



Deliverable 10:

Proposals for improved regulatory procedures for microbial BCAs

REBECA

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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 position paper on the assessment of metabolites from Hermann Strasser which was circulated for comments to experts from regulation authorities, science and industry, (v) a risk index model proposed by Hermann Strasser and Tobias Laengle and (vi) a meeting of the REBECA working group in Ralsdorf September 2007, where the first version of deliverable 10 was produced. This document was discussed during a workshop in Brussels September 2007 and reworked afterwards.

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

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. The proposals for a more balanced regulation of microbial BCAs (MBCAs) are based on a review of their potential risks carried out in 2006 by REBECA. Baculoviruses have been identified to be generally safe for the use as plant protection products. Therefore, REBECA developed proposals for the inclusion of all baculoviruses into Annex I of Directive 91/414. Bacteria and fungi need to be assessed case by case. In the current document REBECA gives guidance for the execution of pre-submission meetings, assessment of risks and delivers the rationale for waivers. The risk assessment methodology for microbials is based on that for chemicals and therefore, in many cases not most appropriate. An adequate risk assessment and the implementation of the REBECA proposals will depend on the development on a methodology better adapted to micro-organisms.

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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 Salzau, 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, Salzau, 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 Salzau, the results of the Innsbruck meeting were presented (see Annex 4: list of presentations Salzau) and afterwards discussed within expert groups (see Annex 3: list of experts microbial products Salzau). 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 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”. 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 consensus view of representatives from regulation was that these proposals could save resources for applicants and MS without reducing safety for humans, animals and the environment.

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 bacterial and fungal contaminants, 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: 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 to identify low risk products.

Comments on data requirements

Data requirements (listed under OECD Series on Pesticides 23. Appendix 11, Point IIM 5 and 2001/36/EC) and methods to assess pathogenicity, infectivity and toxicology have been discussed several times within the Action. The requirements are complex and extensive. It was discussed whether more adapted approaches to test pathogenicity, infectivity and toxicity might produce better data with less effort. However, it becomes very clear in an early stage of this action that the development of such proposals is hindered so far by a significant lack on validated risk assessment methods for microbials and knowledge gaps on natural exposition of humans and other non-target organisms. This is hampering an adequate risk assessment of microbial plant protection products. Therefore, REBECA demands the initiation of a research programme by the EU with the aim to develop more appropriate risk assessment methods for microbials and to reduce whole animal testing and costs.

Human infectivity

Humans are regularly exposed to a wide range of micro-organisms and the human community is spending a lot of resources to identify pathogens. Therefore, human pathogens are well described and documented in the relevant literature and databases (Mölby 1998). 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". However, not explicitly listing the group 1 organisms is a drawback of this Directive, because not listed micro-organisms might be not categorized at all so far. In contrast Germany lists as well the group 1 organisms in the so called technical advises for biological substances (TRBA 466 for bacteria and 460 for fungi). In all EU Member States adaptations of Dir. 2000/54 EC exist. A quite similar categorisation of micro-organisms as used in the EU is used by the WHO (biosafety manual) and many non-European countries. It can be concluded that the risk for infection of humans is very well known for many micro-organisms 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. A correct identification of the micro-organism by the applicant and in the literature will be indispensable prerequisite. 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.

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

- no growth at temperatures $>35^{\circ}\text{C}$
- 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.

Currently the infectivity is assessed by so called clearance investigations. In this investigation the clearance of the micro-organisms from the inner organs of rats or mice is assessed after an intratracheal instillation. This method has several disadvantages and alternative assays should be investigated (for more information see REBECA deliverable 11). REBECA proposes that if all the following criteria are fulfilled, the data requirements for clearance or respectively infectiveness (Dir. 2001/36 EC point 5.2.2) 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 the 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 etc.).

In other words, if humans are already regularly exposed to the micro-organism and no relevant clinical reports exist (risk group 1), the risks of infections is negligible. In addition, if the micro-organism is susceptible to antibiotics, putative infections can be cured.

Genetic stability

In the current data requirements it is demanded that where appropriate, information on genetic stability (e.g. mutation rate of traits related to the mode of action or uptake of exogenous genetic material) under the environmental conditions of proposed use must be provided (OECD Section 1, Point IIM 2.10). Information must also be provided on the micro-organism's capacity to transfer genetic material to other organisms as well as its capacity to being pathogenic for plants, animals or man. If the micro-organism carries relevant additional genetic elements, the stability of the encoded traits should be indicated.

REBECA recommends that data requirements regarding the stability of genetic traits affecting the efficacy of the product should be waived or erased. Changes in the efficacy due to genetic instability will be detected during quality control procedures. Therefore, the applicant should demonstrate that he is using proper quality control measures instead of demonstrating the genetic stability of the beneficial traits.

