Hominick, 1990). They proposed that the infected larvae secrete certain substance that is sensed by IJs and causes them to avoid
penetration into the infected insect. This kind of behavior may have an obvious biological
importance in that it prevents overpopulation of the host, which may lead to a detrimental
competition on food resources. When the two pathogens are applied simultaneously, the
fungus infection might be slower than that of the mobile IJs. The latter may penetrate into the
insect before the occurrence of a pathogenic response associated with secretion of the
hypothesized repelling substance. Hence, there will be no influence on the penetration of the
nematodes.

Effect of *B. bassiana* on the development of IJs to hermaphrodites

Although the simultaneous application of *B. bassiana* and nematodes had no significant effect
on the penetration of IJs into the insect, it did influence their development to hermaphrodites.
Under this preinfection regime only 52 % of the penetrated IJs could develop to hermaphrodites,
compared to 95 % in the control. Similar decrease in IJs development to hermaphrodites was observed when *Galleria* larvae were exposed to *B. bassiana* spores for 24 h before infection with nematodes. However, longer periods of infection with the fungus (48, 72 and 96 h) resulted in a total inhibition of development to hermaphrodites of the small number of IJs that succeeded to penetrate (Table 1). The inhibitory effect of *B. bassiana* on the development of IJs inside the insect could be a consequence of antibiotic activity exerted by the developing fungal mycelium on the nematode symbiotic bacteria. Secretion of toxins and antibiotic substances by *B. bassiana* is a well-documented phenomenon (Krasnoff *et al.*, 1991). When the period of preinfection with *B. bassiana* spores was extended to 48 hours or longer, the well established fungal mycelium may had secreted sufficient amounts of toxins and antibiotic substances that caused a total inhibition of IJs development to hermaphrodites.

Effect of *B. bassiana* on the development to hermaphrodites of the injected IJs

When the number of IJs inside larvae preinfected with *B. bassiana* for 48 and 72 h was
increased, by injection, to 24 and 27 fold respectively, the percent recovery to hermaphrodites
was dramatically increased compared to that of naturally penetrated IJs (Table 1). While the
small number of naturally penetrated IJs failed to develop to hermaphrodites inside the
preinfected larvae, their injected counterparts showed 58 % and 47 % development in insects
preinfected for 48 and 72 h respectively. These results indicate that an increase in number of
IJs, which is accompanied by increased number of symbiotic bacteria released inside the
insect, suppresses the inhibitory effect of *B. bassiana* on IJ development. This suppression
might be attributed to secretion of antibiotics by the symbiotic bacteria (Akhurst, 1982),
which inhibited development of the fungal pathogen.

Effect of *B. bassiana* on the proliferation of the entomopathogenic nematode *H. indica*
inside *Galleria mellonella* larvae

The capacity of one infective juvenile to proliferate inside *B. bassiana*-preinfected *Galleria*
decreased with the prolongation of the preinfection period. One naturally penetrated IJ was
capable of producing 5866 nematodes when it was applied together with the fungal pathogen.
This capability was decreased further when the IJ was inside larva preinfected for 24 h with
the fungal pathogen and did not exceed 1256 nematodes compared to production of 14380
nematodes in the control (Table 1). These results indicate that as the fungal mycelium
becomes more established inside the insect, it exerts a more profound effect on the
proliferation of the nematode. The increased suppressive effect is probably due to secretion of
greater amounts of antibiotic and toxic substances secreted by the fungus (Krasnoff *et al.*, 1991).

In summary, the above observations indicate that the sustainability of the
entomopathogenic nematode *H. indica* as a biocontrol agent might be substantially reduced if
applied together with or after application of the entomopathogenic fungus *B. bassiana*. 
Table 1. Effect of *B. bassiana* on the penetration, development, and total production of *H. indica*, inside *Galleria mellonella* larvae. The insects were infected with nematodes in wet sand for 24 h. The same lowercase letters indicate statistically not significant differences.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Exposure period to <em>B. bassiana</em> (hours)</th>
<th>Mean % penetration of IJs</th>
<th>Number of injected IJs/larva</th>
<th>Mean % recovery of IJs to hermaphrodites</th>
<th>Total nematode production / one naturally penetrated IJ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Natural penetration</td>
<td>Injection</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>7.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.1</td>
<td>94.90&lt;sup&gt;d&lt;/sup&gt;</td>
<td>71.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. bassiana</em> and IJs</td>
<td>24</td>
<td>6.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>52.00&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. bassiana</em> then IJs</td>
<td>24</td>
<td>1.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.7</td>
<td>69.70&lt;sup&gt;f&lt;/sup&gt;</td>
<td>78.80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. bassiana</em> then IJs</td>
<td>48</td>
<td>1.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.3</td>
<td>0.00&lt;sup&gt;g&lt;/sup&gt;</td>
<td>58.30&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. bassiana</em> then IJs</td>
<td>72</td>
<td>1.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.0</td>
<td>0.00&lt;sup&gt;g&lt;/sup&gt;</td>
<td>47.60&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. bassiana</em> then IJs</td>
<td>96</td>
<td>0.00&lt;sup&gt;g&lt;/sup&gt;</td>
<td>36.0</td>
<td>0.00&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Not determined

**Acknowledgements**

This work was supported by a grant (EH 116/3-2) from the DFG, Germany, and in part by a fellowship from UNESCO Biotechnology Action Council.

**References**


Effect of heat shock on the penetration and development of infective juveniles of two entomopathogenic nematodes strains *Heterorhabditis indica* and *H. bacteriophora* inside *Galleria mellonella* larvae

Basma Sandouka, Naim Iraki, Ralf-Udo Ehlers

UNESCO Biotechnology Educational and Training Center for Palestinian Territory and Arab Countries, Bethlehem University, P. O. Box 9, Bethlehem, Palestinian Authority

Dept. for Biotechnology and Biological Control, Institute for Phytopathology, University Kiel, 24223 Raisdorf, Germany

Abstract: In this work we studied the effect of three heat-shock regimes on the penetration of Infective Juveniles (IJ$s$) from two entomopathogenic nematode (EPN) species, *Heterorhabditis indica* and *H. bacteriophora* into *Galleria* larvae and on their capability to develop to hermaphrodites. Heat-shock treatment for one hour at 40 °C resulted in a dramatic decrease in the penetration and development to hermaphrodites of IJ$s$ from both nematode species. The *H. indica* IJ$s$, however, showed a significantly greater penetration rates compared to the *H. bacteriophora* IJ$s$ (6.11 % compared with 0.59 % of the control in *H. bacteriophora*). There was no significant difference between the two nematode species in the reduced recovery of IJ$s$ to hermaphrodites upon treatment at 40 °C. When the IJ$s$ were preconditioned at the sublethal temperature (35 °C) before exposure to 40 °C, their penetration capacities were increased by 3 folds in *H. indica* and by 37 folds in *H. bacteriophora* compared to the penetration values observed after treatment at 40 °C without preconditioning. The improvement in recovery to hermaphrodites of the 40 °C-treated *H. bacteriophora* IJ$s$, upon preconditioning at 35 °C, was lower than that observed for the *H. indica* IJ$s$. While the recovery to hermaphrodites of the latter IJ$s$ reached the same level of the control, the corresponding recovery of the former IJ$s$ did not exceed 72 % of the control. We suggest that the observed improvements in penetration of IJ$s$ and their development to hermaphrodites upon preconditioning at 35 °C are related to an induced synthesis of heat-shock proteins.

Key words: Entomopathogenic nematodes, *Heterorhabditis indica*, *Heterorhabditis bacteriophora*, heat shock, penetration, hermaphrodites

Introduction

Entomopathogenic nematodes (EPN$s$) in the two genera *Heterorhabditis* and *Steinernema* have a great potential in controlling populations of soil-inhabiting insects. However, their success in pest management is limited by several environmental factors that influence the survival, infectivity, reproduction and development of the nematodes (Grewal et al., 1994). Improving the performance of EPN$s$ as biocontrol agents through selection or breeding techniques requires studying their biological traits, such as, infectivity, heat tolerance and dessication tolerance. Studies showed that the capacities of Steinernematides to tolerate elevated temperatures may be modified by the temperature at which they are recycled (Jagdale & Gordon, 1997). Salvan *et al*. (1996) found that infectivity and thermotolerance of the infective juveniles of *H. bacteriophora* were enhanced as a result of preconditioning the IJ$s$ at a sublethal temperature (35 °C) before exposure to the lethal temperature (40 °C). Also they demonstrated correlation between this increased thermotolerance and the synthesis of the heat shock protein 70 kDa.
Heat tolerance of the EPNs is usually measured by determining the survival of IJs at elevated temperatures, and by studying their capability to kill the insect and proliferate inside it. However, the effect of heat on the IJs’ penetration capacities, and their development to hermaphrodites were not studied sufficiently. In this work we investigated the effect of different heat-shock regimes on the penetration and development of IJs to hermaphrodites of two Heterorhabditis EPNs: H. indica, strain Beth. 22, and H. bacteriophora, strain hybrid.

Material and methods

EPN strains
The nematode H. bacteriophora, strain hybrid was provided by Dr. R. Ehlers laboratory. The H. indica, strain Beth 22 was isolated from Bethlehem area in Palestine (Iraki et al. 2000).

Heat-shock treatment
Nematodes of both species were propagated in last-instar wax moth G. mellonella at 25 °C. About 5 ml suspension of one week old IJs were transferred into 100 ml flasks containing 15 ml of deionized water. The flasks were covered with cotton to allow gas exchange, and were held in a water bath with continuous orbital shaking at 200 r.p.m. IJs were exposed for one hour to three heat shock regimes; 35, 40, and 35 °C then a latency period for 3 h at 25 °C before exposure to 40 °C for 1 h. At the end of each heat treatment, flasks were kept at 25 °C for 44 h in the dark before application to Galleria larvae.

Determination of penetration and IJs recovery to hermaphrodites
Larvae of G. mellonella were trapped in multiwell plates containing 10 % ringer-moistened sand and exposed to the heat-shocked IJs at LD 50 concentration; 55 IJs for H. indica, and 160 IJs for H. bacteriophora (Iraki et al., 2000) and incubated for 26 h at 25 °C. Twenty larvae were used in each treatment.

The twenty larvae of each sample were divided randomly into two groups of ten each; the first group was dissected and incubated in pepsin (8g/L) for 1.5 h at 37 °C with continuous shaking at 120 r.p.m. The number of penetrated IJs in the insect was counted under the microscope. The second group of the larvae was left on wet filter paper for 72 h and then dissected to determine the number of hermaphrodites under the microscope.

Statistical analysis
The data were statistically analyzed by ANOVA using the SPSS 9.0 software.

Results and discussion
Exposing the IJs of the two entomopathogenic nematode species (H. indica and H. bacteriophora) to sublethal (35 °C) and lethal (40 °C) temperatures for one hour resulted in a significant decrease in their penetration into Galleria larvae at both temperatures compared to the control (25 °C). The decrease in penetration of IJs’ from both species was more profound at 40 °C than that at 35 °C (Table1). Furthermore, the penetration capacities of the H. indica IJs were significantly greater than those of the H. bacteriophora IJs at both the sublethal and lethal temperatures. When results were expressed as percent of control, the penetration capacities of H. indica IJs were 81.38 %, and 6.11 % at 35 and 40 °C respectively. The corresponding capacities of H. bacteriophora IJs were 63.09 and 0.59 % at 35 and 40 °C respectively (Table 1).

When the IJs of both nematode species were preconditioned at the sublethal temperature before exposure to 40 °C, the inhibitory effect of the lethal temperature on the penetration of IJs of both nematode species was decreased significantly. IJs of both species showed
penetration rates of about 20% of the control. However, when these rates were compared to the corresponding values observed at 40 °C, the penetration of *H. bacteriophora* IJs was improved by 37 folds compared to only three folds improvement in the penetration of the *H. indica* IJs.

Table 1: Penetration into *Galleria* larvae and recovery of IJs to hermaphrodites (expressed as percent of control) of two entomopathogenic nematodes, *H. indica* and *H. bacteriophora*, at three heat shock regimes. IJs were exposed for one hour to the heat shock temperature followed by two days latency period before application to *Galleria* larvae for 26 hours in sand. When two subsequent heat shock treatments were applied, a three-hours latency period was allowed after the first treatment. Figures in brackets represent the actual percent values. The results are means of two independent experiments. Means were tested for significant difference at *P* value of 0.05. The same lowercase letters indicate statistically not significant differences.

<table>
<thead>
<tr>
<th>Heat-shock regime</th>
<th>IJs penetration</th>
<th>IJs recovery to hermaphrodites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>H. indica</em></td>
<td><em>H. bacteriophora</em></td>
</tr>
<tr>
<td>Control (25°C)</td>
<td>100.00 (18.80b)</td>
<td>100.00 (16.80b)</td>
</tr>
<tr>
<td>35°C</td>
<td>81.38 (15.30c)</td>
<td>63.09 (10.60d)</td>
</tr>
<tr>
<td>40°C</td>
<td>6.11 (1.15e)</td>
<td>0.59 (0.10f)</td>
</tr>
<tr>
<td>35°C + 40°C</td>
<td>19.14 (3.60g)</td>
<td>22.02 (3.70h)</td>
</tr>
</tbody>
</table>

Synthesis of heat-shock proteins as a response to heat stress is a well-known phenomenon documented in many organisms, including entomopathogenic nematodes (Hendrick & Hartl, 1993; Hashmi *et al.*, 1997). These proteins are believed to protect the organisms’ functional proteins from heat-induced unfolding and formation of aggregates (Liang & McRae, 1997). Preconditioning of the organism at sublethal temperatures was proven to enhance survival of IJs at the lethal temperature of 40°C (Salvan *et al.*, 1996). Furthermore, the improved survival was accompanied by a *de novo* synthesis of 70KDa heat-shock proteins by the entomopathogenic nematode *H. bacteriophora* (Salvan *et al.*, 1996). In our work, the 37 folds improvement in the penetration of the *H. indica* IJs upon preconditioning at 35 °C (Table 1) might be attributed to an induced synthesis of heat-shock proteins that protected certain functional proteins involved in the penetration process. In the absence of preconditioning, the greater values of *H. indica* IJs penetration at 40 °C compared to those of *H. bacteriophora* indicate that the former IJs are probably protected by a constitutive synthesis of heat-shock proteins. The basis for this conclusion must be proven by documenting a *de-novo* and constitutive synthesis of heat-shock proteins by the two nematode species. Experiments involving quantitative determination of heat-shock protein synthesis in these nematode species are being conducted in our laboratory.

The development of the penetrated IJs to hermaphrodites was reduced dramatically in both nematode species when the IJs were exposed to 40 °C without preconditioning. Although the difference in recovery to hermaphrodites between the two species was not statistically significant, the greater value (*H. bacteriophora*) did not exceed 45% of the recovery in the control (Table 1). However, upon preconditioning the IJs at 35 °C before exposure to the lethal temperature, IJs of the two species showed significant difference in development to
hermaphrodites. While the percent of *H. indica* IJs developed to hermaphrodites was close to that of the control, the development of *H. bacteriophora* IJs was only 72% (Table 1).

These results indicate that the preconditioning treatment was less efficient in reducing the inhibitory effect of the lethal temperature on the development of *H. bacteriophora* IJs to hermaphrodites. Since the survival at 40 °C of the latter IJs was improved upon preconditioning at 35 °C (Salvan et al., 1996), and since their penetration capacity was enhanced under the same conditions (Table 1), we suggest that the biochemical processes involved in the development of *H. bacteriophora* IJs to hermaphrodites are not protected by the same heat-shock proteins protecting the biochemical mechanisms of IJs penetration into the insect.

**Acknowledgement**

This work was supported by a grant (EH 116/3-2) from the DFG, Germany, and in part by a fellowship from UNESCO Biotechnology Action Council.

**References**


Molecular identification of eight isolates of entomopathogenic nematodes from West Bank and Gaza Strip, Palestine

Michael A. Sansour, Naim Iraki, Falah Younis\(^1\), Sonja Hollmer\(^2\), Ralf-Udo Ehlers\(^2\)

UNESCO Biotechnology Educational and Training Center for Palestinian Territory and Arab Countries, Bethlehem University, P.O.Box 9, Bethlehem, Palestinian Authority

Agricultural Experimental Station, Ministry of Agriculture, Gaza, Palestinian Authority

Department for Biotechnology and Biological Control, Inst. for Phytopathology, University Kiel, 24223 Raisdorf, Germany

Abstract: Eight entomopathogenic nematode strains were isolated from Bethlehem area and Gaza Strip in Palestine. Morphological data (tooth and cuticle of dauer juvenile, male spicula and bursa copultrix) enabled us to assign the strains PAL-S-03, 08, 09 and 10 to the genus *Steinernema*, while the strains PAL-H-05 to 07 belong to the genus *Heterorhabditis*. Restriction Fragment Length Polymorphism (RFLP) of the PCR-amplified internal transcribed spacer (ITS) region within the rDNA repeat was used for species identification of the new isolates. The ITS region of each strain was PCR-amplified using the primers described by Vrain et al. (1992). The generated ITS products were digested with restriction enzymes and the fragments were separated by gel electrophoresis. RFLP profiles of the *Steinernema* isolates were compared with other patterns reported in the literature. The strain PAL-S-03 matched with the species *S. feltiae*. The bands obtained from PAL-S-08 were identical to those described for *S. abbasi*. The isolate PAL-S-10 showed unique RFLP profile which does not comply with any reported patterns, which indicate that this isolate might be a new species of the *Steinernema* genus. RFLP patterns of the *Heterorhabditis* isolates were compared with profiles of other reference species. The isolates PAL-H-04 and 05 matched with profiles of *H. bacteriophora*. The patterns of two other isolates, PAL-H-06 and 07 were compatible with *H. taysearae* profiles.

Key words: Entomopathogenic nematodes, *Heterorhabditis taysearae*, *Steinernema abbasi*, ITS-RFLP, Palestine

Introduction

Entomopathogenic nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae are applied successfully as biocontrol agents against soil-borne pests. Since EPNs show host specificity and different biological traits, there is a need for isolating more nematode strains to increase the stock from which we can select an appropriate isolate to control a specific target insect. In contrast to other areas in the world, the Middle East region, particularly Palestine, has not been screened intensively for EPNs. Both Israel and Palestine are a region of diverse climatic conditions, including arid, semiarid, and Mediterranean climates. This increases the chances for isolating EPNs of diverse climatic adaptations.

As more EPNs are added to the nematode stock, there will be a need for reliable methods for identification and classification. The Restriction Fragment Length Polymorphism (RFLP) of the PCR-amplified Internal Transcribed Spacer (ITS) region within the rDNA repeat is a fast method to identify EPN isolates to the species level (Hominick et al., 1997). In this study we applied the ITS-RFLP technique to identify eight EPN strains recently isolated from different locations in Bethlehem and Gaza Strip areas in Palestine. Among these eight strains, four were assigned to the genus *Steinernema* and four to the genus *Heterorhabditis* using morphological characters.
Material and methods

**DNA extraction**

Genomic DNA was extracted from all stages of the nematodes using the Dnasy™ System (QIAGEN). EPN reference strains, *H. megidis* (strain HN1), *H. marelata*, *H. indica* (strain LN2), and *H. bacteriophora* (strain Hybrid) were provided by the laboratory of Dr. Ehlers (Kiel University, Germany). The *H. taysearae* was provided by Dr. M. Shamseldean (Cairo University, Giza, Egypt).

**PCR amplification and restriction digestion**

The ITS region from each isolate was amplified in a sterile 0.5 ml tube using the primers described by Vrain *et al.* (1992). PCR amplifications were performed according to Hominick *et al.* (1997). The amplified ITS regions were digested with five restriction enzymes (*Alu* I, *Hha* I, *Hinf* I, *Rsa* I and *Dde* I) for the *Steinernema* isolates, and three enzymes (*Alu* I, *Hinf* I and *Mbo* I) for the *Heterorhabditis* isolates. The restriction digestion was performed according to the manufacturer's protocol (Promega).

Digestion products were separated by electrophoresis in 2 % agarose (w/v) gels in 1X TBE buffer at 120 V for 1.5 h. RFLP bands were visualized by staining with ethidium bromide.

**Results and discussion**

The RFLP patterns of the *Heterorhabditis* isolates are shown in Figure 1. They were compared with profiles of other reference species: *H. megidis* (strain HN1), *H. taysearae* (Shamseldean *et al.*, 1996), *H. marelata*, *H. indica* (strain LN2) and *H. bacteriophora* (strain Hybrid) (Fig. 2). The RFLP patterns of the isolates PAL-H-04 and 05 were in accordance with profiles of *H. bacteriophora*. The patterns of two other isolates, PAL-H-06 and 07, were compatible with *H. taysearae* profiles.

![Fig. 1. Gel electrophoresis banding patterns obtained from restriction digestion of the PCR-amplified ITS region of four *Heterorhabditis* isolates from Palestine. The PCR-amplified ITS regions were digested with the restriction enzymes indicated on the top. Lower case letters on top of the lanes correspond to the different nematode isolates as follows: "a": PAL-H-04; "b": PAL-H-05; "c": PAL-H-06; "d": PAL-H-07. Lanes "M" = 100 bp DNA marker.](image-url)
RFLP profiles of the *Steinernema* strains (Fig. 3) were compared with other patterns reported in the literature. The strain PAL-S-03 matched with the profile of the species *S. feltiae* reported by Hominick *et al.* (1997). The bands obtained from PAL-S-08 and 09 isolates were identical to those described for *S. abbasi* by Elawad *et al.* (1997).

The isolate PAL-S-10 showed unique RFLP patterns for each of the restriction enzymes. Digestion with *Alu* I gave three fragments of approximately 150, 220 and 300 bp. When the PCR-amplified ITS was digested with *Hha* I, two fragments of about 170 and 650 bp were generated. In contrast, the enzyme *Hinf* I failed to generate any fragment. Two fragments of about 200 and 700 bp were obtained when the ITS material was digested with *Rsa* I. The restriction enzyme *Dde* I produced three fragments of about 180, 300 and 480 bp. These fragmentation patterns did not comply with any reported profile. Hence, this isolate might be a new species of the genus *Steinernema*.

The various strains isolated so far from Palestine belong to a variety of species. In a previous work, we reported the isolation and characterization of two *H. indica* strains (Iraki *et al.*, 2000). They exhibited a relatively high heat tolerance compared to the *H. bacteriophora* hybrid strain. In this work we report the isolation and identification of a probably new *Steinernema* species in addition to strains belonging to another four known species including *S. feltiae*, *S. abbasi*, *H. bacteriophora* and *H. taysearae*. The latter species, which was isolated from Egypt, was identified based on morphological characteristics (Sahmseldean *et al.*, 1996). Our report describes the RFLP profile of this species for the first time.

The diverse climatic conditions and the geographical location of our region are probably the reason for the prevalence of such a diverse collection of EPNs that was isolated so far. This collection of EPNs could be a promising source for selecting appropriate strains for the control of soil-borne pests in this region, as well as for improving biological traits of other strains through cross breeding programs.

---

**Fig. 2.** Gel electrophoresis banding patterns obtained from restriction digestion of the PCR-amplified ITS region of five *Heterorhabditis* species. The PCR-amplified ITS regions were digested with the restriction enzymes indicated on the top. Lower case letters on top of the lanes correspond to the different nematode species as follows: "a": *H. megidis* (strain HN1); "b": *H. taysearae*; "c": *H. marelata*; "d": *H. indica* (strain LN2); "e": *H. bacteriophora* (strain Hybrid). Lanes "M" = 100 bp DNA marker.
Fig. 3. Gel electrophoresis banding patterns obtained from restriction digestion of the PCR-amplified ITS region of four *Steinernema* isolates from Palestine. The PCR-amplified ITS regions were digested with the restriction enzymes indicated on the top. Lower case letters on top of the lanes correspond to the different nematode isolates as follows: "a": PAL-S-03; "b": PAL-S-08; "c": PAL-S-09; "d": PAL-S-10. Lanes "M" = 100 bp DNA marker.

Acknowledgements

This work was supported by a grant (EH 116/3-2) from the DFG, Germany.

References


Identification of bacterial symbionts of entomopathogenic nematodes by the use of universally primed-PCR (UP-PCR)

Otto Nielsen, Peter Stephensen Lübeck
Zoology Section, 1Section for Genetics and Microbiology, Department of Ecology, The Royal Veterinary and Agricultural University, Thornvaldsensvej 40, 1871-Frederiksberg C, Denmark

Abstract: Entomopathogenic nematodes are soil living organisms that are pathogenic to insects. A major reason for the pathogenicity of the nematodes is the bacterial symbionts they carry. These bacteria belong to the genera Photorhabdus or Xenorhabdus. In this study UP-PCR was applied to bacterial strains for the purpose of identification or molecular characterization. UP-PCR is a method similar to RAPD but it is generally more robust in experimental conditions. Different primers were tested and the best results were obtained with the primer L45. The primer gave numerous and intense bands and was used to identify unknown strains. The results were consistent with results obtained by PCR-RFLP and further, the method enabled separation of closely related strains.

Key words: entomopathogenic nematodes, Photorhabdus, Xenorhabdus, identification, molecular characterisation, Universally Primed-PCR (UP-PCR)

Introduction

Entomopathogenic nematodes of the genera Heterorhabditis and Steinernema are soil living organisms that have the potential to infect and kill insects. The infective stage of the nematodes carry within its intestine a pathogenic bacterial symbiont which after infection is released into the hemocoel of the host. Nematodes of the genus Heterorhabditis carry bacteria of the genus Photorhabdus whereas nematodes of the genus Steinernema carry bacteria of the genus Xenorhabdus. Successful infections by this nematode-bacterium complex causes the death of the host and subsequently the host is utilised for nematode propagation.

