REPORT OF THE OECD/KEMI/EU WORKSHOP ON MICROBIAL PESTICIDES: ASSESSMENT AND MANAGEMENT OF RISKS - ANNEX 6 (PRESENTATIONS - PART 3/3)

Series on Pesticides
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This document contains part 3/3 of the Annex 6 of the REPORT OF THE OECD/KEMI/EU WORKSHOP ON MICROBIAL PESTICIDES: ASSESSMENT AND MANAGEMENT OF RISKS. Annex 6 includes slides of all presentations made during the seminar.

The main part of the seminar report, as well as Annexes 1-5, is published under the reference ENV/JM/MONO(2014)2.
PART 3 OF 3

COMPILATION OF PRESENTATION SLIDES
PRESENTED AT THE
OECD/KemI/EU WORKSHOP
on Microbial Pesticides:
Assessment and Management of Risks

17-19 June 2013
Vår Gård, Saltsjöbaden, Sweden

Organised jointly by:
OECD (Organisation for Economic Cooperation and Development)
KemI (Swedish Chemicals Agency)
European Commission
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Swedish Chemicals Agency - Kemi

- Kemi is a Governmental agency responsible for health and environmental risk assessment of active substances and authorisation and enforcement of regulations of chemical products, pesticide and biotechnical organisms
- Kemi endeavour to limit the health and environmental risks associated with chemicals by promoting rules and legislation in Sweden, in the EU and globally that contribute to achieving the environmental quality objective of ´A Non-Toxic Environment´.
Preceeding meetings

• Saltsjöbaden, Sweden October 1991
  – “It is our hope that this meeting could in some aspects act as a catalyst for increased international efforts on co-operation and harmonization” (Kerstin Niblaeus)
  – Main achievement → OECD Working Group on Pesticides

• Stockholm, Sweden October 1998
  – “Our hope is also that it will show that the workshop became a catalyst for increased harmonisation and co-operation in the field of microbial plant protection products between EU member states and OECD member countries” (Vibeke Bemson)
  – Main achievement → EU improved rules for data requirements and principles for product authorisation
Preceeding meetings

• Arlington, USA April 2008
  – The key-word was *Communication*
    • Communication between regulators, scientists, industry, consumer organisations, grower’s organisations and NGO’s should be encouraged
  – Achievement → For example guidance document on environmental risk assessment
  – The Arlington workshop recommended a **central website for biopesticides** to facilitate information exchange – still needs to be further prepared

Urgent need

• To facilitate and streamlining the authorisation process of products containing micro-organisms it is necessary with more robust and harmonised rules and criteria
  – Rules and guidance for product authorisation and criteria for risk assessment are too indistinct
• Biological pesticides is still a small area, however increasing
• New EU regulation on plant protection products push for alternatives to the use of chemical pesticides, microbial pesticides is one alternative
Present meeting

- Saltsjöbaden, Sweden June 2013
  - Possible achievements
    - Establish **EU expert working group on microbial pesticides**
      - To harmonise and facilitate acceptance of risk assessment reports and product authorisations
      - An agency group on micro-organisms for use both in biocidal and in plant protection products.
    - Establish workshop series on **Microbial Pesticides: Assessment and Management of Risks** on a regular biannual basis to continue regulatory discussions like at this workshop.
Science/Academia Views

Scientific support, literature review and data collection and analysis for risk assessment of microbial organisms used as active substance in plant protection products - Lot1-Environmental Risk characterization

By Arena Maria, Pesticides Unit, European Food Safety Authority
Background

Experience gained up to now during the peer review of active substances used in plant protection products (PPPs) has shown that risk assessment for microorganisms is indeed a complex task and differs from assessment of chemical pesticides. Guidance on their assessment is necessary to ensure also their consistent evaluation. Development of such guidance has also been identified as a priority by the Pesticide Steering Committee (PSC).

Collection and evaluation of relevant information is needed to support the preparation of a guidance on how to conduct risk assessments for microbial pesticides within the frame of EU peer review of active substances in pesticides also with a view to gauge the extent of uncertainties associated with the use of such pesticides which will be important in regard to potential precautionary elements to be introduced in a future guidance.

Literature Search on microbials used as a.s

Launching date: March 2012
The call was split into 2 lots:
1. Environmental Risk Characterization
2. Toxicology

After the evaluation of the offers, only lot 1 could be awarded.
Contractor: Bio Intelligence service, a French consultancy company
supported by 2 subcontractors:
• The University of Warwick, UK
• The University of Helsinki, UHEL
The activities started in October 2012.
The scheduled deadline for the draft final report is July 2013
Aim of the literature review microbial a.s.

