



Deliverable 10:

Proposals for improved regulatory procedures for microbial BCAs

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Document History

The document is based on the recommendation, expressed by the participants of (i) the Innsbruck workshop April 2006, (ii) the Salzau workshop in September 2006 and (iii) the Alès workshop in June 2007, (iv) a proposal on the assessment of metabolites from Hermann Strasser which was circulated for comments to experts from regulation authorities, science and industry and (v) a risk index model proposed by Hermann Strasser and Tobias Längle (vi) a meeting of the REBECA working group in Ralsdorf September 2007.

This document reflects the outcome of the REBECA project and contains a number of REBECA recommendations. However it cannot be assumed that all project partners, or even all experts who participated in the REBECA workshops fully agreed with all the recommendations/conclusion."

Document Abstract

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Introduction

Micro-organisms used as active substances in plant protection products in the EU are regulated according to the EU Council Directive 91/414/EEC. This Directive was amended by the Commission Directive 2001/36/EC regarding the data requirements for the Annex I inclusion of micro-organisms as active substances and national authorisation of products (Annex II and III in the directive respectively). The Uniform Principles for evaluation and authorisation of plant protection products containing micro-organisms are laid down in the Council Directive 2005/25/EC.

The objective of the Action REBECA is to accelerate the regulation process for BCAs in Europe and make it more cost-effective without compromising the level of safety. An important part of the Action is the review of potential risks of BCAs. Authorities should introduce regulation based on real risks. Consequently, any recommendation on how to regulate BCAs should be based on existing risks in order to provide proposals for a balanced risk management.

The report is based on the outcome of 3 workshops conducted in Innsbruck, Austria, April 12-13, 2006, in Salzac, Germany, September 18-22, 2006, and in Alès, France, June 6-7, 2007 and a meeting of REBECA experts September 7-9, 2007 in Ralsdorf, Germany. Experts from science, regulatory authorities and industry were involved (see Annex 1: list of participants Innsbruck, Salzac, Alès). At the meeting in Innsbruck, presentations introduced the participants to the potential of microbial plant protection products, experiences with registration of MBCAs, current regulation procedures and requirements (see Annex 2: list of presentations Innsbruck). Afterwards participants split into smaller groups dealing with the different MBCAs (viruses, bacteria or fungi). Risks were categorized according to the possible impact. After the meeting minutes were circulated and suggestions for changes were included into the minutes (see Deliverable D5).

In Salzac, the results of the Innsbruck meeting were presented (see Annex 4: list of presentations Salzac) and afterwards discussed within expert groups (see Annex 3: list of experts microbial products Salzac). Special attention was given to risk assessment strategies and a comment to the OECD paper on contaminant levels. Results were afterwards presented and discussed in the plenum, minutes were circulated and proposals for changes included.

The REBECA meeting in Alès started with a plenary session with presentations listed in Annex 5. Later an expert panel (Annex 6) gathered to focus discussions on the presented proposals on microbial metabolites (by Strasser et al.) and the environmental risk assessment. All information was then gathered by the REBECA experts during a meeting in Ralsdorf, Germany to produce this document.

Virus products

This proposal to the Commission and member states concentrates only on those virus plant protection products containing baculoviruses as the active ingredient. Other virus containing products, e.g. plant viruses used for protection of plants against more virulent virus plant pathogens were not considered. It aims at a facilitation of the procedure for Annex I inclusion and at a facilitation of national registrations.

Baculoviruses represent a family of double stranded DNA viruses that exclusively infect Arthropoda. The vast majority of the known species are confined to insects, predominantly *Lepidoptera*, with fewer species in *Diptera* and *Hymenoptera*. Some baculoviruses are used in plant protection products for the biological control of insect pests in agriculture, horticulture and forestry.

This proposal does explicitly not include genetically modified baculoviruses

The OECD Consensus Document

In 2002, the OECD released the "Consensus Document on information used in the assessment of environmental applications involving Baculoviruses". This document revised all publicly available information relevant for safety assessments of baculoviruses. This includes the biology of baculoviruses, infection mechanisms in the host, host range determination, methods for molecular characterisation of isolates, and the history of use in plant protection products. Extensive information was gathered on effects of baculoviruses on human health including infectivity, replication in vertebrate cells, genotoxicity and carcinogenicity. Ecological information summarized in the OECD consensus document includes persistence and dissemination in the environment, host specificity and effects on non-target organisms.

The following characteristics of baculoviruses were outlined:

- Baculovirus species are extremely host-specific, with their host range limited to one or a few species of the same genus. Larger host ranges covering different genera or even different families are rare (e.g. *Autographa*

- californica* NPV). Baculoviruses probably represent the most specific pesticidal agents, biologicals and chemicals taken together.
- Baculoviruses occur only in arthropods, predominantly in the insect orders *Lepidoptera*, *Diptera*, and *Hymenoptera*.
 - Baculoviruses are not infective for mammals and replication does not occur in mammalian cells.
 - No pathogenic, genotoxic, mutagenic, or carcinogenic effect of baculoviruses was ever observed in mammals.
 - Baculoviruses do not produce metabolites.
 - Effects on non-target species can be excluded, especially for vertebrates, micro-organisms, and plants.