Correct identification of both the nematodes and its symbiont is essential in many studies of the nematode-bacterium complex. PCR-based methods have often shown to be powerful for identification and in this study we applied UP-PCR for molecular characterization of Photorhabdus and Xenorhabdus strains. Universally Primed-PCR (UP-PCR) (Bulat & Mironenko, 1990) is a method similar to RAPD-PCR. The method can separate organisms at the strain level but is in contrast to RAPD relatively robust in experimental conditions. This is due to the design of the primers and the high annealing temperatures (52-56 °C) in the PCR-reaction. The primers are 15-20 bp long and designed to meet two criteria. Firstly, they target the more variable intergenic regions of the genome and secondly, they have a sequence that is universal for any organism (Bulat et al., 1998). UP-PCR can theoretically be applied to any pure culture of organism and have so far been applied for genotyping of fungi (Bulat et al., 1998, 2000; Lübeck et al., 1998, 1999, 2000) and bacteria (Peter Stephensen Lübeck, unpublished data).

The objective of the present study was to evaluate the UP-PCR method for molecular characterization and identification of bacterial symbionts (Photorhabdus and Xenorhabdus).
The results were compared with results obtained by the PCR-RFLP method described by Brunel et al. (1997).

Material and methods

Bacterial strains and DNA extraction
Strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), Ralf-Udo Ehlers, Institut für Phytopathologie, University of Kiel, Germany and Noël Boemare, Laboratoire de Pathologie comparée, Université Montpellier II, France. In addition own strains were obtained from the hemolymph of Galleria mellonella larvae infected with Danish nematode isolates using the method of Akhurst (1980). A kit from Promega Corporation (Wizard® Genomic DNA Purification kit) was used for DNA extraction. One ml bacterial suspension of each strain was used.

Identification by PCR-RFLP
Bacterial strains from the nematodes DK172 (Heterorhabditis sp.) and DK1 (Steinernema feltiae) were identified to species level by comparing restriction patterns of the 16S rDNA unit according to the method of Brunel et al. (1997). The modified primers described by Fisher-Le Saux et al. (1999) were used for amplification. The restriction enzymes HaeIII, AluI and HhaI were used.

UP-PCR
UP-PCR was performed in 20 µl reactions (1×Dynazyme buffer (Finnzymes F-511)), 2 mM MgCl, 0.4 mM dNTP (each), 1 unit primer, 0.6 unit Dynazyme polymerase (Finnzymes) and 10-100 ng total DNA). The primers L45 (5'-GTAAAACGACGGCCAGT-3'), L15/AS19 (5'-GAGGGTGGCGGCTAG-3'), AS15inv (5'-CATTGCTGGCGAATCGG-3') and AS4 (5'-TGTGGGCGCTCGACAC-3') were tested. The reactions were performed on a Perkin Elmer 9600 with a total of 30 cycles. The first cycle was 180 seconds at 94 °C, 70 seconds at 55 °C and 60 seconds at 70 °C. The remaining cycles were 50 seconds at 92 °C, 60 seconds at 55 °C and 50 seconds at 70 °C (extension was prolonged with 2 minutes in the last step). The PCR-products were run on 1.7 % agarose gels in TBE buffer at 100 V for approximately 2 hours and visualised by ethidium bromide staining.

Results and discussion

UP-PCR was applied to both unknown and known strains. The best results were obtained with the primer L45. This primer gave numerous and distinct bands for all strains tested. An example is given in Figure 1 for both Photorhabdus and Xenorhabdus strains.

Identification of unknown strains is exemplified in Figure 2. Strains isolated from the nematode DK172 (Heterorhabditis sp.) had a banding pattern identical to the reference strain HL81 (P. temperata subsp. temperata) and strains isolated from the nematode DK1 (S. feltiae) had several bands in common with the type strain T228 (X. bovienii). These results were consistent with results obtained using the PCR-RFLP method described by Brunel et al. (1997) (results not shown).

Symbionts from S. affine and S. intermedium were compared in Figure 3. The 2 DK15 strains had identical
banding patterns whereas the banding patterns for DK172 strains were not identical. UP-PCR thus enabled separation of strains both from different and the same nematode host.

UP-PCR was here applied to the symbionts of entomopathogenic nematodes for the first time. The method enabled identification of unknown strains by comparison with reference strains and further the method could be used to distinguish between closely related strains (clones). UP-PCR can be used without previous knowledge of DNA sequences and because multiple bands can be obtained with only one primer, UP-PCR could be useful for initial molecular characterization of *Photorhabdus* and *Xenorhabdus* strains.

Fig. 2 (left). Identification of unknown strains. The unknown strains DK172S-a/b had a banding pattern identical to HL81 (*P. temperata* subsp. *temperata*) and the strains DK1S-e/f were similar to T228 (*X. bovienii*). Primer L45 was used.

Fig. 3 (right). Examples of closely related strains: DK15S-1/2 were isolated from DK15 (*S. affine*) and DK72S 1/2 were isolated from DK72 (*S. intermedium*). Primer L45 was used.

**Acknowledgements**

We wish to thank Ralf-Udo Ehlers, University of Kiel, Germany and Noël Boemare, Université Montpellier II, France for providing bacterial strains. The work was financially supported by the Danish Ministry of Agriculture (Contract no. BIO KVL-96-1).

**References**


Liquid culture production of biocontrol nematodes

Ralf-Udo Ehlers, Arne Peters†
Department for Biotechnology and Biological Control, University Kiel,
Klausdorferstrasse 28-36, D-24223 Raisdorf, Germany
† E-nema GmbH, Klausdorferstrasse 28-36, D-24223 Raisdorf, Germany

Abstract: Nematodes of the genera *Heterorhabditis* and *Steinernema* can be produced on artificial media in liquid cultures. The stirred internal loop reactors gave the highest and most consistent nematode yields compared to disk-blade-stirred and air-lift reactors. An evaluation of the nematode yields of 81 processes in 10, 500 and 3000 l stirred internal loop reactors revealed that the dauer juvenile yield is dependent on the number of parental hermaphrodites. A model was fit to the data with 2 parameters: the maximum fecundity and the critical concentration of parental hermaphrodites. The production system was scaled up to a 3000 l reactor with only moderate decline in nematode yields.

Key words: *Heterorhabditis*, *Steinernema*, biotechnology, bioreactor, mass production

Introduction

Insect pathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are widely used to control soil inhabiting insect pests. They are symbiotically associated with insect pathogenic Enterobacteriaceae of the genera *Xenorhabdus* and *Photorhabdus* respectively. After an insect is infected and killed, bacterial and nematode enzymes convert the insect tissue into suitable food for nematode reproduction. Partly due to this symbiotic association, these nematodes can be produced on artificial media in solid or liquid culture (Ehlers et al., 1998). Some characteristics of the liquid production of *Heterorhabditis bacteriophora* are presented.

Culture conditions

For mass production, the liquid medium is pre-incubated with the symbiotic bacteria and monoxenic dauer juveniles are added 24 to 36 h later. The temperature is held at constant 30 °C. The oxygen supply (measured in % O₂-saturation) is PID-controlled to hold constant 40 % by altering the agitation speed. The pH is monitored but not controlled. The whole production process takes 9 to 11 days.

Reactor design

Amongst the different types of bioreactors tested, the conventionally stirred disk-blade reactors gave poor and inconsistent yields (Fig. 1). Air-lift reactors can be used but also gave lower yields than stirred internal loop reactors (Fig. 1).

Production efficiency

The concentration of hermaphrodites 70 h after nematode inoculation is a major factor determining the final yield (Fig. 2). The relation can be modelled by assuming a maximum
The fecundity of each hermaphrodite, \( f_{\text{max}} \), i.e. the concentration of dauer juveniles yielded \( (\text{DJ}_{\text{yield}}) \) related to the concentration of hermaphrodites at the third process day \( (h) \), which decreases with hermaphrodite density down to zero at a critical upper threshold of the hermaphrodite concentration \( (h_{\text{crit}}) \). This is described by the following equation:

\[
\text{DJ}_{\text{yield}} = f_{\text{max}} \cdot \left(1 - \frac{h}{h_{\text{crit}}} \right) \cdot h
\]

Data from processes in 10, 500 and 3000 l reactors were fed to this model (Fig. 2). The maximum fecundity is 118 dauer juveniles per hermaphrodite and the critical concentration of hermaphrodites where fecundity approaches zero is 8030 per ml. This value, however, was not verified in the bioreactors since the maximum hermaphrodite concentration tested was 5800. The decline in nematode yield with very high hermaphrodite concentrations was proven in shaken flasks (unpublished data).

**Scaling up**

The production in stirred internal loop reactors could successfully be scaled up from 10 to 500 and 3000 l reactors with only a slight decrease in nematode yield (Fig. 3).
Fig. 2. Relation between final yield of dauer larvae and concentration of hermaphrodites 3 days after nematode inoculation in liquid cultures of *Heterorhabditis bacteriophora* in different bioreactors (10, 500 and 3000 litres) and grown on different media.

\[ y = x \times \left(1 - \frac{x}{8030}\right) \times 118 \]

Proportion of variance explained by model = 0.39

Fig. 3. Mean yields of *Heterorhabditis bacteriophora* in stirred internal loop reactors of different size.
Conclusion

Mass rearing of insect pathogenic nematodes can be done on a commercial basis in liquid culture. Both *H. bacteriophora* and *S. feltiae* are currently produced in bioreactors of up to 3000 l. Stirred internal loop reactors gave the most consistent and highest nematode yields. Besides occasional contaminations due to the prolonged process time, the major difficulty in getting reproducible nematode yields is the variation in the proportion of the nematodes that develop to hermaphrodites or males and females. Due to this variation, the number of developing nematodes feeding on the bacteria can hardly be controlled.

Acknowledgements

The support of the European Union within the shared COST Action "PRONEMA" (FAIR 3116) is gratefully acknowledged.

References

Integrated control of *Lycoriella solani* (Diptera: Sciaridae) with entomopathogenic nematodes and insecticides

Agnieszka Sznyk-Basalyga, Andrzej Bednarek

*Agricultural University, Department of Animal Biology, Nowoursynowska str. 166, 02-787 Warsaw, Poland*

**Abstract:** Sciarid flies are important pest in mushroom production. The efficacy of combine applications of commercial strains of entomopathogenic nematodes and trigard (an insecticide with cyromazine as active ingredient) was tested for the control of *Lycoriella solani*. Three commercial biopreparations were chosen; they were based either on *Steinernema feltiae*: OVINEMA (Oviplant, Poland) and ENTONEM (Koppert, The Netherlands), or on *Heterorhabditis megidis*: NEMATOP (e-nema, Germany). Nematodes were used at the recommended concentration and the insecticide was applied at a dose ten times lower than the recommended one. Combine treatments caused higher mortality in *L. solani* larvae than treatments with nematodes alone. The efficiency of combine treatments appeared similar to the efficiency of treatments with the insecticide at the recommended concentration.

**Key words:** *Lycoriella solani*, mushroom fly, cyromazine, entomopathogenic nematodes, *Steinernema feltiae*, *Heterorhabditis megidis*, integrated pest management

**Introduction**

Economically important fungus gnats or sciarid flies belong to Sciaridae (Diptera). The larvae have strong chewing mouthparts that enable them to feed on compost, mycelium and into sporophores. The species *Lycoriella solani* is one of the major pests for cultivated mushrooms in Poland and in other European countries (Dmoch, 1984).

Various insecticides are usually used to control populations of these pests. Chemical control became increasingly difficult to achieve due to the development of insecticide resistance. Several biological control agents were applied against sciarids. *Bacillus thuringiensis* var. *israelensis* was demonstrated to be an effective biological factor against *L. solani* and *L. auripila* in mushroom cultivation and greenhouses (Greval & Richardson, 1993; Rinker *et al*., 1995). Also soil dwelling predatory mites such as *Hypoaspis aculeifer* and *H. miles* are applied against fungus gnats (Brodsgaard & Albajes, 1999). Richardson (1987), Tomalak (1994) and Harris *et al.* (1995) found that *Steinernema* and *Heterorhabditis* nematodes are effective in control of sciarids. Biopreparations based on *Steinernema feltiae* have been proved to be useful for biological control of pests such as *L. solani* which occur in mushroom and cover fields cultivations (Bednarek *et al*., 1995).

In this work we investigated the possibility of increasing the effectiveness of nematodes preparations for fungus gnat control in cultivated mushrooms, by combining the biological control agents with an insecticide at low dose. The insecticide trigard, which is commonly used by mushroom growers in Poland to control larvae of *L. solani*, was chosen.
Materials and methods

Three commercial nematode strains were tested. Biopreparations were based either on *S. feltiae*: ENTONEM, mass reared by Koppert Biological Systems B.V. (The Netherlands), and OVINEMA from the Polish company Oviplant, or *H. megidis* NEMATOP supplied by e-nema GmbH (Germany). Nematode IJs were applied together with trigard 5 WB (Novartis Crop Protection AG, Switzerland - active ingredient cyromazine). The usually recommended dose for nematode preparations is 50 x 10^6 IJs per 100 m^2 and in the case of trigard 5 WP the recommend concentration is 3 g/l/m^2.

Mushroom flies *L. solani* were collected by exhauster from a mushroom growing cellar near Warsaw and directly used for the experiment. Biopreparations of nematodes were use direct after delivery by producers.

Plastic pots (diameter 11x16 cm) were filled with 100 g of sterilized damp moss mixed with peat (20 g) and milled Soya beans (5 g). This substrate was given different treatments: 8800 IJs (i.e. the recommended dose) of the appropriate nematode strain, or trigard’s solution at a dose of 3 g/l or 0,3 g/l, or trigard (0,3 g/l) and nematodes (8800 IJs) in combination. As a control pots with substrate were treated with sterile water only. Afterwards 15 adults *L. solani* were put into each pot. Five days later yellow sticky traps were placed on top of the pot to catch new generation of *L. solani* flies. Isolated pots were kept in a dark chamber at a temperature of 24 °C within 35 days. Afterwards the number of emerging adults was counted. The test comprised six replicates for each factor.

Effectiveness of the control measure was evaluated based on the percentage of flies emergence. Result was analyzed by ANOVA.

Results and discussion

Emergence level of *L. solani* treated with trigard (3 g/l) was approx. 1±0,5 % and trigard (0,3 g/l) 22±3,5 % (Figs 1, 2 and 3). In the case of application of nematode IJs alone the emergence was approx. 13±10,5 % (*S. feltiae*) and 85±10 % (*H. megidis*). The most effective biopreparation was OVINEMA (11% flies emerged). When nematode IJs were applied simultaneously with trigard (0,3 g/l) the emergence level was approx. 2,5±2 % (*S. feltiae*) and 30±8,5 % (*H. megidis*). The emergence level was significantly lower in the case of trigard (3 g/l) in comparison to nematodes alone or trigard at a concentration 10 times lower than the recommended one. In tests involving simultaneous application of trigard (0,3 g/l) and *S. feltiae*, fly emergence was not significant different compared to the test with trigard (3,0 g/l) alone. Fly emergence was significant higher in tests with combine treatment of *H. megidis* and trigard (0,3 g/l) than in tests with *S. feltiae* combined with trigard (0,3 g/l) or trigard (3,0 g/l) alone.

It is possible to conclude that the combine treatment of nematode IJs and trigard at low concentration could be effective (reduced fly emergence by 99 %) as is trigard applied at the recommended concentration. Combine treatments are more effective than entomopathogenic nematodes treatment alone. Such a control strategy would lead to a reduction of the amount of chemicals used for the control of fungus gnats.
Fig. 1. Effect of treatment with *Steinernema feltiae* (strain OVINEMA) and/or trigard on the adult emergence of *Lycoriella solani*.

Fig. 2. Effect of treatment with *Steinernema feltiae* (strain ENTONEM) and/or trigard on the adult emergence of *Lycoriella solani*. 
Fig. 3. Effect of treatment with *Heterorhabditis megidis* (strain NEMATOP) and/or trigard on adult emergence of *Lycoriella solani*.

References


Dmoch, J. 1984: Fauna of mushroom growing cellars in Poland. SGGW – AR.


Effects of Steinernematidae and Heterorhabditidae on the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

Elzbieta Pezowicz  
Division of Zoology, Warsaw Agricultural University, Nowoursynowska 166, 02-787 Warsaw, Poland

**Abstract:** The susceptibility of larvae, pupae and imagines of the lesser mealworm to entomopathogenic nematodes was studied under laboratory conditions. Thirteen strains of nematodes belonging to 9 species were tested: *Heterorhabditis bacteriophora* (Brecon), (HP88), *H. indica*, *H. megidis* (Selenter), (Selenter 1), (N2), *H. zelandica*, *Steinernema carpocapsae* (DD-136), *S. affine* (D6), *S. arenarium*, *S. glaseri* (RS 92), (NC). They were compared to 4 biopreparations based on entomopathogenic nematodes: Ovinema and Nemaplus, based on *S. feltiae*, and Nematop and Larvanem, based on *H. megidis*. The susceptibility of the insect varied according its stage, and to species and strain of nematodes. Larvae were found to be most susceptible than adults in the case of several nematode species. The most effective nematodes towards the lesser mealworm appeared to be *S. carpocapsae*, *S. affine*, *H. riobrave*, *H. indica* and the two biopreparations Nemaplus and Ovinema.

**Key words:** *Alphitobius diaperinus*, lesser mealworm, broiler house, biological control

**Introduction**

The lesser mealworm, *Alphitobius diaperinus*, was found for the first time in Europe in 1960 in imported fodder. In Poland it appeared in large numbers at the end of the eighties. Now, it can often be found in chicken farms, where it has optimum conditions for growth. It is an omnivorous beetle feeding on plant and animal food, most willingly when the food is moldy. The insect can transfer dangerous diseases like Marek’s disease, coccidiosis or Gumboro disease. The larvae gnaw through the skin of ill or dead birds causing large economic losses (Axtell & Arends, 1990; Wójcik et al., 2000).

Nowadays the control of the lesser mealworm is based on the use chemical insecticides (Ignatowicz, 1997, Geden et al., 1987).

Nematodes of the genera *Steinernema* and *Heterorhabditis* parasitize a wide range of insect hosts (Poinar, 1979). Most research with entomogenous nematodes has been concerned with their infectivity for coleopteran pests of agricultural importance.

Geden et al. (1985) attempted to use entomogenous nematodes to reduce the numbers of lesser mealworm utilizing three species of nematodes. Geden et al. (1987) applied a dose of $10^6$ per 1 m² in turkey farms. Szczepanik (2000) studied under laboratory conditions the influence of temperature on the effectiveness of *S. feltiae* and *H. bacteriophora* towards *Alphitobius diaperinus*. The aim of this work was to investigate susceptibility of larvae, pupae and imagines of the lesser mealworm to entomopathogenic nematodes under laboratory conditions.
Material and methods

Thirteen strains belonging to nine species of nematodes were tested: *Heterorhabditis bacteriophora* (Brecon), (HP88), *H. riobrave*, *H. megidis* (Selenter), (Selenter 1), (N2), *H. zelandica*, *H. indica*, *Steinernema carpocapsae* (DD-136), *S. affine* (D6), *S. arenarium*, *S. glaseri* (RS92), (NC). They were compared to 4 biopreparations based on entomopathogenic nematodes: Ovinema and Nemaplus, based on *S. feltiae*, and Nematop and Larvanem, based on *H. megidis*. The nematodes were reared using last-instar larvae of *A. diaperinus*.

The experiment was carried out at a temperature of 25 °C in Petri dishes (10 cm diameter) lined with filter paper. A dose of 50 invasive larvae per larva was applied. In the case of adults the applied doses were 200 larvae per insect. The control consisted of insects placed in Petri dishes on filter paper wetted with distilled water. Insect mortality was checked every day for day 8 days. The significance of differences in mortality was checked with the LSD test at p=0.05 after data transformation: arc sin √p for larvae and √y+ 1/2 for imagines.

Results and discussion

Various levels of susceptibility of the lesser mealworm to infection with nematodes were found, according the nematode species and strain, and to the stage of the insect. The data shown in Table 1 are those gained for the 6 most effective nematode species and strains.

Most effective nematodes appeared to be *S. carpocapsae* (DD-136) which caused 49-96 % mortality for various developmental stages, *S. affine* (30-90 %), *H. riobrave* (55-60 %) and *H. indica* (56-80 %) respectively. Adults of *A. diaperinus* were found to be less susceptible than larvae to infection with *S. carpocapsae* and *S. affine*. The effectiveness of *S. feltiae* based commercial preparations was 40-62% for Ovinema, and 70-75% for Nemaplus. For the two latter preparations both larvae and imagines were similarly susceptible. Pupae responded in the same way to all nematode species and strains. The mortality varied around 40%. The significance of differences in the infection of larvae of *A. diaperinus* by nematodes was demonstrated in most cases.

Table 1. Mortality of *A. diaperinus* (different stages) exposed to different nematodes species and biopreparations.

<table>
<thead>
<tr>
<th>Stage of <em>A. diaperinus</em></th>
<th>Mortality %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. carpocapsae</em></td>
</tr>
<tr>
<td>L2</td>
<td>96 ghij</td>
</tr>
<tr>
<td>L6</td>
<td>96 ghij</td>
</tr>
<tr>
<td>adults</td>
<td>49 bcd</td>
</tr>
<tr>
<td>control</td>
<td>5 a</td>
</tr>
</tbody>
</table>

These results allowed us to select nematode species and strains (*S. carpocapsae* (DD-136) and *H. indica*) and biopreparations based on entomopathogenic nematodes (the Polish product Ovinema) for further studies in chicken farms.
Acknowledgements

This study was carried out with the support of the National Committee of Scientific Investigations (KBN), grant KBN 6PO4G02116, 1999-2002. I thank the Committee for the financial support.

References

The influence of cadmium ions on the behaviour of *Steinernema feltiae* Filipjev IJs in soil

Joanna Jarmul, Marta Kamionek

*Department of Zoology, Warsaw Agriculture University, Nowoursynowska 166, 03-787 Warszawa, Poland*

**Abstract:** The influence of cadmium ions on *Steinernema feltiae* was studied in the laboratory. Cadmium nitrate \([\text{Cd(NO}_3\text{)}_2*4\text{H}_2\text{O}]\) was added to sand in concentrations of 1, 5 and 10 ppm Cd. The concentrations refer to the 1\textsuperscript{st} to 6\textsuperscript{th} degree in the soil contamination classification adopted in Poland by the IUNG (Institute of Crops, Fertilization and Soil Science). The experiments lasted 21 days. Invasive larvae of *S. feltiae* were released into the contaminated sand. The test insect (*Galleria mellonella*) was introduced on four dates: immediately after sand contamination and release of the IJs of *S. feltiae*, and after 7, 14 and 21 days. Investigations demonstrated the effect of the contact time of the IJs of *S. feltiae* with cadmium in contaminated sand on the invasive properties of the nematodes.

**Key words:** nematodes, *S. feltiae*, *G. mellonella*, cadmium, soil

**Introduction**

Soil, an important element of the natural environment and the upmost layer of the Earth’s crust, originated as an effect of various soil forming and biological processes. Soil forming factors determine its chemical composition and type.

Functioning of large urban areas is associated with profound transformation of the upper soil layer, with the destruction of natural soils and formation of anthropogenic layers (Kolacz et al., 1997). Soil transformation typically consists in mixing of earth material, in accumulating heavy metals but also in accumulating organic substances and humus in soils restored with peat and compost (Czerwinski et al., 1990). Entomopathogenic nematodes live and actively search for their host in such a habitat (Hara et al., 1982, 1983). Sand is an interesting habitat for nematodes because its low compactness enables invasive larvae to move easily in their search for the host and increases the impact of heavy metal ions on living organisms (Donkin et al., 1994; Jarmul et al., 2000).

Cadmium, one of the trace elements, is often found in soils. It is particularly mobile and active in acidic soils. Cadmium belongs to the elements which do not play any function in metabolic processes of plants. Easily taken up from the surrounding, cadmium is toxic to plants and microorganisms. It is assimilable regardless of the soil properties. Its concentration in the above-ground plants may reach 0.2 ppm and in the case of lettuce, even ten times more. Due to a high toxicity of cadmium ions for living organisms, concentration of 3 ppm in soils has been adopted as a permissible standard (Monitor Polski, 1986).

Easy assimilation of cadmium by living organisms often results in diseases observed in animals and men. Cadmium is especially accumulated in the liver, kidney cortes and bones. It is stored in cells in the form of protein complexes. Kidneys can accumulate up to 50 % of cadmium taken up by an organism (Kabata-Pendias et al., 1997).

Data from Warsaw may illustrate the range of cadmium emissions in large cities. In the Polish capital, the cadmium content in the mouse liver was found to be six and eight times
higher (in spring and in autumn, respectively) than that in the control, which was 10.54 µg/g (Czerwinski et al., 1990).

Most studies carried out until recently on invertebrates (including entomopathogenic nematodes) referred to the influence of chemical compounds, abiotic and biotic conditions on the number, infective properties and survival in the soil habitat (Kamionek, 1990). As an important factor in reducing the number of insects in the natural habitat, entomopathogenic nematodes attracted researchers’ attention to the effects of environmental pollution on these invertebrates (Gospodarek et al., 1998). Many authors underlined a small effect of pesticides and mineral fertilizers on the invasive larvae of nematodes. Jaworska et al. (2000), however, pointed out the importance of heavy metals in soils with regard to the searching and infecting ability of nematode larvae. These authors demonstrated that presence of cadmium compounds in aquatic cultures of Steinernema carpocapsae Fipiljev larvae resulted in the increase of mortality of the IJs of the nematode and markedly affected their infective properties. Similar effects were observed for various trace elements in the habitat of nematode (Jarmul et al., 1999, 2000).