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.

Sensitisation

Data on sensitisation are required in Point IIM 5.3.1. (OECD Series on Pesticides 23. Appendix 11). If no data are provided, the products are likely to be labelled as sensitizing with "Xi - R43 potentially sensitizing through skin contact" or "Xn – R42 potentially sensitizing through eye contact". If both classifications (R42 + R43) are given, the product is labelled as Xn. Currently no reliable test method is available for micro-organisms. As labelling "Xi" or "Xn" 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) and a position paper produced by Strasser et al. 2007 (Annex 7).

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, 2007). 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 minutans* produces antifungal metabolites (e.g. macrospheptide A). But due to irrelevant amounts released by micro-organisms to the environment these MBCAs could be authorised without safety concerns.

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 of metabolites,
- 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*, *Stagonospora* spp.) pose no risk to humans and the environment.

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

1. The biology of MBCAs should be more seriously 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.
2. 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, c; Strasser & Kirchmair 2006; Skrobek et al. 2007).
3. 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. 2007; Seger et al. 2004, Seger et al. 2005a,b; Shah et al. 2005; Skrobek & Butt 2005, Strasser et al. 2000a, b).
4. None of the investigated fungal metabolites entered the food chain in quantifiable amounts, 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. 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.
6. The action of microbial BCAs is in most cases related to the presence of an active living cell. 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 higher than the natural background levels.
7. 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 already 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.

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 plant protection. However, until only limited data are available for other micro-organisms, concerns exist whether these conclusions can be transferred also to fungi and bacteria, which have not

been studied yet to that extent. 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 there is no information on the metabolites it produces?

In the Directive 91/414 toxicity assessments with purified relevant metabolites are required. However, it is not feasible to identify and quantify all metabolites produced or potentially produced by a micro-organism. Methods are needed to detect or exclude the occurrence of relevant metabolites in relevant amounts in a first instance. Most of these metabolites are produced in very little amounts. A purification of sufficient quantities allowing whole-animal testing can be impossible or respectively extremely expensive, discouraging biocontrol companies from developing microbial BCAs (Blum et al., 2003; Zimmermann et al., 2004). Clearly, sensible, simple, better adapted and cost-effective strategies are needed for the risk assessment of metabolites from microbial BCAs.

Genotoxicity assessment is a special part of the toxicity assessment, requiring a particular set of methodologies. Genotoxic effects are often of cumulative nature or can cause germline damage. Therefore, an acute toxicity assessment might be not sufficient to detect hidden genotoxic effects. The data requirements and assessment methods foreseen according Directive 91/414 have been reviewed by MacGregor (2005) and judged as not most appropriate. Better adapted sample preparation protocols, guidelines on test choice and test hierarchy in a tiered system need to be developed in relation to exposure scenarios. The development of assays with crude extracts and culture supernatants should be envisaged detecting or excluding the production of genotoxic metabolites in relevant quantities in a first instance.

REBECA proposes a tiered scheme for bacterial and fungal metabolites (Fig. 1), based on the assessment of supernatants and crude extracts from cultures of the micro-organisms in question in bioassays apart from whole-animal testing. The microbial metabolites may have additive or synergistic toxic effects. It is conceivable that the toxicological risk associated to a particular BCA would be better foreseen by assaying mixtures of metabolites on test organisms characterized by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites. Sensible, high throughput and cost effective bioassay systems would allow the assessment several production batches, determining the toxicity range of a product. Moreover, such test systems would allow the investigation of toxic metabolite production over a broader range of environmental conditions compared to animal testing.

REBECA consortium members are aware that the use of crude extracts also has pitfalls. The crude extracts represent the “worst case” scenario as levels and spectrum of metabolites being assessed are far higher than occurring in nature. Crude extracts are hardly expected to show zero toxicity; therefore it will be necessary to establish tolerance levels of toxicity in biological assays. There are no validated methods available for MBCAs, replacing whole animal testing of purified metabolites. However, a broad range of potential alternatives to the currently used methodology are described in the literature, waiting to get evaluated and validated for risk assessment purposes. Examples for the assessment of MBCAs are given by Favilla et al. (2006), Skrobek & Butt (2005), Skrobek et al. (2006). For more examples and details see deliverable 11.