It should be noted that the document was developed under the OECD Working Group on Harmonization of Regulatory Oversight in Biotechnology and not all countries may have involved specialists for risk assessment concerning plant protection products during the development of the document. Nevertheless, this document was reviewed by a very large number of OECD member states. Taken together, the OECD consensus document concludes, "the use of baculoviruses is safe". Even if the document does not specify which uses are considered safe, human safety is reasonably specified in the document (page 45): "safety tests of more than 51 entomopathogenic viruses including more than 30 baculoviruses resulted in a long and complete safety record. No adverse effect on human health has been observed in any of these investigations indicating that the use of baculoviruses is safe and does not cause any health hazard."

Genetic composition of baculovirus isolates

Micro-organisms are generally registered at strain level. Bacterial and fungal strains used in plant protection products derive from single colonies or spores and are consequently genetically homogenous. Different bacterial and fungal strains from the same species may have significant differences in their biology, especially in the production of secondary metabolites. Concerning their genetics, baculoviruses represent a unique case among micro-organisms used in plant protection products in that they consist of a mixture of different, often very similar genotypes. These variations may influence some biological properties, such as the virulence to their specific target host, but they do not have consequences on the safety towards non-target organisms or on the environment. The composition of this mixture depends among other factors on the genotype of the host used to multiply the baculovirus. Isolation of a single genotype is extremely difficult if not impossible and even not desired since genetic variation is needed to account for variation in the target organisms. Therefore, the demand to evaluate micro-organisms at strain level is not applicable for baculoviruses.

Potential risks from plant protection products containing baculoviruses

Due to the recorded safety of baculoviruses, no risks from the baculovirus itself for man or the environment are expected from plant protection products containing baculoviruses. Potential risks from baculovirus products are minimal and can occur only indirectly through product components other than the baculovirus itself.

All baculoviruses have to be produced *in vivo* in order to be infective to larvae. Host insect or media components might be allergenic as any other biological molecule. Hairs from some lepidopteran larvae (caterpillars) are known for their irritating and sensitizing potential. Sensitisation through baculovirus-containing products was tested and no effects were found for products containing CpGV (produced in *Cydia pomonella* larvae, non-hairy), SpliNPV (*Spodoptera littoralis*, non-hairy larvae), and LdMNPV (*Lymantria dispar*, hairy larvae). To date, all larvae used to produce baculoviruses for use in plant protection products in the EU are not hairy. Also, microbial contaminants cannot be excluded in the products, but have to be controlled. A detailed proposal on contamination thresholds in baculovirus products is given in table 1. Antibiotics potentially included in the media to suppress bacteria and fungi will only end up in very small proportions in the final product.

Current regulatory situation in the EU

Four baculovirus species (all represented by at least one isolate) are at present being evaluated by authorities of EU member states for the inclusion in Annex I of Council Directive 91/414 EEC. *Cydia pomonella* Granulovirus (CpGV) Mexican Isolate is the only one classified as an "existing substance". *Spodoptera exigua* Nucleopolyhedrovirus (SeNPV) strain F1 was recently included in Annex I of Council Directive 91/414. *Adoxophyes orana* Granulovirus (AoGV, Swiss isolate, BV-0001) and *Helicoverpa armigera* Nucleopolyhedrovirus (HearNPV, isolate BV-0003) are at present in the evaluation process as new active substances.

It is expected that after evaluation of the isolates of CpGV, SeNPV, AoGV, and HearNPV by the member states and EFSA, these baculovirus isolates can be included in Annex I of Council Directive 91/414 EEC. Likewise, it is expected that the corresponding products can be used safely under respect of good agricultural practise. As detailed above, baculoviruses represent a very homogenous group concerning their host specificity and effects on humans, non-target organisms and the environment, especially when compared with bacteria or fungi. Thus, all baculovirus

species and all isolates within one species can be treated similarly if not equally in the assessment of risks for man or the environment. Regulation of further baculovirus species and isolates for the use in plant protection products can then be facilitated.

Proposal for facilitated regulation of baculoviruses as active ingredients in plant protection products

Based on the conclusions from the OECD consensus paper and on the expected results of the evaluation of dossiers submitted for the inclusion of isolates of CpGV, AoGV, and SeNPV, we propose that baculoviruses are not evaluated at strain level. The high similarity between baculoviruses justifies a general assessment at the level of the family *Baculoviridae*, considering species-specific information where necessary. In agreement with the OECD consensus document and after comprehensive discussions with REBECA participants and review of latest scientific results on the molecular identification of the group, the authors recommend listing the family *Baculoviridae* on Annex I. Several experts recommend to limit the inclusion into Annex I to "all Lepidoptera-specific Nucleopolyhedroviruses and Granuloviruses". A consensus view of representatives from regulation was that this could save resources for applicants and MS without reducing safety for humans, animals and the environment. However, some representatives of regulation authorities favour the inclusion at the level of individual species. Listing of the complete family was considered to set a precedent and might be abused to list other groups of active ingredients, including chemical substances. Industry supported the inclusion on species level for commercial reasons.

