



**Deliverable 12:**

**Positive list of “low risk” candidates**

**REBECA**

Regulation of Biological Control Agents

Specific Support Action

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### Document History

The document is based (i) on a case by case evaluation of microbial biocontrol agents, assessed by international experts, recognised by REBECA consortium, (ii) the safety data fact sheet published by the US Environment Protection Agency (EPS) and (iii) publication of the European Council regulations, reporting the opinion of the safe use of Annex I listed micro-organisms. The document has been discussed at the Salzau workshop in September 2006, and was made available to the public via REBECA web-page. The discussion was open until September 2007. This document was compiled without the rational arguments discussed in Deliverable 28 “Specification of low risk products”.

### Document Abstract

This document contains the collective views of experts, recognised by REBECA consortium. The written statements on defined microbial BCAs underline that the listed mBCAs are unlikely to cause human disease and will pose only a low risk to human and animal health and the environment, if any. The low risk candidate list is helpful to define a rational argument for a definition for low risk active substances – [see also subsection 4, derogations, article 22, 2006/0136 (COD)].

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## Introduction

Experts from stakeholder industry and academics, recognised by REBECA consortium, were asked in advance of the REBECA workshop, held in Salzau 18-22 September 2006, to provide lists of active substances which they regard as low risk candidate microbials. As international experts, in both fundamental and applied aspects of microbial biocontrol, they were encouraged to present arguments and safety data information for their “favourite” microbial BCAs.

The objective was to decide, whether the listed microbial BCAs, all with a long history of safe use, should be regulated similar to those micro-organisms, which are published by the European Food Safety Association (EFSA) in the “Qualified Presumption of Safety (QPS)” documents as micro-organisms with QPS status. Further, it was assumed that this document will help to define the term “low risk active substances”.

## List of all agents with a history of safe use

In the 3<sup>rd</sup> edition of “The Manual of Biocontrol Agents” edited by Copping (2004)<sup>1</sup>, over 100 active ingredients are based on micro-organisms. All microbial biological control agents (mBCAs) used to control insects, diseases and weed pests are described as “generally to pose little or no risk to man and the environment” (Anonymous, 2007)<sup>2</sup>. This statement is based on the acknowledgement by the EU by the fact that several microbes (active substances) are authorised for the use in plant protection according to EU directive 91/414 Annex I.

Today’s Commission practises regarding the assessment of environment, health and safety risks of BCAs and their “relevant” metabolites is still under discussion (see Presidency proposal of Regulation 2006/0136 dated 22 October 2007). As long as the scientific data remain incomplete, imprecise or inclusive the precautionary principle (COM(2000)1) should be the basis for a risk assessment of plant protection products. A case by case evaluation must be agreed by using our expertise, which is based on a scientific background and a long term experience. Weighing the risks and benefits of the release of a microbial BCA versus other control measurements (chemicals), by maintaining the same level of safety to users and consumers of agricultural products. Currently, no risky microbial BCAs could be identified which is listed by Copping (2004) and should be banned.

1 Copping, L.G. (2004). The Manual of Biocontrol Agents. BCPC *Pesticide Manua*. pp. 752.

2 Anonymous (2007). OECD-Biological pesticide registration.  
[http://www.oecd.org/document/8/0,2340,en\\_2649\\_34383\\_31962760\\_1\\_1\\_1\\_1,00.html](http://www.oecd.org/document/8/0,2340,en_2649_34383_31962760_1_1_1_1,00.html).  
March, 15th, 2007, 4.25 pm

## **Positive list for “low risk” candidates:**

### ***Baculoviruses***

Based on the conclusions from the OECD Consensus document No 20 (2002), and on the expected results of the evaluation of dossiers submitted for the inclusion of isolates of CpGV, AoGV, and SeNPV, REBECA participants, guided by virus experts, propose that all baculoviruses are safe (i.e. all viruses including Dipteran- and Hymenopteran-specific species). In agreement with the OECD consensus document (no 20) and after comprehensive discussions with REBECA participants and review of latest scientific results on the molecular identification of the group, the REBECA consortium recommend listing the family *Baculoviridae* on Annex I (see also deliverables No 10).

### ***Bacterial and fungal products***

International experts reviewed the registration data requirements of microbials based on a list of the following key questions:

- (i) Toxicity/pathogenicity/infectivity (of both the active substance and metabolites/toxins)
  - a) are there metabolites/toxins of concern?
  - b) methods available to quantify the metabolites/toxins which are of toxicological or environmental significance.
  - c) do the metabolites/toxins have any harmful effects on human health (including vulnerable groups), animal health?
  - d) are there unacceptable effects on the environment?

A case by case evaluation has to be made, which was based on a scientific background and a long term experience of the safe use of effective microbials, which are either i) listed in Annex I of European Directive 91/414/EEC, ii) in the process of EU evaluation, iii) applied authorised in OECD countries, or iv) are in the re-registration procedure of RENDER 4.

People from stakeholder industry and academia were also asked to weigh the risks and benefits of the release of a microbial BCA versus other control measurements (chemicals), by maintaining the same level of safety to users and consumers of agricultural products.

Prior to the Salzau conference short written statements were provided to the REBECA workshop participants (Annex I) to be able to discuss the “low risk candidate list”. The documents were also made available to the public via REBECA

web-page. The discussion was open until September 2007. Arguments from stakeholder industry, academia and regulators are summarised in deliverable No 28.

**Table 1** Proposed list of safe bacterial mBCAs

<i>Bacillus subtilis</i> (Serenade):	EPA biopesticide fact sheet
<i>Bacillus thuringiensis</i> :	WHO, Environmental health criteria 217, NPTN General fact sheet, EPA biopesticide fact sheet
<i>Panthoea agglomerans</i> (BlightBan)	EPA biopesticide fact sheet
<i>Pseudomonas chlororaphis</i> (Cedomon)	Margareta Hökeberg
<i>Serratia entomophila</i> (Invade)	Travor Jackson
<i>Serratia plymuthica</i> (Rhizostar)	Gabriele Berg

**Table 2** Proposed list of safe fungal mBCAs.

<b><u>Products</u></b>	<b><u>Nominated experts</u></b>
<i>Ampelomyces quisqualis</i> (AQ10)	Sergio Franceschini
<i>Beauveria bassiana</i> (BotaniGard)	Tobias Längle
<i>Beauveria brongniartii</i> (Melocont)	Hermann Strasser
<i>Coniothyrium minitans</i> (Contans):	EPA biopesticide fact sheet
<i>Gliocladium catenulatum</i> (Prestop)	Marina Niemi
<i>Lecanicillium</i> ( <i>Verticillium</i> ) <i>lecanii</i> (Mycotal, Vertalec)	Willem Ravensberg
<i>Metarhizium anisopliae</i> (GRANMET, BIO 1020):	EPA biopesticide fact sheet
<i>Paecilomyces fumosoroseus</i> (Preferal):	EPA biopesticide fact sheet

### ***Risk index examples***

Selected pest control agents (i.e. biologicals and conventional pesticides) were used to critically review the applicability of a newly developed risk index (RI) system by Längle & Strasser (2008). Five basic components have been proposed for the calculation of the overall environmental risk score: persistence of the substance, dispersal potential, range of non-target organisms that are affected, and direct and indirect effects on the ecosystem. All categories were modified from a widely-accepted model (i.e. ERBIC model). Only one new category was implemented to assess the risks to vertebrate non-target species.

Besides a detailed discussion of the new risk index model (see also Deliverable No 28), the suitability of the model was demonstrated by calculating the risk scores for seventeen selected products.

## Validation of Risk Index using selected pest control agents:

In order to demonstrate the validity of the proposed risk index we applied this system to a number of well-studied biological control agents and selected chemical products used for similar purposes. Organisms scored were *Bacillus thuringiensis*, *Beauveria brongniartii*, *Beauveria bassiana*, *Coniothyrium minitans*, *Metarhizium anisopliae*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Trichoderma harzianum*; conventional pesticides assessed were atrazinechlorpyriphosbenomyl, chlorpyriphos, benomyl, DDT, methyl bromide, phorate and streptomycin. Indices were calculated using open literature and published regulatory documents. The results are displayed in Table 3. The organisms with the lowest risk index were soil applied fungi with very narrow host ranges applied to environments to which they are native. These organisms consistently scored low in all categories. Biocontrol agents with broader host ranges delivered by spray application typically had a higher dispersal potential and also scored higher under direct and indirect effects, but remained about one magnitude or more below their conventional chemical alternatives

The highest scoring substances were DDT, methyl bromide, and chlorpyriphos (data not shown). These scores were largely a consequence of high persistence and dispersal potential, combined with wide target ranges and high values assigned for direct and indirect effects.