This call for tenders was explicitly launched to support the Panel on Plant Protection Products and their residues in developing a future guidance on risk assessment of microorganisms used as a.s. in PPPs focusing on topics like:

- Methodology on the extrapolation or read across among data from a strain to another
- Waiving for experimental data on non-target organisms
- Review of the test guidelines.

Methodology-Literature review microbial a.s.

Objectives of the call for tender

- To perform a systematic literature search and review all available relevant scientific information for each area as specified below
- To present the scientific information in a complete, systematic, clear and concise report written in English.
- To include in the search not only all the publicly available (peer-reviewed) literature but also grey literature (guidelines and government reports) and other relevant information.

The search focused on all the microorganisms used or to be used as active substances in PPPs in the EU which are listed in the Regulation 540/2011 or for which a decision on completeness has been taken in accordance with Article 6(3) of the Directive 91/414/EEC.
### Systematic Reviews vs. Narrative Reviews

<table>
<thead>
<tr>
<th>Category</th>
<th>Systematic Reviews</th>
<th>Narrative Reviews</th>
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<tbody>
<tr>
<td>Study Question</td>
<td>Focused and explicit</td>
<td>Often broad in scope</td>
</tr>
<tr>
<td>Eligibility criteria for inclusion or exclusion of studies</td>
<td>Pre-defined and documented; objectively applied</td>
<td>Not always explicitly stated</td>
</tr>
<tr>
<td>Description of the review method</td>
<td>Reported and also predefined in a protocol</td>
<td>Seldom reported</td>
</tr>
<tr>
<td>Literature search</td>
<td>Structured to identify as many relevant studies as possible</td>
<td>Not always extensive</td>
</tr>
<tr>
<td>Methodological quality assessment of included studies</td>
<td>Included, typically using a quality assessment tool</td>
<td>Variable</td>
</tr>
<tr>
<td>Reporting of study results</td>
<td>Full reporting of relevant results (numerical results)</td>
<td>Selective reporting; often of study author interpretation</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Quantitative synthesis (meta-analysis) when possible</td>
<td>Usually narrative, sometimes selective</td>
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</tbody>
</table>

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**Lot1: Environmental Risk Characterization**

A systematic review should be performed to collect:

- Information on transfer of genetic material from the microorganism to other microorganisms that may lead to unacceptable effects on the environment
- The potential interference with the analytical system for the control of the quality of drinking water as provided for in Council Directive 98/83/EC
- Colonisation, mobility and persistence in different environmental compartments compared to the natural background level
- The mechanisms of toxin/metabolite production, conditions for the stability outside the microorganism and related study designs
- The appropriateness of existing test guidelines for the effect assessment on non-target organisms
Lot 1- Environmental Risk Characterization

A systematic review should be performed to collect:

- Different abiotic and biotic factors/parameters relevant for the evaluation of pathogenicity, infectivity of microorganisms and toxicity of toxins/metabolites to non-target organisms, including specificity of the host. This will support the evaluation of:
  - Whether or not extrapolation or read across between data obtained for one species/strain/isolate to another species/strain/isolate in regard to infectivity, pathogenicity and the different ecotoxicological endpoints/adverse effects (as described in Regulation 544/2011) is possible or scientifically valid for microorganisms used or intended for use in the EU as active substances in PPPs.
  - Whether and which conditions could be set for waiving experimental data on non-target organisms.

According to the aim of the call 6 different topics were identified and for each of them a number of review questions formulated:

1. Genetic stability and transfer (Can transfer of genetic material between MCA under study and other microorganism occur?)
2. Interference with the system for drinking water quality control (Can the MCA under study be present in drinking water?)
3. Fate and behaviour in the environment (What is the persistence of the MCA under study in the environmental compartments of relevance?)
4. Production of metabolites (especially toxins) and potential toxic effect on non-target organisms (Is the MCA under study capable of producing toxic metabolites and can these metabolites affect non-target organisms?)
5. Host specificity range and potential effect of the MCA on non-target organisms (What is the host range of the MCA? Is it broad or narrow? Is the range variable under specific conditions?)
6. Existing test guidelines
### Methodology-Literature review microbial a.s.