A facilitated procedure for the registration of new species or isolates could be performed similarly to the procedure for "equivalence of technical material", as applied for chemical active substances for plant protection products. This would necessitate the submission of an application for national authorisation of a plant protection product containing the new species or the new isolate at member state level. After evaluation and approval of the application the member state then reports this to the Commission. Depending on the level of inclusion, Annex I or the review report needs to be amended.

Formally, each data point for the active substance and the product has to be addressed. However, it is not necessary to submit isolate specific information for many data points. Most of the data formally required are published and equal for all baculoviruses and already assessed by MS and EU authorities. Therefore, it is also possible to refer to already submitted own data or to relevant data already evaluated in other DARs. Species- or isolate-specific data have to be submitted for data points concerning the individual baculovirus species or isolate.

The following species/isolate-specific information -according to Annex II data requirements- has to be provided for the active substance:

- Origin of the isolate
- A molecular identification and characterisation, preferably by restriction length polymorphism (RFLP) analysis of DNA.
- Deposition of the new species/isolate in a recognized culture collection
- Biological properties, especially the host range
- The manufacturing process including threshold levels for contaminants.
- Analytical methods for the detection of the new species/isolate as well as methods for the detection of microbial contaminants

Product-specific data - according to Annex III data requirements - have to be provided including the production method (medium components, larvae hairy or not), information on the amount of non-pathogenic and pathogenic bacteria and fungi, and composition of the product. Changes when compared to methods already submitted for other products have to be declared. Data on toxicology and ecotoxicology should be based on the composition of the product. If the active substance is accepted to be safe without restrictions, risks can only result from other product components. The health and environmental hazards of a preparation should be assessed as described in article 6 and 7 of 99/45/EEC, hence by a conventional (calculation) method or by providing toxicological data on the preparation or its individual components. If the composition of the product is similar to an already evaluated product, applicants can refer to this product (with appropriate justification and, if necessary, bridging studies). Efficacy data have to be submitted for a product containing a new species/isolate according to national regulations.

Data Protection

Large part of the data submitted for the inclusion of a baculovirus species in Annex I are normally covered by data protection. This means that all notifiers applying for national authorisation of a plant protection product containing an active substance, which was included in Annex I, must either prove access to the protected data that were necessary for the Annex I inclusion, or provide equivalent own data. However, this refers only to data still under data protection

(i.e., not to published literature). For submitted studies, for which the notifier claims data protection, the standard EU rules for data protection apply. Likewise, notifiers of products containing a new species have to provide own data or a letter of access to an already submitted dossier.

Proposal on threshold levels for microbial contaminations in Baculovirus products

Baculoviruses for the use in plant protection products are multiplied *in vivo* using living host larvae. As these animals are not sterile, and separation of the virus from any contaminant is not feasible, microbial contaminations cannot be avoided and represent one risk associated to the use of products containing Baculoviruses. A draft OECD document prepared by Canada was discussed as the base for threshold levels. The threshold levels listed in Table 1 were agreed between members of the working group and are proposed as general thresholds for microbial contaminants in plant protection end products containing baculoviruses.

Bacillus cereus represents a particular case for CpGV. *B. cereus* is a common spore forming, motile ubiquitous soil bacterium and an opportunistic human pathogen, causing diarrhoeal or emetic disease through the production of enterotoxins especially during inappropriate storage temperatures. *B. cereus* is frequently isolated as a contaminant of various foods. The consumption of foods that contain more than 10^5 CFU *B. cereus* per gram may result in food poisoning. However, in some outbreaks, lower numbers in the food ($10^3 - 10^4$ CFU/g) were reported. As *B. cereus* is part of the intestinal flora of *Cydia pomonella* larvae, its presence in CpGV products cannot be avoided. CpGV products are highly diluted before application. As *B. cereus* is a soil bacterium, multiplication on fruit surfaces seems minimal due to lack of nutrients.

To estimate the populations of *B. cereus* on apples resulting from application of CpGV products, the following assumptions are made:

- maximum accumulated application rate for CpGV products: 2.7 L/ha per season
- maximum contamination *B. cereus*: 10^{10} CFU/L
- apple yield: 28 t (average for Germany, in France 38-40t)
- 2.7×10^{10} CFU / 28 t = 1000 CFU/g or 10^5 CFU/100 g.

If soil coverage of 60% is considered, maximum contamination levels are 600 CFU/g fruit or 60000 CFU/100 g fruit. This calculation still does not take into account that the majority of *B. cereus* cells will end up on leaves and not on fruits, because fruit surface is still small when compared to leaves at the time of application of CpGV products. In addition, decrease of *B. cereus* between application of the product and harvest through UV radiation or washing of by rain is not considered. Further reduction of *B. cereus* on food can be achieved by washing or peeling. **Table 1:**

Table 1: Threshold levels for Microbial contaminants (per g or mL)

Contaminant	Maximum content
Total mesophiles	10^8 CFU
<i>Bacillus cereus</i>	10^7 CFU
<i>Escherichia coli</i>	none in 1 g or mL
<i>Staphylococcus aureus</i>	none in 1 g or mL
<i>Salmonella spp.</i>	none in 25 g or mL

Yeasts and moulds are visually checked during production.

Bacterial and fungal products

Data requirements for the registration of micro-organisms as active substances and of products based on micro-organisms are laid down in the Council Directive 91/414/EEC, amended by the Commission Directive 2001/36/EC (EC 2001). The Uniform Principles for evaluation and authorisation of plant protection products containing micro-organisms are laid down in the Council Directive 2005/25/EC.