On average, biopesticides had an approximately 40 times (range 9-200) lower risk index than conventional products used for the same purpose. Based on these results it became obvious to us, that the proposed risk index system may serve to define low risk (i.e.,  $RI \leq 100$ ) and reduced risk (i.e.  $500 \geq RI > 100$ ) pesticides.

**Table 3** Risk scores and calculated risk index for selected microbial and pest control products (Länge & Strasser).

Active ingredient (application)	Persistence factor	Dispersal factor		Host range		Direct effect		Indirect effects		Vertebrate effects	Risk index
		Distance	Quantity	Species	Taxonomic level	Likelihood	Magnitude	Likelihood	Magnitude		
<i>Bacillus thuringiensis</i> (foliar spray)	2	2	3	4	4	3	2	2	3	1	280
<i>Beauveria brongniartii</i> (soil)	1	3	1	2	1	1	1	1	1	1	16
<i>Beauveria bassiana</i> (foliar spray)	1	3	3	4	4	3	2	2	2	1	260
<i>Beauveria bassiana</i> (soil)	1	3	1	4	4	3	2	1	2	1	96
<i>Coniothyrium minitans</i> (soil)	1	1	3	1	1	1	1	2	2	1	24
<i>Metarhizium anisopliae</i> (soil)	1	3	1	4	4	3	2	1	2	1	96
<i>Metarhizium anisopliae</i> (foliar spray)	1	3	3	4	4	3	2	1	2	1	240

<i>Pantoea. agglomerans</i> (foliar spray)	1	3	2	3	3	2	1	1	1	2	98
<i>Pseudomonas fluorescens</i> (foliar spray)	1	2	3	3	3	2	1	1	1	1.5	91
<i>Trichoderma harzianum</i> (soil)	2	1	1	3	3	3	2	2	2	1	95

## Appendix:

Reports for REBECA workshop, 18-22 September, 2006, Salzau, Germany

### ***Ampelomyces quisqualis* - AQ 10**

**Sergio Franceschini, Intrachem**

*Ampelomyces quisqualis* isolate M-10 is a naturally occurring hyperparasite of powdery mildews. This parasitism reduces growth and may eventually kill the mildew colony. The mycoparasite is not restricted to powdery mildews. *In vitro* work indicates that it can be parasitic on *Botrytis cinerea* Pers. Ex. Fr., *Alternaria solani* (Ell. & Mart.) Sor., *Colletotrichum coccodes* (Wallr.) Hughes, and *Cladosporium cucumerinum* Ell. & Arth (Jarvis & Slingsby, 1977) There is no information indicating that *A. quisqualis* shows infectivity or pathogenicity to any organisms beyond this relatively narrow taxonomic range of fungal pathogens. Indeed, its life history strategy as a hyperparasite, would tend to preclude a wide host range.

It infects and forms pycnidia (fruiting bodies) within powdery mildew hyphae, conidiophores (specialised spore-producing hyphae) and cleistothecia (closed fruiting bodies of powdery mildews). Having penetrated into the mildew hyphae, the fungus produces pycnidia, in which form the pathogen can survive adverse periods e.g. winter, in and around the host plants of the mildew fungi. The pycnidia produce spores, which require favourable conditions for successful germination e.g. in terms of temperature, moisture and in particular the presence of the appropriate host. Over-wintering pycnidia can also be produced saprophytically in vascular plants e.g. in mildewed clover leaves and in cucumber leaves (Yarwood, 1939). The infectivity of the spores produced by the pycnidia rapidly diminishes under field conditions (e.g. 24 to 48 hours), although this can be extended under appropriate conditions.

Sundheim and Krekling (1982) demonstrated that *A. quisqualis* produced specialised appressorium-like penetration structures on powdery mildew. Penetration of the host cell was probably due to mechanical and enzymatic processes. Furthermore, enzymatic digestion played a major role in the invasion of the host cell and the destruction of cytoplasm. Invading hyphae penetrated the host cells through the septal pores of the host. Beuther *et al* (1981) studied the effects of extracts from the hyperparasite on growth, sporulation and conidial germination of the host. They found no evidence of toxin production.

In terms of long-term exposure, the spores are unlikely to remain viable for long periods of time. Thus, for successful germination the spores need both favourable conditions (e.g. high humidity or moisture, temperature around 25 °C) and the presence of the host. Without the host, viability is rapidly lost e.g. within a few days. While the spores can survive for longer under appropriate conditions (low humidity or lack of moisture and low temperatures), these are unlikely to be the prevailing conditions at the time of application or at least for any prolonged period. However, the *A. quisqualis* pycnidia, which will be produced from the infected mildew, are more resilient and may persist in the environment for relatively long periods (at least into the next season). These in turn, may give rise to viable spores when conditions become favourable again.

The toxicological profile of *A. quisqualis* is environmentally sound. The product shows no adverse effect on humans with no pathogenic or infective effects (acute or chronic) in the different routes of application (oral, dermal, inhalation), tested through experimental studies. No irritating or sensitizer effect on the tested animals and no genotoxicity or mutagenicity activity observed in vitro tests.

Regarding the effect on non target organism, *A. quisqualis* shows very low toxicity to birds. No signs of infectivity or pathogenicity were observed in either of the two species tested.

The generated information indicates that *A. quisqualis* is of low toxicity to non-target organisms, aquatic organisms (fish and aquatic invertebrates) and honey bees. The results of the studies also showed no signs of any infectivity or pathogenicity to the organisms tested.

These findings are consistent with the known mode of action of this fungal parasite, invading the mildew hyphae and penetrating the host cell walls, probably by enzymatic action, resulting in slow death of the host. There is no evidence of any toxin production, which could in turn have effects on other, non-target organisms. In addition, these findings are consistent with the life history strategy of a hyperparasite, having a relatively narrow host range, which in this case is restricted to powdery mildews as well as some other fungal species. On this basis, it would be unlikely to find infectivity or pathogenicity extending to other, non-host groups particularly those that are taxonomically unrelated e.g. birds, mammals, fish, aquatic invertebrates, algae, non-target arthropods and earthworms.

One final consideration that needs to be taken into account, is that *A. quisqualis* is a naturally occurring organism that has been reported from most parts of the world. It is therefore likely that it is present at endemic levels wherever its hosts (the powdery mildews etc) are found with reasonable abundance/persistence. The only difference that the application of AQ-10 is likely to make to this situation, is to the pattern of exposure i.e. short-term increases in the levels of exposure. Taking this into account, suggests that it is even less likely that there will be any significant risk to non-target organisms.

AQ-10, is a water dispersible granule formulation containing the fungal hyperparasite *A. quisqualis* isolate M-10 as the active ingredient (58% viable spores and 42% inert co-formulants). The minimum viable spore content guaranteed is  $5.0 \times 10^9$  / gram of product.

AQ-10 should be applied 2 to 12 times a season at intervals of 7 to 14 days, depending on disease pressure. Earliest application is during shoot emergence, which depending on the region varies from March to April. It is applied at a rate of 35 - 60 g/ha in an adequate volume of water sufficient to have an good coverage of the vegetation. The optimum germination temperature is  $25^{\circ}\text{C}$  and above  $30^{\circ}\text{C}$  germination decreases and eventually stops at  $37^{\circ}\text{C}$ .

The advantage of using AQ10 is multiple. Firstly, the original mode of action, different from any other fungicide, reduced the risks of selection of resistant strains, mostly appearing with sterol inhibitors and strobilurins products. Another important factor is related to the fact that the product shows a higher activity than sulphur at lower temperatures, never determining phytotoxicity on the vegetation.