Despite these investigations, our knowledge on the way these larvae behave in soils, where they are exposed to the direct influence of cadmium ions, is still very limited. The present study was therefore undertaken to analyse the direct effect of cadmium ions on nematode larvae.

Material and methods

Cadmium nitrate [Cd(NO₃)₂·4H₂O] in concentrations of 1, 5 and 10 ppm Cd was added to sand under laboratory conditions. These concentrations refer to the 1st to 6th degree of soil contamination adopted by IUNG (Institute of Crops, Fertilization and Soil Science 1992, see Kabata-Pendias et al., 1999) as a Polish standard. The experiment lasted 21 days. Invasive larvae of S. feltiae were released to the contaminated sand. The test insect (Galleria mellonella) was introduced on four dates: immediately after sand contamination and release of the IJs of S. feltiae and after 7, 14 and 21 days.

To study the abilities of S. feltiae to search and infect the trap insects, we used the method of Bedding & Akhurst’s (1974).

The experiments were designed with the view of showing how the contact time of the S. feltiae IJs with cadmium in contaminated habitat affects 1) the invasive properties of the nematodes, and 2) the sexual structure of the nematode population, which developed in the host.

Results and discussion

Application of water soluble cadmium nitrate enabled to determine its toxicity towards the IJs of S. feltiae. No direct effect of cadmium ions on mortality of the test insects infected by S. feltiae IJs was observed. Mortality was almost 100% regardless of the time of introduction of G. mellonella and concentration of Cd ions. Cadmium nitrate affected, however, the invasive properties of the IJs of S. feltiae by differentiating the intensity of invasion. The intensity of infection did not depend on cadmium concentration itself but rather on the duration the nematodes were exposed to its influence. At the same time interval between soil contamination and nematode release, and the introduction of G. mellonella we obtained similar number of nematodes, which penetrated the insect but at a constant concentration of cadmium ions, the number of nematodes in the insect decreased with the prolongation of the contact time.
After 1, 7 and 14 days of nematodes persistence in contaminated environment, the insect body was penetrated by almost the same number of nematodes, both in the control and in contaminated sand. After 21 days of contact there were almost twice more nematodes in the control than under experimental conditions. Regardless of the cadmium concentration in sand, similar number of the IJs attacked insects after 21 days (Fig. 1).

![Bar chart](image_url)

**Fig. 1.** The influence of cadmium concentration and period of nematode contact with Cd ions on the intensity of infection of *Galleria mellonella* larvae by the IJs of *Steinernema feltiae*.

Duration of the influence of cadmium ions was found to affect the number of females and males, which penetrated the insect body. Concentration was less important. Remarkable differences between the control and other concentrations were found after 21 days. Prolongation of time affected more unfavorably males than females developing in the host (Fig. 2).

Similar phenomenon was observed when the female/male ratio was analysed. Immediately after the contact and for the 7 and 14 days periods the ratio was similar or slightly increased with time. For the 21 days period, however, the ratio increased to 1.5 times compared to the control (Fig. 3).

The results agree with earlier findings on the effect of lead ions on the IJs of *S. feltiae* (Jarmul et al., 1999, 2000). In both cases, toxic ions influenced the sexual structure of the nematodes but differed in the character of the impact. Lead ions acted directly and their concentration was of primary importance for the number of males and females of the nematode. The effect of cadmium ions relied more on the time of the contact than on concentrations. Cadmium appeared to be less toxic though more treachery than lead.
Fig. 2. The influence of cadmium concentration and period of contact of *Steinernam feltiae* with Cd ions on the sexual structure of nematode population that developed in the insect host (*Galleria mellonella*).

Fig. 3. The influence of cadmium concentration and the period of contact of *Steinernema feltiae* with Cd ions on the female/male ratio.

Statistical analysis of the data confirmed earlier observations. The numbers of females and males of *S. feltiae*, and the difference between them were significantly correlated with the time, during which they were exposed to the effect of cadmium nitrate. Much weaker
A correlation was found between the numbers of females, males and the difference between them and the concentration of cadmium nitrate. All the results were significant at the level of 0.01. Regression lines had a 45° slope. (Fig. 4). Interestingly, the statistical program did not reject any data, which confirms the precision of measurements.

Fig. 4. Regression of the *Steinernema feltiae* female/male ratio in the experiments.

The effect of cadmium ions on the intensity of infection of *G. mellonella* with *S. feltiae* IJs in an artificially contaminated sand manifested itself after a longer contact of nematodes with ions (21 days).

The ratio of females to males of *S. feltiae* that penetrated into and developed in the insect body is affected by both cadmium concentration and duration of the contact. This effect is highly regular.

The effect of cadmium ions in the soil habitat is long lasting, so their toxicity is higher than that of other trace elements.

**References**


Entomopathogenic bacteria
Spinosad, a non-synthetic, naturally derived insect control agent

Richard Dutton, Costas Mavrotas¹, M. Miles, Petros Vergoulas²

Dow AgroSciences LLC, Wantage, OX12 9JT, U.K.;
¹ Dow AgroSciences LL, Vouliamenis Ave. 85, City Plaza, Glyfada, 16674 Athens, Greece
² Dow AgroSciences LLC, Buropolis, 1240 Route des Dolines F - 06904 Sophia Antipolis Cedex, France

Abstract: Spinosad is a insect control agent produced by fermentation of the Actinomycete bacterium Saccharopolyspora spinosa Mertz & Yao. It shows exceptional activity on Lepidoptera, Thysanoptera, Diptera and Coleoptera pests and is selective to many beneficials/non target insects. It has a novel mode of action acting on the insect nicotinic, acetyl-choline receptors of the nervous system whilst at the same time showing good selectivity to vertebrates. This action is unique and no cross resistance at the site of action has been found with any other known insecticides. These attributes makes it ideal for use in IPM programmes but in order to conserve this product it is essential that it is used carefully within well planned resistance management strategies.

Key words: spinosad, selectivity, insect pests, resistance management, IPM

Introduction

Spinosad is a new insect control agent obtained by fermentation of the Actinomycete bacterium, Saccharopolyspora spinosa Mertz & Yao and contains two metabolites, spinosyn A and spinosyn D which, together, form the active substance, spinosad. The discovery originated in the Caribbean in 1982 where a chemist from Eli Lilly took a soil sample from a disused rum factory. During the next 3 years the organism was identified followed by resolution of the active metabolites. The product is completely natural and is produced by fermentation of the organism in a carbohydrate medium. Spinosad is extracted from the broth and formulated as suspension concentrates or water dispersible granules. It is the first of a new class of insect control products - Naturalyte™ (TM - Dow AgroSciences LLC).

Not only is spinosad a natural product but its mode of action is completely novel. The mode of entry is primarily by ingestion with some contact action; the main target is the nicotinic acetylcholine receptor in the central nervous system. Secondary effects are also seen on GABA-gated chloride channels.

This novel mode of action makes it ideal for resistance management programmes especially as there is no cross-resistance at the site of action with existing chemistries. In
order to conserve this novel activity Dow AgroSciences is taking a proactive stance in regard to resistance management by developing resistance management strategies for key pests. Specific recommendations will be found on product labels or leaflets and will follow IRAC principles. E.g. optimal use of the product: rotation with other classes of existing and experimental products, integration of use with local, cultural practices.

Ecotoxicology and toxicity

The toxicological properties and environmental fate of spinosad are fully described in the Spinosad Technical Bulletin (Anon., 2001) but may be summarized as being not acutely toxic to birds (EPA classification: practically non-toxic) and wildlife nor to fish and most aquatic invertebrates. Spinosad is not carcinogenic, teratogenic, mutagenic nor neurotoxic and has an excellent acute toxicity profile to mammals. It has a greater affinity for soil than water and is subject to photolysis which minimises any potentially adverse, environmental effects.

Product registration

Owing to its favourable environmental and toxicological profile spinosad was classified by the U.S. Environmental Protection Agency (EPA) as a reduced risk product and granted an accelerated registration review. Reduce risk status was granted because spinosad met the following criteria:

- Reduced environmental load
- Reduced risk to agricultural workers
- Reduced risk to beneficial insects
- Reduced levels of concern on environmental non-target organisms
- No groundwater issues
- Excellent efficacy (fewer treatments), novel action
- Increased options for integrated pest management (IPM), organic farming and resistance management

Globally the product is registered in over 100 crops in 40 countries for the control of caterpillar pests, leaf-miners and thrips. In the EU and Mediterranean countries the key target crops are vines, tomatoes, peppers, melons, lettuce, apples, strawberries, avocado, dates, stone fruits, citrus, cotton, pine trees and ornamentals. Application rates vary between 4.8 and 36 g as/hl depending on the target pest. One novel application is the use of spinosad in a proprietary bait formulation for control of olive and Mediterranean fruit flies, as presented in the case of the olive fruit fly, Bactrocera oleae Gmel., by C. Mavrotas, K. Varikou, D. Alexandrakis, S. Prophetu, D. Michelakis, D. Kovaïos and P. Vergoulas at the 8th European meeting of the IOBC/wprs Working Group “Insect Pathogens and Insect Parasitic Nematodes” which took place in Athens, 29 May-2 June 2001 (this bulletin).

Biological activity

Spinosad is exceptionally active on Lepidoptera, Diptera, Thysanoptera as well as Coleoptera that consume large amounts of foliage, e.g. the Colorado potato beetle. At rates labelled for specific crops and ornamentals spinosad is not phytotoxic and has always shown a high margin of selectivity in commercial use.

Spinosad has been tested on non target insects in both laboratory and field conditions (Tables 1 and 2). When used according to good horticultural practice, it was shown to be
compatible with predatory mites, predatory Heteroptera and Diptera. Some parasitic Hymenoptera are sensitive to spinosad, however toxic effects are short lived due to the short persistence of the product. Consequently species such as *Aphidius colemani*, *Encarsia formosa* and *Trichogramma brassicae* can be introduced to protected crops soon after application (1-2 weeks).

Semi-field studies were conducted on honey and bumble bees. Spinosad was applied when bees were not flying. Bees were allowed to forage on the treated plants when spray deposits had dried. The findings from these studies showed that spinosad was completely safe to foraging worker bees and no significant effects on queen or brood were observed in either species even though activity was seen in Tier 1 laboratory tests.

Table 1. Compared Spinosad and Cypermethrin laboratory Tier 1 tests on a selection of beneficial Arthropods.

<table>
<thead>
<tr>
<th>Beneficial organism</th>
<th>Stage</th>
<th>Exposure Route</th>
<th>Spinosad</th>
<th>Cypermethrin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Encarsia formosa</em></td>
<td>Adult</td>
<td>Contact</td>
<td>29</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Orius spp.</em></td>
<td>Adult</td>
<td>Contact</td>
<td>200</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Hippodamia sp.</em></td>
<td>Adult</td>
<td>Contact</td>
<td>&gt;200</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Chrysoperla carnea</em></td>
<td>Adult</td>
<td>Contact</td>
<td>&gt;200</td>
<td>na</td>
</tr>
<tr>
<td><em>Phytoseilus persimilis</em></td>
<td>Mobiles</td>
<td>Contact</td>
<td>&gt;200</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td><em>Hippodamia convergens</em></td>
<td>Larva</td>
<td>Treated prey</td>
<td>&gt;200</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td><em>Chrysoperla carnea</em></td>
<td>Larva</td>
<td>Treated Prey</td>
<td>&gt;200</td>
<td>na</td>
</tr>
<tr>
<td>Honey bees</td>
<td>Adults</td>
<td>Contact</td>
<td>11</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 2. Spinosad semi-field/field tests (Tier 2) on a selection of beneficial mites.

<table>
<thead>
<tr>
<th>Species</th>
<th>Life stage</th>
<th>Test Conditions</th>
<th>Rate</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phytoseilus persimilis</em></td>
<td>Adults</td>
<td>Semi-field</td>
<td>19.2 g as/hl</td>
<td>Harmless class 1</td>
</tr>
<tr>
<td><em>Amblyseius californicus</em></td>
<td>Adults</td>
<td>Semi-field</td>
<td>19.2 g as/hl</td>
<td>Harmless class 1</td>
</tr>
<tr>
<td><em>Typhlodromus pyri</em></td>
<td>Mobiles</td>
<td>Field studies in vines</td>
<td>96 g as/ha</td>
<td>Safe to weakly toxic</td>
</tr>
</tbody>
</table>

One major opportunity maximizing the three key attributes of spinosad (efficacy against thrips, leafminers and caterpillars, low toxicity to many commercially important beneficials, natural predators and pollinators and exceptional safety to plants) is to use it in IPM programmes in protected crops. One focus of our work has been to define the conditions under which spinosad and beneficials can be used together safely and to define re-introduction periods following treatment with spinosad (Table 3) (Miles & Dutton, 2000).

Recently Dow AgroSciences was given the US Presidential Green Chemistry which recognises the unique contribution of spinosad and also highlights Dow AgroSciences' commitment to producing safer and more effective products for insect control.
Table 3. Re-introduction periods for beneficial Arthropods following spinosad treatment.

<table>
<thead>
<tr>
<th>Beneficial type</th>
<th>Species</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predatory mites</td>
<td><em>Phytoseiulus persimilis</em></td>
<td>Mites may be introduced on day of application when spray deposits are dry.</td>
</tr>
<tr>
<td></td>
<td><em>Amblyseius californicus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Amblyseius cucumeris</em></td>
<td></td>
</tr>
<tr>
<td>Predatory insects</td>
<td><em>Coccinella septempunctata</em></td>
<td>Insect predators may be introduced on day of application when spray deposits are dry.</td>
</tr>
<tr>
<td></td>
<td><em>Hippodamia convergens</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Chrysoperla carnea</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Orius laevigatus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Orius insidiosus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aphidoletes aphidimyza</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Macrolophus caliginosus</em></td>
<td>No restriction spinosad may be applied directly to plants containing <em>M. caliginosus</em></td>
</tr>
<tr>
<td>Parasitic Hymenoptera</td>
<td><em>Aphidius colemani</em></td>
<td>Direct applications of spinosad are harmful to parasitic wasps.</td>
</tr>
<tr>
<td></td>
<td><em>Encarsia formosa</em></td>
<td>Wait at least one week after an application of spinosad before introducing new parasites.</td>
</tr>
<tr>
<td></td>
<td><em>Trichogramma brassicae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Diglyphus isaea</em></td>
<td></td>
</tr>
<tr>
<td>Pollinators</td>
<td><em>Bombus terrestris</em></td>
<td>Do not spray spinosad when bumble bees are flying. Keep bees in the hive.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Open entrance once the spray deposit has dried.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry spray deposits are harmless to foraging bumble bees.</td>
</tr>
</tbody>
</table>

References

Anon. 2001: Spinosad Technical Bulletin. Published by Dow AgroSciences LLC, 9330, Zionsville Road, Indianapolis, IN 4628-1054, USA.

Insect Pathogens and Insect Parasitic Nematodes
IOBC wprs Bulletin 26 (1), 2003
pp. 209-213

Screening Bacillus thuringiensis strains for activity against Dociostaurus maroccanus (Orthoptera: Acrididae)

Enrique Quesada-Moraga, Cándido Santiago-Alvarez
Cátedra de Entomología Agrícola y Forestal, Departamento de Ciencias y Recursos Agrícolas y Forestales, E.T.S.I.A.M. Universidad de Córdoba, Apartado 3048, 14080 Córdoba, Spain

Abstract: Forty Bacillus thuringiensis isolates, from the authors’ collection, representing 20 serotypes, were assayed using the one dose method against third, fourth and/or fifth instar nymphs of the Mediterranean or Moroccan locust, Dociostaurus maroccanus. Three successive experiments were done. The first one allowed to select 4 quite active strains (belonging to serovars aizawai, mexicanensis, soonchoen and kim respectively) which were further assayed with randomly selected strains among the remaining ones. Finally, one strain (N° 5, serovar aizawai) appeared to maintain a high activity towards nymphs in the three performed experiments. This strain could be regarded as an appropriate biological control agent against the Mediterranean locust.

Key words: Dociostaurus maroccanus, Mediterranean locust, Moroccan locust; Bacillus thuringiensis

Introduction

The main goals of locust plague control are the reduction of both expenditure and environmental damage. They are fulfilled through the preventive control measures applied against the Mediterranean locust, Dociostaurus maroccanus, in the Spanish permanent breeding areas (Anónimo, 1999).

Moreover, the environmental concern has led up to an interest in replacing chemical insecticides by biological control agents (Lomer & Prior, 1992). Entomopathogenic micro-organisms are the most valuable biological control agents for locust control (Prior & Greathead, 1989), since they are easily mass-produced and formulated for inundative approach.

The aim of this study was to screen strains of Bacillus thuringiensis for their activity against the Mediterranean locust.

Material and methods

Forty strains of B. thuringiensis, belonging to twenty serotypes (Table 1), were selected from our collection and produced in the standard B. thuringiensis medium (UG) containing Bactopeptone (7 g l⁻¹), glucose and salts (Lecadet & Dedonder, 1971) at 28-30 °C for 72 h. Spore crystal mixtures were washed twice in cold sterile water and centrifuged at 10000 x g for 10 min at 4 °C between washings and the resulting pellet was resuspended in sterile water.

The insects used in this assay came from a stock colony maintained under the following controlled conditions: L13:D11 photoperiod, 26 ± 4 °C and 40-60 % RH, according to the method developed by Quesada-Moraga & Santiago-Alvarez (2001a). The nymphs were placed in plastic cups (40 mm diameter, 20 mm deep), one per cup, and fed on pieces of wheat seedlings (1.5-2 cm) treated by immersion in a spore/crystal suspension of known
concentration, ca $2.3 \times 10^9$ spores/ml. The suspensions were made in sterile distilled water to which 0.1 % of a spreader (AGRAL®) had been added. As a control, pieces of wheat seedlings were immersed in water plus AGRAL.

Table 1. *Bacillus thuringiensis* isolates tested against *Dociostaurus maroccanus* nymphs.

<table>
<thead>
<tr>
<th>H Serotype</th>
<th>Serovar</th>
<th>Abbrev.</th>
<th>N. Isolates</th>
<th>N. Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a3b3c</td>
<td>kurstaki</td>
<td>KUR</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4a4b</td>
<td>sotto</td>
<td>SOT</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>aizawai</td>
<td>AIZ</td>
<td>3</td>
<td>3, 4, 5</td>
</tr>
<tr>
<td>8b8d</td>
<td>nigeriensis</td>
<td>NIG</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>10a10b</td>
<td>darmstadiensis</td>
<td>DAR</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>pakistani</td>
<td>PAK</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>israelensis</td>
<td>ISR</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>18a18b</td>
<td>kumamotoensis</td>
<td>KUM</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>19</td>
<td>tochigiensis</td>
<td>TOC</td>
<td>6</td>
<td>11, 12, 13, 14, 15, 16</td>
</tr>
<tr>
<td>24a24b</td>
<td>neoleonensis</td>
<td>NEO</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>27</td>
<td>mexicanensis</td>
<td>MEX</td>
<td>5</td>
<td>18, 19, 20, 21, 22</td>
</tr>
<tr>
<td>34</td>
<td>konkukian</td>
<td>KON</td>
<td>2</td>
<td>23, 24</td>
</tr>
<tr>
<td>37</td>
<td>andaluaciensis</td>
<td>AND</td>
<td>2</td>
<td>25, 26</td>
</tr>
<tr>
<td>41</td>
<td>soonchoen</td>
<td>SOO</td>
<td>3</td>
<td>27, 28, 29</td>
</tr>
<tr>
<td>52</td>
<td>kim</td>
<td>KIM</td>
<td>4</td>
<td>30, 31, 32, 33</td>
</tr>
<tr>
<td>53</td>
<td>asturiensis</td>
<td>AST</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>55</td>
<td>palmanyolensis</td>
<td>PAL</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>64</td>
<td>azorensis</td>
<td>AZO</td>
<td>3</td>
<td>36, 37, 38</td>
</tr>
<tr>
<td>66</td>
<td>graciosensis</td>
<td>GRA</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>67</td>
<td>vazensis</td>
<td>VAZ</td>
<td>1</td>
<td>40</td>
</tr>
</tbody>
</table>

Eighty nymphs were used in each of the treated and control groups. Nymphs that had not eaten the treated leaf piece after 32 h were discarded, whereas the others were transferred to rearing cages (Quesada-Moraga & Santiago-Alvarez, 2001a) to assess mortality over the 8 days following treatment. Bioassays were conducted under the same conditions as the stock colony.

**Results**

In a first experiment, the forty isolates were assayed against third and fourth instar nymphs. The mortality in the control reached 12.8 % and in the treated groups the corrected mortality range from 3.4 % (strain 25) to 93.6 % (strain 5) as shown in Figure 1. The most active strains (mortality > 60 %) were 5 (AIZ), 29 (SOO), 33 (KIM) and 22 (MEX) while nine other strains gave around 50 % mortality: 20 (MEX), 21 (MEX), 31 (KIM), 32 (KIM), 34 (AST), 35 (PAL), 37 (AZO), 38 (AZO) and 39 (GRA). Interestingly, differences in activity among isolates were noted from the same serotype.
Fig. 1. Response of 3\textsuperscript{rd} and 4\textsuperscript{th} instar nymphs of \textit{Docioastaurus maroccanus} to 40 strains of \textit{Bacillus thuringiensis}.

In a second experiment, eleven isolates were assayed against fourth and fifth instar nymphs: the 4 isolates which appeared the most active in the first experiment, i.e. 5 (AIZ), 22 (MEX), 29 (SOO) and 33 (KIM), and 7 isolates randomly selected: 18 (MEX), 19 (MEX), 26 (AND), 34 (AST), 35 (PAL), 37 (AZO) and 38 (AZO). The mortality in the control reached 15\% while, in the treated groups, the corrected mortality range from 0\% (strain 38) to 82.4\% (strains 5 and 34) as shown in Figure 2. In this assay only the strain 5 (AIZ) exhibited about the same activity as in the first experiment but on the other hand, the strains 18 (MEX) and 34 (AST) exhibited a higher activity.

Fig. 2. Response of 4\textsuperscript{th} and 5\textsuperscript{th} instar nymphs of \textit{Docioastaurus maroccanus} to selected \textit{Bacillus thuringiensis} strains.

Finally, the strains 5 (AIZ), 18 (MEX) and 34 (AST) were assayed in a third experiment against fourth instar nymphs. The mortality in the control reached 28.7\% and in the treated groups the corrected mortality range from 41.5\% (strain 18) to 64.3\% (strain 5) (Fig. 3). The strain 5 (AIZ) maintained an activity higher than 60\%, though lower than in the two previous experiments, and the strains 18 (MEX) and 34 (AST) gave the same results as in assay 1. Thus it appears that strain 5 (AST) shows insecticidal activity against \textit{D. maroccanus}.
Fig. 3. Response of 4th instar nymphs of *Dociostaurus maroccanus* to three selected *Bacillus thuringiensis* strains.

**Discussion**

In previous screening experiments carried out in our laboratory, appreciable mortality caused by five strains of *B. thuringiensis* (among 22 tested) against *D. maroccanus* was reported (Hernández-Crespo *et al.*, 1994). These experiments, however, involved a locust rearing method in which mortality in the control was not negligible. Since then, the rearing method developed by Quesada-Moraga & Santiago-Alvarez (2001a) has drastically reduced the mortality among the control nymphs and allows us now to attribute the observed mortality to the bacterium treatment.

In intensive screening trials specifically carried out to find *B. thuringiensis* strains pathogenic to locusts, neither Zelazny *et al.* (1997) nor Chaufaux *et al.* (1997) found any isolates active against *Locusta migratoria* (L.) or *Schistocerca gregaria* (Forsk.). Given the consistence of our results we can consider the isolate 5 (AIZ), a strain of *B. thuringiensis* serovar *aizawai* isolated from a vermiform larvae of *D. maroccanus* (Aldebis *et al.*, 1994), as the most promise candidate to be used against the Mediterranean locust.

In field trials against Mediterranean locust nymphs, in the former Soviet Union, Safarova (1984) obtained 30-40 % mortality with the commercial formulation of *B. thuringiensis*, “Bitoxibacillin” (Latchininsky & Launois-Luong, 1992), which contains the water soluble β-exotoxin. In our experiments, the observed mortality could be related to the δ-endotoxins and/or the spores since the unspecific water soluble β-exotoxins were eliminated by washing and centrifugation.

The crystalline δ-endotoxins of *B. thuringiensis* acts only after dissolution and proteolytic cleavage in the gut of the host insect into a toxic protein that reacts with specific receptor sites in the gut wall (Lambert & Perferon, 1992). Recently it has been reported histopathological disruptions on the midgut of *D. maroccanus* adults fed with food contaminated by isolate 5 (Quesada-Moraga & Santiago-Alvarez, 2001b). This can explain the presence of bacteria in the hemolymph of *D. maroccanus* nymphs fed with food contaminated by a strain of *B. thuringiensis* serovar *mexicanensis* (Quesada-Moraga *et al.*, 1997).

Since the dissolution of *B. thuringiensis* endotoxins crystals depends on both the bacterial strain and the locust species (Stephan, 1992), its appear that in our system, both the strains and the host meet the requirements for insecticidal activity.
Acknowledgements

This work was partly supported by the Spanish Comisión Interministerial de Ciencia y Tecnología (CICYT), Grant AGF99-0823 and by NewBioTechnic S. A. (Sevilla).

