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Annex to the Working Document on the Risk Assessment of Secondary Metabolites of Microbial Biocontrol Agents

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11. Background document

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GENERAL INTRODUCTION

Definitions

Primary metabolites

"Primary metabolites are involved in growth, development, and reproduction of a microorganism. The primary metabolite is typically a key component in maintaining normal physiological processes; thus, it is often referred to as a central metabolite. Primary metabolites are typically formed during the growth phase as a result of energy metabolism, and are deemed essential for proper growth. Examples of primary metabolites include alcohols such as ethanol, lactic acid, and certain amino acids. Within the field of industrial microbiology, alcohol is one of the most common primary metabolites used for large-scale production. Specifically, alcohol is used for processes involving fermentation which produce products like beer and wine. Additionally, primary metabolites such as amino acids, including L-glutamate and L-lysine, which are commonly used as supplements,- are isolated via the mass production of a specific bacterial species, *Corynebacteria glutamicum*. Another example of a primary metabolite commonly used in industrial microbiology includes citric acid. Citric acid, produced by *Aspergillus niger*, is one of the most widely used ingredients in food production. It is commonly used in pharmaceutical and cosmetic industries as well" (source: Boundless).

Secondary metabolites

The following definition has been formulated by Vinale et al. (2014).

"Although not essential for their primary metabolic processes, microbes, and particularly fungi, produce various secondary metabolites (SMs), including compounds of industrial and economic relevance. SMs are chemically different natural compounds of relatively low molecular weight (in most cases < 3 kDa), that are mainly produced by microorganisms and plants and typically associated to individual genera, species or strains. SMs are biosynthesised from primary metabolites in specialised pathways (e.g. polyketides or mevalonate pathways derived from Acetyl Coenzyme A, or amino acids) and some genes are clustered together. The expression of these genes appears to be induced by one or a few global regulators. SMs show several biological activities possibly related to survival functions of the organism, such as competition against other micro- and macroorganisms, symbiosis, and metal transport".

Hypotheses for formation secondary metabolites

Biological control agents produce a wide array of SMs. The question is often posed why these SMs are being produced. There are several reasons:

- Defence: these SMs are a defence against the immune system of the parasitized insect/plant pathogen.
- Antagonism: these SMs have antibiotic properties, including against competing microbials.
- Competition: these SMs enable a mciroorganism to compete for nutrients and space.
- Pathogenesis: other SMs may be important pathogenicity determinants (Amiri et al., 1999; Bandani et al., 2000). They kill or weaken the host.

Microbial metabolites in perspective

Paterson (2006) designed a useful Venn diagram to show the relationships between fungal metabolites in terms of toxicity (Figure 1). This diagram shows the overlapping relationship between mycotoxins, pharmaceuticals, metabolites from biocontrol agents, and fungal biochemical weapons, which illustrates clearly that the same compound can be represented in different fields. According to this diagram, almost all metabolites from biocontrol agents are considered to be toxic metabolites. The definition of 'toxic metabolites' is however not yet clear. Some metabolites will be toxic such as cytochalasin D, whereas most metabolites have an antibiotic mode of action and are not toxic to non-target organisms. In the follow-up to

this introduction attention will be paid to the definitions of toxic metabolites as this is important for the risk assessment.

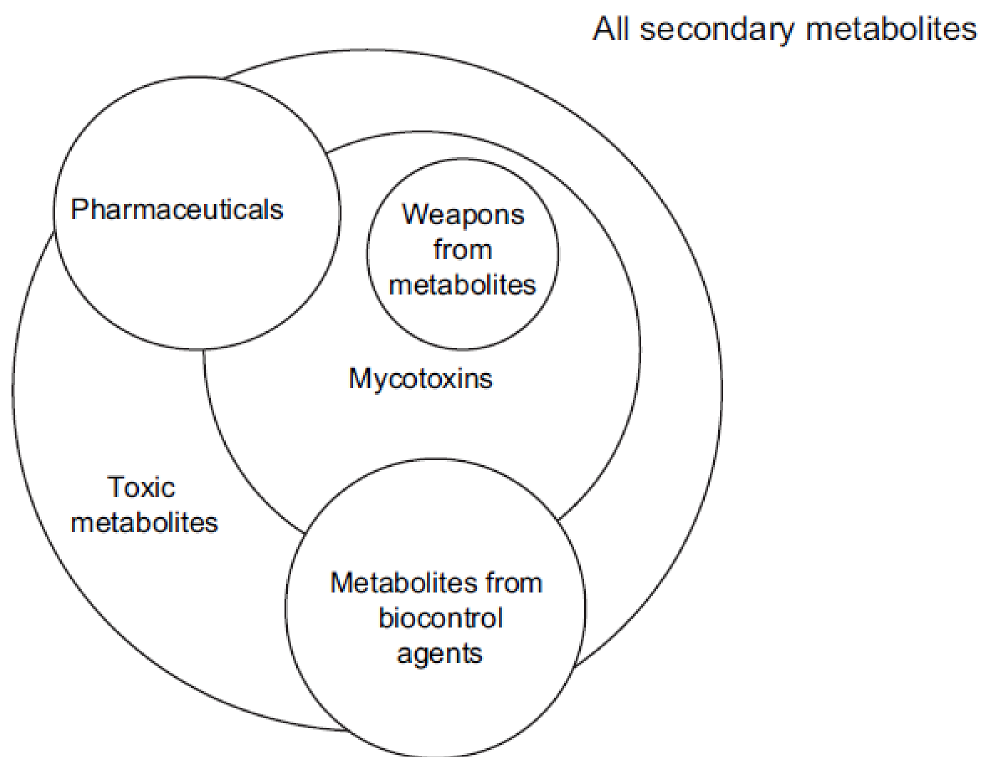

Figure 1. Venn diagram of the relationship between fungal metabolites in terms of toxicity. The sizes of the circles are in proportion to the actual number of compounds only in a general manner (Paterson 2006).

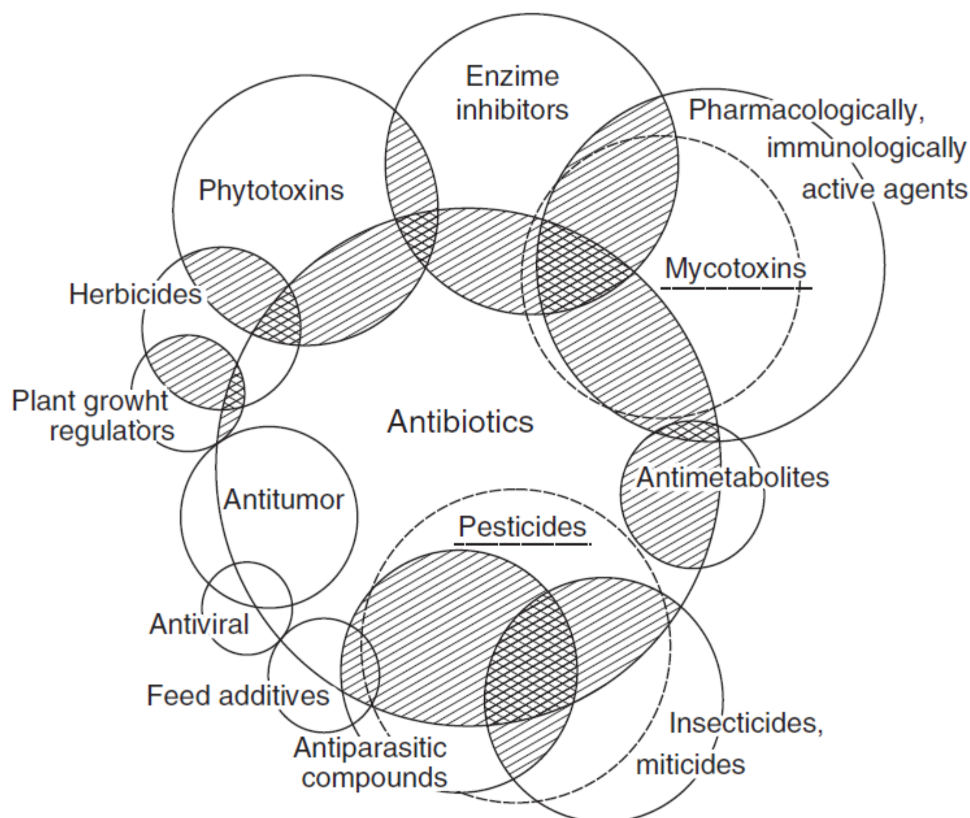

Figure 2. Venn diagram of bioactive microbial metabolites (Bérdy 2012).

Definition of relevant metabolites

According to the EFSA any assessment of safety must consider the possibility and consequences of the added organisms entering and remaining with the food chain as well as the fate of any metabolites.

Herein, the EFSA follows the EU Regulation 283/2013 which says:
'*relevant metabolites (i.e. if expected to be of concern to human health and/or the environment) known to be formed by the micro-organism shall be identified and characterized at different states or growth stages of the micro-organism.*'

In this consideration the following definition will be used:

'Any metabolite known to be formed by the microorganism that can be of concern to human health and/or the environment'

The relevance of the SMs may however be difficult to assess. There may be unknown SMs produced by microorganisms. The risk assessment is therefore performed with known information about the biological organism and its known SMs that are produced in the formulation and after application in contact with the target organisms, as well as that of genetically close taxa.

In a theoretical situation where a microorganism produces an unknown and hazardous SM, the risk assessor would be alerted to this hazardous substance while reviewing the results of toxicological studies. The toxicological data should be judged against known properties of the microorganism and the possible routes of exposure.

Secondary metabolites relevant for risk assessment
For the risk assessment of SMs in MPCAs, different groups have been recognised in the literature as relevant: mycotoxins, phytotoxins and antibiotics.

Mycotoxins

Mycotoxins are defined by Bennett (1987) as ‘‘natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route.’’ Natural routes may include ingestion, skin contact, inhalation, or others[1] as opposed to ‘‘unnatural’’ routes such as injection.

The definition made by Paterson (2006) overlaps more or less: “Mycotoxins are a somewhat exclusive group of low molecular weight compounds that are present in foods, and affect mammals (e.g. humans). They are produced by filamentous fungi, but the fungi may no longer be present in the food.”

A comprehensive review on the major mycotoxins was made by Bennett and Glick (2003):
“*While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins. Whether or not a compound is called a mycotoxin depends on the target and the concentration of the metabolite. Targets are vertebrates including humans and animal groups. The compound must demonstrate significant toxicity at low concentrations.*” Table 1 shows some of these mycotoxins. A longer list is presented in Section 8.2.1, table 3 of the Working document.

Table 1. Production of some mycotoxins known to be associated with human health (Blumenthal, 2004).