<table>
<thead>
<tr>
<th>Topic</th>
<th>Associated key words</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genetic stability and transfer</td>
<td>&quot;genetic stability&quot; OR &quot;gene stability&quot; OR &quot;genetic transfer&quot; OR &quot;gene transfer&quot; OR &quot;genome stability&quot; OR &quot;genetic uptake&quot; OR &quot;DNA stability&quot; OR &quot;DNA transfer&quot; OR &quot;DNA uptake&quot; OR &quot;natural competence&quot; OR mutation</td>
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<tr>
<td>2. Interference with the system for drinking water quality control</td>
<td>(&quot;drinking water&quot;) AND (&quot;quality control OR analysis&quot;)</td>
</tr>
<tr>
<td>3. Fate and behaviour in the environment</td>
<td>(fate OR behaviour OR mobility OR persistence OR interaction OR colonization OR dispersal OR dispersion OR multiplication OR spread OR survival OR scaphology) AND (environment OR air OR water OR &quot;aquatic environment&quot; OR soil OR rhizosphere OR field OR crop OR plant)</td>
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<tr>
<td>4. Production of metabolites (especially toxins) and potential toxic effect on non-target organisms</td>
<td>metabolite OR toxin OR toxic OR &quot;non target organism&quot;</td>
</tr>
<tr>
<td>5. Host specificity range and potential effect of the MCA on non-target organisms</td>
<td>specificity OR pathogenicity OR pathogenic OR infectivity OR virulence OR lethality</td>
</tr>
</tbody>
</table>

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### Eligibility criteria

- The papers are within the scope of the study / of the topic / of the research questions;
- The material covers the right geographic region (EU as a priority).

#### Data sources

**Search engines and databases**
- Web of Science: www.isiknowledge.com
- Science Direct: www.sciencedirect.com
- Cat. inist: cat.inist.fr
- Agricola: http://agricola.nal.usda.gov/
- CAB Abstracts: http://www.cababstract.org/
- BIOSIS
- Scopus: www.scopus.com
- Google scholar
- Google

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### Grey Literature-Databases

OECD
US-EPA
REBECA Project
EPPO
Preliminary Results

Number of Hits combining all the search terms in a single string:
- PubMed: 339
- Web of knowledge: 1577
- Science Direct: 7251

Topic 1: genetic stability and transfer
The conclusions of EFSA often report data gaps related to this point. Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis* subsp. *kurstaki* (strains ABTS 331, PB 54, SA 11, SA 12, EG 2348), EFSA Journal, 2012;10(2):2540.

In the literature, it is reported that transfer of genes between introduced *Bacillus thuringiensis* subsp. *kurstaki* and indigenous Bacillus spp. can occur in soil under field conditions (Dommarum et al., Soil Biology & Biochemistry 42(2010)1329-1337). Authors reported that isolates of *B. mycoides* acquired part of the sequence of the *cry1A* gene from *Bacillus thuringiensis* subsp. *kurstaki*. No cells of *Bacillus thuringiensis* subsp. *kurstaki* or *B. mycoides* carrying the 238-bp fragment of the *cry1A*Ab9 gene were isolated from samples of unsprayed control soil.
Preliminary Results

Preliminary results:

Topic 1: genetic stability and transfer

Conclusion on the peer review of the pesticide risk assessment of the active substance *Bacillus thuringiensis israelensis AM165-52*. EFSA Journal 2013;11(4):3054

A data gap has been identified with regard to the potential for gene transfer

Ankarloo et al., Current Microbiology; 40 (2000), pp. 51–56 reported that the Bt *israelensis* mosquicidal crystal toxins are plasmid encoded and may be transferred by conjugation as showed among different soil isolates of *Bt israelensis*.

Preliminary Results

Preliminary results:

Topic 3: Fate and behaviour in the environment


No studies on persistence and multiplication in soil and aquatic environment of *Bacillus thuringiensis* subsp. *kurstaki* strains PB54, ABTS-351, SA-11, SA-12 and EG-2348 were provided and then a data gap has been identified.

Vettori et al., Soil Biology & Biochemistry 35 (2003) 1635–1642 indicated that *Bacillus thuringiensis* subsp. *kurstaki* is able to survive in soil in which it is not an indigenous bacterium, but they also showed, that *Bacillus thuringiensis* subsp. *kurstaki* could be detected more than 7 yr (the longest time studied) after introduction to natural soils in sprays. Its toxin was detected 28 months after spraying by immunological assay, but at a reduced concentration while the larvicidal activity decreased essentially linearly to 14 months and then decreased markedly between 14 and 28 months.
Conclusions

- The preliminary results highlight the importance of a systematic review of the literature for a complete Risk Assessment of the microbial a.s. A guidance has been provided by EFSA on how to identify and select “scientific peer-reviewed open literature” as required by Article 8(5) of Regulation (EC) No 1107/2009 on the placing of plant protection products on the market and how to report it in a dossier.

- GD on how to do the Risk Assessment for these active substances. This need has also been identified by the PSC.

Literature review microbial a.s.

Thanks for the attention!