References


Isolation and characterization of \textit{Bacillus sphaericus} from soils in Greece

Sophia G. Aptosoglou, Stephanos I. Koliais
Laboratory of General Microbiology, Division of Genetics, Development and Molecular Biology, Department of Biology, Aristotelian University of Thessaloniki, Thessaloniki 54006, Greece

\textbf{Abstract}: Strains of the mosquitocidal bacterium \textit{Bacillus sphaericus} were isolated from soils in Greece and characterized through their whole-cell polypeptide profiles, total DNA restriction patterns and plasmid profiles, and using polymerase chain reaction (PCR). Isolates appeared heterogeneous. Such investigations should facilitate the discovery of novel strains of \textit{B. sphaericus} toxic against mosquitoes.

\textbf{Key words}: \textit{Bacillus sphaericus}, mosquito control, whole-cell polypeptide profiles, heterogeneity

\textbf{Introduction}

Mosquitoes are vectors of diseases such as malaria or dengue and they are also an important nuisance in Mediterranean countries such as Greece, especially during the summer. \textit{Bacillus sphaericus} is a soil bacterium, several strains of which produce proteins that are toxic to mosquitoes (Charles \textit{et al.}, 1996). \textit{B. sphaericus} is therefore used as a biological mosquito-cide. Isolation and characterization studies of local \textit{B. sphaericus} strains could contribute to establish the control of mosquitoes on a local basis. Whole-cell polypeptide profile analysis and DNA restriction endonuclease analysis can enable the comparison of patterns characteristic for each strain (Mew \textit{et al.}, 1985). Plasmid profiles of different isolates may also give an account of the heterogeneity of the species (Aquino de Muro \textit{et al.}, 1992). Polymerase chain reaction (PCR) can enable the fast detection of certain toxin genes in \textit{B. sphaericus} (Priest \textit{et al.}, 1997). A study of the degree of heterogeneity among isolates of \textit{B. sphaericus} will facilitate the search for novel toxic strains and genes of this bacterium for the control of mosquitoes.

\textbf{Materials and methods}

\textit{Isolation of Bacillus sphaericus}

\textit{B. sphaericus} was isolated according to the method of Ohba & Aizawa (1986). The identity of \textit{B. sphaericus} was checked through the sphaerical shape of the spore after transfer of colonies resembling this bacterium (Parry \textit{et al.}, 1988) on NYSM medium (Cokmus & Elcin, 1995) and according to the results obtained in the following three tests: (1) growth in nutrient broth at pH 6.8 (expected result positive), (2) fermentation of glucose (expected result negative), and (3) hydrolysis of gelatin (expected result positive).

\textit{Extraction of polypeptides and SDS-PAGE}

A precipitate from 8 ml of vegetative cell culture in nutrient broth was resuspended in 80 μl of TSM solution (0.03 M Tris- HCl pH 8, 0.003 M MgCl$_2$·6H$_2$O, 27.5 % sucrose, 10 mg lysozyme/ml and 2x 10$^{-7}$ M N- tosyl- L- phenylalanine chloromethyl ketone and N- α-p tosyl-
L-lysine chloromethyl ketone) and incubated at 37 °C for 1 h, followed by a cold shock in ice for 5 min. The cells were then disrupted in 200 µl of disruption buffer (Sambrook et al., 1989). The resulting samples were boiled for 5 min and electrophoresed in a 12 % SDS-polyacrylamide gel. The resulting polypeptide profiles were visually compared in groups.

**Isolation and analysis of total DNA**
Total DNA was isolated from a 5 ml Penassay broth overnight culture (Difco) as previously described (Sambrook et al., 1989). Disruption of the cells was facilitated by the addition of 10 mg lysozyme/ml followed by incubation at 37 °C for 30 min. DNA was purified by two extractions with phenol and three extractions with water-saturated diethylether. About 225 µg DNA/ml were digested with 20 units of the restriction endonuclease EcoRI (Sambrook et al., 1989). Rnase (final concentration 0.1 mg/ml) was included in the reaction to eliminate contaminating RNA.

**Isolation and analysis of plasmid DNA**
Plasmid DNA was isolated from a 500 ml Penassay broth overnight culture as previously described (Sambrook et al., 1989). Disruption of the cells was facilitated by the addition of 10 mg lysozyme/ml followed by incubation at 37 °C for 30 min. DNA was purified by two extractions with phenol and three extractions with water-saturated diethylether. Rnase (final concentration 0.1 mg/ml) was included in the reaction to eliminate contaminating RNA.

**Polymerase chain reaction (PCR) analysis**
PCR analysis was carried out in order to detect the binary toxin (btx) gene and the mosquitocidal toxin 1 (mtx-1) gene. Total DNA from 38 different strains, obtained as described above, was used as template. Total DNA from the *B. sphaericus* strain 2362, harbouring the btx gene, was included in the analysis as a positive control for the detection of the btx gene and detection of a 1000 bp DNA fragment indicates the presence of this gene. Total DNA from the *B. sphaericus* strain SSII-1, harbouring the mtx-1 gene, was included as a positive control for the detection of the mtx-1 gene and detection of a 1493 bp DNA fragment indicated the presence of this gene. The following primers were used: for the btx gene:

- Primer 1: 5´-CCTATACTAATCCACTTACGC-3´.
- Primer 2: 5´-CAGTTTTTGTCTCTTTAC-3´ as previously described (Priest et al, 1997), and for the mtx-1 gene:
- Primer 3: 5´-CCCTCTTAAGCTTCTCCAAATGG-3´.
- Primer 4: 5´-CCATCTTTGATTTATTCCCCC-3´ as previously described (Aquino de Muro & Priest, 1994).

**Results and discussion**

**Isolation of Bacillus sphaericus**
Of a total of 127 soil samples, that were collected from 30 different areas from all over the country, 37 were found to contain *B. sphaericus*. This corresponds to a percentage of 29.1 %. Of a total of 1147 *B. sphaericus*-like colonies, recovered from the different soil samples, 85 were identified as *B. sphaericus*. This corresponds to a percentage of 7.4 %. These results show that *B. sphaericus* occurs regularly although sparsely in Greek soils.

**Whole-cell polypeptide profile analysis**
Many isolates had bands of similar electrophoretic mobility, but many of the overall profiles were different among the isolates (Fig. 1). The SDS-PAGE analysis of 75 isolates revealed 38 different polypeptide profiles (electrophoretypes). This corresponds to a percentage of 50.7 % of different profiles, which shows that about half of the isolates have a unique polypeptide
content, with respect to the given 75 isolates. Among these 38 electrophoretypes, 22 electrophoretypes correspond to a single isolate, and the remaining 16 correspond to more than one isolates. The electrophoretypes varied considerably from those of the entomocidal bacterium *Bacillus thuringiensis* (Aptosoglou et al., 1997). The fact that many different electrophoretypes were obtained, indicates a large degree of heterogeneity in *B. sphaericus*. The 38 polypeptide profiles were considered to represent 38 different *B. sphaericus* strains. This is interesting with regard to a possible exploitation for the control of mosquitoes.

![Fig. 1](image)

**Fig. 1.** Whole cell polypeptide profiles of 11 different Greek isolates of *Bacillus sphaericus*. The isolates of lanes 9 and 10 have identical profiles. Lane 1 shows a profile from a local *Bacillus thuringiensis* strain.

**Total DNA restriction analysis**

Electrophoresis of the EcoRI-digested total DNA revealed a large variability in the restriction patterns, indicating large differences in the DNA content among the strains. Most patterns were unique, as expected by the fact that the corresponding isolates also gave different polypeptide profiles (data not shown). A few isolates with different polypeptide profiles, corresponding to different strains, gave similar restriction patterns, indicating genomic differences that were not revealed by the restriction enzyme used in the present analysis, and these differences were only revealed using whole-cell polypeptide analysis by SDS-PAGE. On the other hand, some strains with similar (but not identical) whole-cell polypeptide profiles gave clearly different restriction patterns, indicating that in this case the restriction analysis revealed more differences between two isolates at the DNA level. Indeed, the combination of two or more methods can often give a better indication of the diversity among bacterial isolates and strains.

**Plasmid DNA analysis**

The analysis revealed a complexity in the number and size of bands (data not shown), suggesting a heterogeneity in the plasmid DNA content in the studied *B. sphaericus* strains. The number of bands varied between 1 and 4. Many strains gave a single band of high electrophoretic mobility. Several other strains gave additional bands of lower mobility while others gave additional bands of higher mobility.
**Polymerase chain reaction (PCR) analysis**

In the present analysis, neither the 1000 bp DNA band corresponding to the btx gene nor the 1493 bp DNA band corresponding to the mtx-1 gene were detected in the Greek isolates. Additional analyses involving soils from rice culture farms, the most important breeding area of mosquitoes, and mosquito larval homogenate samples, are currently carried out in our laboratory. In toxic isolates, novel toxin genes could be expected to exist. PCR analysis involving a larger number of isolates from mosquito breeding areas could therefore lead to reveal the presence of novel interesting toxin genes.

**Acknowledgements**

We thank Dr. Allan Yousten from Virginia Polytechnic Institute and State University for the kind gift of *Bacillus sphaericus* strains 2362 and SSII-1 used as positive controls in the present work.

**References**


Toxicity of wild Bacillus thuringiensis strains from Sardinia on Lymantria dispar (Lepidoptera: Lymantriidae) and comparison with commercial preparations

Emanuela Bazzoni, Marcello Verdinelli
Istituto di Ricerca sul Controllo Biologico dell’Ambiente, CNR, via E. De Nicola, 07100 Sassari, Italy

Abstract: Sardinian wild strains of Bacillus thuringiensis (Bt) were tested in the laboratory for their toxicity towards Lymantria dispar larvae and compared with isolates obtained from Bt commercial preparations. The maximum efficacy of a wild isolate after 48 h was 61 % and only 8 of the 28 wild strains produced statistically different mortality. The isolates obtained from the commercial preparations yielded lower mortality in the same time interval (33.3 % maximum). After 72 h, the highest mortality due to commercial strains was obtained with Rapax® (43.8%) whereas four of the Sardinian wild strains (SS53, 141, 92 and 136) produced % mortality ranging from 72.9 to 94.5 %.

Key words: soil, bacterial isolates, Bacillus thuringiensis, gypsy moth, insecticidal activity, microbial control

Introduction

Bacillus thuringiensis Berliner (Bt) is one of the most widely used biologically based tools for the control of insects pests, particularly Lepidoptera, and some Diptera and Coleoptera as well. The biology, pathogenicity, and mode of action of Bt have been reported by many authors, e.g. Entwistle et al. (1993), Knowles (1994), Navon & Ascher (2000).

Preparations of Bt, usually as sprays containing a mixture of cells, spores and parasporal crystals, have been used as insecticides for more than 30 years. The efficacy of formulations of B. thuringiensis subs. kurstaki was tested against Lymantria dispar, the major cork oak defoliator, at different larval population densities in various forest areas of Sardinia (Luciano et al., 1994).

At present there is a strong interest in collecting, analysing and screening B. thuringiensis strains in order to select new isolates exhibiting a higher toxicity. In this study we tested several strains of Bt, isolated from soils of Sardinia, for their insecticidal activity against larvae of L. dispar under laboratory conditions and made a comparison with some commercial preparations.

Material and methods

Sample collection
Soil samples were collected in different areas of Sardinia. The samples were stored at -20 °C until processed.

Isolation of bacteria
Isolation of the spore-forming bacteria was carried out as described by Ohba & Aizawa (1986).
Preparation of the spore/crystal suspensions
The bacteria were grown according to the method of Karamanlidou et al. (1991), with modifications: the pellet was washed four times to remove water-soluble exotoxin. The washing water was then eliminated from the pellet by overturning the test tube and aspirating the residue.

The pellets were resuspended in water and analysed by spectrophotometry at 600 nm. The suspensions were adjusted with water until the optical density was 1, so that 8 x 10⁹ cells were in 1 ml of suspension.

Further isolates were obtained from the three following commercial B. thuringiensis subs. kurstaki preparations: Foray 48B® (serotype 3A3B, Abbott), DiPel 2X® (serotype 3 HD1, Abbott) and Rapax® (serotype EG 2348, Intrachem Italia), using the same procedure.

Laboratory rearing and insect bioassays
In spring and autumn 1999, egg masses of gypsy moth were collected in Tempio Pausania and Monti (SS) and stored at +4 °C in order to have a supply of larvae for bioassays over a long period of time. The eggs were separated by hand from each mass, cleaned of hairs and counted. They were then left to hatch in an air-conditioned environment at 25±2 °C and 50-60 % relative humidity.

The newly hatched larvae were fed with ICN-Biomedicals S.r.l. artificial diet (the Gypsy Moth Diet, Catalog No. 960294). The larvae were reared up to the 2nd-3rd instar and then used in bioassays.

Preliminary toxicity tests were carried out on groups of 4 larvae to which 0.4 g of diet with 30 µl of Bt suspension were administered. Subsequently the same procedure was applied on groups of 20 second instar larvae fed with 0.5 g of diet with 30 µl of suspension.

Separate controls were run for each bioassay using 35 µl of water instead of Bt suspension. The number of dead larvae was counted after 24, 48 and 72 h from the beginning of tests.

Statistical analysis
A one-way analysis of variance was performed on transformed mortality data (arcsine of the square root); treatment means were separated from the control using Dunnet’s test (Dunnet 1955). Results of all statistical tests were considered significant if P<0.05.

Results and discussion
After an initial screening, the bioassays were replicated for a further comparison between the most effective strains and the commercial products. The results are shown in Table 1, confirming the lower efficacy of the commercial treatments compared with some isolates from Sardinian soils. For instance, after 48 h, the % mortality for the three commercial strains ranged from 20.0 to 33.3 %, while three wild strains caused a mortality higher than 35 % (61 % for the SS136 strain). After 48 h, the mortality did not exceed 43.8 % for the commercial strains (with Rapax® precisely) but was higher than 70 % for four wild strains (with the maximum at 94.5 % for the SS136 strain).

To conclude, these laboratory tests showed a low level of insecticidal activity of the commercial isolates in comparison with the results of treatments in the field with the same strains in commercial formulations (Luciano et al., 1993). The strain SS136 appeared especially active.

The insecticidal activity of products based on Bt, particularly Foray, demonstrated in the control of forest defoliators, suggests that further studies would be useful in order to have a better understanding of some aspects dealing with the formulation of the product.
Furthermore, our results seemed to suppose that the success of the treatments could be at least partly determined by the joint activity of the emulsions, solvents, excipients and wetters used in the commercial preparations.

Table 1. Mortality of *Lymantria dispar* larvae after exposure for 48, 72 h to aqueous suspensions of spores and crystals from Sardinian wild isolates (SS) of *Bacillus thuringiensis* compared to isolates from commercial preparations.

<table>
<thead>
<tr>
<th>Wild (SS) or commercial isolate</th>
<th>Mortality * caused by spore/crystal mixtures after 48 h</th>
<th>after 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS2</td>
<td>3.8</td>
<td>12.0</td>
</tr>
<tr>
<td>SS4</td>
<td>8.0</td>
<td>36.0</td>
</tr>
<tr>
<td>SS6</td>
<td>9.3</td>
<td>40.0**</td>
</tr>
<tr>
<td>SS9</td>
<td>1.9</td>
<td>16.0</td>
</tr>
<tr>
<td>SS19</td>
<td>25.5**</td>
<td>33.3**</td>
</tr>
<tr>
<td>SS27</td>
<td>7.4</td>
<td>16.0</td>
</tr>
<tr>
<td>SS28</td>
<td>9.3</td>
<td>8.0</td>
</tr>
<tr>
<td>SS29</td>
<td>9.3</td>
<td>38.0</td>
</tr>
<tr>
<td>SS30</td>
<td>13.0</td>
<td>28.0</td>
</tr>
<tr>
<td>SS38</td>
<td>21.7</td>
<td>27.5</td>
</tr>
<tr>
<td>SS40</td>
<td>16.1</td>
<td>26.1</td>
</tr>
<tr>
<td>SS53</td>
<td>41.8**</td>
<td>72.9**</td>
</tr>
<tr>
<td>SS58</td>
<td>7.9</td>
<td>19.5</td>
</tr>
<tr>
<td>SS70</td>
<td>16.4</td>
<td>47.9**</td>
</tr>
<tr>
<td>SS71</td>
<td>3.5</td>
<td>50.0**</td>
</tr>
<tr>
<td>SS75</td>
<td>8.7</td>
<td>5.0</td>
</tr>
<tr>
<td>SS79</td>
<td>8.7</td>
<td>22.5</td>
</tr>
<tr>
<td>SS92</td>
<td>35.1**</td>
<td>91.3**</td>
</tr>
<tr>
<td>SS96</td>
<td>10.5</td>
<td>41.3</td>
</tr>
<tr>
<td>SS98</td>
<td>29.1**</td>
<td>41.7**</td>
</tr>
<tr>
<td>SS103</td>
<td>15.1</td>
<td>24.4</td>
</tr>
<tr>
<td>SS136</td>
<td>61.0**</td>
<td>94.5**</td>
</tr>
<tr>
<td>SS140</td>
<td>19.3**</td>
<td>60.9**</td>
</tr>
<tr>
<td>SS141</td>
<td>35.1**</td>
<td>84.9**</td>
</tr>
<tr>
<td>SS143</td>
<td>29.8**</td>
<td>56.5**</td>
</tr>
<tr>
<td>SS148</td>
<td>30.5</td>
<td>52.5**</td>
</tr>
<tr>
<td>SS151</td>
<td>17.9</td>
<td>26.1</td>
</tr>
<tr>
<td>SS152</td>
<td>12.5</td>
<td>21.7</td>
</tr>
<tr>
<td>Dipel</td>
<td>26.4</td>
<td>17.1</td>
</tr>
<tr>
<td>Foray</td>
<td>33.3</td>
<td>40.0**</td>
</tr>
<tr>
<td>Rapax</td>
<td>20.0</td>
<td>43.8**</td>
</tr>
</tbody>
</table>

* percentage of mortality corrected with Abbott formula
** significantly different from the control (P<0.05)

Finally, it would be also important to determine, from the δ-endotoxin genes in the most toxic strains tested, whether their higher toxicity is attributable to the presence of unidentified insecticidal proteins.
References


Characterization and insecticidal activity of *Bacillus thuringiensis* strains isolated from hazelnut fields in Turkey

Remziye Nalcacioglu, Sabriye Dulger, Mustafa Yaman, Ali O. Belduz, Zihni Demirbag
Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, 61080, Trabzon, Turkey

**Abstract**: 70 *Bacillus thuringiensis* isolates were obtained from soil samples collected from various hazelnut fields in Turkey using acetate selection method. All isolates were characterized by morphological, biochemical and molecular methods. For 40 isolates the characterized internally transcribed spacers (ITSs) correspond to known *B. thuringiensis* varieties. Repetitive extragenic palindromic PCR (rep-PCR) results indicated that there is a significant genomic variation among *B. thuringiensis* isolates. Bioassays showed that among the 70 isolates, 25 exhibited a high insecticidal effect against *Hyphantria cunea*, 10 against *Agelastica alni* and 25 against *Neodiprion sertifer*. Two isolates are toxic to the three insect species. Four isolates are pathogenic for both *A. alni* and *H. cunea*, but for *N. sertifer*.

**Key words**: soil, Turkey, *Bacillus thuringiensis*, ITS, rep-PCR, insecticidal activity

**Introduction**

The main purpose of most agricultural studies is to increase the yield of products per hectare. With regard to production and exportation, Turkey is the first hazelnut producing country. However, this country lags far behind many other producing ones in terms of amount harvested per unit area. A main reason for this is that hazelnut has many agricultural pests, which can not be effectively controlled. Great efforts have been made in the development of biological and biotechnical control against these pests. However, until now, these techniques have been sporadically applied and need further research. In such a context, we considered the bacterium *Bacillus thuringiensis*, an ecologically sound microbial control agent specific for target insects.

A number of different phenotypic and genotypic methods are presently employed for microbial identification and classification. Generally, DNA-based methods are emerging as the more reliable, simple and inexpensive tools to identify and classify bacteria. In fact, the assignment of genera/species has traditionally been based on DNA-DNA hybridization methods and modern phylogeny is increasingly based on 16S rRNA sequence analysis. ITS PCR and rep-PCR are DNA amplification based techniques, which have been found to be extremely reliable, reproducible and highly discriminatory (Louws *et al.*, 1996).

In this study we report the isolation of *B. thuringiensis* strains from eleven hazelnut fields during the summer of 1998 in the north-eastern coast of Turkey, and their characterization with biochemical reactions and molecular (ITS and rep-PCR) methods. These strains were also investigated as regards their toxicity towards three species of insects.
Material and methods

Sample collection
Soil samples were collected from hazelnut fields in 11 different areas of North-East coast of Turkey. For each sample about 10 g of surface soil were scraped to a depth of 2 to 5 cm with a sterile spatula. The samples were placed in sterile plastic bags and stored at 4 °C.

Isolation and identification
The selection method described by Hossain et al. (1997) was used. Bacterial colonies were picked up and identified as B. thuringiensis in comparison with standard B. thuringiensis strains (B. thuringiensis var. thuringiensis, B. thuringiensis var. israelensis, B. thuringiensis var. kurstaki). Morphological characters such as vegetative cells, cell arrangement and spores were examined using light microscopy. Biochemical characters were determined according to Logan & Berkeley (1984).

Preparation of DNA for PCR analysis
DNA was isolated from 16-18 h cultures in Luria-Bertani broth. Aliquots of 3 ml were harvested by centrifugation. Further DNA extraction was performed as described by Sambrook et al. (1989).

PCR amplification of intergenic 16S-23S rDNA sequences.
Primers FGPS1490-72 (5’-TGCGGCTGGATCCCTCCTT-3’) (located at nucleotides 1521-1541 of 16S rRNA gene sequence of Escherichia coli) and FGPL132’-38 (5’-CCGGGTTTCCCCATTCGG-3’) (located at nucleotides 114-132 of the 23S rRNA gene sequence of E. coli) were designed previously from multiple alignments of 16S and 23S genes of diverse bacteria. These primers are complementary to the conserved regions 4 (positions 1521-1541 for 16S rRNA gene) and 6 (positions 114-132 for the 23S rRNA gene) within the rDNA operon and match the recommended positions to detect spacer variation at the species level (Gürtler & Stanisich, 1996). Amplification was carried out in a Hybaid thermal cycler for 35 cycles. Each amplification cycle was as follows: 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C.

Rep-PCR genomic fingerprinting
Primers used in this study for rep-PCR genomic fingerprinting are REP 1R 5’-IIIICgICgICATCIggC-3’ and REP 2I 5’-ICgICTATTCCCTCCTC-3’. The cycling program was as previously described (Rademaker et al., 1998).

Insect bioassays
Toxicity of B. thuringiensis isolates was assessed against larvae of three insect species representatives of the orders Lepidoptera, Coleoptera and Hymenoptera. Insects were collected from hazelnut fields. Bioassays involved larvae of Neodiprion sertifer (Hymenoptera: Diprionidae), larvae of Hyphantria cunea (Lepidoptera: Arctiidae), and adults of Agelastica alni (Coleoptera: Chrysomelidae). The mortality of insects was checked every 24 h and all dead insects were removed immediately from containers. Bioassays were repeated 3 times on different days. Mortality data were corrected using Abbott’s formula (Abbott, 1925).

Results and discussion
B. thuringiensis isolates were obtained from almost all considered hazelnut fields in the North-East coast of Turkey. A total of 70 isolates were obtained from 22 soil samples. These results underline the ubiquity of this bacterium in soil and the fact that soil is likely its normal environment (Dulmage & Aizawa, 1982).
Characterization of the Turkish isolates indicate that they have general biochemical characteristics similar to those of *B. thuringiensis* described by de Barjac (1981), with minor differences however.

According to the ITS PCR results, the internally transcribed spacers (ITSs) of 40 of the 70 *B. thuringiensis* isolates correspond to the three *B. thuringiensis* varieties noted above. Accordingly, we can say that these 40 isolates are certainly *B. thuringiensis* strains. On the other hand, the ITS results did not allow us to determine whether the 30 remaining isolates are *B. thuringiensis* strains. Indeed, studies performed earlier by Daffonchio et al. (1998) showed that the 16S-23S ITS of *Bacillus cereus* are well conserved in terms of length, in contrast for example, with those in *Bacillus licheniformis* for which at least two different ITS fingerprints were observed. Two different ITS-PCR patterns have also been observed in *Bacillus subtilis* (Wunschel et al., 1994). Given this we only performed rep-PCR fingerprints for the 40 isolates and we observed that there was a significant genomic variation among Turkish *B. thuringiensis* isolates. There was also a variation between the standard strain of *B. thuringiensis thuringiensis* and the two other control strains.

Bioassays showed that among the 70 isolates, 38.6 % were toxic for *H. cunea*, 21.4 % for *A. alni*, 34.3 % for *N. sertifer* respectively (Table 1). Only 2.9 % of the isolates were pathogenic for the three insect species. Respectively 3, 9 and 12 isolates caused a high mortality (>80%) in *A. alni*, *N. sertifer* and *H. cunea*. Four isolates caused 100 % mortality in *H. cunea* and five isolates in *N. sertifer*. Six isolates are pathogenic for both *A. alni* and *H. cunea*, but for *N. sertifer*.