On extensive field trials, AQ10 demonstrate also an activity against cleistothecia, over-wintering stage of the powdery mildew. Due to the absence of residue, National

Authorities did not set any maximum level of residue (MLR) and consequently did not apply a post harvest interval (waiting period). Another relevant aspect is the no interference on the making and quality of musts and wines.

For effective powder mildew control AQ10 can be applied alone, but it can also be included in IPM strategies, which provide for applications of both conventional agrochemicals and the bio control agents. AQ10 can be applied with any conventional spray equipment on grapes, strawberry, cucurbits, tomatoes, pepper and roses at low powdery mildew infestation levels.

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008; (<http://faolex.fao.org/docs/pdf/eur49704.pdf>)

**COMMISSION DIRECTIVE 2005/2/EC** of 19 January 2005 amending Council Directive 91/414/EEC to include *Ampelomyces quisqualis* and *Gliocladium catenulatum* as active substances (Text with EEA relevance).

## ***Bacillus subtilis***

Internet: last accessed, Jan. 15<sup>th</sup>, 2008

([http://www.epa.gov/pesticides/biopesticides/ingredients/tech\\_docs/tech\\_006479.htm](http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/tech_006479.htm)) and (<http://www.epa.gov/oppt/biotech/pubs/fra/fra009.htm>)

*Bacillus subtilis* Strain QST 713 (006479) Biopesticide Registration Action Document

### **Executive Summary**

*Bacillus subtilis* strain QST 713 as an active ingredient is a biological control agent for use on several minor crops to treat a variety of plant diseases and fungal pathogens including gray mold, powdery mildew, early and late blight, bacterial spot, and walnut blight. *Bacillus subtilis* is a ubiquitous bacteria commonly found in various ecological niches including soil, water and air which does not have a history of pathogenicity from contact in the environment. In addition, there are other strains of *B. subtilis* which are registered as microbial pesticides.

Sufficient data are available to determine that *Bacillus subtilis* strain QST 713 has low toxicity to mammals and is not expected to be pathogenic in humans. Standard personal protective equipment are required to mitigate any risk to pesticide handlers and applicators. No significant risk is expected from the terrestrial ground application of the end-use product to birds, fish, ladybird beetles, green lacewings, honeybees, parasitic wasps, and aquatic invertebrates. Risks to honeybees have been mitigated by requiring that the end-use product not be applied when bees are actively visiting the treatment area and not allowing certain bee-sensitive crops (apples and pears) on the label.

The data submitted to EPA on *B. subtilis* strain QST 713 are adequate to support a 2 year time-limited conditional registration under FIFRA section 3(c)(7)(C). A whole hive honeybee study is being required as a condition of registration. In addition as a term and condition of registration, the Agency is requiring a specific manufacturing process including an analysis of each batch for particular microbial contaminants. Additional product characterization studies, including storage stability studies will be required if the registrant applies to amend their registration to eliminate the required manufacturing process and batch analysis. In addition, the Agency is requiring confirmatory data on some ecological effects studies to further evaluate the pathogenicity potential of this strain of *B. subtilis* to non-target organisms.

The acute oral toxicity, acute dermal toxicity, acute pulmonary, acute intravenous, primary eye irritation, primary dermal irritation, and delayed contact hypersensitivity test are acceptable. The acute pulmonary study showed no mortality and no adverse effects when performed using the technical product. There was also no mortality from the acute inhalation study using the end-use product although some clinical signs and weight loss were noted. However, the acute inhalation study did not measure the actual concentration of the product in the test. Considering that the particle size of the wettable powder poses a low risk of inhalation exposure and the nature of the inerts in the product, the Agency is using the acute inhalation study as confirmatory of the acute pulmonary study and waiving the requirement for a repeated acute

inhalation study. In addition, any risk is mitigated by standard personal protective equipment and the reentry interval required for the end-use product. Extremely high levels of the bacteria were used in the ecological effects testing. The levels far exceeded what will typically be used in the field. These high test concentrations resulted in mortality which complicated interpretation of the studies. Toxicity was not considered significant except for the honeybee study where mortality was seen at all test concentrations. There were numerous reports from researchers and bee keepers indicating that no adverse effects were seen during experimentation. As a condition of registration, EPA is requiring that the registrant conduct a whole hive study following an EPA approved protocol. Until this data can be generated, reviewed by EPA, and a determination is made that honeybees are not at risk from the use of QST 713, use sites where bees would be exposed are prohibited and a honeybee warning statement is required. EPA test guidelines for non-target organisms recommends that if toxicity is found, a pathogenicity test also be conducted. Given the very high doses at which toxicity occurred in the honeybee study, it is unclear whether the effect is from toxicity alone or combined with pathogenicity. For the non-target tests in freshwater fish, aquatic invertebrates, honeybees, and hymenoptera where mortality was reported at high dose levels, EPA is requiring a confirmatory pathogenicity test be conducted and EPA is recommending that the tests be conducted at more reasonable concentrations. In addition, the submitted manufacturing process did not have sufficient quality control required for all fermentation batches. Therefore, as a term and condition of this registration, EPA has established criteria each batch of product must meet in order to be marketed. Results of these batch tests will be submitted to EPA upon request. A listing of the required data are given on pages 27 to 28. The submitted data in support of this registration under section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) have been reviewed and determined to be adequate for a time-limited two (2) year registration. This registration will not cause unreasonable adverse effects to man or the environment.

## ***Bacillus thuringiensis***

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;  
(<http://www.inchem.org/documents/ehc/ehc/ehc217.htm>, )

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the United Nations Environment Programme, the International Labour Organisation, or the World Health Organization.

### **Environmental Health Criteria 217**

### **Microbial Pest Control Agent**

### ***BACILLUS THURINGIENSIS***

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World Health Organization  
Geneva, 1999

### **SUMMARY**

This monograph deals with microbial pest control agents (MCPAs) based on *Bacillus thuringiensis* (Bt). This bacterium is also a key source of genes for transgenic expression to provide pest resistance in plants and microorganisms as pest control agents in so-called genetically modified organisms (GMOs). The potential effects on human health and the environment of GMOs involve several aspects that are only remotely or not at all related to Bt products, and they are therefore outside the scope of this monograph.

## ***Beauveria bassiana* (Bals.-Criv.) Vuill. –**

### **Tobias Längle**

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### **History of *Beauveria bassiana* (Bals.-Criv.) Vuill.**

The origins of microbial pest control date back to the early nineteenth century, when the Italian scientist Agostino Bassi spent more than 30 years studying white muscardine disease in silkworms (*Bombyx mori* L.). He identified *Beauveria bassiana* (Bals.-Criv.) Vuill., named in his honour, as the cause of the disease. His discovery not only laid the foundation for microbial pest control, but also significantly influenced the work Louis Pasteur, Robert Koch and other pioneers of microbiology (Ainsworth, 1956; Porter, 1973; Van Driesche & Bellows, 1996). Bassi himself recognized the potential to use organisms such as *Beauveria bassiana* to control insect pests (Bassi, 1836; cit. in Van Driesche & Bellows, 1996) and by the early 20<sup>th</sup> century, field trials had been conducted with *B. bassiana*, *B. brongniartii* (Sacc.) Petch, and *Metarhizium anisopliae* (Metschn.) Sorokin. Today, over 100 years later, there are no known reports of significant adverse effects that can be attributed to the use of these organisms in biocontrol.

### **Pathogenicity/Infectivity of *B. bassiana***

An extensive literature search was conducted to evaluate risks related to human exposure to *Beauveria bassiana*. A total of 8 distinct reports naming the genus *Beauveria* Vuill. as the alleged cause of fungal infections and disease of humans were identified, but only 4 of these reports could be conclusively attributed to species of the genus *Beauveria*.

Like any micro-organism, *Beauveria bassiana* has the potential to act as an opportunistic pathogen, but as the literature study demonstrates, *Beauveria* infections are extremely rare events. A detailed analysis of case reports allegedly involving *Beauveria bassiana* reveals that extraordinary circumstances, such as a severely compromised immune system or a history of surgery/injury, are required for a *B. bassiana* infection to occur.