감사합니다 Natick Danke Eυχαριστίες Dalu Köszönöm Tack Grazie Спасибо Dank Gracias Merci 谢谢 ありがとう
The SLU Center for Biological Control

By Margareta Hökeberg
Swedish Agricultural University

The SLU Centre for Biological Control - CBC

Margareta Hökeberg

CBC, SLU

Department of Forest Mycology and Plant Pathology

OECD/KemI/EU workshop on Microbial Pesticides, Stockholm 17-19 June 2013

www.slu.se/cbc

Outline of presentation

1. CBC – Aim and organisation
2. CBC research
3. Some future regulatory challenges
4. Concluding remarks

www.slu.se/cbc
CBC

Financed by the Swedish Ministry for Rural Affairs

- Focus is on the use of living organisms to control or restrict damages caused by harmful organisms.
- Research and development in different areas of biocontrol.
- Promote biocontrol research and research collaboration.
- Cooperation with stakeholders – companies, authorities, extension, growers...
- Steering group + Advisory group
- Annual stakeholder meeting on biological control, seminars.

CBC research

- Fundamental and applied research on biological control to strengthen the knowledge base;
- Facilitate the development and implementation of new biocontrol products and approaches.
- Five research areas:
  insects/arachnides, fungi and bacteria for biocontrol;
  microbial stabilisation/formulation; safety and regulation.

Current focus: biocontrol in agricultural and horticultural crops with both augmentation/application and conservation biological control strategies. Biological control in IPM.
**Ingvar Sundh: Safety and regulation**

*Better methodology assessing human safety:*

*in vitro* toxicity tests of model compounds with cell lysates/extracts from microbes

*Ecology of biocontrol agents: Fate in the environment; strain specific SCAR markers*

- Pseudomonad against snow mould in wheat
- Two *Trichoderma* used against plant diseases
- Population ecology of Bti in periodically flooded riverine meadows.

**Sebastian Håkansson: Microbial Stabilization/Formulation**

Microbial formulation
- Treatments of, and additions to, cultured microorganisms to ensure:
  - Long term stability
  - Viability and biological activity
  - Low production costs
  - Purposeful product characteristics
  - Simplicity and safe handling
  - Easy storage and transportation

**Process Engineering**
- Thermodynamics

**Physical chemistry**
- Pharmaceutical galenics

**Biology**
- Stress metabolism
- Bioengineering

**Interdisciplinary field**

Example: Pre- and postformulation mixtures of BCAs, fungi + bacteria
Mattias Jonsson – Insects and arachnides
Conservation biological control in a food web and landscape perspective

Hanna Friberg: Fungi for biological control

Ecological aspects – interactions between crop plant pathogens and their antagonists in their environment

Stimulation of naturally occurring antagonistic organisms – crop rotation, cultural practices etc.

How can introduced organisms be enhanced?

Ex. *F. gramineraum* – Survival on plant residues in different tillage systems, succession av fungi, establishment of antagonists.

(Future: Antagonism as an eco-system services?)
Margareta H: Bacteria for biological control

**Biological seed treatment** – *P. chlororaphis, P. azotoformans*
(Products Cedomon, Cerall, Cedress, AMASE; Lantmannen BioAgri)

**BCA mixtures** – Could mixtures improve disease controlling effects?
Compatibility, interactions, root colonising ability, efficacy spectrum.

**Combination of control measures, crop perspective**: Augmentation and conservation biocontrol, seed and planting material, crop rotation, cultivation practices, chemical control, etc.
Interactions insect and disease biological control.

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Some future regulatory challenges – plant beneficial microorganisms

- Plant strengtheners/stimulators vs MBCAs
- Microbial consortia
- Endophytic microbes
- Plant and microbe integrated breeding
- Closing gap conservation – augmentation biocontrol

Ruppel et al., 2006; Nelson, 2004
Concluding remarks

Biological control is an on-going, competitive processes in organism interaction.

Its efficiency can be increased in various ways, e.g. by promoting beneficial organisms in situ, or by adding them to the system after mass-production.

Current regulation for microbial pesticides is mainly based on a single strain concept. How should microbial consortia be evaluated?

MBCAs often have additional plant beneficial traits to antagonism. Will authorisation as plant stimulants be used as a fast track to the market?

How to make regulatory systems safe and reliable, but still flexible enough to handle new product concepts?

Thank you!
Evaluation of non-viable residues and relevant metabolites

By Ingvar Sundh
Swedish University of Agricultural Sciences

Contents of presentation

1. EU data requirements/uniform principles: examples
   • Relevant metabolites
   • Non-viable residues

2. The ‘fate’ of microbial organic matter/detritus (metabolites/residues) in the environment

3. EFSAs pesticide peer reviews
   Usually identify several ‘data gaps’, why?

4. Conclusions and recommendations

Focus on EU regulation

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EU data requirements (DR)/uniform principles (UP) 1

- ‘Relevant metabolites (i.e. if expected to be of concern to human health and/or the environment)….’ (DR 1.4.2)
  1107/2009: ‘A metabolite is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to the parent substance…..’