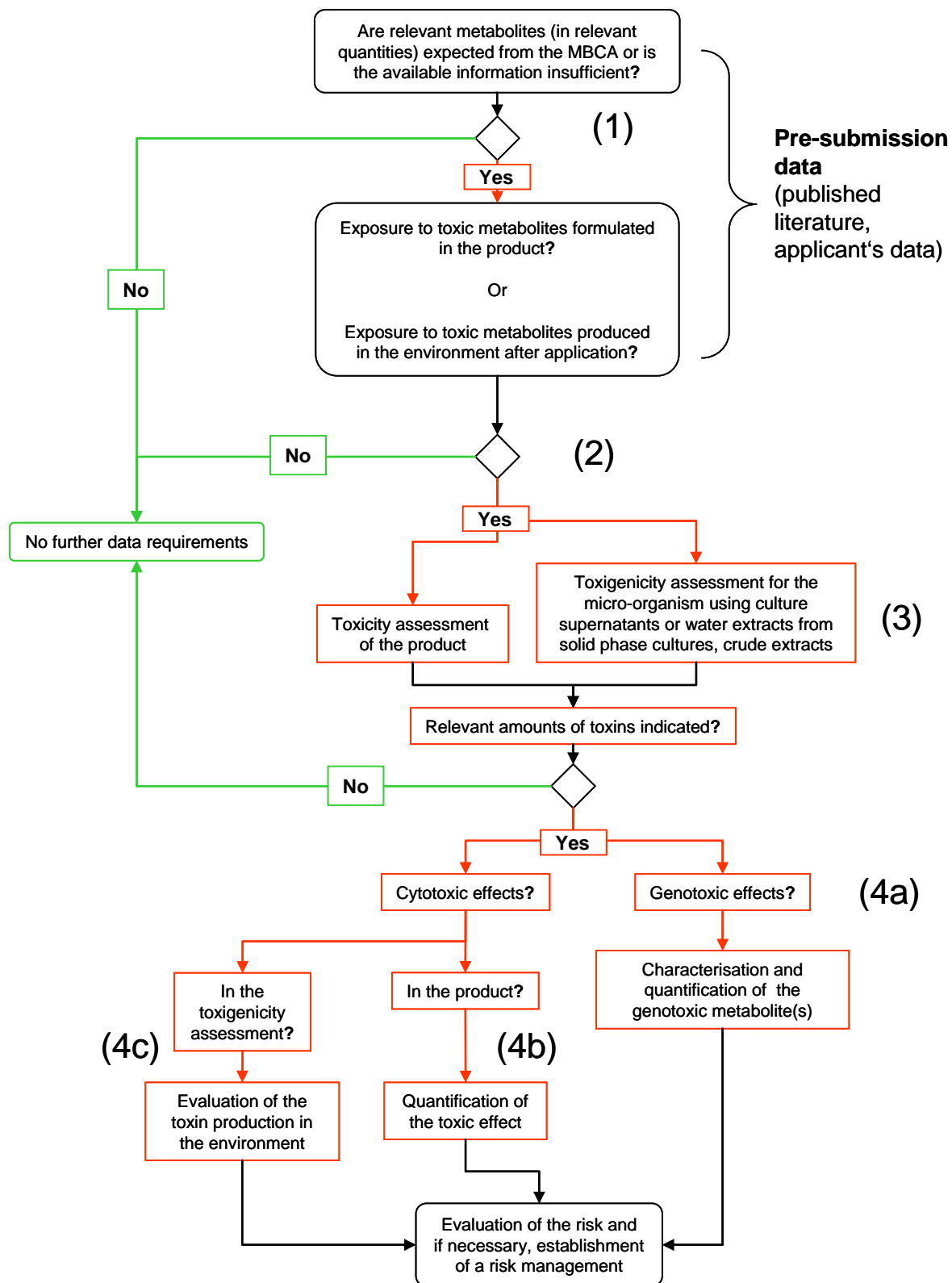


Figure 1: Scheme for assessment of potential relevant metabolites of microbial BCAs.