The most severe human cases of *Beauveria* infections are two recent reports of disseminated mycoses (Henke *et al.*, 2002; Tucker *et al.*, 2004). Both of these infections occurred in severely immuno-compromised patients with acute leukemia. Prior the development of mycoses, one patient underwent 4 full cycles of chemotherapy, the other was in her first cycle of chemotherapy and had been diagnosed with *Streptococcus viridans* in her bloodstream. Despite their poor health, both patients responded well the antimycotic treatments and fully recovered from their mycoses. Further reports of *Beauveria*-related deep tissue mycoses (Freour *et al.*, 1966a-c, Drouhet & Dupont, 1980) could not be substantiated.

While there are some reports of *Beauveria* spp. isolated from patients with corneal keratitis, the *B. bassiana* can certainly not be considered a significant eye pathogen. Of four reports linked to *Beauveria*, only two (Sachs *et al.*, 1985; Kisla *et al.*, 2000) can be conclusively attributed to *Beauveria bassiana*. In these cases the affected eye had undergone surgery following traumatic injury to the eye, and in all reported

cases, the therapy of the injured eye involved corticosteroids and antibiotics, which, according to Sachs *et al.* (1985) can predispose the eye to fungal infections by otherwise non-pathogenic fungi.

None of the studies conducted for the registration of *B. bassiana* stain GHA in the US (acute oral toxicity/pathogenicity; acute dermal toxicity; acute pulmonary toxicity/pathogenicity; acute intraperitoneal toxicity/pathogenicity) showed any pathogenicity of the test organism against the tested mammals (US EPA, 2006). Likewise, eye irritation studies (US EPA, 2006) and literature reports (Ishibashi *et al.*, 1987; Begley & Waggoner, 1992) show no unacceptable effects related *B. bassiana* exposure to the healthy eye. Furthermore, it has been used in biocontrol for over 100 years with no reports of illness related to exposure to *B. bassiana* strains used in biocontrol.

These considerations allow the conclusion, that the label compliant use of *B. bassiana* based products such as Botanigard® will not result in unacceptable risks for applicators and consumers.

### **Metabolites**

Species of the genus *Beauveria* have been reported to produce the secondary metabolites bassianin, bassiacridin, beauvericin, bassianolide, beauverolides, tenellin and oosporein (Strasser *et al.*, 2000; Vey *et al.*, 2001; Quesada-Moraga & Vey, 2004).

It is important to note that the discovery of a certain metabolite during liquid cultivation of a specific strain cannot be extrapolated to all strains of the species. Moreover, it cannot be assumed that these substances will also be produced under natural conditions in the soil or in the target host. Further, it should be kept in mind that entomopathogenic fungi naturally cause epizootics similar to those resulting from artificial inoculations. There are no reports of metabolites entering the food chain or accumulating in the environment as a result of such natural or artificial epizootics or natural metabolite secretion (Vey *et al.*, 2001). In contrast, numerous studies have documented environmental accumulation and food chain contamination with chemical pesticides and antibiotics used in agricultural production.

Specifically, no metabolites of concern have been detected in end-use formulations of Botanigard® products, which are based on *B. bassiana* GHA spores, and toxicological tests (see pathogenicity section), have not shown any adverse effects related to either pathogenicity or toxicity.

The ubiquitous natural occurrence of the species *B. bassiana*, its more long history of use in biocontrol, and the fact that Botanigard® products have been safely used in the United States and other countries for more than 10 years, demonstrate that no unacceptable risks are expected to result from the use of these products.

### **Ecotoxicology**

No negative effects of Botanigard® were found in ecotoxicology studies with mammals, birds or fish (US EPA, 2006). Literature reports of *B. bassiana* infections in captive reptiles (Georg *et al.*, 1962; Fromtling *et al.*, 1979; Gonzalez *et al.*, 1995) can be attributed to inappropriate captivity conditions, and no reports of any vertebrates infected by *B. bassiana* in the wild were found. Further, *B. bassiana* is not known to cause adverse effects to plants or earthworms.

Compared to the second species of the genus, *Beauveria bassiana* has a wider host range and a therefore, in theory, a somewhat higher potential to affect non-target arthropods. It is, however, important to recognize the difference between the physiological and the ecological host range of an organism, i.e. if a non-target species can be infected in the lab this cannot be directly translated into potential adverse effects in the field. Based on the natural occurrence of *B. bassiana* and the low toxicity profile demonstrated by ecotoxicology studies conducted with Botanigard® products, the ecological risk due to exposure to this microorganism, is expected to be minimal (US EPA, 2006).

#### Efficacy

Levels of control reached with biological organisms are generally more dependent on environmental conditions, such as climatic factors, than those achieved with conventional pesticides. *Beauveria bassiana* has been tested in a wide range of pest control scenarios and has been successfully used in many countries. While under suitable conditions, efficacy rates of Botanigard® can exceed 90 %, in many instances, a considerably lower level, but longer-term of suppression can be sufficient to prevent crop damage. It is important to recognize that biological control agents such as *B. bassiana* significantly differ from chemical pesticides in their properties and this should be taken into account when designing and reviewing efficacy studies.

#### Comparison of safety with synthetic plant protection products used

Chlorpyrifos is a common insecticide used against the some of same targets as *B. bassiana*. This substance acts as neurotoxin (acetylcholine esterase inhibitor) and is therefore highly toxic to a wide range of non-target organisms, including mammals and birds, aquatic organisms or bees. Chlorpyrifos can leach into the ground water or enter the food chain through agricultural produce. The risks of this pesticide have been judged acceptable by the European Union, yet, *Beauveria bassiana*, which is by far less problematic than chlorpyrifos, is still awaiting approval for inclusion in Annex I A of Directive 91/414/EEC.

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## ***Beauveria brongniartii* (Saccardo) Petch**

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The enthomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Clavicipitaceae) is used as the selective and virulent microbial pest control agent (MPCA) against *Melolontha* spp. (*M. melolontha* L. and *M. hippocastani* F.; Coleoptera: Scarabaeidae; common European cockchafer). In Austria, *B. brongniartii* has been registered for unrestricted use (all crops) in 2000 (Austrian Plant Protection Product Register, Reg. No. 2582). Presently, the MPCA will be notified in accordance with Article 4 of the Commission Regulation No 1112/2002 by the Austrian Government, assisted by the two companies Agrifutur s.r.l. and Kwizda Agro GmbH (task force *Beauveria brongniartii* - TFBB). For registration purposes of the MPCA *B. brongniartii* (MPCP - MELOCONT<sup>TM</sup> -Pilzgerste), TFBB considers that the dossier is complete. All relevant safety data are available, especially those information, which demonstrates that the product is safe, posing no unacceptable risks to operators applying the formulated product nor to people or animals who might consume treated crops; the product is also safe to the environment. *B. brongniartii* is therefore an effective and safe biological control agent.

*Beauveria brongniartii* can be characterised as follows:

*B. brongniartii* exhibit a very limited host range: the fungus is used to control all developmental stages of *Melolontha* spp., (*M. melolontha* Linnaeus, *M. hippocastani* Fabricius).

The application of an adapted ERBIC Risk Index to *B. brongniartii* resulted in a Risk Index of 15 on a scale of 125 (Längle, 2005). According to van Lenteren *et al.* 2003 a risk index of up to 35 would result in no objection for the use of the assessed agent.

Toxicity/pathogenicity/infectivity: Depending on the risk level of infection (Commission Directive 2000/54/EC), *B. brongniartii*, is classified in the risk Group 1: Biological agents which are unlikely to cause human disease. Experiments with the use of *B. brongniartii* for *Melolontha* control were done over a period of more than 100 years. No published or reported data of side-effects on humans and mammals exist. As *B. brongniartii* is unable to grow at temperatures higher than 33°C, pathogenicity to warm-blooded animals can be excluded.

Under natural conditions *B. brongniartii* is not competitive and suppressed by the natural microbiota (Kessler, 2004). There are no reports on the germination, invasion or growth on plants, plant material or foodstuff. Only after autoclaving these substrates they are easily colonised because no competition with other microorganisms takes place.