- ‘Member states shall evaluate the possibility of exposure of humans or animals to non-viable residues …. the following information should be taken into account:
  - the stage of development of the micro-organism at which non-viable residues are produced,…’ (UP 2.6.2.1)

EU data requirements (DR)/uniform principles (UP) 2

- ‘Methods to determine and quantify residues (viable or non-viable) of:
  - the active micro-organism(s)
  - relevant metabolites (especially toxins)’ (DR 4.2)
    on and/or in crop, food and feedstuffs, animals and humans, soil, water
    and in air where relevant.

- Mobility in the environment (DR 7.2): ‘The possible spread of the micro-organism and its degradation products in relevant environmental compartments has to be evaluated…..’
Environmental fate of metabolites/non-viable residues of microbes

- Recycling: Production new microbial biomass balanced by loss processes
- Dead microbes
  - enter the pool of “microbial detritus”/humus
  - contribute to the input of organic matter
  - substrates for heterotrophic organisms in the detrital degrader/consumer food web
- Very high background of organic matter/residues from other organisms
- Degradation pathways for residues are present!

Graphics: Oak Ridge National Laboratory, US

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Examples of difficulties and problems

EFSAs conclusions on pesticide peer reviews of microbial active ‘substances’

1. Data gaps with respect to information that testify that the organism do not produce metabolites that might fulfill the criteria (CR 7 iv):
   - Stable outside the microorganism
   - Biologically active independently of the presence of the microorganism
   - Intended to be applied at levels above background levels

2. Effects of organism/residues on analytical systems for control drinking water. How likely? Examples?


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Conclusions and recommendations

• Microbials are legally pesticides, but must be evaluated as microbes.

• Less attention to requirements for xenobiotic chemicals, more to requirements for microbes in other areas of utilisation.

• Environment: Focus should be on the living organism, not what it produces. Non-viable residues are highly unlikely to poison the environment!

• Updated data requirements/guidance for non-viable residues/metabolites are urgently needed.

• Best way forward? a) produce guidance for requirements no longer in line with current microbial safety assessment/ecology knowledge?; or b) develop more relevant data requirements?

• How far can the requirement to demonstrate absence of ‘relevant metabolite’ production be drawn? Microbials are “guilty until proven innocent”, but 100% proof of absence is not possible.

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Publications

• ‘Safety and regulation of yeasts used for biocontrol or biopreservation in the food or feed chain’
  (Antonie van Leeuwenhoek, 99: 113-119, 2011)
• ‘Regulating biocontrol agents: a historical perspective and a critical examination comparing microbial and macrobial agents’
  (BioControl2013, DOI, on-line first)
• ‘Beneficial Microorganisms in Agriculture, Food and the Environment: Safety Assessment and Regulation’
  (Book on CABI Publishing, 2012)
• ‘Harnessing the value of beneficial microorganisms: role of regulatory landscapes’
  (CAB Reviews 2013, 8: No. 013)

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Experiences of biological control, risk assessment, fungi.

By Jan Stenlid
Swedish University of Agricultural Sciences

Fungi as biocontrol agents

- Eukaryotes, thus potential to recombine
- Spread by wind, rainsplashes or vectored
- Can effect host innate immunity
- Can persist in the environment
Concerns for risk assessment

- Health challenges
- Efficacy
- Spread
- Impact on non-target flora
- Impact on resident populations
- Impact on other ecosystem services
- Long term aspects

Case study

Controlling *Heterobasidion* root rot of conifers using *Phlebiopsis gigantea*
European forest owners lose ca 2 million Euros daily!

Heterobasidion annosum
Transfer to young trees

Preventing spore infections

Winter cutting, Biological control, pH regulation
Application of spores of a competing fungus – *Phlebiopsis gigantea*

Concerns for risk assessments

- Health challenges – No major, no harmful metabolites, not allergenic
- Impact on non-target flora – Minor
- Impact on resident *Phlebiopsis* - Minor
- Impact on other ecosystem services – None known
- Spread – Little effect
- Long term persistence – Low
- Efficacy - Good also long lasting effects
- Long term effects on target populations - Possibly
Most common fungal species 7 weeks after Rotstop and urea treatments

<table>
<thead>
<tr>
<th>Species</th>
<th>Control %</th>
<th>Rotstop %</th>
<th>Urea %</th>
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<tbody>
<tr>
<td>Morteriella isdell</td>
<td>71</td>
<td>91</td>
<td>29</td>
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<tr>
<td>Morteriella ram</td>
<td>62</td>
<td>33</td>
<td>10**</td>
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<td>Sistotrema brinkmanii</td>
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<td>33</td>
<td>5***</td>
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RESEARCH ARTICLE
Impact of the biological control agent *Phlebiopsis gigantea* on its resident genetic structure in the Baltic Sea area