Mycotoxin	Producers*	Confirmed production in		
		A. niger	*A. oryzae*	*T. reesei*
Aflatoxin B1[a,b,c]	*Aspergillus*	No	No	No
Citreoviridin[c]	*Penicillium*	No	No	No
Fumonisins[b,c]	*Fusarium***	No	No	No
3-Nitropropionic acid[c]	*Aspergillus, Penicillium Arthrinuim****	No	Yes	No
Ochratoxin A[a,b,c]	*Aspergillus, Penicillium*	Yes	No	No
Sterigmatocystin[a]	*Aspergillus, Bipolaris Chaetomium*****	No	No	No
Trichothecenes				
T-2[a]	*Fusarium, Trichoderma*	No	No	No
Deoxynivalenol[b,c]	*Fusarium*	No	No	No
Nivalenol[c]	*Fusarium*	No	No	No
Diacetoxyscirpenol[c]	*Fusarium, Gibberella*	No	No	No
Zearalenone[a,b,c]	*Fusarium*	No	No	No

[a] Testing previously required by JECFA in all fungal-derived food enzyme preparations.
[b] Considered medically important by Pitt (2000).
[c] Postulated human mycotoxicoses by Peraica and Dominjan (2001).

Mycotoxins are not only hard to define, they are also challenging to classify. Due to their diverse chemical structures and biosynthetic origins, their myriad biological effects, and their production by a wide number of different fungal species, classification schemes tend to reflect the training of the person doing the categorizing:

- Clinicians often arrange them by the organ they affect. Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, and so forth;
- Cell biologists put them into generic groups such as teratogens, mutagens, carcinogens, and allergens;
- Organic chemists have attempted to classify them by their chemical structures (e.g., lactones, coumarins);
- Biochemists according to their biosynthetic origins (polyketides, amino acid- derived, etc.); physicians by the illnesses they cause (e.g., St. Anthony’s fire, stachybotryotoxicosis);

[1] Only eye contact can be added

- Mycologists by the fungi that produce them (e.g., *Aspergillus* toxins, *Penicillium* toxins).

Mycotoxins are produced by pathogenic fungi and could theoretically also be produced by related biocontrol species. If such species are considered for their biocontrol properties, production of these mycotoxins should be evaluated. In the overview tables of Background document 1, presenting the SMs in perspective of phylum, class, order, family and species, these mycotoxins are marked clearly in red.

"*There are over 200 recognised mycotoxins, however, the study of mycotoxins and their health effects on humans is in its infancy and many more are waiting to be discovered. Many mycotoxins are harmful to humans and animals when inhaled, ingested or brought into contact with human skin. Mycotoxins can cause a variety of short term as well as long-term health effects, ranging from immediate toxic response to potential long-term carcinogenic and teratogenic effects. Symptoms due to exposure to mycotoxins include dermatitis, cold and flu symptoms, sore throat, headache, fatigue, diarrhea, and impaired or altered immune function, which may lead to opportunistic infection. Historically, mycotoxins have been a persistent problem to farmers and the animal husbandry industry in Eastern Europe and developing countries. Recently, however, research has implicated many toxin-producing fungi, such as Stachybotrys, Penicillium, Aspergillus and Fusarium species, to indoor air quality problems and building related illnesses. Inhalation of mycotoxin producing fungi in contaminated buildings is the most significant exposure, however, dermal contact from handling contaminated materials and the chance of ingesting toxin containing spores through eating, drinking and smoking is likely to increase exposure in a contaminated environment*" (cited from: http://www.iaqm.com/toxins.html).

Phytotoxins

Strictly speaking, phytotoxins are toxins produced by plants. The adjective "phytotoxic" refers to a toxin that is toxic to plants. Apparently, the meaning of phytotoxins was erroneously extended to toxins with herbicidal potential, produced by microorganisms, as can be read in the following paragraph.

Cited from Glare et al. 2012: "Many phytotoxins are produced by microbes with herbicidal potential (Duke and Dayan, 2011), and these may provide more broad host range effects than the microbe alone. For example, the fungus *Phoma macrostom*a, has been found to produce phytotoxic metabolites (novel cyclic tetramic acids) that cause bleaching and chlorosis when applied as extracts to several broadleaf species. The bacterium *Streptomyces acidiscabies* produces a phytotoxin, thaxtomin that is essential for the development of scab symptoms and appear to disrupt the synthesis of cellulose by plant cells. Understanding and characterizing the bioactive compounds and optimizing their production in fermentation can increase the efficacy and consistency of a biopesticide in a way that is more comparable to that of synthetic pesticides, but their use will probably raise some questions around residues on food and potential of resistance development. Major regulatory agencies already require toxicology tests on microbes and their bioactive compounds, especially if the latter are included in the end product. The risk of resistance development can be considered as low if the mode of action is based on a combination of several bioactive compounds and sometimes also the living microorganisms, along with their physiological interaction with the target pest".

A list of SMs with a mode of action against plants is provided in Section 8.2.3, table 4, of the Working document.

Antibiotics

Antibiotics are complex chemical substances produced by microorganisms as SMs. These antibiotics react towards bacteria either by inhibiting their growth or killing them.

A list of SMs with an antibiotic mode of action is provided in Section 8.2.4, table 5, of the Working document.

Views of the European Union, Canada and the USA on relevant secondary metabolites

In this chapter the views of the European Union (EU), Canada and the USA on the definition of relevant metabolites are presented.

European Union

Commission Regulation (EU) 546/2011 of 10 June 2011, amending Annex VI to Regulation (EU) 2009/1107 as regards plant protection products containing micro-organisms says:
"Micro-organisms may produce a range of different metabolites (e.g. bacterial toxins or mycotoxins) many of which may have toxicological significance, and one or more of which may be involved in the mode of action of the plant protection product. The characterisation and identification of relevant metabolites must be assessed and the toxicity of these metabolites should be addressed.

Information on production and/or relevance of metabolites may be deduced from:
(a) toxicity studies,
(b) biological properties of the micro-organism,
(c) relationship to known plant, animal or human pathogens,
(d) mode of action,
(e) analytical methods.

On the basis of this information, metabolites may be considered as possibly being relevant. Therefore potential exposure to these metabolites should be assessed, in order to decide on their relevance."

In Commission Regulation (EU) No 283/2013 the data requirements have been worded as follows:
"If, under the conditions of use,
- relevant metabolites are present in the product or produced by the microorganism,
- and there may be exposure of these metabolites to man and the environment,
- data requirements and the corresponding risk assessment need to be fulfilled if all the following conditions are met:
    - the relevant metabolite is stable outside the microorganism
    - a toxic effect of the relevant metabolite is independent of the presence of the microorganism, and
    - the relevant metabolite is expected to occur in the environment in concentrations considerably higher than under natural conditions."

Canada

In Canada, there is no strict definition for a relevant metabolite, however, a microbial agent is defined in Section 1 of the Pest Control Products Regulations (SOR/2006-124) as a pest control product whose active ingredient is a microorganism. It includes any metabolites and toxins produced by the microorganism. Operationally, Canada has adopted the definition:
"Any metabolite formed by the micro-organism that can be of concern to human health and/or the environment."

The information and data requirements for registering microbial agents (including information on potentially toxic metabolites) are identified in Regulatory Directive, DIR2001-02, Guidelines for the Registration of Microbial Pest Control Agents and Products. Information requirements on potentially toxic impurities and SMs can be found throughout these registration guidelines, i.e., Parts
2.7–Characterization of the Microbial Pest Control Agent,
2.9–Disclosure of Ingredients,
2.10–Analytical Data and Methodology,
4–Human Health and Safety Testing (toxicology),
5–Exposure Assessment,
7–Food and Feed Residue Studies, and

9–Environmental Toxicology.

These requirements cover a large range of information requirements (e.g., biological characterization information as well as unique test requirements such as genotoxicity testing) and other considerations during toxicological testing (e.g., careful consideration of non-target test organisms for environmental toxicology testing).

If the presence of a toxin or other toxic metabolite or impurity has been identified and the petitioner wishes to pursue registration for a specific purpose, however, the product may be subject to the same data requirements as a chemical pesticide, and appropriate data may be required to establish a maximum residue limit (MRL).

USA

The list of data requirements for microbial pesticides is found in the Code of Federal regulations at 40 CFR 158.2100 to 158.2200. Included at the start of this section is a definition of what is considered a microbial pesticide but there is no formal definition of a microbial toxin or SM. The footnote to the tables on Residue Chemistry 158.2130 refer to the requirement for residue testing when the results of toxicity testing indicate the potential to cause adverse human health effects or the product characterization indicates the microbial pesticide has a significant potential to produce a mammalian toxin; and the use pattern is such that residues may be present in or on food or feed.

A definition of toxin is found in the Overview for the Guidelines for Microbial Pest Control Agents (885.0001) which states that a toxin is “a poisonous substance, generated by a microorganism, plant or animal, capable of causing injury or damage when it interacts with host cells.” A related definition is given for toxicity which is “the injury or damage caused by a poison or toxin where infection by and/or replication or viability of the microorganism is not necessarily required.” These definitions link the idea that a toxin is a chemical substance produced by a microorganism and that it can have activity in the absence of microbial reproduction.

Other Guidelines clarify what information is expected on the subject of potential toxin production include Product Identity (885.1100), Discussion of Formation of Unintentional Ingredients (885.1300), Background-Microbial Toxicity/Pathogenicity/Infectivity (885.3000) and the Background for Nontarget Organism Testing for Microbial Pest Control Agents (885.4000).

As reflected by both the EU and Canada, there is no definition of SMs or ‘metabolites of concern’ in the enabling legislation. Secondary metabolites or metabolites of concern are terms of art used to describe compounds differing from the typical products of microbial physiology that may have other biological properties including possible toxicity. The function of these compounds can be part of the mode of action as a microbial pesticide or simply a by-product of that particular microbe. The most important aspects of identifying the presence of a toxin are the results of the toxicity testing indicating an adverse effect and the identified potential of a microbe to produce a known mammalian toxin.

Another example of how a metabolite of concern would be identified is laid out in the EU description in the actual order of importance. The first indication is adverse effects seen in the results from the toxicity studies with the MPCA. Second would be known toxins in the description of the biological properties for the microorganism including any information on taxonomically-related pathogens of plants or animals. Finally, there would be a consideration of the mode of action, if it is well established the activity is the secretion of a biologically active compound.

Some background information on metabolites

Table 2 shows the vast numbers of metabolites that have been discovered (Bérdy, 2005).

Table 2. Bioactivity types of microbial metabolites. Numbers of discovered bioactivities (Bérdy, 2005).