Metabolites/toxins: Data on metabolite production by commercial isolates of the genus *Beauveria* (e.g. MELOCONT<sup>TM</sup> -Pilzgerste, Beauveria-Schweizer,

Engerlingspilz-Andermatt, MELOCONT<sup>TM</sup> -WG) is hard to come by. Oosporein was identified as the only major secondary metabolite in submerged culture, in the final

product and in mycosed pest organisms (Strasser *et al.* 2000b, Seger *et al.* 2005a). Oosporein is a C<sub>2</sub> symmetrical red 2,5-dihydroxybenzoquinone derivative biosynthesized by a broad variety of soil borne fungi and is known for almost six decades. There is no evidence of metabolites transferred to plants. (Seger *et al.* 2005b, Strasser *et al.* 2004). As can be derived from the chemical and physical characterisation of oosporein (Seger *et al.*, 2005c), the metabolite degrades quickly under moderate alkaline conditions. Oosporein is not volatile and, therefore, cannot be inhaled/taken up by workers as MVOCs. An adsorption into soil and charged biological matrices is nearly irreversible; however, oosporein can be washed off from the surface of crops and fruiting vegetables with tap water. Risks of exposure to toxins for workers and users is not relevant because formulated products are free of toxicologically “relevant” *Beauveria* metabolites. *Beauveria* metabolites have no relevant antibiotic activity, no cytotoxic or apoptotic effects (Abendstein & Strasser 2000; Butt *et al.*, 2004). Hypothetically speaking, even if the fungus would show saprophytic growth on plant materials, the quantity of produced metabolites still would be not relevant. Neither the active agent *B. brongniartii* nor metabolites which are produced by the fungus show unacceptable effects on human health and/or the environment during or after application.

**Efficacy:** *B. brongniartii* is an entomopathogenic soil fungus with world-wide distribution. *B. brongniartii* has a narrow host range and has been tested in large-scale field tests in Austria, Italy and Switzerland over the last two decades. The use of this MPCA is intended without restriction in open fields and in agricultural systems because no ecological risk, no risks for the health of humans and animals are to be expected. Survival in host free soils is limited to about a few years. Impact on non-target organisms, especially beneficial insects and earthworms are very unlikely (i.e. no negative results of reports and studies). Dissemination of fungal spores into the groundwater or into the air are very unlikely. The isolates used for control purposes can be identified by classical microbiological techniques and by using microsatellite markers (Enkerli *et al.* 2001). These methods are recommended to monitor the fate of the fungus in the environment.

**Comparison of safety with synthetic plant protection products used for the same purpose:**

Crops treated with synthetic insecticides may be protected against some pests but are harmful to non-target organisms such as mammalian herbivores, and cannot be used immediately for human or livestock consumption. Strasser *et al.* (2000a) reported, that chemical pesticides (i.e. chlorpyrifos, dursban, methylbromide) have had little or no effect in controlling the subterranean pest *Melolontha melolontha*. Chemicals applied to the soil may be ineffective because of the intrinsic buffering properties of soil. Leaching of pesticides into ground water will ultimately contaminate water sources. Presently, chlorpyrifos is the top insecticide used on crop fields. Residues of the chemical are found on dozens of crops, including vegetables and grains. According to the Federal Centers for Disease Control in the U.S.A., 82 percent of Americans already have the insecticide in their bodies (EWG, 2006). So far there have been no reports of *B. brongniartii* or its metabolites having any negative effects on humans and non-target organisms even though they have been deployed extensively in specific crop production systems. *Beauveria* is applied at 30-50 kg/ha in agricultural systems; the amounts of oosporein released by *B. brongniartii* into the soil either from the formulated product or from mycosed insects is relatively

small. The concentration of oosporein detected in the soil is usually 2.5 million times lower than that of the pesticides methylbromide and dazomet (Strasser *et al.*, 2000a). Weighing the risks and benefits of the release of a *B. brongniartii* versus other control measurements (chemicals), one would expect that *MELOCONT*<sup>TM</sup> products could replace many products which harm humans as well as the environment.

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## ***Coniothyrium minitans* strain CON/M/91-08**

(PC Code 028836)

### **BIOPESTICIDES REGISTRATION ACTION DOCUMENT**

U.S. Environmental Protection Agency

Office of Pesticide Programs

Biopesticides and Pollution Prevention Division

*Coniothyrium minitans* strain CON/M/91-08

(PC Code 028836)

Interent: last accessed: Jan. 15<sup>th</sup> 2008

[http://epa.gov/opppd1/biopesticides/ingredients/tech\\_docs/brad\\_028836.pdf](http://epa.gov/opppd1/biopesticides/ingredients/tech_docs/brad_028836.pdf)

### **EXECUTIVE SUMMARY**

The fungal active ingredient *Coniothyrium minitans* strain CON/M/91-08 is a naturally occurring strain of the *Coniothyrium* fungal species. *Coniothyrium minitans* is a highly specialized antifungal agent that targets *Sclerotinia sclerotiorum* and *Sclerotinia minor*, common plant pathogens. The sole end-use product (EP) Contans®WG which contains a minimum of  $1 \times 10^9$  spores per gram, is manufactured by PROPHYTA Biologischer Pflanzenschutz GmbH. This pesticide product is registered in Germany, Switzerland, Austria, Hungary, Luxembourg and Poland. Several acute toxicity studies (eye, dermal, oral, and intraperitoneal) using a dose greater than  $10^7$  colony forming units (CFU) of *Coniothyrium minitans* strain CON/M/91-08 were conducted, with no adverse effects being observed. *Coniothyrium minitans* has not been reported as a pathogen of any organism in public literature other than *Sclerotinia*, and occasionally several other closely related sclerotia producing fungi. Exposure of humans, birds, fish, aquatic invertebrates, and honey bees to Contans®WG is anticipated to be minimal because the product is incorporated into the soil. However, appropriate personal protection equipment is required as prudent to protect applicators, mixers and other pesticide handlers from unnecessary exposure to microorganisms.

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;

[http://eur-lex.europa.eu/smartapi/cgi/sqa\\_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=32003L0079&model=guichett](http://eur-lex.europa.eu/smartapi/cgi/sqa_doc?smartapi!celexapi!prod!CELEXnumdoc&lg=EN&numdoc=32003L0079&model=guichett)

**COMMISSION DIRECTIVE 2003/79/EC** of 13 August 2003 amending Council Directive 91/414/EEC to include *Coniothyrium minitans* as active substance (Text with EEA relevance).

## ***Gliocladium catenulatum* J1446**

**Marina Niemi**

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### 1) Toxicity/pathogenicity/infectivity (of the active substance)

#### a) In animal tests:

- non-pathogenic
- non-infective
- non-toxic (oral, dermal, intracheal, intraperitoneal; in the latter two cases some effects probably caused by the way of exposure)
- non-irritant (dermal, eye)
- skin sensitizing

#### b) In cytotoxicity test (FL cells):

- no toxicity

#### c) In medical surveillance of personnel:

- no adverse effects on personnel involved in production or testing of the MPCA during several years
- no acute or chronic symptoms or other significant findings related to exposure to *G. catenulatum* J1446 in personnel, no details on any occurrence of hypersensitivity or chronic sensitization.

### 2) Metabolites

#### a) presence of metabolites of concern in the product

- no indications from literature or from toxicity tests that *G. catenulatum* J1446 would produce metabolites of toxicological or environmental concern
- nevertheless, perceived risk of gliotoxin due to known production of gliotoxin by other *Gliocladium* and *Trichoderma* species
- demand for analysis of gliotoxin in various samples
  - ☐ no gliotoxin detected in fermentation broth or in rockwool cubes with cucumber seedlings, treated with formulated *G. catenulatum* J 1446 (at requested LOD of 10 µg/l)
  - ☐ analysis of gliotoxin in unformulated cell mass of *G. catenulatum* J 1446 unsuccessful (too low recovery % due to the complex nature of the sample matrix)
  - ☐ no genotoxicity in *Escherichia coli* WP2/CM871 DNA-repair test with samples of fermentation broth

#### b) Availability of analytical method to quantify the metabolite

- no standard method available
- gliotoxin analysis from different methods had to be developed and validated (extraction procedures, HPLC-analysis)
- what is a reasonable Limit of Detection/Quantification?