Table 1: Turkish isolates of *Bacillus thuringiensis* found to be toxic towards *Hyphantria cunea*, *Agelastica alni* and *Neodiprion sertifer*

<table>
<thead>
<tr>
<th>Target insects</th>
<th>Insecticidal effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥80 %</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td></td>
</tr>
<tr>
<td><em>Hyphantria cunea</em></td>
<td>2, 5, 9, 11, 18, 19, 20, 45, 47, 52, 70, 72</td>
</tr>
<tr>
<td>Coleoptera</td>
<td></td>
</tr>
<tr>
<td><em>Agelastica alni</em></td>
<td>26, 27, 9</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td></td>
</tr>
<tr>
<td><em>Neodiprion sertifer</em></td>
<td>16, 22, 24, 37, 47, 60, 69, 70, 73</td>
</tr>
</tbody>
</table>

Numbers in italics and underlined refer to isolates which showed 100% insecticidal effect

Some of the isolates exhibited no toxicity at all towards any of the tested insects. Similarly, Jung et al. (1998) observed that *B. thuringiensis* var. *higo* BT205 isolated from rice bran had no insecticidal activity against *H. cunea*. These authors suggested that this isolate might have novel *cry*-type genes different from *cry* genes. Additional study is needed to predict the toxicity of such isolates against insects of other orders.

The fact that among the isolates, 4 caused 100% mortality in *H. cunea* and 5 in *N. sertifer* is quite promising. The prospects offered by isolation of new *B. thuringiensis* strains with improved efficacy and specificity may be of benefit in developing in Turkey biocontrol
strategies against hazelnut pests, which currently can be controlled only by chemical pesticides.

These toxic isolates are candidates for harboring putative novel cry genes. The identification of putative novel B. thuringiensis strains could be the first step in the way to find novel toxins which could be active on new targets. The isolation and sequencing of novel cry genes should be encouraged once the target insect is identified and more evidence on the potential of novel toxins as biological control agents is available.

References


Entomopathogenic viruses
Abortive infection of the insect parvovirus, *Mythimna loreyi* densovirus (MIDNV) in mammalian cells

Mohamed El-Far, Yi Li, Gilles Fédière1, Said Abol-Ela1, Max Bergoin2, Peter Tijssen
INRS-Institut Armand-Frappier, Laval, QC, Canada H7V 1B7
1 Center of Virology, Faculty of Agriculture-IRD, Cairo University, Egypt
2 Laboratoire de Pathologie Comparée, Université de Montpellier II, 34095 Montpellier, France

Abstract: The *Mythimna loreyi* densovirus (MIDNV) is a promising agent for insect pest control. As for all biological control agents, it is essential to investigate its effect on vertebrates before field applications. As a first step in MIDNV inoculation studies, four different vertebrate cell lines (L cells of mouse, COS-7, 293, and PT cells) were used. These cell lines were infected with the wild type MIDNV or transfected with its infectious clone (pMl28) respectively, the expression of viral protein was then tested by the immunofluorescence (IF) method. No IF positive cells were observed using specific antibody to the MIDNV capsid. However, the infection and transfection of Ld652 insect cells by the same doses of both viral particles and infectious clone, showed strong positive results. The infected Ld652 cells, which lost their fibroblast shape, rounded, and detached from the flask walls, showed clear cytopathic effects (CPE), while such CPEs were not observed in the four tested vertebrate cell lines. Northern blots were performed with mRNAs from both vertebrate and invertebrate cells after 4 days infection or transfection to establish whether the expression of viral proteins in vertebrate cells was due to a transcription or a translation block. The results contradict the findings of Kurstak et al. (1969) who reported that the infection of L cells of mouse with *Galleria mellonella* densovirus (GmDNV), which shares 91% of homology with the MIDNV, showed positive IF and CPE. Experiments to study whether the viral genome integrates into the host cell chromosome are in progress.

Key words: MIDNV, infection, transfection, non-permissive, cell line

Introduction

The use of entomopathogenic viruses for biological control of insect pests has become critical in order to reduce risks of the harmful chemical pesticides. Densoviruses, classified within the subfamily Densovirinae (family Paroviridae), are potential candidates for pest control due to their high pathogenicity and specificity to their hosts (Bergoin & Tijssen, 1998). As a result of the characteristic accumulation of viral particles in nuclei in susceptible host cells, the name of densoviruses was coined (Vago et al., 1966). Generally, the first symptoms of infected larvae are anorexia and lethargy followed by flaccidity and inhibition of molting and metamorphosis. During the infection, larvae become first whitish and are progressively paralyzed, followed by a slow melanization. Prior to death, the hind legs are paralyzed and their movements become uncoordinated. Recently, using the cloned genome of the JcDNV, typical infections have been initiated both *in vitro* and *in vivo* resulting in complete viral particles (Jourdan et al., 1990; Li et al., 1996). The densovirus of the maize worm *Mythimna loreyi* (MIDNV), which was first isolated in Egypt, is promising for the control of the maize pest and also for a world-wide pest, the cotton worm *Spodoptera littoralis* (Fédière et al., 1995).
The complete genome (about 6 kb) of the MIDNV has been recently cloned and sequenced (unpublished results). Its genome organization and the mechanism of transcription and translation are very similar to that of GmDNV and JcDNV. Before the use of such a virus in the field application it is essential to study its effect on the vertebrates. Prior to animal studies, cultured cells provide a useful tool to study the viral pathogenesis (Flint et al., 2000). In an early investigation on the effect of densovirus on mammalian cells, it was reported that the densovirus of the wax moth *Galleria mellonella* (GmDNV), which is closely related to the MIDNV (Tijssen et al., 1999), could multiply in the L929 cells of mouse and resulted in different CPEs (Kurstak et al., 1969). In this report, we present preliminary results about the effect of MIDNV on some mammalian cells, including the L929 cells, by both infection with purified viral particles and transfection with its infectious clone.

**Material and methods**

**Infectious clone**

The complete viral genome was previously cloned in pEMBL19+ (unpublished data).

**Cells**

Five cell lines were used: four mammalian cells (L929, COS-7, 293, and PT cells) were maintained in DMEM medium supplemented with 8% serum, and one insect cell line Ld652 was maintained in Grace medium supplemented with 10% serum.

**Infection and transfection**

Infections and transfections were carried out 18-20 h post-plating of cells (10⁶ cells/well) in 6-well plates. Cells were infected with virus (corresponding to 8x10¹⁰ particles) diluted in 1 ml medium. In addition, mammalian and insect cells were transfected by MIDNV infectious clone (pML28) at 1.5 µg DNA/well, using the LipofectAmine Kit (GIBCO BRL), according to the manufacturer’s instructions. After 6 h of incubation with virus, cells were washed twice with PBS, then supplied with fresh complete medium. Cells were harvested 6, 12, 24, 36, 48, 72, and 96 h later.

**Immunoflorescence (IF)**

Infected and transfected cells were fixed in 3% of formaldehyde in IF buffer (Diagnostic Pasteur Sanofi), then permeabilized by 3% Triton X-100 in IF buffer. Cells were first incubated with the antibody prepared against the viral structural proteins at a dilution of 1/5,000, followed by anti-IgG-antibody labelled with fluorescein isothiocyanate. Finally, the cells were examined by UV microscopy.

**Quantitative PCR**

The amount of viral genome in infected cells (Ld652 and L929 cells) was established by quantitative (MIMIC) PCR. Both control and infected cells were harvested at the different time points after infection, and volumes were re-adjusted to 2.5 ml by fresh medium and kept at –70 °C until use. The cells were frozen and thawed three times to release virus. After centrifugation at low speed, the supernatant was diluted with distilled H₂O. The quantification was performed using standard concentrations of internal competitor MIMIC DNA.

**Western Blot**

Extracts from infected L929 and Ld625 cells were subjected to Western blot analysis using the MIDNV capsid-specific antibody, 6, 12, 24, 36, 48, 72 and 96 h postinfection.
Results and discussion

Detection of viral protein synthesis in MIDNV infected cells

The Ld652 insect cell line was chosen based on its relatively high susceptibility to densovirus. COS-7, PT, and 293 were chosen to represent different types of mammalian cell lines, whereas the L929 mouse cell line was chosen to confirm or infirm previously reported results that claimed about its susceptibility to densovirus infection. Infections were carried out using highly purified virus as described in Material and Methods. During the first 48 h, there were no observed cytopathic effects (CPE) in both insect and mammalian cells. After 48 h, more than 50% of Ld cells started to show remarkable CPE with cell rounding, nucleus swelling and detachment from the flask. By 96 h, the majority of cells lost their attachment and floated in the culture medium. No CPEs were observed in any of the tested mammalian cell lines.

To ensure the entry of the viral genome into cells, transfections were carried out on both Ld and L929 cells using the infectious clone, pML28. CPEs were also obtained in Ld cells, starting by 48 h post-transfection, whereas no CPE was observed even after 4 days post-transfection in the case of mammalian cells. These results were confirmed by immunofluorescence. Ld652 insect cell line was the only cell line that showed clear IF for both infection by the wild type virus and transfection by its infectious clone. The nuclei of infected cells were completely fluorescent due to the presence of newly synthesized viral capsid proteins. No IF positive cell was observed on any of the other tested mammalian cells, both for infections and transfections. In infections, entry of the virus particles was observed in the cytoplasm of Ld652 and the cell membrane of L929 cells 6 h post-infection. However, the fluorescence of L929 cells disappeared 48 h post-infection, which suggests that no productive infection was established in the mammalian cells.

Western blotting, using the MIDNV capsid-specific antibody, also clearly showed that 48 h postinfection the capsid proteins increased significantly in Ld652 insect cells whereas its level remained unchanged in L929 mouse cells during the infection.

Quantification of viral DNA replication in infected cells

Viral DNA multiplication was monitored using the quantitative MIMIC-PCR in both Ld652 and L929 cells. Samples were obtained from infected cells after 6, 12, 24, 36, 48, 72 and 96 h of infection, and subjected to the quantitative PCR. The viral DNA remained at the same level during first 36 h of infection in infected Ld652 cells and increased rapidly from 48 h post-infection. However, L929 cells, while showing uptake of input virus, did not support multiplication and the level of viral DNA stayed at the same level throughout the experiment.

In an assay to follow the presence of MIDNV DNA replication in subsequent passages of initially infected cells, the Ld652 cells could not be passaged since the cells were lysed after infection, whereas L929 cells could undergo at least 9 passages without any detectable CPEs. The viral DNA could be detected in the first passage because of the input of virus, but decreased dramatically in the second passage while it disappeared completely after the third passage. Taken together, these results strongly suggested that mammalian cells are not permissive to densovirus infection and contradict previous reports by Kurstak et al. (1969).

Acknowledgements

This work was supported by the following grants: Fonds International de Coopération Universitaire, FICU (Agence Universitaire de la Francophonie) et Fonds de Contrepartie de l’Aide Alimentaire Francaise en Egypte (Bureau de Liaison Agricole Franco-Egyptien, BLAFE).
References


A nuclear polyhedrosis virus of the lackey moth, *Malacosoma neustria* (Lepidoptera: Lasiocampidae) in Turkey

Mustafa Yaman, Remziye Nalcacioglu, Ali O. Belduz, Zihni Demirbag
Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey

**Abstract:** A Turkish isolate of *Malacosoma neustria* nuclear polyhedrosis virus (MnNPV) was investigated in the perspective of biological control. The virus occurred in populations of *Malacosoma neustria* in 1999-2000. Microscopic observations showed that the virus developed in nuclei of hypodermis, lymphocytes, tracheal matrix and fat body. The dimension of polyhedra was 1.64±0.52 µm. The presence of the virus was also demonstrated by DNA-DNA hybridization assay using polyhedrin gene specific probe of *Autographa californica* nuclear polyhedrosis virus (AcNPV). The biossays carried out to determine the infectivity of the virus showed its high activity on the first-second instar larvae of *M. neustria*. The young larvae infected with the nuclear polyhedrosis virus died more rapidly than older larvae, and the total mortality was greater. On the other hand, horizontal transmission of MnNPV appeared very high. The infected larvae contaminated the healthy larvae very speedly. In some population of *M. neustria*, up to 98% of first instar larvae died due to polyhedrosis. Finally the Turkish isolate of *Malacosoma neustria* nuclear polyhedrosis virus looked very promising in biological control.

**Key words:** *Malacosoma neustria*, nuclear polyhedrosis virus, biological control

**Introduction**

*Malacosoma neustria* (Lepidoptera: Lasiocampidae) is one of the important pest of hazelnut as well as fruit trees in Turkey. This insect is very susceptible to its nuclear polyhedrosis viruses (MnNPV), which were the subject of investigations in Europe (Bergold, 1943; Lipa et al., 1968; Jankevica et al., 1998).

The present work reports on a nuclear polyhedrosis virus found on *M. neustria* in Turkey. Such a study is of great importance with regard to the potential of the virus as a biological control agent against this pest. Furthermore, to study a virus originated from Turkey is also of biological interest since this country represents a bridge which joins Asia to Europe.

**Material and methods**

*Collection of insects*
Diseased and dead infected larvae of *M. neustria* were collected in May and June in 1999 and 2000 during the mass occurrence of the insect in Turkey.

*Microscopic observations*
For microscopic observation, a small piece of infected insect tissue was smeared on a microscope slide and stained 25-60 min in 10 % Giemsa in 0.02 M phosphate buffer. After staining, occlusion bodies (OBs) of nuclear polyhedrosis viruses (NPVs) were examined with an oil immersion objective. The polyhedral inclusion bodies (PIBs) were also studied under scanning electron microscopy.
**Virus purification and characterization**

Polyhedra were purified by centrifugation, from crude extracts of field-collected *M. neustria* larvae. In brief, cadavers of virus-infected larvae were ground with 0.5 ml of STE-C buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylene-diamine tetracetic acid (EDTA), 50 mM NaCl, and 10 mM cysteine). The resulting powder was transferred to 2 ml microfuge tubes and 1 ml STE-C containing 1 mg/ml sodium docecyl sulfate (SDS) and 0.066 mg/ml Dnase for each sample. The samples were incubated on a rotary shaker for 20 min at room temperature and then centrifuged at 145 g for 5 min to pellet large pieces of insect debris. The supernatant was transferred to new tubes, and the viral polyhedral inclusion bodies (PIBs) were pelleted by centrifugation at 15.000 g for 10 min. Viral DNA extracted according to Levin *et al.* (1997).

**Production of AcNPV DNA probe**

Polyhedrin gene specific probe of *Autographa californica* nuclear polyhedrosis virus (AcNPV) was produced by Polymerase Chain Reaction (PCR). The coding region of AcNPV polyhedrin gene was targeted as template DNA. Primers for PCR were selected according to Moraes & Maruniak (1997). The DNA sequence for the set of primers was: 5’ TAC GTG TAC GAC AAC AAG T-3’ (forward) and 5’ TTG TAG AAG TTC TCC CAG AT-3’ (reverse). The amplification products were analyzed by 0.7 % agarose gel electrophoresis in TAE (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0) buffer, stained with ethidium bromide. Probes were labeled using the procedure recommended by the manufacturer of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics GmbH) (Germany).

**DNA-DNA Slot-blot hybridization assay**

MnNPV DNA was vacuum blotted onto nylon membrane using a Slot-Blot apparatus (Hoefer Scientific Instruments, San Francisco, California, USA). The prehybridization and hybridization procedures were carried out according to protocols modified from Ward *et al.* (1987) and Roche Diagnostics GmbH (Germany) using DIG High Prime DNA Labeling and Detection Starter Kit I (for color detection with NBT/BCIP) (Roche Diagnostics GmbH). For all experiments, each slot-blotted membrane contained positive and negative controls. Positive control was DNA from AcNPV. The negative control was uninfected *Malacosoma neustria* larval suspension treated with 6 M NaOH and centrifuged.

**Pathogenicity and host specificity of MnNPV**

Semi-purified polyhedra were used to infect larvae. The concentration of MnNPV polyhedra in the suspension was counted in a haemacytometer. The suspension was standardized to contain 1 x 10^8 polyhedra/ml (Stairs, 1964) and tested against 20 first-instar *M. neustria* larvae. A film of virus suspension was applied to the surface of fresh leaves and the larvae were allowed to feed on these diets. On the other hand, determination of host range was an important part of the studies of MnNPV. For host specificity studies larvae of insect species, *Gypsonoma dealbana* and *Euproctis chrysorrhoea* were used. Twenty larvae were used for each assay. The control larvae were fed with virus-free diet. The larval mortalities were recorded daily and dead larvae were removed. All bioassays were repeated 3 times. Data were evaluated by using Abbott’s formula (Abbott, 1925).

**Horizontal infection of MnNPV**

Three experimental groups were set in the laboratory to detect the horizontal infection of MnNPV. First group (positive control) included 50 first instar larvae fed with MnNPV-contaminated leaves in first day and then with fresh leaves. The second group (experimental group) was set as the first one but after 5 days 50 healthy larvae were added. Total 100 larvae were fed with fresh leaves for 21 days. Third group (negative control) included 50 healthy larvae fed with fresh leaves. The larval mortalities were recorded daily and dead larvae were removed for the three groups.
Results and discussion

In 1999 and 2000 viral infections were observed in Turkish populations of *Malacosoma neustria*. Microscopic observations and Slot-blot hybridization showed that the virus was a nuclear polyhedrosis virus (MnNPV). The virus developed in nuclei of hypodermis, lymphocytes, tracheal matrix and fat body. The dimension of polyhedra is 1.64±0.52 µm. The polyhedra vary in size 0.76-3.85 µm. Viral infection was also showed by DNA-DNA Slot-blot hybridization. The probes produced from *Autographa californica* nuclear polyhedrosis virus (AcNPV) hybridized with DNA from field-collected MnNPV-infected lackey moth larvae and AcNPV DNA (positive control). Hybridization was not observed between probes and DNA from uninfected lackey moth samples (negative control).

Bioassays indicated that MnNPV has high infectivity (98 %) against first instar *M. neustria* larvae (Table 1).

Table 1. Infectivity and host specificity of Turkish MnNPV.

<table>
<thead>
<tr>
<th>Insects tested</th>
<th>Number of larvae tested</th>
<th>Viral concentration (polyhedra/ml)</th>
<th>Mortality (%) caused by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>virus</td>
</tr>
<tr>
<td>Lasiocampidae</td>
<td>40</td>
<td>1 x 10^8</td>
<td>97.5</td>
</tr>
<tr>
<td><em>Malacosoma neustria</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymantriidae</td>
<td>25</td>
<td>1 x 10^8</td>
<td>0</td>
</tr>
<tr>
<td><em>Euproctis chrysorrhoea</em></td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Tortricidae</td>
<td>25</td>
<td>1 x 10^8</td>
<td></td>
</tr>
<tr>
<td><em>Gypsonoma dealbana</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Horizontal transmission of MnNPV in *Malacosoma neustria* larvae (A: 50 infected larvae; B: 50 non infected larvae).
It is interesting to note that Lipa et al. (1968) were not able to infect *M. neustria* with nuclear polyhedrosis virus of *B. mori*, although Hukuhara & Hashimoto (1966) were able to infect *M. neustria testacea* with two strains of cytoplasmic viruses of *B. mori*. Benz (1963) found that *Borrelinavirus alpicolae* is not infectious for *M. neustria*.

As shown in Table 1, *G. dealbana* become infected but *E. chrysorrhoea* was resistant to MnNPV. Lipa et al. (1968) noted that the viruses from *Malacosoma* spp. are host specific. To determine the host range is an important part of the studies on MnNPV. Clearly, further investigations are needed by taking into account other lepidopterans species.

On the other hand, infection experiments showed the importance of horizontal transmission. On a total of 50 larvae which did not feed contaminated leaves but were in contact with 50 larvae which fed on contaminated leaves, 28 died from viral infection through horizontal transmission (Fig. 1).

To conclude, the Turkish isolate of *Malacosoma neustria* nuclear polyhedrosis virus looked very promising for biological control. Further studies would be focused on its molecular characterization and comparison with other isolates.

**References**


Record of densoviral diseases occurring among the noctuid populations in lucerne fields at El-Bahareya oasis in Egypt

Gilles Fèdière, Mohamed A.K. El-Sheikh, Rabab El Mergawy, Moguib Salah, Maha Masri, Said Abol-Ela, Max Bergoin¹, Peter Tijssen²
Center of Virology, Institut de Recherche pour le Développement (IRD) - Faculty of Agriculture, Cairo University, Guiza, Egypt
¹Laboratoire de Pathologie Comparée, Université de Montpellier II, Place Eugène Bataillon, CC 101, 34095 Montpellier, France;
²Centre de Microbiologie, INRS – Institut Armand-Frappier, Université du Québec, Laval, QC, Canada H7V1B7

Abstract: Screenings for entomopathogenic viruses in the Noctuid fauna of lucerne fields revealed the presence of viral strains belonging to the Parvoviridae family (Densovirus genus) from larvae of Agrotis ipsilon, A. spinifera, Spodoptera exigua and S. littoralis. These species were similar to those attacking the cotton fields in the Delta and Nile Valley. The DNA genome, characterized using 6 restriction endonucleases, and the proteins of the capsid, were identical with those of the Mythimna loreyi Densovirus (MlDNV), previously isolated in Egypt. This fact illustrated the wide host range of this virus. This biocontrol agent is currently under investigation for use as a microbial pesticide.

Key words: Spodoptera littoralis, Spodoptera exigua, Agrotis ipsilon, Agrotis spinifera, Densovirus, Parvoviridae, biological control, lucerne, Medicago sativa, cotton

Introduction

The present work was planned to record naturally occurring densoviral diseases in the Noctuid fauna of lucerne and their role in the control of insect pest populations of El-Bahareya Oasis, Giza Governorate, Egypt.

The survey was undertaken from April, 2000 to March, 2001 on clover, which is the most important forage crop in Egypt (the Egyptian clover, Trifolium alexandrinum) during the winter and spring seasons, and on lucerne, Medicago sativa, all year round, at two different localities, namely El-Jaffara and El-Kassa villages.

Unlike the Delta and Nile valley, the El-Bahareya Oasis is an isolated area were insect pest problems may be less important. The study was based on weekly observations.

Screening for any entomopathogenic virus by nucleic probe and PCR tests can revealed the presence of different denso viral strains. These isolates may be important natural regulating factor of noctuids pest populations in cotton and lucerne fields in Egypt.

Materials and methods

Virus isolates
All the new strains of Densovirus (DNV) were sampled from larvae collected during pest infestations of lucerne field and from natural dead-larvae from the mass-rearing of the different pests.
For biochemical comparison, the strain of *Mythimna loreyi* DNV designated as *Ml* DNV was used, as the original strain propagated for several years in our laboratory (Fédière *et al*., 1995).

**Virus purification**

The infected larvae were homogenized in Tris (0.05M)-SDS (0.06 %) Buffer, pH 7.8. After filtration through cheese cloth and clarification (9.000 g for 5 min), the virus was concentrated by high speed centrifugation (Ti 55 Beckman rotor, 35.000 rpm for 1 h 30). The viral pellets, resuspended in Tris Buffer were dispersed by ultrasonication and then clarified 9.000 g for 5 min. The resulting supernatant containing virus particles was layered onto a 15-45 % sucrose gradient and ultracentrifuged (SW 28 Beckman rotor, 27.000 rpm for 2 h 30). The virus band was collected and the purified virus were concentrated as above in Tris-Buffer, and the concentration of the final suspension was measured on the spectrophotometer at 260 nm. The purified viral suspensions were stored at –20 °C in Tris Buffer.

**Electron microscopy**

Purified viral suspension was negatively stained in 2 % Uranyl acetate, pH 7.4 and examined through an electron microscope.

**Electrophoresis of viral polypeptides**

Molecular weight and number of virus structural proteins were assessed by comparing their electrophoretic mobilities in polyacrylamide 12 % gel, with the following standard molecular weight markers: phosphorylase b (Mw: 94.000 Da), bovine serum albumine (Mw: 67.000 Da), ovalbumine (Mw: 43.000 Da), carbonic anhydrase (Mw: 30.000), trypsin inhibitor (Mw: 20.100 Da), and lactalbumine (Mw:14.000 Da).

**Nucleic acid (DNA) extraction**

The extraction of nucleic acid from the purified virus was carried out using the suspension of virus, mixed with proteinase K (2 mg/ml ) for a final concentration of 2 %, then lysed with Sarcosyl 10 % from the final volume. This mixture was incubated in water bath at 50 °C for 1 h 30. The DNA solution was deproteinized by mixing with a suspension of a phenol/chloroform and gentle shaking at room temperature. The mixture was centrifuged for 5 min at 5.000 rpm .The nucleic acid was precipitated by addition of 2 volumes of iced absolute ethanol in presence of sodium acetate (0.3M final) for 14 h at –20 °C. After centrifugation (28,000 rpm, 10 min) the pellet was washed in 70 % ethanol. The pellet was dried by centrifugation under vacuum, and incubated in TE (15 mM Tris-HCL, 1 mM EDTA, pH 7.5). Concentration of the nucleic acid was finally measured according to its optical density through 260 nm wavelength. This suspension was kept at –20 °C until required.

**Restriction enzyme digestion and electrophoresis of the viral DNA**

1 µg of viral DNA (1 O.D._260_ of DNA = 50 µg/ml) was digested in a volume of 20 µl as recommended by the suppliers (Roche Diagnostics). Electrophoresis was carried out using 1 % agarose gel in Tris-EDTA-Phosphate buffer (TEP) (90mM Tris-phosphate, 20mM EDTA, pH 8.0). Electrophoresis was conducted at 50 V for 2 h. The gel was visualized and photographed under a short wave UV transilluminator. The size of the DNA fragments was estimated by comparison with standard marker DNA (Roche Diagnostics): Marker III and Marker VII.