Type of activity	Numbers of discovered bioactivities
ANTIBIOTIC ACTIVITIES: (16500 compounds)	
Antimicrobial Activity:	
Antibacterial: Gram-positive	11000~12000
Gram-negative	5000~5500
Mycobacteria	800~1000
Antifungal: Yeasts	3000~3500
Phytopathogenic fungi	1600~1800
Other fungi	3800~4000
Antiprotozoal:	~1000
Chemotherapeutic activity:	
Antitumor (cytotoxic)	5000~5500
Antiviral	1500~1600
OTHER BIOACTIVITIES: (11500 compounds)	
Pharmacological Activity:	
Enzyme inhibitor	3000~3200
Immunological activity (suppressive, modulatory)	~800
Biochemical activity (DNS, tubulin, mitotic, *etc.*)	~1000
Other (antagonistic, modulatory, antiinflammatory, *etc.*) activities	2000~2500
Agricultural Activity:	
Pesticide (antiparasitic, algicide, amoebicide, *etc.*)	900~1000
Herbicide (phytotoxic, plant growth regulatory, *etc.*)	1800~1900
Insecticide/Miticide/Larvicide/Deterrent	1100~1200
Feed additive, preservative	300~400
Other Activities	~1000
Microbial regulators (growth factors, microbial hormones, morphogens)	~500
Biophysical effects (surfactants, *etc.*)	~300

Bérdy (2005) describes the ever increasing rate of the discovery of new bioactive SM: "In 1940 only 10 to 20, in 1950 300 to 400, in 1960 approximately 800 to 1000 and in 1970 2500 antibiotics were already known. Since 1970, the total number of known bioactive microbial metabolites has doubled in every ten years. In 1980 about 5000, in 1990 about 10000 and in 2000 almost 20000 antibiotic compounds were known. By the end of 2002 over 22000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature".

Table 3 gives an idea of the numbers of bioactive metabolites among bacteria, actinomycetales and fungi.

Table 3. Numbers of discovered bioactivities per source (Bérdy, 2005).

Source	Antibiotics	"Other bioactive" metabolites	Total bioactive metabolites	Practically used (in human therapy)	Inactive metabolites
Bacteria	2900	900	3800	10~12 (8~10)	3000 to 5000
Atinomycetales	8700	1400	10100	100~120 (70~75)	5000 to 10000
Fungi	4900	3700	8600	30~35 (13~15)	2000 to 15 000
Total	16500	6000	22500	140~160 (~100)	20000 to 25000

It is expected that the expansion of new screening methods will speed up the discovery of new metabolites, including metabolites produced by species that are already successfully marketed. The vast numbers of bioactive metabolites that exist show us that a Working document on SMs for registration purposes cannot focus on individual metabolites. Any attempt would be extremely time-consuming and the contents of the GD would be outdated rapidly by the discovery of new metabolites. The aim of the following chapters is to provide background to the development of the Working document for the registration process. The Working document will focus on those groups of SMs that are described in the above paragraph titled 'Secondary metabolites relevant for risk assessment'.

Restrictions on the contents of this work

Considering the amount of literature that is available, the data and information presented in the Background documents cannot be exhaustive. This also means that not all relevant genera have been included. It was decided to:

- Restrict the scope to the data found in an earlier literature search performed by Jacqueline Scheepmaker (see also the Appendix of this introduction that shows which data gaps on SMs were identified for several microorganisms in preparation for the workshop in Saltjobaden, June 2013 in Sweden).
- Restrict the scope to some important entomopathogens: *Metarhizium anisopliae, Beauveria bassiana, Isaria fumosorosea and B. brongniartii, Aschersonia aleyrodes, Nomuraea sp., Nomuraea rileyi, Paecilomyces lilacinus, Verticillium lecanii, Paecilomyces fumosoroseus.*
- Accept that information and values contained in this document may not be representative of all isolates of entomopathogens.
- Restrict the scope to the genera of *Pseudomonas, Bacillus, Agrobacterium, Burkholderia, Streptomyces, Erwinia, Serratia, Xanthomonas* for bacterial biocontrol species.

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Appendix

The following table presents the data gaps on SMs that were identified for several microorganisms. This table is an excerpt of the table that was prepared by Sweden in the preparation for the workshop in Saltjobaden, June 2013 in Sweden. The table had been adapted to focus on SMs as the original table also included other information.

Table 4. Some published conclusions on microorganisms.

Tricky question	Published problem	Reference	Micro-organism	Resulting concern	strains
Production of secondary metabolites	Can Bt subspecies strains produce enterotoxins?	Conclusion peer review Bt subspecies strains	All Bt subspecies strains	Operator risk assessment	
Production of secondary metabolites	Can Bta produce enterotoxins after application?	Conclusion peer review Bta ABTS 1857, GC-91	Bta ABTS 1857	Consumer risk assessment	Bta ABTS 1857
Production of secondary metabolites	Can Bta produce enterotoxins after application or in the technical product?	Conclusion peer review Bta ABTS 1857, GC-91	Bta GC-91	Consumer risk assessment	Bta GC-91
Production of secondary metabolites	Can Btk produce enterotoxins after application?	Conclusion peer review Btk ABTS 351, PB54, SA 11, SA 12 and EG 2348	Btk ABTS 351	Consumer risk assessment	Btk ABTS 351
Production of secondary metabolites	Can Btk produce enterotoxins after application?	Conclusion peer review Btk ABTS 351, PB54, SA 11, SA 12 and EG 2348	Btk EG 2348	Consumer risk assessment	Btk EG 2348
Production of secondary metabolites	Can Btk produce enterotoxins after application?	Conclusion peer review Btk ABTS 351, PB54, SA 11, SA 12 and EG 2348	Btk PB 54	Consumer risk assessment	Btk PB 54
Production of secondary metabolites	Can Btk produce enterotoxins after application?	Conclusion peer review Btk ABTS 351, PB54, SA 11, SA 12 and EG 2348	Btk SA 11	Consumer risk assessment	Btk SA 11
Production of secondary metabolites	Can Btk produce enterotoxins after application?	Conclusion peer review Btk ABTS 351, PB54, SA 11, SA 12 and EG 2348	Btk SA 12	Consumer risk assessment	Btk SA 12
Production of secondary metabolites	Can Btt produce enterotoxins after application?	Conclusion peer review Btt NB-176	Btt NB-176	Consumer risk assessment	Btt NB-176
Production of metabolites	Production of toxins during production	Conclusion peer review *Metarhizium anisopliae*	*M. anisopliae* var. *anisopliae* BIPESCO/F52	Human health risk assessment	
Production and persistence of metabolites	Production and persistence of toxins in the environment	Conclusion peer review *Metarhizium anisopliae*	*M. anisopliae* var. *anisopliae* BIPESCO/F52	Environmental risk assessment	

Tricky question	Published problem	Reference	Micro-organism	Resulting concern	strains
Production and persistence of metabolites	Production of toxins during production and in infected insects is unclear	Conclusion peer review *Metarhizium anisopliae*	*M. anisopliae* var. *anisopliae* BIPESCO/F52	Groundwater contamination	*M. anisopliae* var. *anisopliae* BIPESCO/F52
Production of secondary metabolites	Production of toxins during production	Conclusion peer review *Phlebiosis gigantea*	*Phlebiosis gigantea*	Operator risk assessment	*Phlebiosis gigantea*
Production of secondary metabolites	Production of toxins during production and in the environment	Conclusion peer review *Phlebiosis gigantea*	*Phlebiosis gigantea*	Environmental risk assessment	
Metabolites	Production of relevant secondary metabolites/toxins	Ta T34 Conclusion peer review	Ta T34	Human health risk assessment	*Trichoderma asperellum* T34
Metabolites	Groundwater contamination	Ta T34 Conclusion peer review	Ta T34	Groundwater contamination	
Metabolites	Production of relevant secondary metabolites/toxins	Conclusion peer review Tg ICC080	Tg ICC080	Human health risk assessment	*Trichoderma gamsii* ICC080
Metabolites	Groundwater contamination	Conclusion peer review Tg ICC080	Tg ICC080	Groundwater contamination	

1. IDENTIFICATION OF RELEVANT BIOCONTROL AGENTS WITH THE POTENTIAL TO PRODUCE SECONDARY METABOLITES

1.1 Research question to be answered

Identification of relevant biocontrol agents with the potential to produce SMs:
Review the literature on related species and strains known to be used as biocontrol agents and, for example, organize them into a phylogenetic tree (so that grouped approaches to SM production can be explored when they are produced by more than one (or more) species/strain).

1.2 Introduction

There are some biocontrol fungi and bacteria which produce SMs that may play a role in the toxic potential of the organism.

The risks of the SMs should be addressed separately if, under the conditions of use, relevant metabolites are present in the product or relevant metabolites are formed in contact with the pathogen and there is a potential for exposure to humans and/or the environment.

1.2.1 Goals to be achieved

1. Prepare an oversight of the phylogeny of fungal and bacterial biocontrol agents and link each species to the range of metabolites produced.

   Phylogenetic trees can be based on information on genes encoding rRNA or intergenic spacers between these genes, depending on the resolution of the tree. The SM profiles do not seem to match with phylogenetic trees constructed in that way.

   According to Frisvad et al. (2008) "it is not yet possible to use SMs in phylogeny, because of the inconsistent distribution throughout the fungal kingdom. Nevertheless, it is important to have an overview on phylogeny as a backdrop for the risk assessment.

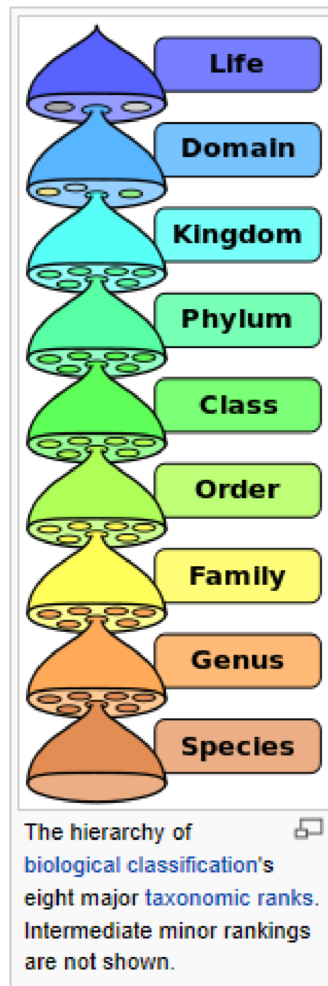

Figure 3. Taxonomic ranking.

2. Preparation of tables with categories of quantities of SM being produced. In this way it becomes visible which SMs are produced most frequently and whether or not the most frequent SMs are also being produced in highest quantities.