Until now, the use of microbial BCAs in plant protection did not pose any hazards to humans and the environment, even though some have been used extensively for decades (e.g. *Bacillus thuringiensis*, *Verticillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*, *Trichoderma spp.*).

Proposal to simplify registration

Information check list for pre-submission

Based on the currently available experience with the use of MBCAs and scientific information on their risks we can conclude that it seems that MBCAs pose little risk for humans, non-target organisms and the environment. In order to simplify the registration procedure it is therefore recommended to summarize the available data and to discuss relevant data requirements in a pre-submission meeting with the Rapporteur MS prior to submission of the dossier. The decision on the relevant data to be provided shall be based on the following information, which can be derived from the applicant's data and/or published literature:

- Identification and taxonomic position of the MBCA
- Natural distribution of the species in particular on food and feed and in agriculture environments
- Modes of action and host range
- Toxicity data
- Metabolites produced by the MBCA
- Intended use of the product (target organisms)
- Formulation of the product
- Site and method of application
- Health and medical reports
- Absence from the list provided in Dir. 2000/54 EC concerning worker's protection from micro-organisms
- Maximum growth temperature
- List of available effective antibiotics

Data provided shall be the basis for a decision on the provision of additional data in the dossier and the definition of waivers. Should no relevant potential risks be identified from this information, no further information should be required on metabolites, toxicology and non-target effects. For the following risk assessment of the MBCA it is essential to refer to the above listed data, which is basically contained in Section 1 according to the OECD format (OECD, 2005). These data might be sufficient to estimate a risk index as proposed in the REBECA deliverable 28.

Comments on data requirements

Human infectivity

REBECA experts agreed that human pathogens are well described and documented in the relevant literature and databases. On the basis of this knowledge microbes are categorised into 4 risk groups (Directive 2000/54 EC). This Directive is aiming at protection of workers against risks to their health and safety, including the prevention of such risks, arising or likely to arise from exposure to biological agents at work. If a biological agent is included in risk group 1, it is unlikely to cause human diseases. In that case no special measures are required according to the Directive to prevent or reduce the risk of exposure to such an organism (article 4, clause 1). Only general principles of good occupational safety and hygiene should be followed. All micro-organisms used in registered plant protection products to date are not listed in the risk groups 2-4.

In Dir. 2000/54 only organisms categorized into the groups 2-4 are listed. This means: "In line with the scope of the Directive, only agents, which are known to infect humans are to be included in the classified list. Animal and plant pathogens which are known not to affect man are excluded". It can be concluded that the risk for infection of humans by micro-organisms is very well known and that the EU and the Member States already made a decision concerning this risk regarding the exposure of workers. This classification should also be applied to micro-organisms used in plant protection products.

REBECA experts concluded that more emphasis should be given to the clinical findings and published reports on adverse effects of the species of a MBCA during the risk assessment procedure. It was questioned whether the classification of a micro-organism into group 1 delivers at least the rationale to waive the risk assessment requirements regarding extensive infectivity studies of the micro-organism or in other words to waive the clearance investigations in the Tier I assessment.

In the EU Member States adaptations of Dir. 2000/54 EC exist. For example in Germany the so called Directive TRBA 466 for bacteria and 460 for fungi are used, listing as well group 1 organisms. A quite similar categorisation of micro-organisms as used in the EU is used in the USA and by the WHO.

Despite the group 1 classification further key indicators for the human (mammalian) safety of MBCAs are:

- no growth at $>35^{\circ}\text{C}$
- no clinical reports and indications in relevant scientific literature or databases
- data on susceptibility of MBCA to antibiotics

The potential of nosocomial infections of immune-compromised patients by MBCAs was discussed. These infections are a result of treatment in a hospital or a healthcare service unit, but secondary to the patient's original condition. Nosocomial infections are alarming as antibiotic resistance has widely spread. Data on the susceptibility of the MBCA to common antibiotics can minimize the risk of nosocomial infections. Reports on infections of immune suppressed patients, however, should not hamper registration of a micro-organism for use in PPP since contact of immune-suppressed patients to PPP should be avoided in any case.

REBECA proposes that if all the following criteria are fulfilled, the data requirements for clearance (Dir. 2001/36 EC point 5.2.2 acute toxicity, pathogenicity and infectiveness) should be waived:

1. No (or few) clinical reports and indications in relevant scientific literature or databases. A low number in most cases is a wrong identification or an indication for an opportunistic infection. This can be assessed from the data provided with the record.
2. Point 1 criteria should be crosschecked with Directive 2000/54 EC or equivalent Member State documents.
3. Data on susceptibility of MBCA to antibiotics, indicating that strain is susceptible to several available compounds.
4. Data on distribution and occurrence of species, which underpin the regular exposure of humans to the micro-organism in question (e. g. common on food and feed, common on food and feed plants foliage or roots, common in the soil ect.).

Genetic stability

"Genetic stability (mutation rate of traits related to the mode of action), factors affecting genetic stability and micro-organism's capacity to transfer genetic information to another population" (OECD Section 1, Point IIM 2.10) has been discussed intensely. Experts recommend that this point is erased. Changes in the efficacy due to genetic instability will be detected during quality control.