#### c) Possible harmful effects of metabolites/toxins on human health (including vulnerable groups), animal health (taking into account known cumulative and synergistic effects when the methods to assess such effects are available)

- not relevant in the case of *G. catenulatum* J1446, since no gliotoxin production was detected

#### d) Possible unacceptable effect on the environment

- not relevant, see above

### 3) Ecotoxicology, environmental hazards and potential waivers for data requirements

- the toxicity of *G. catenulatum* J1446 to non-target organisms is considered 'slight' (?)
- the current ecotox test methods are not suitable for microbial PPPs and the results are accordingly difficult to interpret
- it is highly unlikely that a common soil fungus added to soil should have adverse effects on other soil organisms, considering the amount and diversity of microbes present in soil
- it is unlikely that terrestrial and aquatic organisms would become exposed to this PPP, which is applied in a very targeted way in very small quantities to the growth substrate or the foliage of the crop to be protected
- it should not be necessary to provide extensive data on persistence in the environment, considering the fact that despite a temporary dominance of an applied microbial PPP, the ecosystem will regain its ecological balance with time.

#### 4) Comparison of safety with synthetic plant protection products used for the same purpose

Table 1. Comparison of human health and environmental risk assessment of one microbial and two chemical active substances used for the same purpose

PPP	Active substance	Target pathogen	Toxicological effects	Ecological effects
Prestop Mix	<i>Gliocladium catenulatum</i> J1446	<i>Pythium</i> , <i>Botrytis</i>	No acute toxicity, pathogenicity, infectivity, no irritation, skin sensitizer. No genotoxicity test requested because no toxins produced.	Natural soil fungus, indigenous in soils throughout the world, unmodified wild strain isolated from field soil. Slight (?) toxicity to non-target organisms.
Previcur N	Propamocarb hydrochloride	<i>Pythium</i>	No acute or chronic toxicity, no reproductive effects, no genotoxicity or carcinogenicity, not a skin sensitizer.	Non-toxic to birds and earthworms, very low toxicity to aquatic organisms and bacteria, no adverse effects on soil enzyme activities, rapid degradation in soil and water.
Rovral 75WG	Iprodione	<i>Botrytis</i>	Moderate to intermediate single-dose toxicity, relatively low repeated-dose toxicity. No reproductive effects, no genotoxicity or carcinogenicity, not a skin sensitizer.	Low toxicity to terrestrial organisms, some risk to bees and birds, intermediate to high acute toxicity to aquatic organisms. Slow decomposition, risk for bioaccumulation.

Table 2. Comparison of the classification and safety information for one microbial and two chemical active substances used for the same purpose.

PPP	Classification	R-phrases	S-phrases	Safety period	Re-entry period	Environmental restrictions
Prestop-Mix Xi (irritant)	- R43-May cause sensitization by skin contact	S2-Keep out of the reach of children S13-keep away from food, drink and animal feedingstuff S22-Do not breathe dust S24-Avoid contact with skin S29-Do not empty into drains	S37-Wear suitable gloves	None	None	None
Previcur-N - -	R52-Harmful to aquatic organisms	S2-Keep out of the reach of children S13-Keep away from food, drink and animal feedingstuff	S29-Do not empty into drains 14 days (radish)	21 days (lettuce, endive, cabbage, cauliflower)	12 hours May not be used closer than 10 m from water courses	May not be used on groundwater areas and closer than 30-100 m from drinking water sources
Rovral-75WG Xn (harmful)	N (dangerous for the environment) R36-Irritating to eyes R40-Limited evidence of a carcinogenic effect	R50/53-Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment S2-Keep out of the reach of children S13-Keep away from food, drink and animal feedingstuff S25-Avoid contact with eyes	S29-Do not empty into drains 10 days (general) 4 days (glasshouse cucumber) 7 days (glasshouse tomato) 21 days (raspberry) 30 days (onion)	35 days (carrot)	1 day	May not be used closer than 25 m from water courses. Because slowly degraded and may accumulate in soil, must not be used in two succeeding years

### 5) Efficacy

- Zonal approach in efficacy testing a good improvement
- A reasonable data package in support of selected intended uses should be enough, since it is not possible (nor economically feasible) to provide efficacy data for all crop/pathogen combinations with all different cultivation systems.
- Demand for statistically significant treatment effects in all tests will limit the range of uses, since it is seldom possible to have trials large enough to allow statistical analysis.

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008; (<http://faolex.fao.org/docs/pdf/eur49704.pdf>)

**COMMISSION DIRECTIVE 2005/2/EC** of 19 January 2005 amending Council Directive 91/414/EEC to include *Ampelomyces quisqualis* and *Gliocladium catenulatum* as active substances (Text with EEA relevance).

## Metarhizium anisopliae **strain F52 (029056)**

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;  
[http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet\\_029056.htm](http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_029056.htm)

### **Summary**

*Metarhizium anisopliae* strain F52 is a fungus that infects insects, primarily beetle larvae. It has been approved as a microbial pesticide active ingredient for non-food use in greenhouses and nurseries, and at limited outdoor sites not near bodies of water. Many strains of *Metarhizium anisopliae* have been isolated worldwide from insects, nematodes, soil, river sediments, and decomposing organic material. No harm is expected to humans or the environment when pesticide products containing *Metarhizium anisopliae* strain F52 are used according to label instructions.

## **Pantoea agglomerans strain C9-1 (006470) Fact Sheet**

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;  
([http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet\\_006470.htm](http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_006470.htm))

### **Summary**

Originally isolated in 1994 by researchers at the U.S. Department of Agriculture, Agriculture Research Collection, this strain is derived from apple stem tissue. This naturally occurring bacterium has streptomycin and rifampicin resistance that is not derived through genetic engineering.

### **Regulatory Information**

Registered on September 8, 2006 with a commercial FIFIRA section 3 registration and an exemption from the requirement of a tolerance for the bacterium *Pantoea agglomerans* strain EC9-1, applied to apples and pears.

***Paecilomyces fumosoroseus* (Apopka strain 97, PFR 97 or CG 170, ATCC20874)**

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;  
(<http://www.legaltext.ee/text/en/PH0026.htm>)

**COMMISSION DIRECTIVE 2001/47/EC** of 25 June 2001 amending Annex I to Council Directive 91/414/EEC concerning the placing of plant protection products on the market to include *Paecilomyces fumosoroseus* (Apopka strain 97, PFR 97 or CG 170, ATCC20874) as an active substance.

## ***Pseudomonas chlororaphis* MA 342**

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**COMMISSION DIRECTIVE 2004/71/EC** of 28 April 2004 amending Council Directive 91/414/EEC to include *Pseudomonas chlororaphis* as active substance (Text with EEA relevance).

### **Background**

Lantmännen BioAgri AB produces and sells two seed treatment products for cereals, Cedomon and Cerall, which are both based on the soil bacterium *Pseudomonas chlororaphis*, strain MA 342, as the active organism. Cedomon is an oil-based formulation used in barley and oats. Since 1997 barley and oats seeds for sowing of approximately 1,8 million hectares have been treated with Cedomon, mainly against seed-borne *Pyrenophora* sp. Cerall is a new formulation of MA 342 suitable for treatment of wheat, rye and triticale against seed-borne *Tilletia caries*, *Fusarium* sp., *Microdochium nivale* and *Septoria nodorum*.

The Annex I listing of *P. chlororaphis* strain MA 342 was approved in 2004. Cedomon is so far registered in Sweden, Norway, Finland, Austria, Italy, Denmark and Poland, and Cerall is registered in Sweden, Austria and Finland.

#### **1) Toxicity/pathogenicity/infectivity (of both the active substance and metabolites/toxins if necessary)**

*P. chlororaphis* has not been reported as a disease causing agent in humans. MA 342 does not grow at temperatures above 32 °C, and was as expected rapidly cleared from the test animals in the GLP animal tests performed. There was no indication that MA 342 caused any effect in mammalian systems. The toxicological endpoints can be summarised as follows:

Acute oral toxicity: low toxicity (LD<sub>50</sub> >2×10<sup>10</sup> bacteria/kg b.w. in the rat)

Acute pulmonary toxicity/pathogenicity: No signs of ill health or adverse effects in the rat

Skin irritation: Non-irritating in the rabbit

Eye irritation: Non-irritating in the rabbit

Skin sensitisation: Non-sensitising in the guinea pig

In a Japanese study, a *P. chlororaphis* strain was reported to kill trout, carp and eel after injection.