Nicklas Samils*, Rimvydas Vasaitis, and Jan Stenlid

[Graph showing genetic similarity to the P. gigantea strain in Rotstop®]
- A) Traps in treated area after 7 years
- D + E) From Rotstop original area
- F) Swedish 4 years after treatment 2 km away
- G+H) Resident Swedish before treatment
- I+J) Resident Lithuanian
- B) Traps 200 m from treated area after 7 years
- C) Resident population 200 m from treated area

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<td>0.091</td>
<td>0.03163</td>
<td>0.828</td>
<td>0.455</td>
<td>0.00102</td>
</tr>
<tr>
<td>Gåsholmen</td>
<td>0.643</td>
<td>0.409</td>
<td>0.10898</td>
<td>0.490</td>
<td>0.400</td>
<td>0.57001</td>
<td>0.765</td>
<td>0.889</td>
<td>0.37482</td>
</tr>
<tr>
<td>Lusen</td>
<td>0.612</td>
<td>0.400</td>
<td>0.16835</td>
<td>0.278</td>
<td>0.200</td>
<td>0.58292</td>
<td>0.805</td>
<td>0.300</td>
<td>0.00006</td>
</tr>
<tr>
<td>Räberg</td>
<td>0.747</td>
<td>0.636</td>
<td>0.39906</td>
<td>0.444</td>
<td>0.364</td>
<td>0.58964</td>
<td>0.720</td>
<td>0.364</td>
<td>0.00048</td>
</tr>
<tr>
<td>Palanga</td>
<td>0.717</td>
<td>0.867</td>
<td>0.19775</td>
<td>0.332</td>
<td>0.267</td>
<td>0.58884</td>
<td>0.809</td>
<td>0.64</td>
<td>0.12105</td>
</tr>
<tr>
<td>Druškininkai</td>
<td>0.737</td>
<td>0.867</td>
<td>0.25476</td>
<td>0.231</td>
<td>0.000</td>
<td>0.03372</td>
<td>0.733</td>
<td>0.867</td>
<td>0.10485</td>
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<tr>
<td>USA</td>
<td>0.729</td>
<td>0.231</td>
<td>0.00005</td>
<td>0.298</td>
<td>0.077</td>
<td>0.08190</td>
<td>0.852</td>
<td>0.333</td>
<td>0.00003</td>
</tr>
</tbody>
</table>

Values are based on three microsatellite loci.
Persistence and long-term impact of Rotstop biological control agent on mycodiversity in *Picea abies* stumps

R. Vasiliauskas, E. Larsson, K.-H. Larsson, J. Stenlid

![Graph showing changes in fungal species diversity with Rotstop treatment.](image)

**Most common fungal species 4-6 years after stump treatment with Rotstop**

<table>
<thead>
<tr>
<th>Species</th>
<th>Av frequency</th>
<th>Significant changes after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phialocephala sp.</td>
<td>33.1</td>
<td>NS</td>
</tr>
<tr>
<td>Sistotrema brinkmanii</td>
<td>30.0</td>
<td>NS</td>
</tr>
<tr>
<td>Heterobasidion parviporum</td>
<td>29.2</td>
<td>Lower</td>
</tr>
<tr>
<td>Recinicium bicolor</td>
<td>27.7</td>
<td>NS</td>
</tr>
<tr>
<td>Trichoderma polysporium</td>
<td>20.0</td>
<td>NS</td>
</tr>
<tr>
<td>Phlebiopsis gigantea</td>
<td>16.2</td>
<td>Higher</td>
</tr>
<tr>
<td>Asccoryne sarcocides</td>
<td>13.1</td>
<td>Lower</td>
</tr>
<tr>
<td>Asccoryne alyliphium</td>
<td>13.1</td>
<td>NS</td>
</tr>
<tr>
<td>Hypholoma capnoides</td>
<td>12.3</td>
<td>Higher</td>
</tr>
<tr>
<td>Stereum sanguinolentum</td>
<td>10.8</td>
<td>NS</td>
</tr>
<tr>
<td>Lecytophora sp.</td>
<td>10.8</td>
<td>NS</td>
</tr>
<tr>
<td>Phialophora fastigiata</td>
<td>10.8</td>
<td>Lower</td>
</tr>
</tbody>
</table>
The capacity in *Heterobasidion annosum* s.l. to resist overgrowth by the biocontrol agent *Plethiomax gigantea* is a heritable trait

N. Samuś*, Å. Okin, J. Stanisł

Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Box 760, SE-750 07 Uppsala, Sweden

Received 10 October 2007; accepted 5 March 2008

Available online 25 March 2008

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**Variation against *P. gigantea* overgrowth among progenies derived from a hybrid *H. annosum* s.l. strain**

![Graph showing variation against *P. gigantea* overgrowth among progenies derived from a hybrid *H. annosum* s.l. strain.](image)

- **Strain ID**
- **Broad sense heritability = 0.336**

---

**Fig. 5.** The variation among 91 *H. annosum* s.l. hybrid progenies to resist overgrowth by the *P. gigantea* strain in the biocontrol agent *H. annosum*. The average growth rate of the *P. gigantea* strain over the 91 progenies was 3.36 mm/day.
Thanks!