3. Can the biocontrol agent can be related to a fungus that is known to be toxic?

1.3 Material and methods

Information used for this Background document was derived from the EFSA scientific report on microbial organisms (EFSA, 2013a). The Appendix A to this EFSA report, containing all microorganisms listed in the EU pesticide database, was used as a source. This list was updated with fungi developed or being developed for the biological control of diseases (Butt et al., 2001). Further, all metabolites and toxins produced by fungal MPCAs presented in chapter 7 of the EFSA report (2013a), chapter 1.6 of the EFSA report (2015) and (Vey et al., 2001a) and were included in the tables below. This information was updated with metabolites mentioned in Strasser et al. (2011).

All tables are organized by order.

A complete list of SMs produced by biocontrol agents, with known or suspected toxicity concerns for non-targets other than humans and higher vertebrates, is not available in the published literature.

The tables given in chapter 1.4 give an overview of the major SMs that can be produced by biocontrol agents and their pathogenic relatives. The Nonribosomal peptides (NRPS[2]) (Norine) database can be referred to for a more exhaustive list of SMs formed (Caboche et al., 2008): this database contained 1164 peptides (July 2013). An interesting asset of the Norine database is that it can list SMs by their activity (antibiotic, toxin, siderophore, antitumor etc.). Currently, 642 antibiotics are in the database and 263 toxins. Secondary metabolites listed as mycotoxins in the Section 8.2.1 of the Working document or toxins in this Norine database are coloured red and orange, respectively. Antibiotics (see Section 8.2.4 in the Working document) are in yellow.

The handbook of secondary fungal metabolites (Cole and Schweikert, 2003) can be referred to for specific information about SMs such as molecular formula and weight, information on isolation/purification, biological activity. This handbook contains all major groups of secondary fungal metabolites.

Regarding the quantities of metabolites being produced, some (entomopathogenic) fungi and bacteria will be reviewed in chapter 1.4.4.

1.4 Results

Kingdom of fungi

Figure 4 shows the phylogeny of the kingdom of fungi.

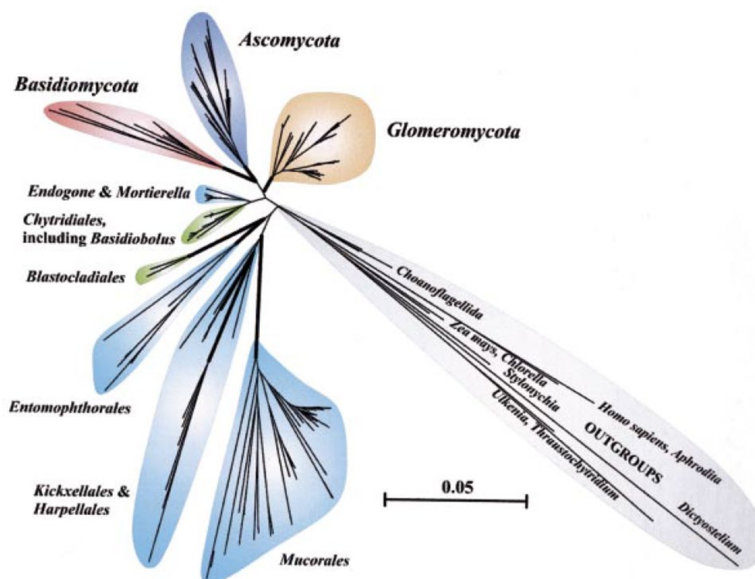

Figure 4. Phylogeny of fungi based on SSU rRNA sequences. Thick lines delineate clades supported by bootstrap values above 90%. The Zygomycota (Entomophtorales) and the Chytridiomycota do not form monophyletic clades and therefore are shown as the respective taxa representing the clade (Schussler et al. 2001).

[2] The reader is referred to Table 33 of Chapter 3 for an overview of metabolites formed in different pathways including the pathway with NRPS synthesis

According to Frisvad et al. (2008) “it is not yet possible to use secondary metabolites in phylogeny, because of the inconsistent distribution of secondary metabolites throughout the fungal kingdom. However, this is the very quality that makes secondary metabolites so useful in classification and identification”. “Fungal chemotaxonomy based on secondary metabolites has been used successfully in large ascomycete genera such as *Alternaria, Aspergillus, Fusarium, Hypoxylon, Penicillium, Stachybotrys, Xylaria* and in few basidiomycete genera, but not in Zygomycota and Chytridiomycota”.

Phylum Ascomycota

Figure 5 shows the main orders and classes of the Ascomycota (Hibbett, 2007).

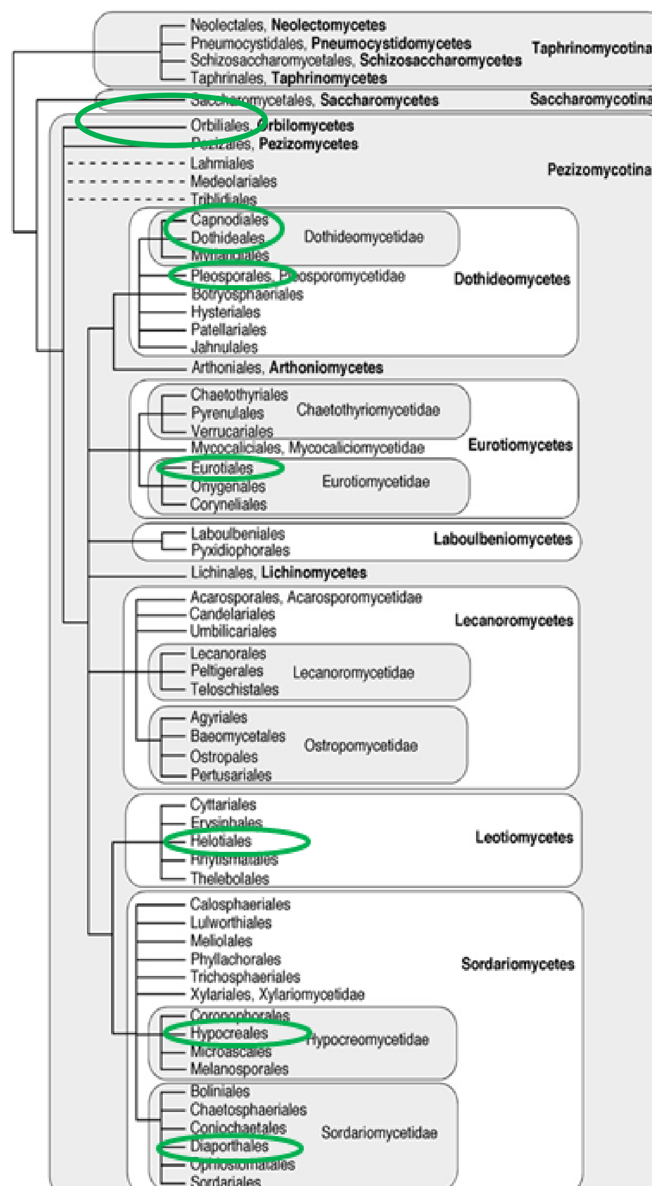

Figure 5. Phylogeny and classification of the kingdom of the Fungi. Phylum Ascomycota (Hibbett, 2007). The orders with the green circles are the ones containing species having biocontrol capacities.

According to the modern classification of Ascomycota (http://en.wikipedia.org/wiki/Pezizomycotina) there are three subphyla that are described and accepted: the Taphrinomycotina, the Saccaromycotina and the Pezizomycotina.

For example: the well-known entomopathogenic genera *Metarhizium* and *Beauveria* belong to the subphyla Pezizomycotina/class Sordariomycetes/order Hypocreales.

In the following tables fungal biocontrol species are arranged according to the newest classification. SMs were given, if available in the literature. All species in bold are pathogenic/herbicidal fungi. They are incorporated in this table as they are known to produce high amounts of certain SMs.

For the genus *Isaria* the classification in the ARSEF collection was followed (see the note of Wheeler (2011) with references therein).

Subphylum Saccharomycotina
Class Saccaromycetes

Table 5. Class Saccharomycetes.

Order	**Family**	**Genus**	**Species**	**Type of action**	**Secondary metabolites**	**Ref.**
Saccharomycetales	Saccharomycetaceae	*Candida*	*C. sake*			
			C. oleophila *C. oleophila* isolate I-182 Product Aspire	Fungicide (against *Botrytis/Penicillium)*		(EFSA, 2013a) (Strasser et al., 2011)

Subphylum Pezizomycotina
Class Dothideomycetes

Table 6. Subclass Dothideomycetidae.

Order	**Family**	**Genus**	**Species**	**Type of action**	**Secondary metabolites**	**Ref.**
Capnodiales	Mycosphaerellaceae	*Cercospora*	*C. rodmanii* Product: ABG 5003	Herbicide against *Eichhornisc crassipes*		(Agropedia)
Dothideales	Dothioraceae	*Aureobasidium*	*A. pullulans*	Acting against pathogenic fungi infesting pome fruits, stone fruits and grapes	Areobasidin A, B, C, E, S2b, S3, S4	(EFSA, 2013a) (EFSA, 2013a)

Table 7. Subclass Pleosporomycetidae (species in bold are known as pathogenic/herbicidal fungi; red = mycotoxin; orange = toxin).

Order	**Family**	**Genus**	**Species**	**Type of action**	**Secondary metabolites**	**Ref.**
Pleosporales	Incertae sedis[1]	*Stagonospora*	*S. convolvuli*	herbicide	Leptosphaerodione, Elsinochrome A, Cercosporin	(Ahonsi et al., 2005)
		Phoma	*P. macrostoma*			

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
	Leptosphaeriaceae	*Coniothyrium*	*C. minitans*	hypoparasite growing on sclerotia of plant-pathogenic fungi.	Macrosphelide A Palmarumycin C1	(EFSA, 2013a) (Strasser et al., 2011)
			C. minitans CON/M/91-08 Product Contans WG	biological activity against bacteria, fungi and garden cress	Palmarumycin C1 to C6, Palmarumycin C8 to C16	(Strasser et al., 2011)
	Phaeosphaericeae	*Ampelomyces*	*A. quisqualis* Product AQ 10	Fungicide For AQ10 the Standing Committee concluded that the metabolites are not relevant (EU, 2005a)	7-hydroxy-5-hydroxymethyl-2Hbenzo[1,4]thiazin-3-one, 2,5-dihydroxy-3- Methanesulfinylbenzyl alcohol, Ampelopyrone; Desmethyldiaportinol; Desmethyldichlorodiaportim; Macrosporin-7-O-sulfate; 3-O-Methylalaternin-7-O-sulfate; Ampelanol	(Zhang HY, 2008) (Aly et al., 2008) (Strasser et al., 2011)
	Pleosporaceae	*Alternaria*	*A. zinnae*	pathogen/herbicide against lettuce seedlings	brefeldin A; dehydrocurvularin	(Liu and Li, 2004)
			A. eichhornia	pathogen/herbicide		
			A. brassicae	pathogen/herbicide	homodestruxin B; destruxin B, destruxin B2; desmethyl destruxin B	
			A. destruens Product Smolder G, Smolder WP	pathogen/herbicide *A. destruens* is minimally toxic and nonpathogenic to mammals. Consequently, no cumulative effects from the residues of this product with other related microbial pesticides are anticipated (US EPA 2005a)	dehydroaltenusin	(Strasser et al., 2011)
			A. cassiae product: CASET	pathogen/herbicide against *Cassia obustifolia*		(Agropedia)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
			A. alternaria	Herbicide against *Datura innoxia* Grasses,broad-leaved weeds	Tenuazonic acid; Tentoxin	(Liu and Li, 2004)
			A. alternaria		Altenuic acid; Alternariol	List mycotoxins in appendix Working document
			A. alternata f. sp. maculosa	Herbicide against spotted knapweed	Maculosins	(Liu and Li, 2004)
			A. alternata f. sp. lycopersici	Herbicide against Jimsonweed, duckweed, and northern jointwetch	AAL toxin	(Liu and Li, 2004)
			A. solani, alternata, triticina, arborescens, cucumerina, dauci, kikuchiana	pathogen	AALs (*Alternaria alternata Lycopersici*)	
	Lophiostomataceae	*Ophiosphaerella*	*O. herpotrichia*	stub	destruxin	