**DNA probe tests**

The preparation of the *Ml* DNV DNA probe, original template DNA was used extracted from *Ml* DNV was at concentration of 4 O.D._260_. The digoxygenin-labelled DNA probe was applied according to the protocol recommended by the supplier (Roche Diagnostics). The final concentration of the probe is 2 µgDNA/100 µl i.e. 20 ng/µl.
PCR methods
One round of PCR amplification is performed using two composite primers (Ml 225 and Ml 264) attached to a 400 nucleotides stretch of sequence.

The temperature conditions were as follows:
- Predenaturation: 95 °C, 2 min, 1 cycle
- Hot start, Denaturation: 94 °C, 30 sec
- Anneling, Polymerisation, Amplification: 55 °C, 30 sec. 35 cycles
- Additional Polymerisation: 72 °C, 2 min
- Final: 72 °C, 8 min

PCR products stored overnight: 4 °C

Results and discussion

Results indicated remarkable infestations with different noctuid species, probably since insecticides are not commonly used in the oasis. The turnip moth, *Agrotis segetum* Denis & Schiff., the black cutworm, *Agrotis ipsilon* Hfn., the brownish cutworm *Agrotis spinifera* Hbn., the large cutworm *Agrotis pronuba* L., the African cotton bollworm, *Helicoverpa armigera* Hbn., the lucerne caterpillar, *Spodoptera exigua* Hbn., the Egyptian cotton leafworm, *Spodoptera littoralis* Boisd. and the African armyworm *Spodoptera exempta* Walker, were the most common and abundant species that occurred. These species were similar to those attacking the cotton fields in the Delta and Nile Valley.

Screenings attempts for any entomopathogenic viruses have revealed the presence of Densovirus (Densonucleosis Virus, DNV)(Paroviridae) from dead and dying specimens of *Spodoptera* and *Agrotis* genera. Four viral strains were isolated, from larvae of *A. ipsilon*, *A. spinifera*, *S. exigua* and *S. littoralis* respectively.

The Densovirus has icosahedral non-enveloped particles of 22 nm diameter. Four capsid proteins of 91, 63, 53 and 47 KDa were identified and were undistinguishable from that of the previously reported Densovirus isolated in Egypt (*Mythimna loreyi* DNV, (*Ml*DNV)). The DNV genome, a single-stranded DNA molecule of size 5.95 Kb was characterized using 6 restriction endonucleases. The restriction profiles were identical with those of *Ml*DNV. Molecular studies by nucleic probe and PCR tests doesn’t bring significant difference.

Genome sequencing is underway for the new isolates of DNV from *S. littoralis*, *S. exigua*, *A. ipsilon* and *A. spinifera* naturally found in the same region of Bahareya Oasis. Furthermore, epidemiological studies should be going on all over the year 2002.

These results suggested that the four DNV strains are new isolates of *Ml*DNV. This fact illustrated the wide host range of this virus.

The confirmation of the natural polyspecificity of *Ml*DNV in the field completed its biological characterization and would provide investigators a more completely known pathogen for use as microbial agent against *S. littoralis* larvae on cotton.

This work is considered as a part of the program concerning the inter-relationship between the different viruses isolated from *S. littoralis* and their host, and the biodiversity of the viral strains regarding as well as the geographical distribution of this insect in Egypt and the host crops.

Possibly *Ml*DNV could represent an important complementary agent alongside with *SnPV* (Nuclearpolyedrosis Virus) and *S/GV* (Granulosis Virus) in the IPM programme dedicated to this important pest.
Acknowledgements

This work was supported by two grants: Fonds International de Coopération Universitaire, FICU (Agence Universitaire de la Francophonie), et Fonds de Contrepartie de l’Aide Alimentaire Francaise en Egypte (Bureau de Liaison Agricole Franco-Egyptien, BLAFE).

References

Preliminary results on the biological activity of a Polish isolate of nucleopolyhedrovirus against *Panolis flammea* Schiff.

Iwona Skrzecz  
*Forest Research Institute, Bitwy Warszawskiej 1920 R. nr 3, 00-973 Warsaw, Poland*

Abstract: The biological activity of a Polish isolate of nucleopolyhedrovirus, PfNPV, against the pine beauty moth, *Panolis flammea* Schiff., was evaluated. Laboratory experiments demonstrated the high infectivity of PfNPV (32-81% mortality) and the lower feeding intensity of *P. flammea* caterpillars infected with the virus. In field trials the highest mortality (68%) due the pathogen occurred at the stage of prepupae and pupae.

Key words: *Panolis flammea*, nucleopolyhedrovirus, biological activity

Introduction

Control of forest leaf-eating insects with chemical insecticides has been gradually replaced with integrated pest control methods, particularly ones associated with biological control programs that involve the use of entomopathogenic microorganisms. Special attention has been paid to the application of insect pathogenic viruses from the family Baculoviridae against forest defoliators (Hunter-Fujita *et al*., 1998). In this context, research on biological activity of insect pathogenic viruses for control of economically important defoliators have been conducted in the Forest Research Institute in Warsaw (Skrzecz, 2000).

The present study was carried out in order to evaluate the insecticidal efficiency of a Polish isolate of nucleopolyhedrovirus virus, PfNPV, against the Pine beauty moth, *Panolis flammea* Schiff., a pest of Scots pines, *Pinus sylvestris* L., which occur in cyclical outbreaks in Poland.

Material and methods

**Laboratory experiments**

Under laboratory conditions, caterpillars of *P. flammea* were fed with Scots pine shoots that were sprayed with 5 different doses (ranging from $3.8 \times 10^5$ to $3.8 \times 10^9$ PIB/ml) of a Polish isolate of PfNPV. The insects reared on uninfected diet act as a control. Mortality in caterpillars was evaluated every day as well as the intensity of feeding determined by the dry mass of frass produced in average by caterpillar over a 24 h period. We also compared the weight of pupae. Observations on the presence of virus in dead insects were made using a light microscope (phase contrast, 1000 x magnification).

The LC$_{50}$ (concentrations of pathogen causing mortality of 50 % insects) was evaluated and optimum concentrations of pathogen were assessed for fields tests.

**Field experiment**

Small scale ground application was performed in a 40-years-old Scots pine forest threatened with mass occurrence of *P. flammea*. The suspension of PfNPV at a concentration $4 \times 10^{12}$ PIB/ha mixed with additive Olejan 85EC was applied in a area of 2 ha. The assessment of the
caterpillars mortality was done 2 and 4 weeks after treatment. Results were calculated based on:
- the number of caterpillars dropped on linen cloths (size 1 m²) placed beneath randomly selected model trees,
- the number of caterpillars found in the crowns of the cut model trees,
- the number of prepupae in forest litter in the area of crown projection of randomly selected trees.

During each assessment larvae were collected for microscopic studies. Additionally, after 4 months, *P. flammea* pupae were searched in the treated and untreated areas.

**Results**

The laboratory results indicated that the mortality of Pine beauty moth caterpillars increased systematically and proportionally to the pathogen concentrations (Fig. 1). The highest mortality (77-81 %) was obtained in the group of insects feeding on the pine shoots treated at the concentrations of $3.8 \times 10^8 - 3.8 \times 10^9$ PIB/ml. In the groups that fed on shoots treated at concentrations $3.8 \times 10^5 - 3.8 \times 10^7$ PIB/ml, the mortality reached 32-53%. The lowest mortality rate (4%) was observed in the control group. The LC₅₀ was $3.0 \times 10^8$ PIB/ml. Microscopic observations indicated numerous virus polyhedra in dead infected caterpillars; less numerous polyhedra were observed in insects from the control area.

![Fig. 1. Mortality of *Panolis flammea* caterpillars infected under laboratory conditions with different concentrations of a Polish isolate of nucleopolyhedrovirus, PfNPV.](image)

The intensity of caterpillar feeding is shown on the Figure 2. During two weeks a increasing feeding intensity was noticed. Afterwards the highest weight of dry mass of frass...
(48-63 mg/day) was obtained in the groups infected with the lowest concentrations of virus (3.8 x 10^5 – 3.8 x 10^7 PIB/ml). The dry mass of frass excreted by insects feeding on the shoots treated with the highest concentrations of pathogen (3.8 x 10^8 – 3.8 x 10^9 PIB/ml) was 25-27 mg/day. The weight of frass in control caterpillars reached 65 mg/day.

Fig. 2. Mean weight of frass of *Panolis flammea* caterpillars infected with different concentrations of a Polish isolate of nucleopolyhedrovirus, PfNPV.

All living larvae pupated and no statistical differences (female: F=1,188, df=5, P=0,127; male: F=0,559, df=5, P=0,731) were found between the weight of infected and uninfected pupae. However, within six months, mortality occurred in infected pupae proportionally to the virus concentration of the treatment (100 % at the maximum concentration) (Table 1).

Table 1. Mortality in *Panolis flammea* pupae (resulting from larvae infected with a Polish isolate of nucleopolyhedrovirus, PfNPV) (data from laboratory experiments).

<table>
<thead>
<tr>
<th>Virus concentration (PIB/ml)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,8 x 10^5</td>
<td>0</td>
</tr>
<tr>
<td>3,8 x 10^6</td>
<td>12</td>
</tr>
<tr>
<td>3,8 x 10^7</td>
<td>29</td>
</tr>
<tr>
<td>3,8 x 10^8</td>
<td>54</td>
</tr>
<tr>
<td>3,8 x 10^9</td>
<td>100</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
</tr>
</tbody>
</table>
In the field trial, 2 and 4 weeks after treatment, mortality due to infection with PfNPV was higher in plots treated with the viral preparation (Table 2). Four weeks after the treatment, the search for \textit{P. flammea} caterpillars in forest litter beneath virus treated and untreated trees showed that the mortality of the pest reached 68 and 24 \% respectively (Table 2).

Table 2. Mortality of \textit{Panolis flammea} caterpillars infected with PfNPV, in field plots treated with a PfNPV preparation.

<table>
<thead>
<tr>
<th>Time of checking</th>
<th>Stage of \textit{P. flammea}</th>
<th>Variant of the experiment</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks after treatment</td>
<td>caterpillar</td>
<td>infection with PfNPV control</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>control</td>
<td></td>
</tr>
<tr>
<td>4 weeks after treatment</td>
<td>caterpillar</td>
<td>infection with PfNPV control</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>prepupa</td>
<td>infection with PfNPV control</td>
<td>68</td>
</tr>
</tbody>
</table>

Microscopic studies indicated numerous polyhedra in the tissue of live and dead caterpillars collected from treated and untreated trees.

High reduction of pupae number was noticed in the treated area, where 8-19 individuals were found compared to the value from untreated areas (43-57 pupae).

Conclusions

In laboratory experiment, infection with a Polish isolate of PfNPV caused high mortality (32-81\%) and a lower feeding intensity in \textit{P. flammea} caterpillars. All caterpillars that survived the infection pupated but 12-100 \% mortality was observed in the resulting pupae.

Field trial data indicated an almost 2-times higher mortality in treated insects with an application of high amount of PfNPV, while the death of untreated caterpillars was caused by naturally occurring virus. The pathogen caused the highest mortality in \textit{P. flammea} in the prepupa and pupa stages.

References


Persistence of the biological effect of codling moth granulovirus in the orchard – a preliminary field trial

Jutta Kienzle1, Christof Schulz1, Claus P.W. Zebitz1, Juerg Huber2
1 University of Hohenheim, Institute of Phytomedicine, 70593 Stuttgart, Germany
2 Institute for Biological Control, BBA, Heinrichstr. 243, 64287 Darmstadt, Germany

Abstract: In a field trial, the persistence of the biological effect of codling moth granulovirus (CpGV) was investigated. With one treatment on May 23, with full concentration of CpGV (MADEX 3, 100 ml/ha), a considerable reduction of CM population was achieved over the whole vegetation period. This may indicate that over a considerable period of time after a single treatment, a biological effect of CpGV sufficient for an increased mortality of the larvae was present in the orchard. However, the onset of mortality was not fast enough to protect the fruit from damage.

Further research must be done to gain more experience about this effect. For intelligent IPM strategies a long term effect in population control of codling moth could be very important.

Key words: Codling moth, Codling moth granulovirus, persistence

Introduction

The use of codling moth granulovirus (CpGV) in IPM strategies as a tool for codling moth (CM) control is increasing in Southern Germany. Main interest is directed towards the efficacy of the virus in CM population control – which on a long term also means damage control.

In this context, the question of the persistence of the biological effect of CpGV in the orchard was raised. In the past, the effect of CpGV was assessed as reduction of damage, i.e. mortality of larvae before they could damage the fruit to a degree that its market quality was downgraded. For our purpose, the interest was directed towards control of CM population, i.e. the mortality of the larva also at later larval stages.

Since observations in the field seemed to indicate a rather persistent effect of CpGV applications in population control, a preliminary field trial was realized in 2000.

Material and methods

The experimental orchard of 0.4 ha was located at the research station of the University of Hohenheim. The variety Elstar on M9 was planted in 1986, the trees were vigorous. The orchard was divided into two equal parts with an untreated buffer zone of 20 m. One part was treated with CpGV in full concentration (MADEX 3, Andermatt Biocontrol, 100 ml/ha) on May, 23, 2000. The second part served as untreated control. After this, the whole orchard remained without any insecticide treatment until the end of the season.

Fruit damage was assessed several times during the year (1000 fruits per plot). Since the trees were also provided with trap bands for the assessment of the survival of the larvae, the fruits remained on the trees and were not opened. Thus, only deep entries and superficial stings could be distinguished. At harvest, 2000 fruits per plot were checked. They were split open to identify „stopped damage“ even if the entry was deeper. „Stopped damage“ means,
that the entry did not reach the seed cavity, i.e. the larva did not complete its development in the fruit.

Corrugated cardboard belts were applied around the trunks of the trees and assessed at regular intervals (100-50 trees per plot). Thus, the effect of the single treatment on the diapausing larvae could be observed from interval to interval. The belts were applied at the end of June and checked on July 26, August 3, August 23 and September 9.

The probable hatching period of the larvae found in the belts in each interval was calculated using sum of degree-days >8.1 °C (Welte, personal communication). For completion of larval and pupal development 100 degree days each were assumed.

Results

In 2000, at Hohenheim, the CM flight started during the first decade of May. Nevertheless, first injured fruits could not be found until the last days of June, i.e. four weeks after the treatment. On 5th of July there was a slight difference in injured fruits between the treated and the untreated part (Table 1). In the treated part, there were also some “stings” on the fruits. At harvest, fruit damage was even slightly higher in the treated part (29.5 %) than in the untreated control (28.4 %). The number of fruits with „stopped damage“, however, was higher in the treated part than in the untreated part.

Table 1: Fruit damage in % in early summer and at harvest in the treated and untreated plot.

<table>
<thead>
<tr>
<th>Date</th>
<th>Plot</th>
<th>Superficial stings</th>
<th>Deep entries</th>
<th>Total damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.7.00</td>
<td>Treated</td>
<td>0.3</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>0</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>21.7.00</td>
<td>Treated</td>
<td>0</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>0</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Harvest</td>
<td>“stopped” damage</td>
<td>17.4</td>
<td>12.1</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>Complete damage</td>
<td>12.8</td>
<td>15.6</td>
<td>28.4</td>
</tr>
</tbody>
</table>

At each sampling date of the corrugated card board belts a distinct reduction of the number of larvae in the CpGV treated plot could be observed (Fig. 1). The efficacy of this single CpGV treatment in population control at the different assessment dates is shown in Table 2.
Fig. 1: Reduction of CM population in the treated and untreated plot: Number of larvae in the corrugated card board belts at the different assessment dates.

Table 2: Effect of CpGV on the diapausing larvae and probable hatching period (in days after treatment) of the corresponding larvae from the egg

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Hatching period of the larvae (DAT)</th>
<th>Reduction of population in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.7.</td>
<td>38-48</td>
<td>64.2</td>
</tr>
<tr>
<td>3.8.</td>
<td>49-60</td>
<td>74.2</td>
</tr>
<tr>
<td>23.8.</td>
<td>61-83</td>
<td>67.8</td>
</tr>
<tr>
<td>23.9.</td>
<td>84-97</td>
<td>46.4</td>
</tr>
</tbody>
</table>

**Discussion**

The reduction of CM population over the whole season by a single treatment with CpGV in May suggests that over a considerable period of time after the treatment, a biological effect of CpGV sufficient for an increased mortality of the larvae was present in the orchard. Obviously, the larvae did not die fast enough to prevent fruit damage. Since the fruit damage in both plots was similar, it can be excluded that the reduced population in late summer is simply due to the effect of CpGV on the first generation, or to a difference in fruit attack. With regard to the small size of the plots, this was also not to be expected.

These first results can confirm the findings of Huber (1980) and of Glen & Payne (1984) that most of the CpGV applied with a treatment is inactivated rather quickly by UV-irradiation (half life about two days).
However, the inactivation curve is bi-shaped: a small part of the CpGV applied (about 1 %) persisted for a much longer time in the orchard. This corresponds to laboratory findings of Huber & Lüdcke (1996).

At first, these findings seemed to be of little interest, since high concentrations of CpGV are necessary for damage control. A normal synthetic product in such a low concentration would also be totally ineffective. However, for CpGV the low slope of the dose-effect-curve must be considered. Since with 1/10 of the normal concentration also rather good effects in damage control can be achieved, it seems realistic, that 1/100 of this concentration (1 % of the CpGV applied) could be enough to cause a considerable, but slow larval mortality – as observed in this experiment.

These one year results certainly must be followed by further experiments. However, if further research confirms the trend shown in this first trial, this would be an important finding for the use of CpGV in intelligent CM control strategies. It would mean that a high concentration of CpGV, applied at the beginning of the hatching period of CM larvae, will conduct to an acceptable population control effect over the whole vegetation period or at least for the first generation. Thus, such an application could give a certain security to the grower for this period. If problems arise with insecticide resistance or imprecise timing of the treatments, there will be no outbreak of the CM population as consequence.

It is not known, as to what happens with the possible persisting fraction of CpGV, if several treatments are applied at rather short intervals. Glen & Payne (1984) supposed that the residuals of several treatments sum up. Further studies are necessary to examine these effects and their possibilities for use in intelligent IPM strategies.

Acknowledgements

We wish to thank the Volkswagen-Stiftung, Hannover, for funding of the project (II/74 037).

References


Codling moth granulovirus –
An efficient tool for codling moth control in IPM

Jutta Kienzle¹, Hubert Gernoth², Markus Litterst³, Claus P.W. Zebitz¹, Juerg Huber⁴
¹ University of Hohenheim, Institute of Phytomedicine, 70593 Stuttgart, Germany
² Amt für Landwirtschaft, Prinz-Eugen-Str. 2, 77654 Offenburg, Germany
³ Obstgrossmarkt Oberkirch, Konrad Adenauer Str. 16, 77704 Oberkirch, Germany
⁴ Institute for Biological Control, BBA, Heinrichstr. 243, 64287 Darmstadt, Germany

Abstract: The joint action of low concentrations of codling moth granulovirus (CpGV), synthetic
insecticides and the „Attract & Kill“-method was tested in the field. It was the aim of these studies to
elucidate whether the impact of the conventional treatments on codling moth population could be
increased by addition of CpGV. Two orchards, situated side by side, served as test plots. In the
orchard additionally treated to the synthetic insecticides and the „Attract & Kill“-method with CpGV,
population control was obviously more efficient than in the orchard without additional treatment
(additional reduction of larvae in trap bands of 86 - 78 % in the first generation). This resulted also in
a better control of damage by the second generation of codling moth. A long term strategy on large
plots is proposed to reduce codling moth populations in areas of high infestation.

Key words: Codling moth, Codling moth granulovirus, long term strategy, joint action,

Introduction

During the past five years, in certain regions of Southern Germany, increasing population
densities of *Cydia pomonella* L. (CM) were observed. In 1999, in addition to the usual
applications of synthetic insecticides like Fenoxycarb, Parathion-Methyl and Tebufenozid,
mating disruption was applied to large areas. Nevertheless, mean infestation of fruit at harvest
was about 8 %. In this situation, the control strategy must focus not only on the immediate
prevention of CM damage but also on the long term reduction of the CM population to an
acceptable level. In the growing season of 2000, codling moth granulovirus (CpGV) was
tested as an additional tool in the strategy for CM population control, since the reduction of
the population was not possible even with the combination of mating disruption and synthetic
insecticides. For a long time CpGV has been considered a rather unattractive product for
Integrated Production (IP). This was mainly due to the high costs of the frequent applications
with the high dosages recommended. Moreover, the efficacy of CpGV in damage control was
rather low in comparison to synthetic insecticides (Höhn et al., 1998). Though it was known
that the effect of CpGV on CM population was definitely higher than the effect on fruit
damage, (Huber & Dickler, 1976; Charmillot, 1998), no attempts were made to use this
potential in control strategies in practice. For CM control, long term effects were considered
to be of inferior importance. In organic fruit growing however, CpGV was used for the past
ten years. In the regions with high infestations of CM, it was observed that in the organic
orchards treated with CpGV for a long time, the infestation seemed to be much lower than in
the orchards with integrated control. In 1999, a field test showed a better efficacy in
population control of CpGV than of phosphoric esters (Kienzle et al., 2001) in combination
with mating disruption. On the basis of these results and experiences, in 2000 a field trial was
started in a region of Southern Germany with very high infestation of CM. The tests should
elucidate whether the use of CpGV in addition to synthetic insecticides and the „Attract & Kill“ method would increase the effectiveness of CM population control.

**Material and methods**

Two orchards of ca. 1 ha each with the variety Jonagold, situated side by side and separated only by a small strip of stone fruit, were treated both with synthetic insecticides and the „Attract & Kill“ method. In one orchard, CpGV was added in low concentrations (6 times 1/10, one time 1/2 and one time 1/3 of the recommended concentration) to each insecticide treatment against CM. On May 18, CpGV was added also to a fungicide spray (Table 1). Summed up, 540 ml Granupom/ha/year (= 1.83 full concentrations/ha/year) at the cost of ca. DM 140/ha/year were used.

Particular attention was paid to the effect of the CpGV treatments on the population of CM. Thus, 100 - 160 trees per orchard were provided with corrugated cardboard belts to monitor descending larvae. To observe the effect at different periods, these belts were controlled three times during the year.

Table 1: Application schedule of the two orchards in comparison. (The normal concentration of the product Granupom® used in the treatments is 0,15 l/ha/m tree height. Thus, 10 % of the normal concentration means 0,015 l/ha/m tree height)

<table>
<thead>
<tr>
<th>Application date</th>
<th>Treatment</th>
<th>kg/l/ha/m tree height</th>
<th>Percentage of the normal dosage of CpGV</th>
</tr>
</thead>
<tbody>
<tr>
<td>06. May</td>
<td>Appeal®</td>
<td>3000 drops/ha</td>
<td>10 %</td>
</tr>
<tr>
<td>13. May</td>
<td>Parathion-Methyl (ME 605®)</td>
<td>0,25</td>
<td>10 %</td>
</tr>
<tr>
<td>18. May</td>
<td></td>
<td></td>
<td>50 %</td>
</tr>
<tr>
<td>08. Jun</td>
<td>Fenoxycarb (Insegar®)</td>
<td>0,2</td>
<td>10 %</td>
</tr>
<tr>
<td>28. Jun</td>
<td>Tebufenozid (Mimic®)</td>
<td>0,25</td>
<td>10 %</td>
</tr>
<tr>
<td>06. Jul</td>
<td>Appeal®</td>
<td>3000 drops/ha</td>
<td>10 %</td>
</tr>
<tr>
<td>21. Jul</td>
<td>Fenoxycarb (Insegar®)</td>
<td>0,2</td>
<td>10 %</td>
</tr>
<tr>
<td>02. Aug</td>
<td>Fenoxycarb (Insegar®)</td>
<td>0,2</td>
<td>33 %</td>
</tr>
<tr>
<td>08. Aug</td>
<td>Parathion-Methyl (ME 605®)</td>
<td>0,25</td>
<td>10 %</td>
</tr>
<tr>
<td>19. Aug</td>
<td></td>
<td></td>
<td>50 %</td>
</tr>
</tbody>
</table>

Fruit damage was assessed on the June 7, June 15, July 18, and August 7 (1000 fruits/plot). All injured fruits were counted (even those with stings). At harvest, in each orchard, 4 x 3 trees were marked and completely harvested. All fruits were assessed for injuries by CM, also the windfall fruit under the marked trees. Stings were considered as injuries.

In 2000, in the region investigated, the first generation of CM appeared very early and in high density. The flight started during the last days of April; the first oviposition was observed on May 8, first larvae on May 16. The unusually high temperatures at the end of April and in May were favourable for the first generation. On the other hand, the cold and
rainy weather in July (first adults of second generation at second decade of July) was rather adverse for the start of the second generation. Later on, the temperatures increased, however, the abundance of the second generation was lower than in other years. The last oviposition was observed on August 20.

Results

In June, the differences in fruit damage were small. In July, due to thinning by the fruit grower, the damage decreased slightly, the differences remained insignificant. Already, during egg deposition of the second generation, a great difference in the number of eggs between the orchard „with GV“ and „without GV“ could be observed. On August 7, the fruit damage in the plot „with GV“ was considerably lower than in that „without GV“ (Fig. 1). At harvest, the difference was very evident.

At each assessment date of the corrugated cardboard belts, in the untreated orchard showed significantly more CM larvae than in the treated one (Fig. 2). On the last assessment date (12.10.00) the additional efficacy of the CpGV treatments in population control was 94.2%.