Genetic variations occur spontaneously. Statements on the stability of the MBCAs can only be based on investigations on their mutation rate, but the relevance of such studies for the assessment of risks is questioned. Results of mutations cannot be predicted. As MBCAs are not expected to be different from other micro-organisms, also in their capacity to transfer genetic information to other populations, data specific to the MBCA will not add more information on its safety.

Toxicological and exposure data and information on the MBCA

Data requirements (OECD Section 3, Point IIM 5) and methods to assess pathogenicity, infectivity and toxicology have been discussed several times within the Action. Several points have been identified, which should be modified. For instance, the absence of an immune-response in toxicology studies as required in 2005/25 EC 2.6.1.1. (a) is inadequate. Should any organism not develop an immune-response against the MBCA, the micro-organism would be able to infect, colonize and propagate in the non-target organism. An immune-response is an essential step for the clearance of any kind of non-self body or organism. Thus it would be more appropriate to detect and evaluate an immune-response against the MBCA.

The requirements are complex and extensive. It was discussed whether more adapted approaches to test pathogenicity, infectivity and toxicology might produce better data with less effort. However, the workshop participants acknowledged a lack of expertise in toxicology and medicine within their group. Experimental data with MBCAs to provide more detailed proposals for changes of the data requirements and methods are still missing.

Sensitisation

Data on sensitisation are required in Section 3, Point IIM 5.3.1. If no data are provided, the products are likely to be labelled as sensitizing (Xi - R42). Currently no reliable test method is available for micro-organisms. As labelling "Xi - R42" is excluding the use of the MBCA from e.g. home gardening markets, applicants want to avoid this label. It is therefore necessary to develop test systems.

Proposal for the risk assessment of metabolites

This proposal is based on the REBECA recommendations, which also discussed results of the EU funded research project RAFBCA (acronym for "Risk assessment of Fungal Biological Control Agents", QLK1-CT-2001-01391).

There has been much interest in microbial BCAs (i.e. bacteria, fungi, viruses) over the past two centuries. Numerous peer reviewed scientific articles and text books have been published on different aspects of their biology. Scientists have studied their ecology, laboratory- and commercial-scale production, formulation, and safety to humans and the environment as well as in-field performance (efficacy).

Bacterial and fungal BCAs secrete a wide range of metabolites, mostly products of secondary metabolism. These metabolites serve different functions depending on the ecological niche of the microbe. Some metabolites may be antibiotics that protect the BCA against antagonistic micro-organisms whereas others may prevent growth of saprophytic microbes on the host after it is killed by the BCA and thus improve survival of that BCA. Some bioactive metabolites are also important pathogenicity determinants and others have antifeedant/repellent properties that presumably deter mycophagous organisms. Quantities normally detected in target hosts or the environment are usually too low to be of major concern and therefore these metabolites pose no risk to humans and the environment.

In the 3rd edition of "The Manual of Biocontrol Agents" edited by Copping (2004), over 100 active ingredients are based on micro-organisms. All microbial biological control agents (BCAs) used to control insects, diseases and weed pests are described as "generally to pose little or no risk to man and the environment" (Anonymous, 2005). This statement would still apply even if these agents secreted toxic metabolites. This is acknowledged by the EU by the fact that several microbes (active substances) authorised for the use in plant protection according to EU directive 91/414 Annex I are known to produce toxic metabolites. These include: (i) *Pseudomonas chlororaphis* which produces the toxic (mutagenic) substance 2,3-didehydro-rhizoxin (DDR); (ii) *Gliocladium* spp. is known to secrete viridian, gliovirin, glioprennins, and heptelidic acids, (iii) *Paecilomyces fumosoroseus* produces beauvericin, beauverolides, and pyridine-2,6-dicarboxylic acid, (iv) *Coniothyrium minitans* produces antifungal metabolites (e.g. macrosphelide A).

In the case of *P. chlororaphis* a consultation of the Scientific Committee on Plants (SCP) was required to assess the potential risks of DDR as well as to other possible antibiotic metabolites. Based on SCP comments the Commission concluded "that no major concern exists for consumer and operator safety, even if more studies would be needed for a more complete assessment of the mutagenicity potential of DDR" (SCP/PSEUDOM/002-Final).

However, companies wishing to commercialise microbial BCAs find that data requirements for metabolites often pose the biggest hurdle in the registration process.

RAFBCA generated data that provide a better understanding of the major metabolites secreted by selected fungal BCAs including their distribution and regulation. Particular attention focused on

- The development of sensitive tools (e.g. biosensors) and methods for rapid and accurate detection of fungal metabolites
- The assessment of their putative mutagenicity and cytotoxicity
- Biochemical and molecular studies to elucidate their mode of action
- Monitoring of fungal BCAs in the environment with molecular markers
- Investigation whether metabolites enter the food chain and, if so, identify the route of entry and type and quantities present

The results indicated that metabolites of fungal BCAs (e.g., *Metarhizium anisopliae*, *Gliocladium* spp., *Beauveria brongniartii*, *Trichoderma harizanum*, *Verticillium lecanii*, *Stagnospora* spp.) pose no risk to humans and the environment.