1: "*Incertae sedis*" seems to be a collection of genera that were not assigned to an existing family, and not to a proper family

Class Eurotiomycetes

Table 8. Subclass Eurotiomycetidae (species in bold are known as pathogenic/herbicidal fungi; red = mycotoxin; orange = toxin, yellow = antibiotic).

Order	Family	Genus	Species	Type of action	Metabolites	Metabolites in common between families	Ref.
Eurotiales	Trichocomaceae	*Aspergillus*	*A. spec.*[1]	Pathogenic saprophyte producing mycotoxins	Aflatoxins (B1, B2, G1, G2), Xanthomegnin, Penitrem A, Patulin, Ochratoxin A (OTA),		(Oliveira et al., 2014)
			A. spec		Aflotoxin Alfatrem; Austdiol; Austamide; Austocystin; Brevianamide; Citrinin;		List mycotoxins in Section 8 of the Working document

Order	Family	Genus	Species	Type of action	Metabolites	Metabolites in common between families	Ref.
					Citreoviridin; Ctochalasin E; Cyclopiazonic acid; Destruxin B; Fumagilin; Gliotoxin; Malformin; Maltoryzine; Ochatoxin; Patulin; Sterigmatocystin; Tryptoquivaline; Verruculogen; Viomellein; Viriditoxin		
			flavus products: *A. flavus* AF36 *A. flavus* NRRL 21882	Nonaflatoxin-producing strains that outcompete the aflatoxin-producing wild strains	Ditryptophenaline Aflavinine; (antiinsectan activity) Seven indol derivates, e.g. 20-Hydroxyaflavinine, Aflazvazole; (antifeeding activity) Paspalinine and two more related compounds, e.g. Alfatrem; (tremorgenic in rodents) Parasiticol (cause biliary hyperplasia in organs) O-Methylsterigmatocystin Dihydro-O-methylsterigmatocystin (negligible effects in cell culture) Aspertoxin (in developing chicken embryo – 100% killed after injection of 2.0 µg/egg) Ergosta-4,6,8(14),22-tetraen-3-one 24-Ethylcholesta-4,6,8(14),22-tetraen-3-one Aspervalvin		{Strasser et al., 2011b}

Order	Family	Genus	Species	Type of action	Metabolites	Metabolites in common between families	Ref.
					Cyclopiazonic acid (potent inhibitor of Ca2+ activated ATPase)		
		Penicillium	*P. spec.*[1]		Xanthomegnin, Ochratoxin A (OTA), Citreoviridin, Citrinin (CTN), Cyclochlorotine, Cyclopiazonic acid, Patulin		(Oliveira et al., 2014)
		Byssochlamys	*B. nivea*		Patulin		(Oliveira et al., 2014)
		Emericella	*E. astellata, olivícola*		Aflatoxins (B1, B2, G1, G2)		(Oliveira et al., 2014)
Onygenales	Arthrodermataceae	*Microsporum*	*M. cookei*	Infections on skin	Xanthomegnin		(Oliveira et al., 2014)
		Trichophyton	*T. megninii, mentagrophytes, rubum, violaceum*	Infections on skin	Xanthomegnin		(Oliveira et al., 2014)

1: mycotoxins were produced in different combinations of cereal crops and species. The exact species can be found in the publication of Oliveira et al. (2014).

Class Leotiomycetes

Table 9. Order Helotiales.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Helotiales	Orbilaceae	*Duddingtonia*	*D. flagrans*	Nematicide in animals	Uncharacterised metabolites	(Butt et al., 2001)

Class Sordariomycetes

According to Molnar et al. (2010) the classification of the Hypocreales has been changed considerably. In Table 10 this new classification has only been adopted by assigning *Beauveria* to the Cordycipitaceae.

Table 10. Subclass Hypocreomycetidae (species in bold are known as pathogenic/herbicidal fungi; red = mycotoxin; orange = toxin, yellow = antibiotic).

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
Hypocreales	Clavicipitaceae	*Aschersonia*	*A. aleyrodes*	entomopathogen	Uncharacterised metabolites	
			A. spec.		Destruxin A1, A4, A5; Homodestruxin B	(Krasnoff et al., 1996)
		Metarhizium	*M. anisopliae*	entomopathogen	E diol; Cytochalasin C; Swainsonine; Destruxins A, A1, A2, A3, B, B1, B2, C2, D1, D2, E, E1, E2, F, Chlorohydrin; Desmethyl destruxins A, C, D Two analogues of fusarin C (i.e. 7-desmethyl and (8Z)-fusarin C) Serinocyclins A and B (cyclic heptapeptides)	(EFSA, 2013a) (Strasser et al., 2011)
			M. anisopliae F52 Products Taenure, Tich-Ex (G, EC) *M. anisopliae* ESF1 Product Bioblast *M. anisopliae* Bipesco Product GRANMET-P (GR), WP		Cytochalasin C (strong inhibitor of mammalian cellular functions) Zygosporin A (Teratogenicity effects in chicken, see also cytochalasin C) see above see above	(Strasser et al., 2011)
			M. flavoviride Product: Green Muscle	entomopathogen/locusts/grasshopper	Destruxins A, B, E E diol; cytochalasin C; swainsonine	
			M. robertsii	entomopathogen	NG-391, NG-393, 7-desmethyl analogues of fusarin C and (8Z)-fusarin C	(Donzelli et al., 2010), (Krasnoff et al., 2006)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
		Nomuraea	*N. rileyi*			
		Isaria	*I. spec*		Isaridin A, B, C1, C2, D, E, F1, F2, G1, G2	(Ravindra et al., 2004), (Sabareesh et al., 2007)
			I. fumosorosea (first described as *Paecilomyces fumosoroseus*[1])	insecticide, acaridide	Beauvericin Beauveriolides	(Luangsa-Ard et al., 2009)
			I. farinosa			
			I. tenuipes		Isariotin A, B, C, D Isariotin E, F Beauvericin	(Haritakun et al., 2007) (Bunyapaiboonsri et al., 2009) (Luangsa-Ard et al., 2009)
		Pochonia	*P. chlamydosporia*	nematicide	Aurovertins; Pochonins; antiviral and antiparasitic resorcylic acid lactones	(Manzanilla-Lopez et al., 2013)
		Claviceps	*C. purpurea*		Ergot alkaloids (ergolines)	
	Cordycipitaceae	*Beauveria*	*B. bassiana*	entomopathogen	Vivotoxins; Bassianin; Tenellin; Beauveriolides; Bassianolide; Beauvericin; Dipicolinic acid. Novel beauvericin derivate (beauvericins G1–3, beauvericins H1–3) cytotoxicity (haptotaxis).	(EFSA, 2013a),(EFSA, 2013a), (Molnár et al., 2010) (Xu et al., 2007)
			B. bassiana ATCC 74040 Product Troy, Boverin, Fermone, Naturalis L-225 *B. bassiana* GHA Product Mycotrol ES, Botanigard 22WP, Organigard ES		Isoleucylisoleucyl anhydride cyclo-(L-Isoleucyl-L-valine) cyclo-(L-Alanyl-L-proline)	(Strasser et al., 2011)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
			B. bassiana 447 "Baits motel stay a while-rest forever" *B. bassiana* HF23 *B. bassiana* HF23			
			B. brongniartii	entomopathogen	Bassianin; Tenellin; Oosporein; Beauvericin; Dipicolinic acid	(EFSA, 2013a), (EFSA, 2013a), (Molnár et al., 2010)
		Lecanicillium	*L. muscarium (formerly Verticillium lecanii)* Products: Mycotal, Vertalec	entomopathogen	Dipicolinic acid No metabolites mentioned 23,24,25,26,27-Pentanorlanost-8-ene-3beta,22-diol	(Molnár et al., 2010) (Strasser et al., 2011)
			L. longisporum	entomopathogen	Destruxin A, A2, B, B2, D, E, E2; Desmethyl Destruxin E	(Butt et al., 2009)
		Isaria	*I. farinosa,* *I. tenuipes,* *I. cicadae*		dipicolinic acid	(Molnár et al., 2010)
			I. fumosorosea	Nematicide Insecticide	beauverolides; Pyridine-2,6-dicarboxylic acid; beauvericin	(Molnár et al., 2010) (EFSA, 2013a)
			P. fumosorosea Apopka strain 97 or PFR 97 or CG 170, ATCC20874 Product PFR-97 (Preferal) *P. fumosorosea* Strain Fe 9901 Product NOFLY WP	Insecticide	Paecilospirone No relevant metabolites	(Strasser et al., 2011) (Strasser et al., 2011)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
	Hypocreaceae	*Gliocladium*	*G. catenulatum*	fungicide	**Gliotoxin;** Viridin; Viridian; Gliovirin; Glioprennins; Heptelidic acid.	
			G. catenulatum J1446 Product Primastop, Prestop		No relevant metabolites	(Strasser et al., 2011)
			G. spec. Strain FTD-0668		Argifin	Ref. in (Liu and Li, 2004)
			G. virens	fungicide	Gliotoxin; Gliovirin; Viridin, Dimethylgliotoxin, Viridiol	Ref. in (Liu and Li, 2004) (Lumsden et al., 1992b)
			G. virens GL- 21 Product WRC-AP-1	fungicide	Viridin Viridol	(Strasser et al., 2011)
		Trichoderma	*T. asperellum (*formerly *harzianum)*	fungicide, bactericide	Acid and neutral Trichotoxin Acetic acid	(EFSA, 2013a)
			T. asperellum T11, TV1, T25 Products TV1 and T11	fungicide	No relevant metabolites	(Strasser et al., 2011)
			T. gamsii (formerly viride) Product Remedier	fungicide	Trichodermin, (strong inhibitor of protein synthesis in mammalian cells, antifungal antibiotic) Viridin (strong antifungal activity) Viridol	(Strasser et al., 2011)
			T. harzianum	fungicide	6-pentyl-2H-pyran-2-one (6PP); 6-pentyl-alpha-pyrone (6PAP); Viridiofungin A; Peptaibiotics (Alamethicin) Trichostromaticins A,B,C,D,E; Trichothecene mycotoxins (Trichodermin A)	(EFSA, 2013a)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
			T. harzianum RIFAI ITEM 908 Product Tricover *T. harzianum* RIFAI T-339 Product: Trichodex	Fungicide, several modes of action including antibiosis using enzymes	Koninginin A and C, (inhibits growth of etiolated wheat coleoptiles at 10^{-3} M) See above	(Strasser et al., 2011)
			T. polysporum ATCC 20475 +(= IMI 206039)+ (*T. atroviride* ATCC 20476 (= IMI 206040) Product BinapProducts: Binab T WP, Binab TF WP	fungicide	Viridin, (strong antifungal activity) Viridol	(Strasser et al., 2011)
			*T. atroviride (*formerly *harzianum)*	fungicide	6-pentyl-2H-pyran-2-one (6PP); viridiofungin A; peptaibiotics (alamethicin); trichostromaticins A,B,C,D,E; trichothecene mycotoxins; trichodermin A	(EFSA, 2013a)
			*T. atroviride (*formerly *harzianum)* T-11 Product Binap T pellets component	Fungicide, several modes of action including antibiosis using enzymes	No relevant metabolites	(Strasser et al., 2011)
			T. viride	Bactericide	Trichodecenins-I and –II; Tichorovins; Tichocellins; Tricholin	Ref. in (Liu and Li, 2004)
			T. virens	Fungicide against *Rhioctonia solani*	Gliotoxin	Ref. in (Pal and McSpadden Gardener, 2006)
			T. brevicompactum		Trichodermin; Harzianum A	(Nielsen et al., 2005)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
			T. longibrachiatum		Longibrachin LGA and LGB	Ref in (Liu and Li, 2004)
		Acremonium	*A. diospyri*	herbicide		
			A. crotocinigenum		Crotocin mm	List mycotoxins in appendix Working document
	Incertae sedis	*Verticillium*	*V. albo-atrum* Strain WCS850 (formerly *Verticillium dahliae)*	Fungicide, Inducing an immune response in elm trees	No relevant metabolites	(Strasser et al., 2011b),
			lecanii	insecticide	Destruxins, Bassianolide, Cyclosporin, Aphidicholin, Verticillin	
		Verticillium	*V. chlamydosporium*	nematicide		(Butt et al., 2001)
			V. biguttatum	Fungicide against *Rhizoctonia solani*	Bigultol; Methylbigutol	Ref. in (Liu and Li, 2004)
		Myrothecium	*M. verrucaria* Strain AARC-02555 Product DiTera	nematicide	Not mentioned	(Butt et al., 2001)
		Myrothecium	*M. verrucaria*		Roridin E, Verrucarin	Section 8.2.1 in Working document
	Nectriaceae	*Fusarium*	*F. oxysporium*	pathogen/herbicide against Jimsonweed and duckweed	Fusaric acid	(Liu and Li, 2004, Butt et al., 2001)
			F. spec. [2]	pathogen	Avenacein Y, phomasins (B1, B2, B3), Fusaproliferin (FUS), Butenolide, Culmorin and derivates, Trichothecenes (Deoxynivalenol (DON)) and Derivatives, Diacetoxyscirpenol (DAS), Beauvericin; Naphthazarins (e.g. fusarubin and anhydrofusarubin), Fusaric acid, Enniatins (ENNs) (A, A1, B, B1) and cyclic peptides, Zearalenone (ZEA),	(Oliveira et al., 2014) See the list of mycotoxins in Section 8 of the Working document for more mycotoxins