Fig. 1: Fruit damage in the orchards treated and not treated with GpGV

![Bar chart showing fruit damage in % (including stings) from 07. Jun to Harvest. A: without CpGV, B: with CpGV.](chart1.png)

Additional effect: 86.2 % 78.7 % 94.2 %
(compared to the treatment without CpGV)

Fig. 2: Larvae of CM in the corrugated cardboard belts in the treated and untreated plot

![Bar chart showing number of larvae in the corrugated cardboard belts per tree.](chart2.png)
Discussion

These first results confirm the practical relevancy of the effect on the CM population found by Huber & Dickler (1976). In the first generation, the population density in the CpGV plot was considerably reduced.

With a lower population density, the „Attract & Kill“ method applied in the orchard, certainly could be more effective in the second generation. Thus, in the CpGV-plot, the number of eggs deposited by the adults of the second generation was remarkably lower. This was the most important reason why the fruit damage caused by the second generation was significantly lower in the CpGV treated plot. Again, in the second generation, the additional efficacy of CpGV in population control (94.2 %) was much higher than the efficacy calculated on the fruit damage at harvest (75 %). These one year results were corroborated by practical experiences in the region, in the same year (reduced catches in trap bands) and support the idea of a long term strategy on larger areas in the regions with high infestation.

The aim could be to reduce the population of CM during several generations to an acceptable level. Used in such a kind of strategy, CpGV, generally considered a very expensive and rather inefficient product to use only in areas with low infestation potential, could become one of the most efficient tools to control the current outbreaks of CM. The change of strategy consists in the change of goal from damage control to long term population management. CpGV is used in concentrations and with a frequency of treatments insufficient for damage control but sufficient for population control. Thus, the cost of the CpGV is reduced to ca. DM 100 to DM 140 which is economically acceptable. Additionally, the joint action with mating disruption or „Attract and Kill“ is used. In the first years, for damage control, synthetic insecticides are applied in addition. If the growers learn to effectuate economic calculations not only for the period of one year, but to consider the possibility to reduce insecticide use over several years, they will realize the economic advantages of this kind of strategy.

Further research in commercial orchards is necessary to gain more experience on this subject and to demonstrate to the fruit growers the effect on population control which is very hard to observe in the field. Since the effect is evident only in the second or third generation after the beginning of the treatments, in some regions it can be observed only in the second year. Demonstration projects over several years with the use of corrugated cardboard belts in commercial orchards will be necessary to introduce such a strategy in common practice. In Southern Germany, in 2001, the addition of CpGV to other control methods will be recommended by the extension service for a long term strategy for CM population control in high infestation areas with problems of insecticide resistance.

Acknowledgements

We wish to thank the fruitgrower for his cooperation and the Volkswagen-Stiftung, Hannover, for the funding of the project (II/74 037).

References


Miscellaneous
Occurrence of microsporidia and other pathogens in associated living spruce bark beetles (Coleoptera: Scolytidae) in an Austrian forest

Bernhard Haidler, Rudolf Wegensteiner1, Jaroslav Weiser2

Institute of Zoology, University of Vienna, Austria;
1 Institute of Forest Entomology, Forest Pathology and Forest Protection, Univ.-BOKU-Vienna, Austria;
2 Insect Pathology, Institute of Entomology, CAV, Ceske Budejovice, Czech Republic

Abstract: Eight bark beetle species (Coleoptera: Scolytidae) were recovered from trap log sections in a coniferous forest in the Central Alps of Austria. One to four different pathogen species (mainly microsporidia) were found in all these coleopteran species. Several bark beetle species were found infected by three pathogen species. Differences were observed in infection rates comparing male and female beetles as well as comparing parental and offspring beetles. In addition, differences were found in occurrence and in abundance of pathogens in beetles emerging from log sections of the first trap log series in comparison with those emerging from sections of the second trap log series resp. from branches.

Key words: spruce bark beetles, Scolytidae, pathogens, microsporidia

Introduction

Bark beetles are known to be one of the economically most important insect pests in Norway spruce forests (*Picea abies*) in central and northern Europe. Some of the involved species show a preference for storm-felled or snow broken trees. Especially blow-down areas are a predominant source of outbreaks in many cases. Bark beetle populations periodically undergo rapid increase, starting usually in storm-felled or snow-broken trees, and especially under favourable climatic conditions. As a consequence of intense outbreaks in fallen trees, bark beetles may attack and kill apparently vigorous trees, especially in secondary spruce stands.

In spite of the importance of bark beetles, there is a great lack of knowledge about their pathogens. Especially, very little is known about their potential as pathogens as biological control agents. Several recent investigations focused on the pathogen complex of the economically most important spruce bark beetle, *Ips typographus* (Wegensteiner et al., 1996), but there are only a few reports about pathogens in other spruce bark beetle species (see references in Mills, 1983; Wegensteiner et al., 1996). No examinations were performed with focus on bark beetles living sympatrically on spruce logs (Haidler, 1998). The aim of this study was to investigate the pathogen assemblage in associated spruce bark beetles in an Austrian forest, using trap log series from different parts of freshly felled trap trees (trunk and limbs).

Material and methods

Log sections infested with bark beetles were collected in the year 1996 at one locality in a managed forest in the central Alps of Austria (Tamsweg, Salzburg), south aspect, at a plateau in an altitude of 1600 m (90 % *Picea abies*, 10 % *Larix decidua*). The last five years before
the study bark beetle attacks were registered in this area even on living trees. As a consequence trap trees have been felled and used during this period for concentration of bark beetle attacks. Trap logs of freshly felled trees (the age of trees varied from 50 years up to 150 years) were exposed to infestation by bark beetles for seven weeks, from 05.05.1996 till 23.07.1996 and from 24.06.1996 till 13.08.1996 respectively. In a first series log sections were cut mostly in the commencement of the crown (10 to 20 cm in diameter). In the second sample series, attacked limbs (from tree tops, 1.5 to 4.8 cm in diameter) were acquired too.

Infested material was incubated in the laboratory within breeding chambers, material of the 1st series at 23 ± 3 °C, material of the 2nd series at 20 ± 3 °C and at long day conditions (L:D = 16:8). Emerged beetles were removed daily with aid of an adapted accu-vacuum-cleaner respectively by using a normal exhaustor. Log segments and limbs were sprayed every day with tap water to prevent rapid desiccation and to favour development of bark beetles (see references in Postner, 1974). Removed beetles were stored in Petri dishes in an incubator at 15 ± 1°C, without light, till inspection. Beetles were determined and individuals were counted separately. Evaluation of pathogen infections of all emerged beetles was performed separately for each trap tree resp. concerning sections from the trunk or from limbs. After the expected emergence of beetles, bark was peeled off from log sections to look for dead specimens. For calculation of number of emerged beetles and of infection rates, daily counts were added to get weekly sums. Infection rates were calculated using $\chi^2$ test.

Results and discussion

More than 3000 specimens of bark beetles were dissected during the entire period of investigation (Haidler, 1998). Eight different scolytid species and six different pathogens were found (Table 1). Highest pathogen diversity was found in Hylurgops glabrat us and Ips typographus (4 different pathogens). Dryocoetes autographus, Pityogenes chalcographus and Ips amitinus had three different pathogens. Other bark beetle species had two (Hylastes cunicularius, Pityophthorus pityographus) or one pathogen (Hylurgops palliatus).

Table 1. Pathogens found infecting different spruce bark beetle species in an Austrian forest and abundance of pathogens (in %): ItEPV = Ips typographus Entomopoxvirus, M.s. = Malamoeba scolyti, Neogr. = neogregarinid species, Gregarina = Gregarina sp., Chytrid. = Chytridiopsis cf. typographi, Micros. = unidentified microsporidia; N = number of inspected beetles; pd = pathogen diversity; No. inf. spp. = number of infected bark beetle species.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H. cunicularius</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30.8</td>
<td>7.7</td>
<td>–</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>H. glabrat us</td>
<td>–</td>
<td>1.0</td>
<td>–</td>
<td>1.5</td>
<td>0.5</td>
<td>0.7</td>
<td>403</td>
<td>4</td>
</tr>
<tr>
<td>H. palliatus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>12.5</td>
<td>–</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>D. autographus</td>
<td>–</td>
<td>38.3</td>
<td>–</td>
<td>3.3</td>
<td>–</td>
<td>0.8</td>
<td>120</td>
<td>3</td>
</tr>
<tr>
<td>P. pityographus</td>
<td>–</td>
<td>–</td>
<td>11.4</td>
<td>–</td>
<td>11.4</td>
<td>–</td>
<td>44</td>
<td>2</td>
</tr>
<tr>
<td>P. chalcographus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>28.3</td>
<td>2.0</td>
<td>1.9</td>
<td>1652</td>
<td>3</td>
</tr>
<tr>
<td>I. typographus</td>
<td>21.3</td>
<td>0.4</td>
<td>–</td>
<td>37.8</td>
<td>16.6</td>
<td>–</td>
<td>577</td>
<td>4</td>
</tr>
<tr>
<td>I. amitinus</td>
<td>–</td>
<td>–</td>
<td>0.9</td>
<td>15.9</td>
<td>7.8</td>
<td>448</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>No. inf. spp.</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Pathogen diversity in *I. typographus* was not as high as found in previous investigations (Wegensteiner *et al.*, 1996). In the case of other bark beetle species only few data are available (see references in Wegensteiner *et al.*, 1996). Infections with ItEPV occurred only in *I. typographus*, while one unidentified neogregarinid species was found in *P. pityographus*. *M. scolyti* was found in three different bark beetle species. *Gregarina* sp. and *Chytridiopsis* cf. *typographi* were found in 6 different bark beetle species. Spores of different microsporidian species, up to now totally unknown and not exactly identified yet, occurred in the adipose tissue of five different host species. Surprisingly, *Nosema typographi* and *Unikaryon montanum* were not observed in *I. typographus* (Wegensteiner *et al.*, 1996; Weiser *et al.*, 1997, 1998). Infection rates were relatively high in *D. autographus* with *M. scolyti* (38.3 %), in *I. typographus* with *Gregarina typographi* (37.8 %) and with the ItEPV (21.3 %), in *H. cunicularius* with *Gregarina hylastidis* (30.8 %), and in *P. chalcographus* with *Gregarina* cf. *typographi* (28.3 %) respectively (Table 1).

Furthermore, differences were found in *Gregarina* sp. and *Chytridiopsis* cf. *typographi* infection rates of male and female beetles as well as in infection rates of parents and offspring. The observed situation was depending on the bark beetle species and the pathogen species (Table 2).

### Table 2. Occurrence and abundance (in %) of selected pathogens in males and female parents and offspring bark beetles in an Austrian forest.

<table>
<thead>
<tr>
<th></th>
<th><em>Gregarina</em> sp.</th>
<th><em>Chytridiopsis</em> sp.</th>
<th>Microsporidia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>males</td>
<td>females</td>
<td>males</td>
</tr>
<tr>
<td><em>H. glabratius</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>offspring</td>
<td>3.9</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td><em>D. autographus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>5.9</td>
<td>4.3</td>
<td>–</td>
</tr>
<tr>
<td>offspring</td>
<td>0.0</td>
<td>0.0</td>
<td>–</td>
</tr>
<tr>
<td><em>P. pityographus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>–</td>
<td>–</td>
<td>40.0</td>
</tr>
<tr>
<td>offspring</td>
<td>–</td>
<td>–</td>
<td>0.0</td>
</tr>
<tr>
<td><em>P. chalcographus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>58.1</td>
<td>37.4</td>
<td>4.3</td>
</tr>
<tr>
<td>offspring</td>
<td>15.3</td>
<td>15.0</td>
<td>2.0</td>
</tr>
<tr>
<td><em>I. typographus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>38.7</td>
<td>48.0</td>
<td>19.4</td>
</tr>
<tr>
<td>offspring</td>
<td>30.1</td>
<td>30.1</td>
<td>1</td>
</tr>
<tr>
<td><em>I. amitinus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parents</td>
<td>3.4</td>
<td>2.2</td>
<td>16.9</td>
</tr>
<tr>
<td>offspring</td>
<td>0.0</td>
<td>0.0</td>
<td>22.3</td>
</tr>
</tbody>
</table>

With regard to the origin of the recovered beetles (first or second trap log series), it is interesting to note that three different pathogens, *G. typographi*, *C. typographi* and microsporidia, were found in *P. chalcographus* from all the trap material. Three of the four different pathogens, i.e. *M. scolyti*, *G. typographi* and *C. typographi*, were found only in *I. typographus* from log sections. The ItEPV was found in *I. typographus* from logs of the 1st log series and from branches of the 2nd trap log series only. *Chytridiopsis* sp. and microsporidia were found in *I. amitinus* from all the trap material, *Gregarina* sp. in beetles from log sections only (from both series) (Table 3).

Significantly more *P. chalcographus* and *I. typographus* were found infected with *G. typographi* (p < 0.05 and p < 0.005) from sections of the first trap log series compared to
sections of the second trap log series. In contrast, *C. typographi* was found rather more frequently in beetles from the second trap log series. Microsporidia in the adipose tissue were found more frequently in *P. chalcographus* from branches in comparison with beetles from log sections (*p* < 0.025) (Table 3).

Table 3. Occurrence and abundance (in %) of selected pathogens (for abbreviations see Table 1) in three bark beetle species from log sections (log 1 = 1st trap log series, log 2 = 2nd trap log series) and from branches (branch; from the 2nd trap log series) in an Austrian forest (*n* = number of inspected beetles).

<table>
<thead>
<tr>
<th></th>
<th>ItEPV</th>
<th>M.s.</th>
<th>Gregarina</th>
<th>Chytrid.</th>
<th>Micros.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chalcographus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log 1</td>
<td>–</td>
<td>–</td>
<td>32.9</td>
<td>1.8</td>
<td>1.8</td>
<td>510</td>
</tr>
<tr>
<td>log 2</td>
<td>–</td>
<td>–</td>
<td>23.1</td>
<td>2.5</td>
<td>0.4</td>
<td>512</td>
</tr>
<tr>
<td>branch</td>
<td>–</td>
<td>–</td>
<td>28.9</td>
<td>1.8</td>
<td>3.2</td>
<td>630</td>
</tr>
<tr>
<td><em>I. typographus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log 1</td>
<td>26.4</td>
<td>0.2</td>
<td>45.4</td>
<td>17.9</td>
<td>–</td>
<td>463</td>
</tr>
<tr>
<td>log 2</td>
<td>–</td>
<td>0.9</td>
<td>7.1</td>
<td>11.6</td>
<td>–</td>
<td>112</td>
</tr>
<tr>
<td>branch</td>
<td>50.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td><em>I. amitinus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log 1</td>
<td>–</td>
<td>–</td>
<td>0.9</td>
<td>11.5</td>
<td>9.4</td>
<td>234</td>
</tr>
<tr>
<td>log 2</td>
<td>–</td>
<td>–</td>
<td>1.3</td>
<td>25.6</td>
<td>5.1</td>
<td>156</td>
</tr>
<tr>
<td>branch</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6.9</td>
<td>8.6</td>
<td>58</td>
</tr>
</tbody>
</table>

Acknowledgements

We want to thank Dipl. Ing. Müller and OFö Thomasberger from the Austrian Federal Forest Administration in Tamsweg (Salzburg) and A. Stradner as well as R. Wanjek from the Institute of Forest Entomology, Forest Pathology and Forest Protection (Univ.-BOKU-Vienna).

References


Ultrastructure and temperature sensitivity of bacteriomes in *Prostephanus truncatus* (Horn)

R. G. Kleespies, C. Nansen,¹ T. Adouhoun,¹ A. M. Huger

Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Heinrichstrasse 243, 64287 Darmstadt, Germany;
¹ International Institute of Tropical Agriculture, Biological Control Center for Africa, B.P. 08-0932, Cotonou, Republic of Benin

Abstract: In these studies it was shown that both larvae and adults of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae) have a pair of bacteriomes dorsally located in the fat body parallel to the midgut. Bacteriomes development was shown mainly to occur during larval stages. Bacteriome size was not found to be associated with body size in adults, but in larvae reared at 30 °C bacteriome size increased progressively with body length. As heat treatment elsewhere has been used to eliminate endosymbionts in other insect orders, the potential heat sensitivity of symbiotes was evaluated. For this purpose, the bacteriome dimensions of larval and adults reared at temperatures of 30, 35 and 37 °C were measured. The symbiotes could not be eliminated but a significant reduction of bacteriome size was found in females reared at 35 and 37°C as compared to specimens grown at 30 °C.

Key words: bacteriomes, bacteroids, endocytobiosis, *Prostephanus truncatus*, temperature

Introduction

Since the accidental introduction of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae) first to East Africa in 1981 and a few years later (1984) to West Africa, it has become a major pest on maize and cassava and it is still spreading into new areas. Little is known about possible limiting factors regarding the establishment of *P. truncatus* and/or its confinement to ambient climate conditions. Only a few studies have been carried out with regard to intracellular symbiotes in bostrychids. In the present work light microscope studies of the symbiotes are shown and the potential implications of heat sensitivity of these symbiotes will be discussed in an integrated pest management context.

Material and methods

**Insect cultures**

*P. truncatus* specimens originated from the laboratory culture on maize kernels at the International Institute of Tropical Agriculture (IITA) station in Cotonou, Benin. Rearing was conducted at 30 ± 5 °C and 65 ± 10 % RH.

**Light microscopy**

Larvae, adult males and females were dissected for isolation of bacteriomes. Wet mount preparations of squashed bacteriomes were examined with phase contrast. Larvae and adults were fixed with Dubosq-Brazils alcoholic Bouin’s and embedded in histosec.
**Bacteriome dimensions**

*P. truncatus* bacteriome diameter was determined from dissection under light microscope (x 160). Bacteriome diameter were obtained from a total of 150 females, 84 males and 114 larvae reared at 30, 35 or 37 °C. Mixed model procedure in SAS 6.12 for Windows was used to examine the effect of temperature on bacteriome diameter.

**P. truncatus reproduction at 30 °C**

In order to assess the effect of temperature conditions on *P. truncatus* reproduction, adults <7 days old and of unknown mating status were transferred to jars containing 200 g maize kernels and reared at 30 °C. For each temperature culture, ten replicates comprising 20 unsexed *P. truncatus* specimens were established (total 30 jars). Differences in reproduction of these new cultures at 30 °C would be attributed to the previous rearing conditions. The reproduction was assessed after two months, which according to Bell & Watters (1982) is equal to approximately two generations, by counting the number of larvae and adults after excluding the initial 20 individuals.

**Results and discussion**

**Stereoscopy and light microscopy**

Dissection studies showed that both larvae and adults had a pair of bacteriomes (Fig. 1) with the same position in males, females and larval stages. The bacteriomes were located in the fat body parallel to the midgut with the anterior bacteriome at caeca and the posterior near the ventriculus, both on the dorsal side. The bacteriomes were anchored with connective tissue and trachea (Figure 1). In wet mount preparations of larval, female and male bacteriomes, bacteroids and nuclei were found to be spherical- to oval-shaped (Fig. 2).

**Bacteriome dimensions and reproduction at different temperatures**

In larvae reared at all three temperatures, the size of bacteriomes increased progressively with body length. However, the diameter of bacteriomes in larvae reared at 35 and 37°C only reached about half the size as in larvae reared at 30 °C (Fig. 3). Where examining bacteriome diameter within the larval length classes, larvae reared at 30 °C had significantly larger bacteriomes than larvae reared at 35 °C (*F*<sub>1,102</sub> = 97.33; *P* < 0.001); however, there was no significant difference between bacteriome diameter in larvae reared at 35 °C compared to 37 °C (*F*<sub>1,102</sub> = 0.46; *P* = 0.501).

There was no significant difference in bacteriome size in males from the three temperature cultures (*F*<sub>2,81</sub> = 1.64; *P* = 0.20). In females, bacteriome size (*F*<sub>2,147</sub> = 110.00; *P* < 0.001) varied significantly among specimens reared at the three temperatures. Females reared at 30 °C had significantly larger bacteriomes compared to females reared at 35 and 37 °C (*F*<sub>1,147</sub> = 166.26; *P* < 0.001), but there were no significant differences in the bacteriome sizes of females reared at 35 °C compared to 37 °C (*F*<sub>1,147</sub> = 2.57; *P* = 0.111).

The reproduction varied significantly among temperature cultures (*F*<sub>2,27</sub> = 8.30; *P* = 0.002), and it was, on average, twice as high for the 30 °C cultures compared to 35 °C cultures (Fig. 4). There was no significant difference in reproduction of cultures reared at 35 °C compared to 37 °C (*F*<sub>1,27</sub> = 3.29; *P* = 0.081).

**Size of bacteriomes**

In aphids (Lamb & Hinde, 1967), coccids (Koch, 1960) and the bostrychid *Rhyzopertha dominica* (Huger, 1956) it has been shown that the size of organs harbouring intracellular symbiotes was linearly correlated with larval length, while Lamb & Hinde (1967) showed that the size of mycetocytes among adult stages of aphids did not vary. A progressive increase in bacteriome diameter was also found in the present study in larvae reared at 30 °C, but in larvae reared at 35 and 37°C the bacteriome diameter was markedly reduced, and bacteriomes
only reached about half the size compared to larvae reared at 30 °C. It is therefore suggested
that constant temperatures above 30 °C may suppress bacteriome development. Bacteriomes
in the largest larvae, reared at 30 °C, were slightly smaller than bacteriomes of adult females
but larger than in adult males. This suggests that most of the bacteriome development
occurred during larval stages, and that during the pupal stage and early imago, bacteriomes
started to degenerate in males while they continued to increase in size in females.

Fig. 1. Two bacteriomes (B) prepared from a *P. truncatus* female adjacent to the midgut
(mg). Note: Bacteriomes are anchored to the midgut (mg) by tracheae (t) and
connective tissue (ct). Bar = 0.2 mm.

Fig. 2. Squash preparation of a larval bacteriome from *P. truncatus* with myriads of
bacteroids (b) in various stages of division and several nuclei (n) of the syncytium.
Phase contrast, bar = 20 µm.

Elimination of intracellular symbiotes is difficult in insects with transovarial transmission
of symbiotes (Anand & Pant, 1980), but rearing at elevated temperatures is known as a
treatment to obtain aposymbiotic insect strains (Houk & Griffiths, 1980; Douglas, 1989).
Bacteriomes in *P. truncatus* could not be eliminated with the tested constant temperatures,
and in males there was no temperature effect on the bacteriome diameter. However in
females, bacteriomes were significantly smaller among specimens reared at 35 and 37 °C
compared to 30 °C. A possible explanation for the difference in bacteriome heat sensitivity
between sexes is that bacteriomes are probably more metabolically active in females than in
males and therefore also more susceptible to high temperatures.
Fig. 3. Larval length classes collected from laboratory cultures at 30, 35 and 37 °C: Class 1 = 1 mm - < 2.3 mm (25); class 2 = 2.3 mm - < 3.15 mm (31); class 3 = 3.15 mm - < 3.77 mm (27); class 4 = 3.77 mm - ≤ 6 mm (31). Each bar represents the average of 5-10 larvae with corresponding standard deviation.

Fig. 4. Reproduction of *P. truncatus* previously reared at 30, 35 and 37 °C for three months and then transferred to 30 °C for two months. Bars represent the average of 10 replicates with corresponding standard deviation.

*P. truncatus* specimens transferred from either 35 or 37 °C to 30 °C had a significantly lower reproduction compared to the culture which remained at 30 °C throughout the experiment. Changing the temperature from either 35 or 37 °C to 30 °C may affect reproduction, but not necessarily reduce it. Bell & Watters (1982) showed that mortality of
eggs, larvae, and pupae of *P. truncatus* was about the same when reared at 32 °C compared to 30 °C, but it increased markedly between 32 and 35 °C. It may be hypothesized that this increase in mortality, when reared at temperatures above 32 °C, is at least to some extent associated with the heat sensitivity of intracellular symbiotes. Similarly, we find it reasonable to assume that the lower reproduction of specimens originally reared at 35 and 37 °C but transferred to 30 °C was related to their significantly smaller bacteriomes.

The indirect influence of ambient temperature on reproduction of *P. truncatus* (through its affect on bacteriome development) may also be tested in an IPM context, as increasing the internal temperature in small-holders maize stores may be a way to reduce the fitness of this pest insect, and thereby the losses of stored products associated with its presence in stores.

**Acknowledgements**

We wish to thank the Danish Council of Development Research for financing the present study. Dr F. Schulthess and Dr W. G. Meikle are thanked for their comments on the manuscript, and Dr Dietrich Stephan for the discussion of the statistical data.

**References**

Bell, R.J. & Watters, F.L. 1982: Environmental factors influencing the development and rate of increase of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) on stored maize. J. Stored Product Pest Res. 19: 131-142.
A new canestriniid mite (Acari, Astigmata, Canestriniidae) associated with *Blaps polychresta* Forsk. (Insecta, Coleoptera, Tenebrionidae)

Sh. M. O. El Bishlawy, S. F. M. Allam
Department of Agricultural Zoology and Nematology, Faculty of Agriculture, Cairo University, Egypt

**Abstract**: Several specimens of a new mite species were collected from the beetle *Blaps polychresta* Forsk. found in a palm farm in Ismaielia Governorate, Egypt. This new species, which belongs to the genus *Percanestrinia* (Berlese, 1911) (Acari, Astigmata, Canestriniidae) is described and illustrated.