- (1) + (2) In case the available (public) information demonstrates that no relevant (toxic) metabolites are produced by the micro-organisms in relevant amounts or no exposure to relevant metabolites will occur no data on metabolites should be required. Natural background levels and the related natural exposure to the micro-organism should always be taken into account. If no hazards are known from a regular exposure of humans and other non-target to the micro-organism no risks can be expected from the application of the same organism as a PPP in amounts comparable to background levels.
- (3) In case questions (1) and (2) can not be answered with no, in a first instance, it should be investigated if relevant amounts of microbial toxins are contained in the product and the toxigenicity of the micro-organism should be evaluated. At this stage the toxicity assessment should be carried out using maximum hazard doses (at least 10 fold higher than the maximum expected environmental exposure). The toxigenicity or potential of the micro-organism to produce toxins under different environmental conditions should be evaluated by using culture supernatants and crude extracts produced under different growth conditions. The growth conditions (temperature, substrate) after application of the MBCA should be taken into consideration. A prerequisite for these investigations will be the development and validation of sensitive, high throughput and cost effective standard bioassays for the cyto- and genotoxicity assessment. Whole animal (vertebrate) testing with single purified metabolites would not be feasible and not appropriate. Examples for bioassays, which can be potentially used, are given in deliverable 11. If no relevant amounts of toxins can be detected in the product or in the toxigenicity assessment no further data requirements on metabolites would be necessary. For the identification of relevant amounts the application rate, growth conditions at the application site, natural background levels and the fact that metabolites are biodegradable should be taken into consideration.
- (4)
- a. In case genotoxic effects were detected under (3), indicating relevant amounts of toxins in the product or potentially produced in the environment, hidden risks due to the application of the MBCA might exist. Consequently, the genotoxic metabolites need to be identified and characterised. In order to build up a risk management the amount of these metabolites needs to be quantified in the product and the production in the environment needs to be evaluated.
 - b. In case cytotoxic effects were detected under (3) in the product, the toxic effect should be quantified using validated bioassays. Until now single relevant toxins were purified and assessed. The toxin content was quantified in several batches in order to identify maximal exposure rates. However, it is conceivable that the toxicological risk associated to a particular MBCA would be better foreseen by assaying the mixtures of all metabolites/compounds in the product instead of assessing the toxicity of single metabolites. With validated sensible and low cost bioassay systems a quantification of the toxicity of many product samples would be possible, less expensive and more reliable than the current practice.
 - c. In case it was indicated under (3) that the micro-organism might produce cytotoxic compounds in relevant amounts in the environment related exposure rates need to be evaluated. For this evaluation, data should be submitted allowing a worst case estimation. These data are the application rate, persistence and growth rate and growth place of the micro-organism in the environment and the toxin production under different relevant environmental conditions (temperature, substrate). It should

be checked if additional data to that collected under (3) and to public data are necessary for this assessment.

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 8, 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.

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 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. Most MBCAs can be considered to be part of the “background population” (Annex II, 2001/36/EC). Natural habitat and application site for MBCAs are in most cases identically or similar (e. g. Damgaard 2000, Ramos 2004, Meyling & Eilenberg 2007). In these cases persistence should be not recognized as a risk. The density of microbiota often heavily fluctuates depending on host, seasonal and micro-climatic conditions and agricultural measures. Therefore, the introduction of a relatively high and persistent population of an indigenous organism in the environment should not be a major concern. Application of microbial species to any particular environment results usually in a temporary increase of its population followed by a gradual decrease to background levels.

Most micro-organisms have a world-wide distribution. However, some might be non-indigenous in the habitat they were applied. For those micro-organisms release and persistence in the environment might pose a risk to potential non-target organisms that have never been exposed to the micro-organism before. Data on non-target effects will add to the assessment of potential risks due to persistence in a defined habitat.

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. Many animals, including mammals, birds, fish and crustaceans are under intensive animal husbandry. Therefore, the pathogens of those animals are under extensive investigation. Consequently, possible non-target effects could be identified by a literature research and the absence of hazard reports in combination with a regular exposure of the non-target organisms can indicate a negligible risk.

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 (Nannipieri et al. 2003). 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 practices 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. Hazards have not been observed so far. 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 (Alabouvette 1998, Steenwerth et al. 2002, Buckley et al. 2003, Clegg et al. 2003, Johnson et al. 2003, Garbeva et al. 2004, Grayston et al. 2004, Salles et al. 2006, Meyling & Eilenberg, 2007). 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. Data on the effect of the release of MBCAs on other micro-organisms in the soil should therefore not be requested.

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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
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[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
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Industry:

Ravensberg, Willem, Koppert, Netherlands
Serk, Guido, Biobest N.V., Belgium
Donat, Christina, Bio-ferm, Austria
Kron-Morelli, Roberto, Agrifutur, Italy
Niemi, Marina, Vedera Oy, Finland
Witthaker, Mark, JSC International, United Kingdom
Lüth, Peter, Prophyta, Germany
Peters, Arne, E-nema, Germany
Luyten, Agnieszka, Redebel, Belgium
Gwynn, Roma, Rationale Biopesticide Consultants, United Kingdom
Knauf, Werner, Liederbach, Germany

Virus Workshop

Academia

Jürg Huber, BBA Darmstadt, Germany
Johannes Jehle, DLR Rheinpfalz, Neustadt, Germany

Regulation

Susanne Guske, BVL, Germany
Richard Davis, PSD, United Kingdom
Jeroen Meeussen, CTB, The Netherlands
Heli Nommsalu, PPI, Estland

Industry

Philip Kessler, Andermatt BiocontrolAG, Switzerland
Antoine Bonhomme, NPP, France
Claudia Mochen, Sipcam, Italy
Rüdiger Hauschild, GAB Consulting GmbH, Germany