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
					T-2 toxin and HT-2 toxin, Fusarenon-X (FUS-X), Moniliformin (MON), Nivalenol (NIV)	
	Bionectriaceae	*Clonostachys*	*C. rosea (formerly Gliocladium roseum*	fungicide	Gliotoxin; Glisoprenin C, D and E	Ref. in (Liu and Li, 2004)
	Ophiocordycipitaceae	*Tolypocladium*	*T. spec.*		Cyclosporin; Efrapeptins (C, D, E, F, G)	(Strasser et al., 2000b)
		Hirsutella	*H. thompsonii* Product: Mycar	insecticide	Hirsutellin A	(Mazet and Vey, 1995)
		Purpureocillium	*P. lilacinum*[3]	Herbicide nematicide	Paecilotoxin	(Khan et al., 2003)
	Chaetomiaceae	*Chaetomium*	*C. aureum*	pathogen	oosporein	(Taniguchi et al., 1984)
	Halosphaeriaceae	*Halosarpheia*	*H. spec.*		Enniatins (ENNs) (A, A1, B, B1) and cyclic peptides	(Oliveira et al., 2014)

1: read (Zimmermann, 2008)
2: mycotoxins were produced in different combinations of cereal crops and species. The exact species can be found in the publication of Oliveira et al. (2014).
3: formerly *Paecilomyces lilacinus*

Table 11. Subclass Sordariomycetidae.

Order	Family	Genus	Species	Type of action	Metabolites	Ref
Diaporthales	Cryphonectriaceae	*Cryphonectria*	*E. parasitica*	fungicide/formerly *Endiothia parasitica* (chestnut blight)		(Butt et al., 2001)
Glomerellales	Glomerellaceae	*Colletotrichum*	*C. orbiculare*	herbicide		
			C. spec.	herbicide	colletotrichin	
			C. gloeosporioides f. sp. aeschynomene Products: Collego, LUBOA2 (China), Biomal	Herbicide against *Aeschynomene sp.,* *Cuscuta sp.,* *Malva f. sp. malvae pusilla*	No relevant metabolites Tetraol (-)-1 Cis-4-hydroxy-6-deoxyscytalone and (4R)-4,8-dihydroxy-a-tetralone	(Strasser et al., 2011) (Femenia-Rios et al., 2006) (Agropedia)

Table 12. Subclass Xylariomycetidae.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref
Xylariales	Xylariaceae	*Muscodor*	*M. albus* Products Arabesque, Andante, Glissade	Inhibit and kill plant pathogenic and other organisms	A number of volatiles, mainly alcohols, acids, and esters	Strasser et al., 2011b)

Phylum Basidiomycota
Class Agaricomycetes

Table 13. Class Agaricomycetes.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Polyporales	Merluiaceae	*Chondrostereum*	*C. purpureum* HQ1 Product Aspire *C. purpureum* strain O Product Bionext	herbicide	No relevant metabolites See above	(Strasser et al., 2011b)
	Phanerochaetaceae	*Phlebiopsis*	*P. gigantea* Products ROTSTOP, ROTEX	fungicide	No relevant metabolites	(Butt et al., 2001) (Strasser et al., 2011b)
	Polyporaceae	*Polyporus = Laetiporus*	*P. sulphureus*	saprophyte	Beauvericin	(Deol et al., 1978)

Table 14. Class Urediniomycetes.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Uredinales	Pucciniaceae	*Puccinia*	*P. monoica* Product Woad Warrior	Herbicide, acting by competition	No relevant metabolites	(Strasser et al., 2011)

Class Tremmellomycetes

Table 15. Class Tremellomycetes.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Tremaliales	Tremellaceae	*Cryptococcus*	*C. albidus*	fungicide/ (against *Botrytis/ rhiz)*		(Butt et al., 2001)

Class Ustilaginomycetes

Table 16. Class Ustilaginomycetes.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Ustilaginales	Ustilagniaceae	*Pseudozyma*	*P. flocculosa*	fungicide/ antagonist	cis-9-Heptadecenoic acid (CHDA); flocculosin	(EFSA, 2013a), (Mimee et al., 2005)
			P. flocculosa PF-A22 Product Sporodex L	Acting by fatty acid metabolism	Three fungitoxic unsaturated C-17 fatty acids: -(9-heptadecenoic acid, 6-methyl-9-heptadecenoic acid and 4-methyl-7,11-heptadecadienoic acid) -fungitoxins -acyclic norterpene (2, 6, 10, 14,18-pentamethyl-2, 6, 8, 10, 12, 14,17-nonadecaheptene-1,19-diol)	(Strasser et al., 2011)

Phylum Zygomycota

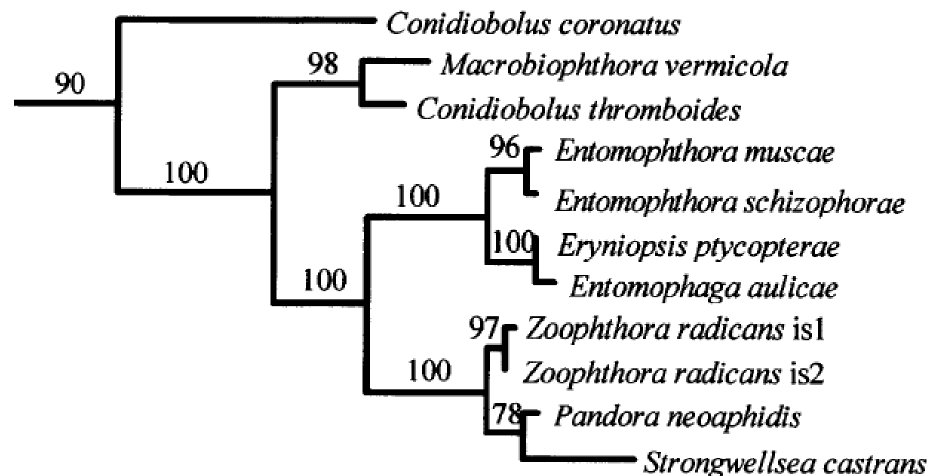

Figure 6. Phylogenetic relationships within Entomophthorales inferred from parsimony analysis of 1581 nucleotides of nuclear SSU rDNA. Bootstrap percentages over 50% from 200 replicates are shown above each supported branch (Jensen et al., 1998).