In detail, the following general conclusions were drawn by REBECA experts based on the discussions of the RAFBCA results:

1. Fungal BCAs investigated produced metabolites in extremely small amounts both *in vitro* and *in vivo* and are, therefore, unlikely to pose a threat to humans and the environment (Boss et al. 2007c; Seger et al. 2004, Seger et al. 2005a,c; Shah et al. 2005; Skrobek & Butt 2005, Strasser et al. 2000a, b).
2. None of the investigated fungal metabolites entered the food chain, even when applied ten times higher than the recommended application rate. Metabolite risks were assessed at all stages of the production and application cycle, i.e. in fermenters, unformulated inoculum, formulated product, on crops and in harvested crops (Skrobek & Butt, 2005, Skrobek et al. 2006; Boss et al. 2007c).

3. Aspects of the biology of MBCAs should to a larger extent be taken into account when assessing the risks. For instance, in most cases MBCAs are already in the environment. Although their density increases immediately after application, MBCAs and their metabolites decline over time returning to the naturally occurring levels in the field.
4. Toxins are usually produced under inducible conditions within or in contact with the host or target. Their concentrations are low and they cannot be easily detected in amounts in the crop or the environment to monitor their presence or fate. Therefore, these toxins are of minor concern (Boss et al. 2007c; Seger et al. 2005a, b; Strasser & Kirchmair 2006; Skrobek et al. 2007).
5. Purification of any metabolite is time consuming and requires the use of several analytical methods. Only few of the several possible metabolites produced by these organisms could be isolated. Therefore, a risk assessment investigation based on single metabolites is not feasible.

What has been demonstrated for several fungal BCAs on different crops suggests that the results can possibly be extrapolated to other micro-organisms used in PPP. However, since only limited data may be available for other micro-organisms, concerns exist whether these conclusions can be transferred also to fungi and bacteria, which have not undergone the same examination regarding metabolites yet.

Two important questions need to be addressed. (1) How should we deal with the regulatory requirements for a microbial BCA that has only recently been described? (2) How should we deal with the regulatory requirements for a microbial BCA, for which no information on the metabolites produced exist?

The action of microbial BCAs is related to the presence of an active living cell. The mode of action is complex and may involve metabolites. Metabolites of microbial origin are biodegradable. They are produced in situ by the cell and are active within a limited time and space. They are not accumulated in the environment and consequently residues are not to be expected. Should the acute oral toxicity study not show any effect at maximum levels, a relevant metabolite is considered to be absent in the product. Should the pre-submission data set not provide any indication on the presence of relevant metabolites, no further data should be requested.

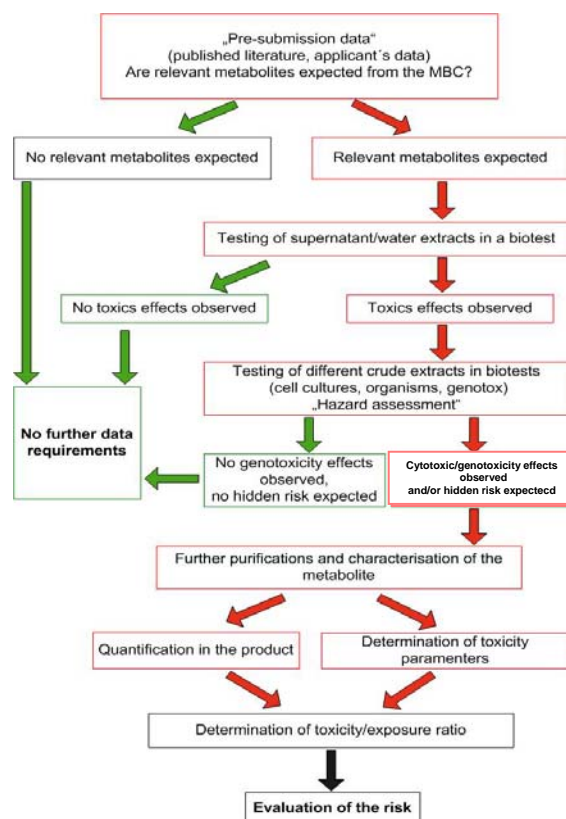


Figure 1: Scheme for assessment of potential relevant metabolites of microbial BCAs

It is often argued that metabolites produced in situ after application might enter the food chain or affect non-target organisms through secondary poisoning. These substances would not have been encountered in the acute oral toxicity test. Should pre-submission data indicate that the MBCA is member of the microbial community at the application site, no major risk is expected because non-targets including consumers of the plants are and always have been naturally exposed to these organisms and their metabolites. The argument that higher densities of the MBCA above the natural level might produce higher amounts of metabolites is not applicable as populations of MBCAs rapidly decline after application. Spore-forming micro-organisms are an exception, as spores are often surviving longer periods. However, the metabolism of resting stages is down-regulated and metabolites are not expected to be produced.

Should the review of the pre-submission data reveal that a potential toxic metabolite exists and might be of concern, in that case the scheme presented in Figure 1 is proposed as a possible procedure. To determine potential risks, culture supernatants from liquid cultures or water extracts from production on solid media of the MBCA should be tested in bioassays as described in Favilla et al. 2006 and Skrobek et al. 2006. The analysis of isolated substances is often not feasible as they are unknown. Should the tests indicate toxic effects, further investigations are necessary. They should be performed with crude extracts consisting of the secreted metabolites extracted with different solvents and concentrated by evaporating the solvent.