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

Participants

Altomare, Claudio, ISPA, Italy
Ehlers, Ralf-Udo, Christian-Albrechts-University of Kiel, Germany
Gustavsson, Kersti, KEMI, Sweden
Hansen, Bjarne Munk, National Environmental Research Institute, Denmark
Hansen, Vinni Mona, National Research Centre for the Working Environment, Denmark
Hauschild, Rüdiger, GAB Consulting GmbH, Germany
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Klingen, Ingeborg, Norwegian Crop Research Institute, Norway
Mensink, Hans, RIVM, The Netherlands
Niemi, Marina, Veedra, Finland
Nutti, Marco, University of Pisa, Italy
Strasser, Hermann, Leopold-Franzens-University-Innsbruck, Austria
Strauch, Olaf, Christian-Albrechts-University of Kiel, Germany
Sundh, Ingvar, SLU, Sweden
Typas, Milton, University Athens, Greece

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 Consulting GmbH, Germany)

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

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

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

Annex 7: Position paper on microbial metabolite assessment

Policy Support Action

The Risk Assessment of Metabolites produced by Micro-organisms in Plant Protection Products

April 2007

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1. Introduction

This document was initiated by a working group in the REBECA Policy Support Action in January 2007. The document is intended as a proposal to the European Commission and EU member states in order to facilitate the registration procedure for plant protection products containing micro-organisms as the active ingredient. It aims at a facilitation of the procedure for Annex I inclusion and at a facilitation of national registrations. Micro-organisms used as active substances in plant protection products in the EU are regulated according to the EU Council Directive 91/414/EEC. 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.

2. Potential risks related to microbial metabolites

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" (Anonymus, 2005). This statement would still apply even if these agents secreted produced 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). Another persuasive precedent was set by the Commission with the active ingredient *Gliocladium*. The review of *G. catenulatum* (strain J1446) was said by the Commission to be complete and precise (see also Commission Directive 2005/2/EC).

Today’s Commission practises regarding the assessment of environment, health and safety risks of BCAs and their “relevant” metabolites – (see also Appendix 2) is in accordance with REBECA interest. A case by case evaluation is 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, there is no “relevant metabolite” identified – secreted/accumulated during and after application of the potential BCAs -, which can prohibit the use of microbial BCAs.

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- EC (2004). European Commission Directive 2004/71/EC of 28 April 2004, amending Council Directive 91/414/EEC to include *Pseudomonas chlororaphis* as active substance. Official Journal of the European Communities L 127/104.
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3. Microbial BCAs are effective and safe

There has been much interest in microbial BCAs (i.e. bacteria, fungi and virus) over the past two centuries. Numerous peer reviewed scientific articles and text books have been published on different aspects of their biology. Scientists have looked at their ecology, production, formulation, and safety.

Microbial BCAs are mostly soil-borne organisms found throughout the world. They are listed in the risk group 1 category, which is defined as biological agents which are unlikely to cause human disease (Commission Directive 2000/54/EC). There is one exception, *Pantoea agglomerans*, which currently belongs to risk group 2 but its risk status is being reevaluated. The question “how to evaluate human and environmental safety of microbial BCAs and their metabolites” is still under discussion. Nevertheless, RAFBCA* experts have generated data that provide a better understanding of the major metabolites secreted by selected fungal BCAs including their distribution and regulation (see RAFBCA reference list – Appendix 1).

* RAFBCA, acronym for Risk assessment of Fungal Biological Control Agents, was an EU-funded project (QLK1-CT-2001–01391) with the aim to establish whether metabolites produced by fungal BCAs entered the food chain and if they posed a risk to human and animal health (see <http://www.rafbca.com>). European scientists and industrial partners from different European countries participated in this multidisciplinary, multifaceted project. Particular attention focused on (i) the development of sensitive tools (e.g. biosensors) and methods (including high throughput assays like ELISA, AMES and the Vitotox test) for rapid and accurate detection of fungal metabolites and access their putative mutagenicity and cytotoxicity, (ii) biochemical and

molecular studies to elucidate their mode of action of metabolites, (iii) to monitor fungal BCAs in the environment with molecular markers (iv) to determine if metabolites enter the food chain and, if so, identify the route of entry and type and quantities present. **The RAFBCA experts concluded that metabolites of fungal BCAs posed no risk to humans and the environment.**

4. Occurrence of metabolites with regard to the safety of plant protection products containing micro-organisms as the active substance

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. The quantities normally detected in target hosts or the environment are usually too low to be of concern. In other words these metabolites pose no risk to humans and the environment.

There is considerable mitigating evidence that microbial BCAs do not pose a risk to humans and the environment. Some examples are listed below:

(i) No introduced beneficial microbial BCA has been reported to have harmed humans even though some have been used extensively for decades (e.g. *Bacillus thuringiensis*, *Verticillium lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*). Most reported cases of infections are by opportunistic species usually infecting immuno-compromised patients.