- Nicklas Samils
- Rimvydas Vasaitis
- Magnus Thor
- Ellen Larsson
- Karl Henrik Larsson
- Åke Olson
- Vaidotas Lygis
Secondary metabolites: a literature study - Key stones for risk assessment methodology

By Jacqueline Scheepmaker
the Netherlands

Secondary metabolites
Results of a literature study

National Institute for Public Health
and the Environment
Ministry of Health, Welfare and
Sport

1

Jacqueline Scheepmaker, June 18, 2013

Short introduction
Setup literature review
6 representative graphs
Conclusions
How to proceed?

National Institute for Public Health
and the Environment
Ministry of Health, Welfare and
Sport

2

Jacqueline Scheepmaker, June 18, 2013
Role literature review

Development
Risk assessment
(fungal metabolites)

OECD Guidance
EFSA Guidance
General statements

Review open literature

Relevant recent work

Data requirement of persistence:

Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. Biocontrol Science and Technology 20: 503-552.
Purpose of this literature study

1. Provide basic information on secondary metabolites for registration purposes

2. Make a review of the range of metabolites produced by entomopathogenic fungi (EPF)

3. Determine factors influencing the production of key metabolites

Importance of metabolites

Opportunities:
1. pharmaceuticals
2. taxonomic markers
3. biopesticides (spinosad: metabolite of soil bacterium *Saccharopolyspora spinosa*; abamectin: metabolite of soil bacterium *Streptomyces avermitilis*)
Reasons for data requirements:

Fears:
- Secondary metabolites can be mycotoxins (aflatoxin by *Aspergillus*)
- Metabolites present in formulated product and stable after application
- Uncontrolled production of metabolites
- Secondary poisoning

7. Fate and behaviour in the environment

Data requirements and the corresponding risk assessment needs to be fulfilled if all the following conditions are met:

1. The relevant metabolite is stable outside the microorganism
2. A toxic effect of the relevant metabolite is independent of the presence of the microorganism
3. The relevant metabolite is expected to occur in the environment in concentrations considerably higher than under natural conditions
Description relevant metabolite in Regulation 544/2011? implementing Regulation no. 1107/2009

“where they are of significance for human health and/or the environment”

depends on the combination of
• quantities produced and
• their toxicity

If both are not known beforehand this would need investigation

Problem for industry, risk assessment and registration

Contents database
• 78 references with measured quantities (120 others used for manuscript)
• Different EPF species and strains
• Different culture methods (solid state, liquid fermentation, insects)
• Variety of growing media,
• Different conditions (pH and temperature)
• Fermentation periods/timing of sampling
• Different extraction methods: 1-butanol, acetone, acetonitrile, dichloromethane, ethyl acetate, methanol, methylene dichloride
Restrictions

• Focus on environment only

• Only well known entomopathogenic fungi (EPF)
  \( \text{(Metarhizium, Beauveria, Isaria, Lecanicillium)} \)
  
  o non-EPF微生物s are included in case they produce the same metabolites

  o Maximum quantities + days of incubation

• Literature search on EPF was not exhaustive

Frightening messes of stray yarns and swatches all entangled together into fabulously gruesome disasters
Time course for production of destruxins by *M. anisopliae* V245 in liquid medium

From: Anvari-Bashahi et al., 2000

Different quantities by different strains

Destruxin A production by different strains of *Metahrizium anisopliae* var. *anisopliae*

Culture media
Different quantities by different strains

Destruxin A production by different strains of *Metarhizium anisopliae* var. *anisopliae*

![Graph showing destruxin A production by different strains with pH level indicated](image)

Yield of metabolites depends on the content of the medium

Destruxin production by *Metarhizium* in two different Czapek Dox Broth media

![Graph showing destruxin production in different media](image)

From: Hsiao et al, 2001
In liquid fermentors, Destruxin A, B and E major metabolites

Quantities metabolites of *Metarhizium anisopliae* [mg/L]

Dtx A and B based on 14 studies
Dtx E on 11 studies

In insects: only Destruxin A, B and E

Destruxin production by *Metarhizium anisopliae* in insects

Dtx E only in *Carpocapsa pomonella*
Dtx A + B *Carpocapsa pomonella* + *Galleria*
Beauvericin produced by several species

Conclusions of this literature search

• Range and quantities depend on contents of fermentation medium and vary between species/strains
  o Only three metabolites of Metarhizium in insects, Destrains A, B and E
  o These three are dominant in liquid fermentation
  o Destrain E is not always present
  o All others are incidental and at low concentrations

The same metabolite can be produced in different genera

  o Quantities of Beauvericin: in Fusarium >> EPF

• Data from literature are incoherent and incomplete:
  o Studies differ in all parameters: difficult to create coherent subsets
  o Incomplete descriptions of culture methods
Standard Test Method??