Supernatants and crude extracts have been tested within the RAFBCA project (Favilla et al. 2006; Skrobek & Butt, 2005; Skrobek et al. 2006). Methods and strategies have been developed to standardise the toxicity testing of microbial biological control agents with emphasis on fungal metabolites. Crude extracts from different mycoparasitic, entomopathogenic or phytopathogenic fungi (used as MBCA) and their selected metabolites displaying different structural nature and mode of action were used to assess the acute toxicity. Insect, invertebrate and cell line bioassays are widely used for studies of ecotoxicology as well as of general toxicity of chemicals and natural compounds. The testing of supernatants, if really necessary, requires less time and resources than the analysis of single metabolites and subsequently would be more cost effective for companies wishing to register BCAs.

REBECA consortium members are aware that the use of crude extracts also has pitfalls:

1. The crude extracts represent the “worst case” scenario as levels and spectrum of metabolites being assessed are far higher than occurring in nature.
2. Crude extracts are hardly expected to show zero toxicity; therefore it will be necessary to establish tolerance levels of toxicity (genotoxicity, cytotoxicity) in biological assays.

The investigations have been performed with a limited number of fungal BCAs. Results with PPP based on bacterial agents are limited. Additional information on the extraction methods, solvents and test systems are needed to develop innovative procedures to assess the possible risks of microbial metabolites.

Fate and behaviour in the environment

Experience with past dossiers indicates that data requirements on the fate and behaviour of MBCAs have been of minor concern in the risk assessment and information from public data have often been accepted.

Persistence

Persistence of an organism in the environment is an important factor in determining its risk because it strongly influences the likelihood for non-target organism exposure. Clearly, living organisms can have an entirely different behaviour in the environment than chemicals as they can proliferate in the environment. It is important to note that, from a risk assessment perspective, an organism or substance naturally present in the environment must be regarded differently than a new species or substance introduced to the ecosystem.

Microbial BCAs occur naturally, are airborne or soilborne and are often associated with food crops. However, it appears that any risk these organisms may pose is low and therefore acceptable from a regulatory perspective. Most MBCAs can be considered to be part of the “background population” (Annex II, EC 2001).

Naturally occurring micro-organism will pose no additional risk to the environment if introduced into a comparable system at similar densities. The density of microbiota often heavily fluctuates depending on host, seasonal and micro-climatic conditions and agricultural measures and is often comparable to the density of a MBCA after artificial introduction. Therefore, the introduction of a relatively high and persistent population of an indigenous organism in the environment does not necessarily add an environmental risk. Consequently persistence should not be a major concern. It is well accepted that application of any microbial species to any particular environment results in a temporary increase of its population followed by a gradual decrease to background levels.

The occurrence of micro-organism species and their survival much depends on the environmental conditions at a certain spot. Most organisms have a world-wide distribution. However, some might be non-indigenous. For those

micro-organisms release and persistence in the environment might pose a risk through exposure of potential non target organisms that have never before been exposed to the micro-organism. However, this does not necessarily imply that a non-indigenous organism does not follow the same population decline as an indigenous species when released into that environment. Any potential longer persistence deviating from the population decline which is normally observed with any micro-organism after release, will depend on an available food source or host. So data on the non-target effects will add to the assessment of potential risks.

Ecotoxicological studies and effects on non-target organisms

Current regulation practice requests studies on effects on non-targets in case of a potential exposure. If evidence exists that the micro-organisms causes no hazards to non-target organisms waivers can be accepted. REBECA proposes to waive the data requirements for effect on earthworms. No pathogens of earthworms have been described in the scientific literature. Thus it is most improbable that effects on earthworms will be detected and any positive control for tests cannot be provided. REBECA also proposes to waive data requirements on non-target effects on micro-organism in the soil. Soil seems to be characterised by a redundancy of functions. The functional characteristics of component species are at least as important as the number of species for the maintenance of essential processes. Therefore, an expedient assessment of environmental risks caused by different agricultural practises should not be focused on possible changes of the abundances of particular species; attention should be paid to preserve the functionality of the soil and keep the different functional groups of organisms in balance. Directive 2005/25 EC mentions that micro-organisms may pose risks because of their potential to interfere with nitrogen and carbon mineralization in the soil. It is also mentioned that experimental data are not normally required (point 2.8.6.1). Carbon mineralization is the consequence of microbial activity in the soil. It was questioned whether the release of comparatively low numbers of additional micro-organisms pose a risk to the other soil micro-organism community responsible for carbon mineralization. Major hazard have not been observed.

Changes in the soil microbiota are regularly occurring, particularly in agricultural soil ecosystems. Severe impacts on the composition and quantities of soil micro-organisms are observed during irrigation, tillage, application of organic or synthetic fertilizers or simply by crop rotation. Agricultural measures with negative impacts on the functional soil characters are not regulated, but are always more severe than the release of comparatively few microbial plant protection organisms. Scientific evidence supports the observation that more severe impacts are caused by common agricultural practice than by the use of microbial BCAs. Data on the effect of the release of MBCAs on other micro-organisms in the soil should therefore not be requested.