**Key words**: canestriniid mite, *Percanestrinia*, new species, tenebrionid, *Blaps polychresta*

**Introduction**

Canestriniid mites are found on many families of insects. The genus *Percanestrinia* (Berlese, 1880) was established for one species *Alloptes blaplis* collected from Sardinia. This species was found also in Hungary and Bulgaria (Samsinak, 1970a). According to Samsinak (1970b) the genus *Percanestrinia* is synonym of the genus *Canestrinia*. Haitlinger (1990) found 9 genera and 28 species of canestriniid mites found on passalid beetles. The same author, in 1992, described 3 species belonging to the genus *Percanestrinia* obtained from tenebrionid beetles.

**Material and methods**

Mites were collected from the beetle *Blaps polychresta* Forsk. (Coleoptera: Tenebrionidae) from palm farm in Ismaielia governate, Egypt. The mites were beneath the elytra of the insect host. Mites were mounted in Hoyer's media. All measurements (in micrometers, µm) are given as holotype (one female) or allotype (one male). We followed the nomenclature for body setation according to Griffiths et al. (1990).

**Results**

*Percanestrinia egypti* n. sp. (description)

**Female**: (Figs 1a, 1b and 1c, and Table 1)

Body pear shape; length: 1.38 µm, width: 1.003 µm.

**Gnathosoma**: chelicerae robust, fixed and movable digit with teeth, distal palpal segment with sensella. Palpas length (0.466 µm), basal segment with one dorsal and one ventral setae.

**Dorsum**: largely covered by irregular sclerites shaped, sejugal furrow absent. Vi setae triforcate, Ve setae filiform, Scx setae barbed, Sci setae biforcate, Sce very long filiform setae. Dorsal setae (d1-d4) filiform, d5 and L5 long filiform setae located on the ventral side. L1, L2 and L4 diforcate setae, L3 and L5 setae filiform. Humeral (h) long filiform setae.

**Ventrum**: anterior apodemes of coxal fields I fused medially forming Y shape. Posterior apodemes of coxal fields I short. Anterior apodemes of coxal fields II thickened, the posterior...
apodemes of the same coxa short. Apodemes of coxal fields III arched, posterior apodemes III fused with anterior apodemes IV. Posterior apodemes of coxal fields IV short. Subhumeral setae (sh) biforcate, anterolateral of leg III. Coxal fields I, III and IV each with a pair of filiform setae. Genital region with three pairs of small simple genital setae (ga, gm and gp) and two pairs of genital papillae each with two segments. Ovipore between coxal fields III-IV elongate, with genital valves forming inverted V shape. Anal region with four pairs of filiform setae (a1, a2, a3 and a4).

Legs: with all five segments free.

Chaetotaxy: tarsi 7-7-5-5; tibiae 0-0-0-0; genua 2-2-0-0; femora 1-1-0-0; trochanters 0-0-0-0.

Solenidiotaxy: tarsi 3-1-2-1; tibiae 1-1-1-1; genua 1-1-1-0; femora 0-0-0-0. Pretarsi I-IV with empodium and membranous ambulacra; condylophores present. Setae hT and kT absent on tibia I and III.

Male: (Figs 2a, 2b and 2c, and Table 1)
General morphology as in female; length: 1.17 µm, width: 0.9775 µm.

Gnathosoma as in female.

Dorsum as in female but the dorsal setae 4 (d4) is biforcate, L4 long filiform setae, located ventrally. d5 and L5 very long filiform setae, found on the ventral side also.

Ventrum: anterior apodemes of coxal field I fused as in female. Anterior apodemes of coxal field III arched, posterior apodemes of the same coxa fused with anterior one of coxa IV. Coxal field III and IV each with a pair of filiform setae. Anal capulated suckers sitting in the base of rounded opisthosomal lobes, with three pairs of anal setae a1, a2 and a3. Genital region located on the level of the base of coxa IV with two pairs of gential desicke.

Legs: Chaetotaxy: tarsi 6-4-3-4; tibiae 0-0-0-0; genua 2-2-1-0; femora 1-1-0-0.

Solenidiotaxy: tarsi 3-2-1-1; tibiae 1-1-1-1; genua 1-1-0-0; femora 0-0-0-0. Pretarsi I-IV as in female.

Types
Holotype: one female collected from the beetle Blaps polychresta Forsk. found in a palm farm in Ismaielia Governorate, Egypt, and kept in the Acari Collection of the Faculty of Agriculture, Cairo University. Allotype: one male collected in the same situation as the holotype.

Paratypes: several females and males collected in the same situation as the holotype and the allotype.

Discussion
The genus Percanestrinia was distinguished by several characters. Idiosoma are not ornamented or weakly ornamented by transversal or longitudinal lines. Opisthosoma are narrow (males) but with posterior part enlarged. Setae vi, hi and sci are enlarged. Males have two suckers, below them 5-6 setae, at least one of them thick. Setae a are long, with basis somewhat enlarged, many times longer than gp. Legs III-IV are thicker than legs I-II; seta on genua III enlarged. Ventral setae on tarsi I, II are thin. Females have 6 pairs of setae below genital region (except d5, L5).

The new species Percanestrinia egypti is closely related to P. maroccana Cooreman. It can be separated from the latter by the following differences: sex setae barbed; sci, L1, L2, L4 (female) and d4 (male) setae biforcate; genital region in both male and female with 3 pairs of setae; female anal region with 4 pairs of setae. The position of a4 differs.
Table 1: Size (in µm) of idiosomal setae in *Percanestrinia egypti* n. sp.

<table>
<thead>
<tr>
<th>Character</th>
<th>Female (mean)</th>
<th>Male (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vi</td>
<td>0.18</td>
<td>0.90</td>
</tr>
<tr>
<td>Sci</td>
<td>0.15</td>
<td>0.74</td>
</tr>
<tr>
<td>Sce</td>
<td>1.43</td>
<td>0.76</td>
</tr>
<tr>
<td>Scx</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td>H</td>
<td>0.50</td>
<td>0.16</td>
</tr>
<tr>
<td>d1</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>L1</td>
<td>0.10</td>
<td>0.06</td>
</tr>
<tr>
<td>d2</td>
<td>0.012</td>
<td>0.02</td>
</tr>
<tr>
<td>d3</td>
<td>0.04</td>
<td>0.0124</td>
</tr>
<tr>
<td>L3</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>d4</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>d5</td>
<td>0.30</td>
<td>0.1</td>
</tr>
<tr>
<td>L5</td>
<td>0.45</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Acknowledgements

This study was supported by a grant from the Applied Center for Entomonematodes, Faculty of Agriculture, Cairo University. We thank very much Dr Barry M. Oconnor, Museum of Zoology and Department of Biology, University of Michigan, Ann Arbor, Michigan, USA, for his help in identification and collecting researches which were very important in this study. We also thank Dr Ryszard Haitlinger, Katedra Zoologii Akademii Rolniczej, Ul. Cybulskiego, Poland for his help in this study.

References


Figure 1a. *Percanestrinia egypti* n. sp., female: dorsal.

Figure 1b. *Percanestrinia egypti* n. sp., female: ventral.

Figure 1c. *Percanestrinia egypti* n. sp., female: legs I to IV.
Figure 2a. *Percanestrinia egypti* n. sp., male, dorsal.

Figure 2b. *Percanestrinia egypti* n. sp., male, ventral.

Figure 2c. *Percanestrinia egypti* n. sp., male: legs I to IV.
Utilization of essential oils and chemical substances alone or in combination against Varroa mite (Varroa destructor), a parasite of honeybees

S. F. M. Allam, M. F. Hassan, M. A. Risk¹, A. U. Zaki¹
Department of Agricultural Zoology and Nematology, Faculty of Agriculture, Cairo University, Egypt
¹ Plant Protection Institute, Ministry of Agriculture Research Center of Agriculture, Fayoum, Egypt

Abstract: The acaricide efficacy of essential oils, i.e. jasmine oil, neem oil, and black cumin oil, and a chemical insecticide, Mavrik, with formic acid, used alone or in combination, was tested against the honeybee parasitic mite, Varroa destructor Oudemans. The potency of these substances was evaluated in honeybee colonies during August, September and October 2000. Cloth stripes saturated with the tested substances were suspended between the middle combs in all treatments during the study. Levels of infestation with Varroa destructor in both the brood and adult honeybees were estimated before and after the treatment. The tested natural four substances are safe to both honeybees and humans.

Key words: Varroa destructor, honeybee mite, control, essential oils, jasmine oil, neem oil, black cumin oil, Mavrik, formic acid

Introduction

Varroa is a dangerous pest for beekeeping and for crops that require insect pollination (Witherell & Herbert, 1988). The control of Varroa mites is especially difficult as the majority of mites stay in the sealed brood for reproduction and are therefore well protected from different forms of treatment (Hoppe et al., 1989). Acaricides appeared effective against Varroa mites but their application within the hives tends to contaminate the wax and honey. Furthermore, the mites can develop resistance against these chemicals (Ifantidis, 1987). For these reasons, Dung & Huan (1992) suggested that the control of Varroa mite in the future should be based rather on biotechnical control methods such as trapping of mites in the drone brood and using heat treatment (42-44 °C for 20-30 min) to reduce the mite population inside the colony. On the other hand, several people used essential oils to control the pest (Fathy & Fouly, 1993, 1995; El-shemy et al., 1997). In such a context, the present work was carried out to evaluate the efficacy of three essential oils, namely jasmine oil, neem oil and black cumin oil, and a chemical pesticide (Mavrik and formic acid), used alone or in combination, in controlling the Varroa mite, Varroa destructor as identified by Anderson & Trueman (2000).

Materials and methods

Tests for controlling Varroa mites were carried out during August, September and October 2000. Twenty one naturally infested honeybee colonies nearly similar in their strength and headed by hybrid Egyptian queens (Apis mellifera lamerkii) which mated with hybrid carnio lan drones (Apis mellifera carnica) (H. L. H.), were used.
The colonies were divided into seven stocks (3 colonies as replicates for each treatment and 3 colonies as control). Preliminary tests were carried out to decide the appropriate dose of oil, and to estimate the side effects of oils on bees and queens.

**Products**
- Jasmine oil (*Jasminum grandiflorum*, from the Family Oleaceae) extract of flowers.
- Neem oil (*Azadirachta indica*, from the Family Melioidae).
- Black cumin oil (*Nigella sativa*, from the Umbellifereae) extract of seeds.
- Mavrik (22.3 % fluvalinate) (Mavrik aquaflow Novartis).
- Formic acid (85 % concentration).

**Preparing essential oils and mixtures**

Jasmine oil was prepared by mixing 15 ml oil with 0.5 ml tritonx (emulsifier) and sufficient water to obtain 100 ml of solution.

Neem oil was prepared by mixing 15 ml oil with sufficient water to obtain 100 ml of solution.

Black cumin oil was prepared by mixing 15 ml oil with 0.5 ml triton x (emulsifier) and sufficient water to obtain 100 ml of solution.

The first mixture was 5 ml jasmine oil, neem oil and black cumin oil with water added to obtain 100 ml of solution.

The second mixture was 7.5 ml mavrik and 7.5 ml formic acid with sufficient water added to obtain 100 ml of solution. Each colony received 10 - 15 ml of solution.

The third mixture was 5 ml mavrik, jasmin oil and black cumin oil with 85 ml water added to obtain 100 ml of solution.

**Experimental procedures**

The queen of each colony was caged in a small cage to be protected from bees during the day of application and was released in the next day.

All colonies were treated with strips of cotton material (20 x 2.5 cm x 5 mm) which saturated with oils (10-15 ml) from solution. One strip was hanged between the middle combs every week in all treatments for 11 weeks.

**Assessment of the efficacy levels**

The infestation levels in all experiment colonies were determined before and after each application. The following data were recorded:
- number of dead mites fallen down on a white cardboard (coated with vaseline) located under the colony.
- number of mites in random sample of approximately 50 live bees.
- number of mites in 10 cells of each worker and drone brood (if available) or in 20 workers cells if drone brood was not available.
- the area of sealed worker brood (colony strength).

The efficacy of the tested essential oil was calculated after each application according to the equation of Girdani & Leporati (1989):

\[
\text{rate of efficacy} \% = \frac{\text{No. of dead Varroa mites}^{*}}{\text{Total No. of Varroa mites}^{**}} \times 100
\]

* Dropped mites as a result of the treatment + natural mortality.
** Dropped mites + natural mortality in control + No. of mites on 50 live bees + No. of mites in 10 cells of brood.
Results and discussion

All experimental data are presented in Table 1.

**Jasmine oil**
The efficacy rate ranged from 12.4 to 40.8 % (average: 24.4 %) through all treatments. Number of dead mites ranged from 4 to 58.3 mites/col. weekly (average: 17.7 mites/col.). These results are similar to those of Fathy & Fouly (1993) who used different concentrations of camphor oil to control *Varroa* mites. Also El-shemy *et al.* (2001) used clove oil in 1996-97. They found that rate of efficacy ranged from 45.75 to 53.98 %.

**Neem oil**
The efficacy rate ranged from 18.5 to 70.7 % (average: 35.7 %). Number of dead mites ranged from 5.3 to 115.3 mites/col. weekly (average: 29.7 mites/col.). These results agree with those of Liu (1995) who reported that the use of a small amount of neem extract as an additive to the sugar syrup may be a promising approach to treat chalkbrood and *Nosema* diseases of the honeybee. He also observed no long-term effect on the honeybee. Naumann & Isman (1996), when studying the toxicity of neem oil to larval honeybees, observed that no dead larvae still laying in the cells, no deformed pupae or adults and no significant effects on adult longevity in the survivors.

**Black cumin oil**
The efficacy rate ranged from 6.5 to 43.1 % (average: 24 %) through all treatments. Number of dead mites ranged from 5.7 to 36.3 mites/col. weekly (average 14.4 mites/col.). The black cumin oil is commonly found in Egypt. The plants were cultivated around beekeeping for this purpose. It was observed more activity in colonies treated with blackcumin oil (with regard to the brood area and number of combs).

**Mixture of jasmine, neem and blackcumin oils**
The efficacy rate ranged from 20.8 to 66.9 % (average: 47.6 %). Number of dead mites ranged from 18.3 to 158 mites/col. weekly (average: 44.20 mites/col.). These results were the best ones, with regard to the efficacy rate and the mortality. These results could be compared to those of Imdrof *et al.* (1999) who reported that an aerosol treatment of a thyme-sage oil mixture had been used successfully for mite control.

**Mixture of Mavrik and formic acid**
The efficacy rate ranged from 19.3 to 61.2 % (average: 44.2 %). These relatively low values could be due to the development of resistance to fluvalinate in mites. These results are in agreement with those of Hilleshem *et al.* (1996) in Switzerland who recorded a mite resistance to pyrethroids compounds. Also El-shemy *et al.* 2001) found that the efficicacy rate was higher during 1996 (79-95 %) than 1997 (49.6 %) when they used apistan strips in controlling *Varroa* mites. Number of dead mites mortality ranged from 6.7 to 95.7 mites/col. weekly (average: 44.8 mites/col.). No side effect was observed towards bees and queens.

**Mixture of Mavrik, jasmine and blackcumin oils**
The efficacy rate ranged from 11.3 to 80.9 % (average: 38.2 %). Number of dead mites ranged from 7.7 to 59.3 mites/col. weekly (average: 24.7 mites/col.). No hazards towards bees or queen were observed during the application. These results are in accordance with those of Mutinelli *et al.* (1993). These authors used the Apilifevar formulation which consist of 74.1 % (as a carrier) to control the *Varroa* mite. They found an efficacy rate of 78.7 %. Similarly, Imdrof *et al.* (1999) reported that only a combination of wintergreen oil and thermal treatment, an aerosol treatment of thyme-sage oil mixture and the passive evaporation of thymol, oregano oil and marjoram oil in combination with diluted formic acid have been used successfully for mite control.
Table 1. Effects of various essential oils and Mavrik chemical pesticide, alone or in combination, on *Varroa destructor* infestation in honeybee colonies (M: No. of dead mites on the cardboard; W: No. of mites on 10 pupae (workers); B: No. of mites on 50 bees).

<table>
<thead>
<tr>
<th>Date (2000)</th>
<th>Jasmine oil</th>
<th>Neem oil</th>
<th>Black cumin oil</th>
<th>Jasmin + neem + black cumin oils</th>
<th>Mavrik + formic acid</th>
<th>Mavrik + jasmine + black cumin oils</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>23/8</td>
<td>M: 5.0 W: 0 B: 1 R%: 40.0</td>
<td>M: 15.7 W: 0 B: 0 R%: 70.7</td>
<td>M: 9 W: 0 B: 5.4 R%: 43.1</td>
<td>M: 18.3 W: 2.5 B: 1.2 R%: 64.2</td>
<td>M: 6.7 W: 1.5 B: 0.5 R%: 44.1</td>
<td>M: 7.7 W: 2.0 B: 1.5 R%: 43.5</td>
<td>Toc: 6.5 W: 2 B: 0.6 R%: 36</td>
</tr>
<tr>
<td>29/8</td>
<td>M: 5.7 W: 1 B: 2 R%: 33.3</td>
<td>M: 11.7 W: 0 B: 1.9 R%: 51.8</td>
<td>M: 8.3 W: 0.5 B: 3.5 R%: 38.9</td>
<td>M: 24.3 W: 2 B: 1 R%: 66.9</td>
<td>M: 12.7 W: 2.5 B: 0 R%: 52.5</td>
<td>M: 15.7 W: 0.5 B: 1.5 R%: 58.5</td>
<td>Toc: 9 W: 2 B: 1 R%: 31</td>
</tr>
<tr>
<td>5/9</td>
<td>M: 6.0 W: 0 B: 27.0 R%: 12.4</td>
<td>M: 5.3 W: 1.5 B: 5.6 R%: 19.0</td>
<td>M: 11 I: 1 B: 3 R%: 36.1</td>
<td>M: 19.7 W: 0.2 B: 4 R%: 50.0</td>
<td>M: 13.3 W: 0 B: 1.5 R%: 43.9</td>
<td>M: 11.5 W: 1.5 B: 37.3 R%: 15.5</td>
<td>Toc: 1.5 W: 4.7 R%: 35</td>
</tr>
<tr>
<td>12/9</td>
<td>M: 19.3 W: 1 B: 1.5 R%: 33.7</td>
<td>M: 11.7 W: 2 B: 4.1 R%: 22.7</td>
<td>M: 6.2 W: 2.5 B: 0.7 R%: 13</td>
<td>M: 20.3 W: 0.5 B: 1 R%: 35.4</td>
<td>M: 39.0 W: 0.5 B: 2 R%: 50.7</td>
<td>M: 18 W: 0.8 B: 33.2 R%: 35.5</td>
<td>Toc: 0.5 W: 3.9 R%: 32</td>
</tr>
<tr>
<td>19/9</td>
<td>M: 10.7 W: 4.5 B: 1.5 R%: 19.0</td>
<td>M: 14.7 W: 1 B: 0.6 R%: 263</td>
<td>M: 11 I: 3 B: 3.5 R%: 3.5</td>
<td>M: 19 I: 4.5 B: 1 R%: 47.6</td>
<td>M: 14.7 W: 0 B: 0.8 R%: 26.8</td>
<td>M: 14.7 W: 0.5 B: 4.5 R%: 25</td>
<td>Toc: 39.5 W: 5 B: 8.1 R%: 29</td>
</tr>
<tr>
<td>26/9</td>
<td>M: 11.3 W: 1 B: 0 R%: 13.6</td>
<td>M: 26.7 W: 0.5 B: 0 R%: 27.2</td>
<td>M: 8.7 W: 1 B: 0 R%: 11</td>
<td>M: 37.7 W: 3 B: 0 R%: 33.4</td>
<td>M: 23.3 W: 3 B: 0 R%: 23.9</td>
<td>M: 9 W: 0 B: 11 R%: 3</td>
<td>Toc: 71.0 W: 10 B: 0 R%: 29</td>
</tr>
<tr>
<td>3/10</td>
<td>M: 4.0 W: 3 B: 2.5 R%: 4.6</td>
<td>M: 25.3 W: 2.5 B: 2.5 R%: 23.3</td>
<td>M: 5.7 W: 2 B: 1 R%: 6.5</td>
<td>M: 23.7 W: 7.5 B: 4.5 R%: 20.8</td>
<td>M: 19.0 W: 0.5 B: 0.6 R%: 19.3</td>
<td>M: 15 W: 1.5 B: 2 R%: 15.5</td>
<td>Toc: 78.5 W: 7 B: 6 R%: 29</td>
</tr>
<tr>
<td>10/10</td>
<td>M: 6.7 W: 0.5 B: 5.1 R%: 11</td>
<td>M: 27 W: 2 B: 3 R%: 32.3</td>
<td>M: 16.3 W: 1 B: 3 R%: 24</td>
<td>M: 27 W: 7.5 B: 0.5 R%: 32.3</td>
<td>M: 69.0 W: 0.5 B: 1.5 R%: 57.7</td>
<td>M: 45.7 W: 1.5 B: 1 R%: 47.0</td>
<td>Toc: 48.5 W: 6 B: 6 R%: 29</td>
</tr>
<tr>
<td>17/10</td>
<td>M: 31.3 W: 0.5 B: 2.9 R%: 32.4</td>
<td>M: 51 W: 1 B: 4.5 R%: 43.0</td>
<td>M: 36.3 W: 1 B: 5.5 R%: 35</td>
<td>M: 79 W: 8 B: 3.5 R%: 51.8</td>
<td>M: 75.3 W: 0 B: 0 R%: 54.8</td>
<td>M: 59.3 W: 3.5 B: 3.5 R%: 46.2</td>
<td>Toc: 62.5 W: 5 B: 10 R%: 28</td>
</tr>
<tr>
<td>24/10</td>
<td>M: 58.3 W: 2.5 B: 5.0 R%: 40.8</td>
<td>M: 115.3 W: 0 B: 6.0 R%: 58.1</td>
<td>M: 30.3 W: 10 B: 3.0 R%: 25</td>
<td>M: 158 W: 3 B: 6.6 R%: 64.6</td>
<td>M: 123 W: 0 B: 1 R%: 61.2</td>
<td>M: 49.7 W: 0 B: 4.0 R%: 80.9</td>
<td>Toc: 77 W: 6 B: 23 R%: 22</td>
</tr>
<tr>
<td>31/10</td>
<td>M: 36.3 W: 2.5 B: 1.7 R%: 27.4</td>
<td>M: 22.7 W: 4 B: 4.3 R%: 18.5</td>
<td>M: 15.3 W: 5.5 R%: 3.9</td>
<td>M: 37.3 W: 6 B: 5.4 R%: 57.0</td>
<td>M: 95.7 W: 0 B: 0.9 R%: 50.7</td>
<td>M: 26.0 W: 2.0 B: 4.5 R%: 20.9</td>
<td>Toc: 92 W: 13 B: 27 R%: 24</td>
</tr>
<tr>
<td>Mean</td>
<td>M: 17.7 W: 1.5 B: 4.6 R%: 24.4</td>
<td>M: 29.7 W: 1.3 B: 3.0 R%: 35.7</td>
<td>M: 14.4 W: 2.6 B: 2.9 R%: 24</td>
<td>M: 44.2 W: 4.1 B: 2.6 R%: 47.6</td>
<td>M: 44.8 W: 0.8 B: 0.8 R%: 44.2</td>
<td>M: 24.7 W: 1.1 B: 2.3 R%: 38.2</td>
<td>Toc: 48.6 W: 5.4 B: 8.2 R%: 29</td>
</tr>
</tbody>
</table>
Overall comparison
The comparative results between Mavrik and formic acid, mixture of Mavrik and oils, mixture of oils, and oils alone in controlling Varroa mites could be summarized in the following points.

Fluctuations were noted in the efficacy rate between applications. The efficacy of all treatments during the first, second, third and fourth applications ranged from 33.5 to 70.7%. It ranged from 4 to 47% for the fifth, sixth and seventh applications, and from 20.9 to 80.9% for the eighth, ninth, tenth and eleventh applications. These fluctuation may be due to higher temperature in the first month (32-36 °C) than in the second month (22-29 °C). Temperatures could act as a biotechnical treatment against Varroa mites. On the other hand, there is an optimum temperature for reproduction of mites. These results agree with those of El-shemy et. al. (2001) who found that the higher efficiency of natural materials in 1996 than in 1997 could be due to the higher temperatures in the former (34-41 °C) and in the latter year (31-35 °C).

According to our results, the mixture of oils was the more efficient of all the tested mixtures. Neem oil was the best of the three tested oils through all treatments during eleven applications.

These results agree with those of Eshbah et. al. (1995) who found that spraying German chamomile, tyme and pudding pipe extracts caused reduction in Varroa mite infestation at a range of 35-45%. However, dusting neem oil and German chamomile reduced the percent of infestation at 34-39% in two seasons.

Essential oils were safe to human and bees, residues in honey are low even after long term treatments (Imdorf et. al., 1999). Terpenes (mainly mono-terpenes) were the main components of essential oils, making up about 90% of the total. Most essential oils were mixtures of more than 50 components and many essential oil components naturally occur in honey.

Essential oils may be more efficient in colonies when used in strips. When sprayed, the bees were more excited because possible disturbance in queen pheromone inside colonies. Also, resistance to essential oils could possibly develop, as it is the case with synthetic pesticides. Since oils mix poorly with water, addition of an emulsifier such as tritonx is necessary. The use of essential oils can not be considered as the panacea. Considering the economic injury level, only an integrated pest management strategy combining cutting out of drone brood, thermal treatment, application of essential oils, good apicultural management practices and use of queens hybrid between Apis mellifera carnica (drones) and Apis mellifera lamerkii (virgins), could be an effective and reliable method for the control of V. destructor.

References