(ii) Microbial BCAs occur naturally, are airborne and are often associated with food crops. However, it appears that any risks these organisms may pose is acceptable. They are viewed as the “background population” (Annex II, EC 2001).

(iii) 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 mycorrhizal fungi (85 % flowering plants have symbiotic relations with these fungi), N-fixing bacteria (nodule and free living species) as well potential human pathogens (see presentation by Gabriele Berg at REBECA Meeting in Innsbruck 2006; www.rebeca-net.de).

(iv) Many microbes are killed by antagonists and UV-irradiation or inhibited by antibiotics produced by other microbes. For these reasons microbial BCAs have to be applied frequently or at relatively high levels. Examples of soil biota feeding on fungal BCAs include mycophagous nematodes, amoebae, rotifers, and collembolans (e.g. see BIPESCO report <http://www.uibk.ac.at/bipesco/>).

(v) Most introduced microbial BCAs may only temporarily dominate – and are unlikely to displace natural biota (including indigenous related species) which is already in flux.

(vi) EU funded project RAFBCA demonstrated using selected fungal BCAs that they do not pose a risk to humans and the environment (See <http://www.rafbca.com> as well as RAFBCA reference list; Appendix 1) - RAFBCA impacts on Directive 91/414/EEC and Directive 2001/36/EEC (data required on microbial BCAs in Annexes II and III Part B) by showing that the evaluation of microbial metabolites during registration of BCAs could be simplified.

5. Risk assessment of metabolites produced by micro-organisms in plant protection products

The EU-approach to microbial metabolites is still under discussion although a lot of information and experience has been gained within funded EU projects (e.g. BIPESCO, RAFBCA).

The following general conclusions were drawn by RAFBCA* experts:

- (1) Purification of any metabolite - even in very small amounts - is time consuming and requires the use of several analytical methods. Even under these conditions 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.**

- (2) A possible solution to overcome this problem is to study and assess the risks by using **crude extracts**, which is a mixture of all possible metabolites. Crude extracts may come after growth of BCAs on minimal and complete media. Minimal media show the realistic aspect while complete media show the worst case scenario where all metabolites can act synergistically.
- (3) RAFBCA results can be extrapolated to other similar micro-organisms since data from field experiments (using several different crops) clearly showed that BCAs are safe. It is noted that experiments were performed through all stages from production to final products and the worst case scenario was also examined (applied ten-times higher than normal dose; Skrobek & Butt, 2005, Skrobek et al. 2006; Boss et al. 2007c).
- (4) In consequence very low amounts of metabolites were detected (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). Therefore, RAFBCA results should be considered as a model and more effort should be put in dissemination of these results, particularly regulatory people.

In the assessment of risks that fungal BCAs may pose to the ecology and the biology of BCAs should be taken seriously into account.

- (1) In most cases these BCAs are already in the fields. After application of the fungal BCA its population is increased in the soil, but after a while, the population decreases and goes back to the naturally occurring levels in the field (trials/studies done in BIPESCO and RAFBCA project).
- (2) Sprayed conidia do not pose a risk because they germinate only after the contact to the target (i.e. on treated pest insects). Toxins are produced under inducible conditions within the host, therefore they exist in extremely low amounts elsewhere and crops are safe (see also worst case scenario of RAFBCA results, metabolites did not enter the food chain; Boss et al. 2007c; Seger et al. 2005a, b; Strasser & Kirchmair 2006; Skrobek et al. 2007).

How to deal with a registration of a microbial BCA that has only recently been described/introduced? - and for which there are no information about metabolites available?

- (1) **Crude extracts should be the solution:** Strictly control the process of the production, use the crude extract produced in the production facility in approved bioassays (Fig. 1).
- (2) This approach does not require the set up of analytical methods with high sensitivity for each of the known toxic metabolites of a particular microbial BCA (which is a very time-, labour- and money-consuming task).
- (3) Strain level information should be requested – do not extrapolate data between species (see also SANCO document: Guidance developed within the Standing Committee on the Food Chain and Animal Health on the taxonomic level of micro-organisms to be included in Annex I to Directive 91/414/EEC (Sanco/10754/rev. 5).
- (4) Find its genetic relation with other known BCA (i.e. BCA already in the market) – basic research (see also item 6).