Development of a standard test method seems impossible due to different responses of EPF.

Development of a standard test method is not useful: the obtained metabolite profile may differ from the profile obtained in the commercial fermentation method.

How to continue?

- We need to think out of the box and invent other strategies

- Construct simple risk assessment schemes for low, medium and high risk groups

- Identify key questions and perform literature studies where needed
  - Are metabolites present in the formulated product?
  - Low and high yielding fermentation media?

- Identify the most important route of exposure
Fate of metabolites in product application on plant surface

Metabolites in the sprayed EPF product?

If so, relevant in comparison with known L(E)C50 values?

---

### Toxicity (in microgram/L)

<table>
<thead>
<tr>
<th>Route of exposure</th>
<th>Parameter</th>
<th>Organism</th>
<th>Stage</th>
<th>Crude extract</th>
<th>Dextoxin A</th>
<th>Dextoxin B</th>
<th>Dextoxin E</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact</td>
<td>Mortality</td>
<td>LC50</td>
<td>Nymph</td>
<td>89.8</td>
<td>95.5</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>Ha et al., 2009</td>
</tr>
<tr>
<td>Contact</td>
<td>Mortality</td>
<td>LC50</td>
<td>Larval</td>
<td>87</td>
<td>&gt;500</td>
<td>50</td>
<td>30</td>
<td>Amini &amp; Rehman, 1999</td>
</tr>
<tr>
<td>Contact</td>
<td>Mortality</td>
<td>LC50</td>
<td>Larval</td>
<td>56</td>
<td>376</td>
<td>53</td>
<td>30</td>
<td>Amini &amp; Rehman, 1999</td>
</tr>
<tr>
<td>Contact</td>
<td>Mortality</td>
<td>LC50</td>
<td>Larval</td>
<td>165.4</td>
<td>184</td>
<td>90</td>
<td>33</td>
<td>Amini &amp; Rehman, 1999</td>
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<tr>
<td>Oral</td>
<td>Activity</td>
<td>A150</td>
<td>Larval</td>
<td>5</td>
<td>182</td>
<td>33</td>
<td>33</td>
<td>Amini &amp; Rehman, 1999</td>
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<tr>
<td>Oral</td>
<td>Activity</td>
<td>A150</td>
<td>Larval</td>
<td>10</td>
<td>182</td>
<td>33</td>
<td>33</td>
<td>Amini &amp; Rehman, 1999</td>
</tr>
<tr>
<td>Cell cultures</td>
<td>Altered</td>
<td>K50</td>
<td>Pupae</td>
<td>138</td>
<td>13</td>
<td>1397</td>
<td>1397</td>
<td>Marx et al., 2009</td>
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<tr>
<td>Cell cultures</td>
<td>Viability</td>
<td>LC50</td>
<td>Pupae</td>
<td>&gt;500</td>
<td>5</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>Sirobot &amp; Blatt, 2005</td>
</tr>
</tbody>
</table>

LC = Lethal Concentration
EC = Effective Concentration

Under construction, your input is necessary!

<table>
<thead>
<tr>
<th>Statement</th>
<th>Lacking info</th>
<th>In what way simplifying data requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Makes sense to differentiate into low, medium and high risk micro-</td>
<td>Prepare general overview of genera/species/metabolites</td>
<td>No further data requirements for low risk microorganisms</td>
</tr>
<tr>
<td>organisms (EPF = low risk)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Many commercial microbial products only contain spores or biomass</td>
<td>General overview. Contribution to industry?</td>
<td>Tool to differentiate into low, medium and high risk</td>
</tr>
<tr>
<td>Culture methods can be made either low or high yielding in metabolites</td>
<td>Determine what is low yielding. Comparison with LC50 values if present in</td>
<td>If low yielding no further data requirements on metabolites</td>
</tr>
<tr>
<td></td>
<td>literature</td>
<td></td>
</tr>
<tr>
<td>Secondary metabolites are degraded rapidly outside the micro-organism,</td>
<td>Literature search necessary</td>
<td>No further data requirements on metabolites for fate and behaviour. Domino effect for other data points?</td>
</tr>
<tr>
<td>therefore the condition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;The relevant metabolite is stable outside the microorganism&quot; is never met</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Thank you for your attention!