Class Entomophthoromycotina

Table 17. Phylum Incertae sedis, class Entomophthoromycotina.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Entomophthorales	Ancylistaceae	*Conidiobolus*	*C. thromboides*	insectide		
			C. coronatus	insectide	coronatin-1	(Wieloch et al., 2011)
		Entomophthora	*E. muscae*			

Phylum Microsporidia
Class Dihaplophasea

Table 18. phylum Microsporidia, class Dihaplophasea.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Dissociodehaplophasida	Nosematidae	*Nosema*	*N. locustae* Product: NoLo Bait	Insecticide against Acrididae	Not known to be produced	(BCPC, 2014)

Kingdom Chromalveolata (or Strameopila)
Phylum Heterokontophyta
Class Oomycota

Table 19. phylum Heterokontophyta, class Oomycota.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Lagenidiales	Lagenidiaceae	*Lagenidium*	*L. giganteum* Product: Laginex	insecticide	undetermined	(Vyas et al., 2006)
Pythiales	Phytiaceae	*Phytphthora*	*P. palmivora* Product: Devine	Herbicide against *Malva f. sp. malvae pusilla* and *Morrenia odorata* (milk weed vine/strangler vine)		(Agropedia)
		Pythium	*P. oligandrum* Strain DV74 Product DV 74	Fungicide acting by mycoparasitism, hyphal interactions, antibiosis, enhancement plant resistance, competion	No relevant metabolites	(Strasser et al., 2011)

Kingdom Bacteria
In the figure below the phylogenetic tree of bacteria is detailed.

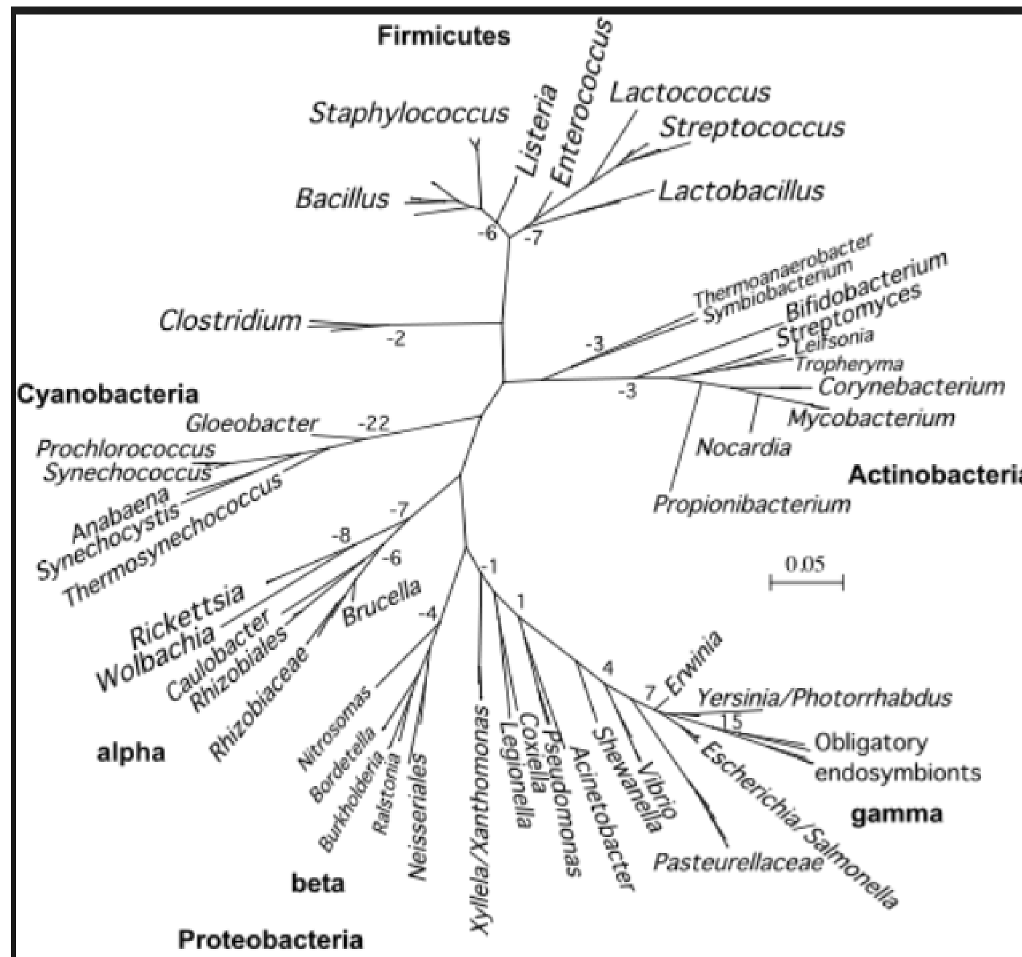

Figure 7. Unrooted phylogenetic tree with the relative stability of ancestral states (multiplied by 100), inferred with Compare (Martins and Hansen, 1997), using the linear model (corresponding to brownian evolution). http://wwwabi.snv.jussieu.fr/erocha/research/ordervsdisorder.html.

Phylum Proteobacteria
Class Alphaproteobacteria

Table 20. phylum Proteobacteria, class Alpha Proteobacteria.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Rhizobiales	Rhizobiaceae	*Agrobacterium*	*A. tumefaciens*	suppression competitive bacteria and fungi within the plant rhizosphere	Surfactin, Bacillomycin D, Fengycin, Putative peptide, Bacillibactin, Bacilysin/anticapsin, Macrolactin, Bacillaene, Difficidin, Oxydifficidin	(Chen et al., 2008)
		Agrobacterium	*A. radiobacter* Strain K-84	Active against *Agrobacterium tumefaciens,* the causal agent of crown gall disease	Agrocin 84	Ref. in (Pal and McSpadden Gardener, 2006)

Class Betaproteobacteria

Table 21. phylum Proteobacteria, class Betaproteobacteria.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Neisseriales	Neisseriaceae	*Chromobacterium*	*C. subtsugae*	Insecticide against foliar-feeding insect pests, acting as a stomach poison	Violacein, deoxyviolacein	US Patent Application Pubblication, 2012/0100236 A1.
Burkholderiales	Burkhoderiaceae	*Burkholderia*	*B. cepacia*	Fungicide against *R. solani* and *Pyricularia oryzae* causing damping off and rice blast	Pyrrolnitrin, Pseudane	Ref. in (Pal and McSpadden Gardener, 2006)
		Burkholderia	*B. cepacia*	Seed treatment in which seeds of peas, maize, soybeans and other crops.		(Wozniak),

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
			Product: Deny, Blue Circle, Intercept	After reevaluation registration was cancelled in the USA in 2000[3]		(Parke and Gurian-Sherman, 2001)
		Burkholderia[3]	*B. cepacia sp.*	Pathogenic to human, animals, and plants, as well as species involved in promoting plant growth and biodegradation of pollutants extracellular enzymes[1], siderophores[2], toxins, antimicrobials, and other SMs produced by the members of this very diverse genus	Antifungals: Pyrrolnitrin, Xylocandin Complex (cepacidines), Quinoline Derivatives (or pseudanes), Glidobactins, CF661, 2-Hydroxymethyl-chroman-4-one, Altericidins, Cepacins A and B, Cepaciamides A and B, Phenylpyrroles, Phenazine Toxins: Toxoflavin, tropolone, Rhizobitoxine, Rhizoxin, Bongkrek acid, Rhizonin	(Vial et al., 2007)
			*B.*spp.	Insecticidal and miticidal activities	Toxoflavin, Fervenulin, Rhizobitoxin, Rhizoxin, Bonkrekic acid, Rhizonins A and B, tropolone	(Cordova-Kreylos et al., 2013)

1: these were not considered to be SMs and were not included in the column at the right.
2: siderophore: Iron is one of the most abundant element on earth and one of the most important nutrients of bacteria. However, in the presence of oxygen and at neutral pH, Fe2+ is rapidly oxidized to Fe3+, which is not readily available to bacteria. Bacteria have developed ways to scavenge iron with high affinity by producing siderophores, low-molecular-weight chelating molecules that sequester iron from other iron containing molecules present in the surroundings. Members of the *Burkholderia* genus produce many kinds of siderophores which, depending on the chemical structure of their chelating group, are mostly classified into hydroxamates (based on hydroxamic acid) and catecholates (containing a catechol ring) (Vial et al., 2007).
3: As it is not yet possible to determine precisely which members of the *B. cepacia* complex present a risk to patients who have cystic fibrosis, the medical community has raised objections to its agricultural use; as a result, a moratorium has been placed on its development for foliar spraying until more is known about the specific bacterial factors that predict risk to vulnerable human hosts.

Class Gammaproteobacteria

Table 22. phylum Proteobacteria, class Gammaproteobacteria.

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*P. chlororaphis* Strain PA23		Pyrrolnitrin, Phenazine, HCN, proteases, lipases and siderophores	(Selin et al., 2012)
			P. fluorescens F113	Fungicide against *Pythium spp.*	2, 4-diacetylphloroglucinol (2,4-DAPG)	Ref. in (Pal and McSpadden Gardener, 2006)
			P. spec.	Antibiosis, Iron competition, Induced resistance, Root colonization, nutrient competition, inactivation of pathogenicity and germination factors (Keel and Défago, 1997)	Phenazines[3], Pyroles[4], Pterines[5], Siderophores[6], Miscellaneous group[7] 2, 4-diacetylphloroglucinol (2,4-DAPG), Pyoluteorin, Oomycin A, Agrocin 84, Hydrogen cyanide Pseudobactin B10, 2,3,de-epoxy-2,3-didehydro-rhizoxin, 2,5-dialkylresorcinol, Quinolones, Gluconic acid, Rhamnolipids, Various lipopeptides[8], rhizoxins	(Dowling and O'Gara, 1994) (Ligon et al., 2000) (Raaijmakers and Mazzola, 2012)
			P. syringae Strain ESC 10 and 11	Probably acting by outcompetition of the wild plant pathogenic strain.	Syringomycin E	(Bull et al., 1998)
			S. entomophila	Insecticide against Coleoptera		
Enterobacteriales	Enterobacteriaceae	*Serratia*[1]	*S. entomophila* Product: Invade	Sep proteins which are related to the Tc toxins produced by *Photorhabdus* and other bacteria		Jackson, Rebeca
			S. marcescens	Insecticide against Coleoptera, Hymenoptera, Lepidoptera and Diptera. Activity due to immune	No metabolites in insect pathogenic species reported	(Petersen and Tisa, 2013)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
				suppresive properties, invasive capabilities, exoenzymatic activities. Lecithinase, proteinase, and chitinase are reported (Grimont and Grimont, 2006)		
			S. marcescens	Pathogenic against fungi and bacteria	Prodiogiosin[2]	(Rahul et al., 2014)
			P. agglomerans Strain C9-1	Bactericide against *Erwinia amylovora* causing fire blight	Herbicolin	Ref. in (Pal and McSpadden Gardener, 2006)
		Pantoea	*E. herbicola* B247	Suppression of *Fusarium culmorum* and *Puccinia recondita* f.sp. *tritici* on wheat	Herbicolin A	Ref, in (Raaijmakers and Mazzola, 2012)
		Erwinia	*Lysobacter sp.* Strain SB-K88 No products	Fungicide against *Aphanomyces cochlioides* causing damping off	Xanthobaccin A	Ref. in (Pal and McSpadden Gardener, 2006)
Xanthomonadales	Xanthomonadaceae	*Lysobacter*				