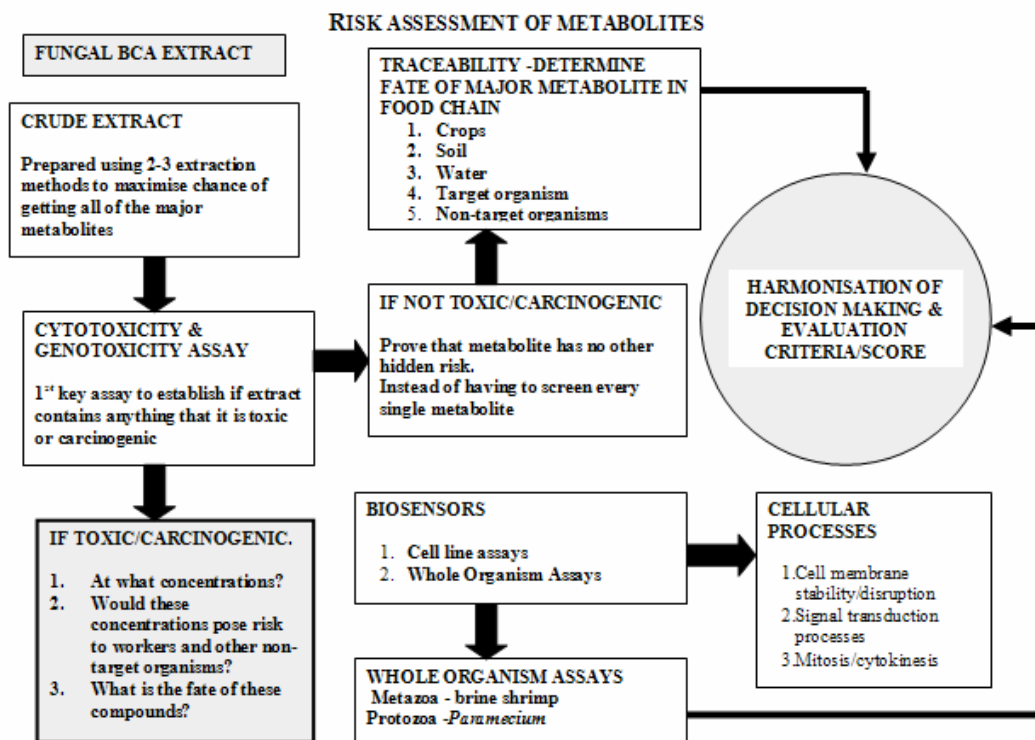
RAFBCA experts are aware that the use of crude extracts has also pitfalls.

- (1) There is a need to set up standard procedures for the production of crude extracts (cultivation method, extraction protocol, bioassays) for each microbial BCA that meets the requisite of maximum expression and detection of potential toxicity of extracts.
- (2) Crude extracts are usually very concentrated as they come from pure cultures of the fungus grown in the best condition for metabolite production. This means that in biological assays someone will use

concentrations of metabolites that hardly occur in nature. This actually is a “hazard assessment” and not a “risk assessment”, as the latter would require an evaluation of the probability that hazardous levels of metabolites occur in the reality after in situ production, translocation and degradation.

- (3) Crude extracts are hardly expected to show zero toxicity; therefore it will be necessary to establish tolerance levels of toxicity (genotoxicity, cytotoxicity, ecotoxicity) by biological assays. This should be done, of course, on the basis of scientific criteria and procedures that we have to define.
- (4) Also, other possible concerns of EU representatives could stem from the following considerations: It evaluates only acute toxicity and not toxicity due to sub-lethal doses (chronic toxicity) and it is based only on in vitro testing (no tests on laboratory animals are required).

RAFBCA expert (2006) came to the conclusion that based on the risk decision scheme risk assessors should argue on a case by case basis which of the tests and results are essential for microbial BCA registration (Fig.1).



Butt T.M. (2006)

Fig. 1 Microbial metabolite decision scheme for microbial plant protection predicts.

The proposed risk assessment decision schema has already been proofed by RAFBCA* experts on case studies (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 extract from different mycoparasitic, entomopathogenic or phytopathogenic fungi (used for BCA) and their selected metabolites displaying different structural nature and mode of action were used to assess the acute toxicity. Insect, invertebrate and cell culture were the models that have been widely used for studies of ecotoxicology as well as of general toxicity of chemicals and natural compounds.

Our findings suggest that the cytotoxic activity of crude extracts from BCAs represent a “worse case scenario”. It is conceivable that the toxicological risk associated to a particular BCA would be better foreseen by assaying

mixtures of metabolites, like those in crude culture extracts, on test systems characterised by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites.

The testing of crude extract, if really necessary, requires less time and resources and subsequently would be more cost effective for companies wishing to register BCAs.

6. Potential of the fungal BCAs to produce metabolite

With the exception of genes involved in trichothecene production by *Trichoderma* species (c-15, sequences EE27895; EE257851, 2006), no other genes responsible for the production of any metabolite of fungal BCAs is yet known. This topic urgently needs further research because should such gene sequences become available, primers amplifying the complete gene region by PCR can be used : (i) to screen for BCAs carrying intact the responsible genes, (ii) to examine the gene expression levels by RT-PCR, (iii) to over-express the gene in recombinant vectors and examine directly its product effects – at controlled concentrations - with several bioassays.