The bioactive metabolite DDR (see below) in high concentrations have been shown to have an aneugenic effect (affecting the mitotic spindle) in the flow cytometer-based *in vivo* mouse micronucleus assay.

### **2) Metabolites**

#### **a) are there any metabolites/toxins of concern in your product?**

The substance 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) is produced in low concentrations during exponential bacterial growth. In the products DDR is under LOQ.

**b) are any methods available to quantify the metabolites/toxins which are of toxicological or environmental significance?**

Methods for quantification of DDR have been developed and validated by Lantmännen BioAgri AB and approved by the authorities. The DDR concentration is analysed in every batch to secure that the concentration is kept below the safety concentration level decided by the authorities.

**c) do the metabolites/toxins have any harmful effects on human health (including vulnerable groups), animal health (taking into account known cumulative and synergistic effects when the methods to assess such effects are available)?**

At concentrations above a certain threshold value, aneugenic substances, like DDR, cause non-disjunction and chromosome loss. Under the threshold, no effects occur and hence a NOEL can be established. DDR decomposes rapidly, especially at low pH. No cumulative or synergistic effects have been shown.

**d) do they have any unacceptable effect on the environment?**

The DDR concentration in the product is very low. On the germinating seed in soil, DDR is produced by multiplying MA 342 bacteria, as part of the anti-fungal mode of action. DDR has been shown to decompose rapidly, at low pH (e.g. birds stomach) the half life is less than 15 minutes. As the bacteria stay in the spermosphere and decrease to non-detectable levels in a couple of weeks, the effect is very restricted both in time and space. The effect on the environment is regarded acceptable.

### **3) Ecotoxicology**

MA 342 is a good spermosphere but a poor rhizosphere and phyllosphere coloniser; MA 342 applied to seed does not colonise the shoot and roots. In the spermosphere, the strain is out-competed to non-detectable levels in the 3-4 leaves plant stage. No residues of MA 342 or DDR have been detected in the shoot or in the grain yield at harvest.

As MA 342 belongs to a group of commonly occurring soil bacteria and the degradation of DDR is rapid, the impact of MA 342 and DDR on soil non-target micro-organisms, earth worms and soil-dwelling arthropods is likely to be negligible. Being used for seed treatment, the exposure and risk for honey bees and above ground arthropods is considered acceptably low. A study on the survival of MA 342 in fresh stream water showed that it was a very weak competitor compared to bacteria normally occurring in the water. An acute toxicity test on rainbow trout showed no effect. Feed consumption, growth and mortality of the birds were not affected in a chicken feeding test with MA 342-treated seed.

### **4) Comparison of safety with synthetic plant protection products used for the same purpose**

Many (most) of the fungicidal cereal seed treatment chemicals are dangerous to inhale and consume. They are also eye and skin irritating. Some are skin sensitising and some harmful for the foetus development. Common environmental risks are toxic effects for fish and other water-dwelling organisms, as well as harmful long-term effects on water environments. Some show toxic effects on earth- worms and other soil-dwelling organisms.

Many show a high persistency in soil (half-lives up to three years).

### **5) Efficacy**

The biological efficacy dossiers of Cedomon and Cerall consist of several hundred GEP trials conducted in various climatic zones in Europe. Results from these trials show that Cedomon and Cerall give an equal yield and have a disease controlling efficacy in level with chemical seed treatments in standard seed.

## ***Pseudomonas chlororaphis* strain 63-28 (006478) Fact Sheet**

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;  
([http://epa.gov/oppbpd1/biopesticides/ingredients/factsheets/factsheet\\_006478.htm](http://epa.gov/oppbpd1/biopesticides/ingredients/factsheets/factsheet_006478.htm))

### **Summary**

*Pseudomonas chlororaphis* strain 63-28 is a naturally occurring bacterium that can be used in controlling various fungi that attack crop roots. The bacterium has shown no toxicity or pathogenicity to humans, wildlife, or the environment. Its use is limited to vegetables and ornamental crops in containers in greenhouses.

## ***Serratia entomophila* (Enterobacteriaceae)**

**Trevor A. Jackson**

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*Serratia entomophila* is a native soil bacterium found in New Zealand. Strains bearing a specific plasmid (pADAP) are able to cause amber disease in the New Zealand grass grub *Costelytra zealandica*. The bacterium has been developed as a biopesticide and has been used for 15 years as a commercial microbial control for grass grub. The bacterium was firstly applied as a liquid product Invade but has recently been developed for application as a solid granule Bioshield. The bacterium has been registered for sale in New Zealand after carrying out required safety testing. Tier 1 testing (high dose challenge) was carried out against small mammals and no detrimental effects were noted following application by oral, intraperitoneal, dermal and ocular routes. Environmental safety was assessed by direct challenge of closely related scarabs and beneficial organisms. Sheep and chickens were tested as representative vertebrates likely to be exposed to the bacterium. No infectivity was demonstrated for any non-target animal tested, indicating high specificity of *S. entomophila* to grass grub. The bacterium has been used in New Zealand for 15 years with no indications of safety problems or unexpected environmental effects from widespread application. Despite widespread testing, no other insect species have been shown to be susceptible to the plasmid bearing strains of *S. entomophila*.

### **Toxicity**

Once ingested by the grass grub larva, *S. entomophila* causes a rapid cessation of feeding and gut clearance associated with colonisation by the bacteria. This leads to a prolonged period of chronic infection followed by invasion of the haemocoel and death of the insect by sepsis. Gut clearance is associated with a reduction in gut protease titre. The disease state is permanent once triggered by bacterial ingestion.

- Jackson, T.A., Christeller, J.T. McHenry, J.Z. and Laing, W.A. (2004). Quantification and kinetics of the decline in grass grub endopeptidase activity during initiation of amber disease. *Journal of Invertebrate Pathology* **86**: 72-76.
- Jackson T.A., Boucias D.G., and Thaler J.O, (2001). Pathobiology of amber disease, caused by *Serratia* spp., in the New Zealand grass grub, *Costelytra zealandica*. *Journal of Invertebrate Pathology*, **78**: 232-243.
- Jackson, T.A., Huger, A.M. and Glare T.R. (1993). Pathology of amber disease in the New Zealand grass grub *Costelytra zealandica* (Coleoptera:Scarabaeidae). *Journal of Invertebrate Pathology*. **61**: 123-130.

### **Metabolites**

Grass grub pathogenic strains of *Serratia* produce Sep proteins which are related to the Tc toxins produced by *Photorhabdus* and other bacteria. Sep proteins must be induced, with induction occurring within the grass grub gut. While Tc toxins have shown cytotoxic effects direct toxicity of the Sep proteins is unknown.

- Hurst M R H., Glare T R., Jackson T A., Ronson C W. (2000). Plasmid-located pathogenicity determinants of *Serratia entomophila*, the casual agent of amber disease of grass grub,

show similarity to the insecticidal toxins of *Photobacterium luminescens*. *Journal of Bacteriology*, **182**: 5127-5138.

### **Ecotoxicity**

Plasmid bearing strains of *S. entomophila* occur naturally in New Zealand pastures and are most commonly associated with populations of *C. zealandica* which have been resident in a pasture for some years. Bacterial populations are disrupted by cultivation which leads typically to outbreaks of healthy grass grub in pastures two to three years from sowing. Plasmid bearing strains will become established in high density grass grub populations leading to a disease build up and levels of bacteria of up to 10<sup>6</sup> bacteria per g of soil. As the population collapses from disease there is little new contribution of plasmid bearing strains to the soil and numbers of pathogens decline to low levels. Commercial application typically takes place in young pastures or where the disease cycle has been disrupted by drought. The objective of application is to mimic the natural process but to provide inoculum and initiate disease in an earlier part of the population cycle before damage has occurred. Levels of applied bacteria never reach higher numbers than occur in natural population events but the timing of the interaction is altered to prevent damage.

As the activity of plasmid bearing *S. entomophila* is mono-specific, it would be surprising if there were non-target effects. Other insects seem unaffected by *S. entomophila* challenge and a wide range of species have been tested. No significant effects on non-target organism have been identified following commercial applications of the bacteria. In total more than 25,000 ha of pasture have been treated with *S. entomophila* with no indications of hazardous effect.