Assessment of metabolites in the environment

Bacterial and fungal BCAs have the potential to secrete a wide range of metabolites, mostly products of secondary metabolism. The quantity normally detected in target hosts or the environment is usually too low to measure significant effects. The relevance of metabolites in the environment can in many cases be assessed by the available knowledge on the biology properties (mode of action) and application methods for the micro-organism in question. RAFBCA results showed that most fungal BCAs produced metabolites in extremely low amounts both in vitro and in vivo. Metabolites are, therefore, unlikely to pose a threat to humans and the environment (see also RAFBCA reference list - Appendix 1, Boss et al. 2007c; Seger et al. 2004, Seger et al. 2005a,c ; Shah et al. 2005; Skrobek & Butt 2005, Strasser et al. 2000a, b). In addition to species exploited as BCAs, numerous other species of fungi and bacteria are associated with the rhizosphere and phylloplane. These include beneficial species such as mycorrhizal fungi (85 % flowering plants have symbiotic relations with these fungi), N-fixing bacteria (nodule and free living species) as well as potential human pathogens. In case a species of a MBCA is regularly isolated from these microbiota, hazards related with the impact of its potential metabolites on the environment and non-targets can probably be neglected. However, the use of endophytic micro-organisms need attention as higher amounts of metabolites might be transferred into the plant. If the MBCA is known to grow inside of food and feed plants and no hazards have ever been reported, the risk must be regarded as negligible.

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Annex 1: List of participants Innsbruck Meeting

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Annex 2: List of presentations Innsbruck

All presentations are available on the REBECA webpage (www.rebeca-net.de).

Short introduction in current MBCAs in market (problems in registration, potential hazards, fate and behaviour)

[Welcome and introduction](#) *Hermann Strasser*, LFU-Innsbruck, Austria
[Experience in microbial registration](#) *Guido Sterk*, Biobest N.V., Belgium
[Fungi against insects](#) *Hermann Strasser*, LFU-Innsbruck, Austria
[Fungi against plant pathogens](#) *Marina Niemi*, Verdera Oy, Finland
[Bacteria against insects](#) *Sergio Franceschini*, Intrachem s. r. l., Italy
[Bacteria against plant pathogens](#) *Margareta Hökeberg*, BioAgri AB, Sweden
[Virus](#) *Martin Andermatt*, Andermatt Biocontrol AG, Switzerland
[Objectives of REBECA and the group work](#) *Ralf-Udo Ehlers*, CAU-Kiel, Germany

Presentations on risks

[Fungal metabolites](#) *Claudio Altomare*, ISPA Bari, Italy
[Risks of bacterial MBCAs](#) *Gabriele Berg*, TU-Graz, Austria
[Risks of using virus](#) *Jürg Huber*, BBA Darmstadt, Germany
[Human risks of Pantoea](#) *Joel Vanneste*, HortResearch Ltd., Australia

Comparison of indicated risks, suggested assessment strategies and current registration requirements

[Current regulating system and data requirements within the EU](#) *Anita Fjelsted*, Danish EPA, Denmark
[Current regulating system and data requirements in non EU countries](#) *Rüdiger Hauschild*, GAB Consulting GmbH, Germany
[GENOEG Breed](#) *Tycho Vermeulen*, CLM, Netherlands

Annex 3: List of participants of microbial workshops Salzau

Bacteria workshop:

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Fungi workshop

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Virus Workshop

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Annex 4: List of presentations Salzau

All presentations are available on the REBECA webpage (www.rebeca-net.de).

Potential of Microbial Control Agents:

Virus *Martin Andermatt*, Andermatt Biocontrol AG, Switzerland

Bacteria *Trevor Jackson*, AgResearch, New Zealand

Fungi *Sebastian Kiewnick*, Agroscope, Switzerland

Comparison of Registration Requirements in Different Countries

Rüdiger Hauschild, GAB Consulting GmbH, Germany

Summary of the Results of the Innsbruck Workshop (11.-13.04.2006)

Virus *Jürg Huber* (BBA Darmstadt, Germany)

Fungi: Consequences of the EU Project RAFBCA on the Regulation of Fungi

Tariq Butt, University Wales, United Kingdom

Bacteria/Fungi: Ranking of Risks and Suggested Waivers

Hermann Strasser, University Innsbruck, Austria

Annex 5: List of participants of microbial workshop in Alès

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Annex 6: List of presentations Alès

Regulation of Microbial BCAs in Europe – Results of the REBECA Policy Support Action. *Ralf-Udo Ehlers* (Christian-Albrechts-University of Kiel, Germany)

Current data requirements for the environmental and ecotoxicological risk assessment. *Rüdiger Hauschild* (GAB, Germany)

REBECA Proposal on the assessment of microbial metabolites. *Hermann Strasser* (Ludwig-Franzen-University-Innsbruck, Austria)

The impact of Plant Protection Products (PPPs) on non-target organisms: soil microbiota. *Marco Nuti* (University of Pisa, Italy)

How to evaluate the environmental safety of microbial pest control products? A proposal. *Hans Mensink* (RIVM, The Netherlands)