7. Alternative approach to assess relevance/significance of metabolites produced by micro-organisms in plant protection products

The actual E.U. approach to microbial metabolites is discussed in the OECD Issue Paper on “Microbial Metabolite Residues in Treated Food Crops” (Rochon & Belliveau, 2006, see Appendix 4), but no real innovative regulatory approach for assessing their risks has been postulated.

Political willingness is announced to open ways and strategies to get microbial BCAs on the European market. We have to learn that not all minimal risks can be excluded. If experts believe that microbials are safe, then we should register them. (see List of safe microbial BCAs - REBECA homepage <http://www.rebeca-net.de>). If there is evidence that they are not safe, then we should use specific precautions or even omit the species or the isolate.

8. Proposal for a tiered system for data requirements on microbial metabolites

It is public knowledge that the major hurdle for prevention of the use of these products is the current legislation following the Councils Directive 91/414/EEC (EC 2001), which was originally developed to register synthetic chemical compounds. It is a fact, until microbials became more attractive to be used where chemical pesticides are banned or being phased out, or where pests have developed resistance to conventional pesticides, stakeholder groups insisted to treat biologicals in the same manner as synthetic chemicals. This approach is in many cases overdrawn, not to say a wrong strategy.

At the Salzau conference in September 2006, organised by REBECA, an important recommendation on how to deal with fungal metabolites was agreed by stakeholders from academia, industry and regulation: “As the result of the RAFBCA Studies on production of metabolites by certain fungal BCAs (1), the potential for exposure to these metabolites is considered to be low. There is scientific evidence (2) that no major concern exists for human and environmental safety. Therefore, it is recommended that REBECA produce a guidance document on fungal metabolites using the RAFBCA project and other relevant information for the registration process.”

(1) All those fungal products are included, which are already listed in Annex I (according to EU directive 91/414 Annex I) and notified as fungal species (in accordance with Article 4 of Commission Regulation (EC) No 1112/2002) These fungal strains are group 1 classified MPCAs, biological agents which are unlikely to cause human disease (Article 13, EU regulation 2229/2004).

(2) Term “opinion” replaced with “evidence” by facilitator Dr. Strasser

RAFBCA experts suggests that the detection of “relevant” metabolites should be based on TIER I toxicity tests. However, this studies should only be requested when a clear positive result could be estimated with the help of a sensitive test systems (see also item 5 and reference list - Appendix I). Otherwise metabolite and residue data may not be required and an exemption from the requirement of a tolerance may be recommended for products intended for use on food/feed crops.

Looking at our model fungal organisms *Beauveria* and *Metarhizium* – are the key data on metabolites really necessary when it became clear that

- The lethal dose (LD₅₀) of oosporein is achieved to kill 50 percent of *Paramecia*, if 21 Mio. kg Melocont® (active ingredient *Beauveria brongniartii*) has to be applied in a swimming pool system, with a dimension of 50 to 10 to 2 meters. Recommended application rate of Melocont® is 50 kg / ha.
- 3500 kg tomatoes treated with *Metarhizium* conidia powder (containing enough Dtx A) are necessary to cause fifty percent mortality of the most sensitive biosensor system (i.e. HL60 human leukemic cells).

For this particular information the “Austrian Science Fund“ and the “EU- Commission“ has spent > 1.2 Mio € for the set up of the analytic to monitor relevant *Beauveria* and *Metarhizium* metabolites from production, formulation to field use. The output of these studies were that RAFBCA experts could confirm that used fungal isolate and their relevant metabolites do not harm humans and environment. This information, however, has been available to experts for more than twenty years because both BCAs have been used in large amounts to control soil dwelling pests in Europe for many years. The findings were realised in two different EU funded projects (i.e. BIPESCO- FAIR6-CT98-4105- and RAFBCA) and kept two teams busy for five years. There will be a need of six person months per crop or biological matrix in minimum to adapt the sample preparation technique and analytic to demonstrate whether metabolites enters the food chain.

Currently, there is still an agreement of rapporteur member state representative that every batch should be checked on relevant metabolites (= part of residue definition; e.g. in the case of *Beauveria* on beauvericine, see also Appendix 2). The assessors claim to use the best available (routine) techniques and to define a limit of quantification lower than the current 50 ppm (Minutes of List 4 MO meeting, Feb. 2007).

A ‘lessons learned’ document“ should be elaborated in the near future by risk assessors. This document is appreciated by REBECA, because it will help to identify when expert judgement is necessary to decide whether studies for risk assessment are useful or not.

9. Acknowledgements

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