Jackson, T.A. (2003). Environmental safety of inundative application of naturally occurring biocontrol agent, *Serratia entomophila*. In: *Environmental impacts of Microbial Insecticides: Need and Methods for Risk Assessment*. HMT Hokkanen and AE Hajek (eds). Kluwer Academic Publishers, Netherlands, pp 169-176.

### **Alternatives**

*S. entomophila* is the most widely used material for grass grub control in New Zealand aligning with the national priority for “clean-green” agriculture. There is minor use of organophosphates (Diazinon, Chlorpyrifos) and neonicotinoids (imidacloprid) for seed treatment.

### **Efficacy**

*S. entomophila* application establishes disease within a grass grub population usually leading to 20% infection within two months of application. Recycling of disease within the population will lead to a 50% reduction in the population in the year of application but most benefit is obtained through continued recycling of bacteria in subsequent generations providing long-term pest suppression.

Townsend, R.J., Jackson, T.A., Ferguson, C.M., Proffitt, J.R., Slay, M.W.A., Swaminathan, J., Day, S., Gerard, E.M. O'Callaghan, M. and Johnson, V.W. (2004). Establishment of *Serratia entomophila* after application of a new formulation for grass grub control. *NZ Plant Protection* **57**, 310-313.

## ***Serratia plymuthica* HRO C-48 – safety issues**

**Gabriele Berg, TU Graz, Austria**

*Serratia plymuthica* is grouped into risk group 1 by the DSMZ (German Collection of Microorganisms and Cell Cultures), which means that there is no risk for humans and the environment caused by the bacteria. This classification based on an international approved German directive (TRBA 466). The risk group is species specific. In contrast to a known pathogen of the genus *Serratia*, *Serratia marcescens* (risk group 2); there is no evidence that *S. plymuthica* strains can cause infections in humans. Furthermore, no pathogenicity factor is known for *S. plymuthica*. The red pigment prodigiosin, which is characteristic for *S. marcescens* and responsible for the antifungal and anti-eucaryotic activity of *S. marcescens* is not produced in *S. plymuthica* HRO C-48. In an alternative animal model to assess the pathogenicity of facultative pathogenic strains, the *Caenorhabditis elegans* assay, no pathogenicity for our biocontrol strain was found. For the strain themselves, a low level of antibiotic resistance was measured. In addition, the impact of a *Serratia* treatment on the indigenous microflora in terrestrial ecosystems was analysed. No treatment specific differences were obtained in the composition of species of the non-target microorganisms in the rhizospheres<sup>1</sup>.

<sup>1</sup> Scherwinski, K., Wolf, A. und G. Berg, 2006. Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bacteria. **Biocontrol** 52:87-112.

BioControl (2007) 52:87–112 \_ Springer 2006 DOI 10.1007/s10526-006-9006-8  
Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bacteria.

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**Abstract.** The phytopathogenic fungus *Verticillium dahliae* Kleb. causes high yield losses in strawberry production. As effective chemical control of this fungus is no longer available, biological control based on natural antagonists might provide new control strategies. The aim of this study was to assess the impact of the two biological control agents *S. plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 on the rhizosphere community of the *Verticillium* host plant strawberry in field trials at two different sites in Germany. Therefore, we determined the abundances of culturable bacteria and investigated the community structure of the total rhizosphere microbiota by PCR-single strand conformation polymorphism analysis of the 16S rRNA and fungal ITS1 region. The abundances of culturable rhizobacteria on R2A medium as well as the proportion of in vitro *Verticillium* antagonists did not differ significantly. Additionally, no treatment specific differences were obtained in the

composition of species of the non-target antagonistic bacteria in the rhizospheres. The culture-independent analysis revealed only transient differences between the bacterial communities not due to the treatments rather than to the plant growth stage. Fungal and bacterial community fingerprints showed the development of a microbiota, specific for a field site. However, no sustainable impact of the bacterial treatments on the indigenous microbial communities was found using culture-dependent and -independent methods.

Key words: biocontrol, rhizosphere, risk assessment, *Serratia*, SSCP, *Streptomyces*

## **Comments on registration of *Verticillium lecanii* and *Trichoderma harzianum***

**W. Ravensberg, Koppert BV**

### **A) *Verticillium lecanii* (Mycotal and Vertalec)**

- many studies (acute package) have demonstrated that there are no harmful effects, some studies were done with the MPCA, some with the MPCP. Some countries still require a full set of studies with the MPCA as well as with the MPCP. What is the rationale behind this? Also considering the food approved ingredients one set should be enough.
- Even though countries follow the same EU directive re requirements different countries require different tests. This should be harmonized and accepted from each other.

Metabolites

- In Rafbca it was found that *V. lecanii* under certain circumstances can produce destruxins. This will be published soon. The quantity and the circumstance under which the fungus makes these metabolites indicates that this is not of any significance related to tox or the environment

Methods are available, but not in companies.

### **B) *Trichoderma harzianum* (Trianum)**

It is questionable whether this trichoderma should be seen as a PPP. Its effects are very close to effects shown by mycorrhizae.

Metabolites

- *Trichoderma*'s make numerous metabolites, but none of any significance related to tox or environment. So identification and so on are non-relevant and should not be required. Methods are not available.

### ***Trichoderma harzianum* Rifai Strain T-22**

See also Interent: last accessed: Jan. 15<sup>th</sup> 2008;

[http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet\\_119202.htm](http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_119202.htm)

### **Summary**

*Trichoderma harzianum* Rifai Strain T-22 is a naturally occurring fungus that is used to protect crops and seeds from various fungi that cause plant diseases. It is used primarily in greenhouses and nurseries, as well as by consumers. The active ingredient is not expected to cause adverse effects to humans, pets, or the environment. There are certain crops where it is not approved for use.

**Table 4****MPCPs: Experts nominated to defend the MPCA at the REBECA workshop**

Microorganism	Type	Commercial Name	Category	Rapporteur	Expert / Type of document
<i>Ampelomyces quisqualis</i>	Fungus	AQ10	Fungicide	France	Sergio Franceschini (Intrachem, IT); Commission directive
<i>Bacillus subtilis</i>	Bacterium	Serenade	Fungicide, Bactericide	Germany	EPA document; Commission directive
<i>Bacillus thuringiensis kurstaki, israelensis, aizawai</i>	Bacterium	Dipel, XenTari, Vectobac ect.	Insecticide	Italy Denmark	Collective view of international group of experts (WHO document, 1999)
<i>Beauveria bassiana</i>	Fungus	Botanigard	Insecticide	Germany	Tobias Längle (Agriculture and Agri-Food, CAN)
<i>Beauveria brongniartii</i>	Fungus	Melocont	Insecticide	Germany	Hermann Strasser (LFU, AT)
<i>Coniothyrium minitans</i>	Fungus	Contans	Fungicide	Germany	EPA document; Commission directive
<i>Gliocladium catenulatum</i>	Fungus	PreStop	Fungicide	Finland	Marina Niemi (Vedera, FI); Commission directive
<i>Verticillium (Lecanicillium) lecanii</i>	Fungus	Mycotal	Insecticide	Netherlands	Wilhelm Ravensberg (Koppert, NL)
<i>Metarhizium anisopliae</i>	Fungus	GRANMET/F 52	Insecticide	Netherlands	EPA document
<i>Pantoea agglomerans</i>	Bacterium	Strain C9-1	Bactericide	New Zealand, USA	EPA document
<i>Paecilomyces fumosoroseus</i>	Fungus	Preferal	Insecticide	Belgium	EPA document; Commission directive
<i>Pseudomonas chlororaphis</i>	Bacterium	Cedomon	Fungicide	Sweden	Margaretha Hökeberg (Bioagi, SE); Commission directive
<i>Serratia entomophila</i>	Bacterium	Invade	Insecticide	New Zealand	Travor Jackson (Ag Research NZ)
<i>Serratia plymuthica</i>	Bacterium		beneficial microbes; Plant growth promotor		Gabrielle Berg (University Graz, AT)
<i>Trichoderma harzianum</i>	Fungus	Tricover, T-22	Mycoparasite/ Plant growth promotor	Sweden	Wilhelm (Koppert, NL) EPA document