1: The genus *Serratia* is very diverse with insect pathogenic species, plant-growth promoting species and bacterial antagonists.
2: Prodiginines share a common pyrrolyldipyrromethene core structure which can also be isolated from, *Vibrio psychroerythrous, Alteromonas rubra, Pseudomonas magneslorubra, S. rubidaea*, Actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus ruber* (Rahul et al., 2014). In the genus *Serratia*, prodigiosin is only produced by strains of *S.marcescens, S. plymuthica, and S. rubidaea*. In *S. marcescens*, prodigiosin is produced by biogroups A1 and A2/6 and never by biogroups A3, A4, A5/8, or TCT (unpigmented groups).
3: Phenazine-1-carboxylic acid, Pyovanine, Hemipyocanine, Idoinin, Chlororaphin, Oxychlororaphin, Aeruginosin A + B.
4: Pyroles, Pyrrolnitrin, Phenylpyrolles, Isopyrrolnitrin, Aminopyrrolnitrin, pyluteorin.
5: Pterine, Aminopterine, Ribilyllumazine, Putidolumazine.
6: Pyochelin, Pyoverdine, Pseudobactin- B10, M114, A214, 7SR1, A112, B117, Ferribactin, Phytosiderophores, Ferrichrome, Ferroxamine B.
7: Cuanhydric acid, Aeruginoic acid, Magnesidin, Pseudomonic acid, Antibiotic P2563, Amino-2 acetophenone, c-Acetyl phloroglucinols, Antibiotic DB-2073, Fluopsin C+F, Sorbistin A1+ B, Salicylic acid.
8: Lipopetides: orfamide, viscosin, syringafactin, entolysin

Phylum Actinobacteria

Class Actinobacteria (gram positive)

Table 23. phylum Actinobacteria class Actinobacteria (= Actinomycetes).

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Actionomycetales	Microbacteriaceae	*Clavibacter*	*C. michiganensis*	Bactericide against foliar diseases	Not mentioned	(BCPC, 2014)
	Streptomycetaceae	*Streptomyces*	*S. griseoviridis* Strain IFO 13350		Streptomycin, Grixazone,	(Ohnishi et al., 2008)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
					Isorenieratene, HPQ melanin, Alkylresorcinol, 2-methylisoborneol Many more	(Tarkka and Hampp, 2008)
			S. coelicolor		Actinorhodin, Methylenomycin, Prodiginines, CDA, Coelichelin, Coelibactin, Tetrahydroxynaphtalene, Geosmin, Hopanoids, Isorenieratine, Desferrioxamines	(Challis and Hopwood, 2003)
			S. clavuligerus, jumonjinensis, katsurahamanus		Clavulanic acid and cephamycin C (coproduced in these species)	(Challis and Hopwood, 2003)
			S. aureofaciens	antagonist of *Colletotrichum musae* and *Fusarium oxysporum*	5,7-dimethoxy-4-p-methoxylphenylcoumarin, 5,7-dimethoxy-4-phenylcoumarin	(Taechowisan et al., 2005)
			S. acidiscabies	herbicidal	Thaxtomin A	EPA registrered

Phylum Firmicutes

Class Bacilli

Table 24. phylum Firmicutes , class Bacilli (gram positive).

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
Bacillales	Bacillaceae	*Bacillus*[1]	*B. amyloliquefaciens* Strain FZB42	Stimulation plant growth and suppression plant pathogenic organisms. Fungicidal action against *Fusarium oxysporum*	Surfactin Bacillomycin D, Fengycin, Bacillibactin, Bacilysin, Macrolactin Bacillaene,	(Chen et al., 2008)

Order	Family	Genus	Species	Type of action	Secondary metabolites	Ref.
					Difficidin Oxydifficidin	
			B. subtilis Strain AU195	Fungicidal action against *Aspergillus flavus*	Bacillomycin D	Ref. in (Pal and McSpadden Gardener, 2006)
			B. licheniformis Strain ATCC 14580		Bacitracin, Lichenicidin	(Rey et al., 2004)
			B. pumilus	Pumilacidin, a lipopeptide, has antimicrobial, antiviral and antiulcer activity	Pumilacidin A, B, C, D, E Datagap for metabolites in EU	(Melo et al., 2009)
			Bacillus spec	Effects on plants	Indole-acetic acid, 2,3-butanediol	Ref. in (Raaijmakers and Mazzola, 2012)

1: Within the genus *Bacillus*, the lipopeptides iturin, surfactin, fengycin and the polyketide antibiotics bacillaene, difficidin and macrolactin have received most attention (Raaijmakers and Mazzola, 2012).

1.4.1 Quantities of secondary metabolites produced by some mBCA species

1.4.1.1 Quantities of secondary metabolites produced by some entomopathogens (Ascomycota)

Following paragraphs present a snapshot of the information that is available in the literature. Nevertheless, they give an impression of the variety and quantities of SMs produced. Some SMs stand out against others, irrespective of the type of media and the particular culture conditions.

1.4.1.1.1 Metarhizium anisopliae

Metarhizium anisopliae (note that there have been recent taxonomic changes for this species) is well known for production of some SMs and there has been extensive research to try and identify all SMs. The review of Liu and Tzeng (2012) lists 39 destruxins that have been identified. The tables below categorizes the SMs according to the quantities produced in submerged fermentation (Table 25) and in their hosts (Table 26).

Table 25. Quantities produced in submerged fermentation of strains of *Metarhizium anisopliae* (mg/L) (D= destruxin).

Strain		100-1000	10-100	1-10	0.1-1	Nr. observations	Ref.
several	DA		42 ± 63			14	(Amiri-Besheli et al., 2000), (Moon et al., 2008), (Seger et al., 2006), (Wang et al., 2009), (Liu et al., 2007), (Hsiao and Ko, 2001), (Loutelier et al., 1996), (Wang et al., 2004), (Wang et al., 2003), (Wahlman and Davidson, 1993), (Seger et al., 2004), (Chen et al., 1999), (Liu et al., 2000), (Païs et al., 1981)
	DA2		14 ± 19			3	(Loutelier et al., 1996), [12], [16]
V275	DA3				0.7	1	[12]
V275	Desmethyl destruxin A			2		1	(Wahlman and Davidson, 1993)

Strain		100-1000	10-100	1-10	0.1-1	Nr. observations	Ref.
ARSEF1095	Dihydro DA			5		1	(Jegorov et al., 1992)
several	DB		60 ± 123			24	(Amiri-Besheli et al., 2000), (Moon et al., 2008), (Seger et al., 2006), (Wang et al., 2009), (Liu et al., 2007), (Hsiao and Ko, 2001), (Loutelier et al., 1996), (Wang et al., 2004), (Wang et al., 2003), (Wahlman and Davidson, 1993), (Seger et al., 2004), (Chen et al., 1999), (Liu et al., 2000), (Païs et al., 1981)
V275, Ma23	DB2			6 ± 9		3	(Loutelier et al., 1996), [12], [16]
V275	Desmethyl destruxin B			3.4 ± 3.4		4	(Wahlman and Davidson, 1993), (Hsiao and Ko, 2001), (Païs et al., 1981)
V275	DC			3.1 ± 3.3		2	[12], [16]
V275	Desmethyl destruxin C			1.9		4	(Wahlman and Davidson, 1993)
Ma23	DD		44 ± 62			1	(Loutelier et al., 1996), [16]
several	DE	97 ± 146				22	(Amiri-Besheli et al., 2000), (Seger et al., 2006), (Wang et al., 2009), (Hsiao and Ko, 2001), (Loutelier et al., 1996), (Wang et al., 2004), (Wang et al., 2003), (Wahlman and Davidson, 1993), (Seger et al., 2004), (Païs et al., 1981)
Ma23, V275	DE diol		40 ± 26			2	(Loutelier et al., 1996), (Wahlman and Davidson, 1993)
V275	DE chlorohydrin			3.8		1	(Wahlman and Davidson, 1993)
ARSEF1095	DE diol1			1		1	(Jegorov et al., 1998)
V275	DF			1.4		1	(Wahlman and Davidson, 1993)
FKL-1079	Fungerin			4.3		1	(Uchida et al., 2005)
FKL-1079	Hydroxy fungerin A				0.2	1	(Uchida et al., 2005)
FKL-1079	Hydroxy fungerin B				0.13	1	(Uchida et al., 2005)
IMI152487	Swainsonine		51 ± 30			6	(Patrick et al., 1996), (Tamerler and Keshavarz, 1999), (Tamerler et al., 1998), (Tamerler-Yildir et al., 1997, Patrick et al., 1993)
HF260	Aurovertin D		53			1	(Azumi et al., 2008)
HF260	Aurovertin F			3.5		1	(Azumi et al., 2008)
HF260	Aurovertin G		15			1	(Azumi et al., 2008)
HF260	Aurovertin H			3.5		1	(Azumi et al., 2008)
-	Cytochalasin		21.5			1	(Fujii et al., 2000)
-	Deacetylcytochalasin C	565				1	(Fujii et al., 2000)
-	Zygosporin D		61.5			1	(Fujii et al., 2000)

The collated data from Table 25 clearly show that *M. anisopliae* produces mainly destruxin B and E in quantities of 60 and 97 mg/L fermentation broth, respectively (22-24 studies). Destruxin A is produced in mean quantities of 42 mg/L, in 14 studies. The standard deviations are, however, very high. This can be explained by the fact that the conditions under which experiments have been performed differed considerably. Destruxin A was detected in 14 experiments while in only a few of these experiments other metabolites such as destruxin C and F were detected. This is a strong indication that destruxin B and E are regular metabolites that are always formed at higher quantities while others may only be formed under particular conditions.

Deacetylcytochalasin C, for example, was produced at a very high quantity but this has only been observed in one experiment. Zygosporin, Swainsonine, Aurovertin D, Destruxin E Diol are also produced in quantities between 40 and 61 mg/L, but only in 1 to 6 studies.

Therefore, it can be concluded that these collated data inform about the potential of production of (major) metabolites but do not reflect the metabolites profile of the MPCAs.Very few studies are available that measured the quantities of SMs produced in the host. In Table 26 the mean quantities of destruxins A, B and E range between 0.7 and 1.3 µg/insect. It is however difficult to relate these quantities with quantities produced in growth media.

Table 26. Quantities of destruxins produced by strains of *Metarhizium anisopliae* in hosts (µg/insect).

Strain		100-1000	10-100	1-10	0.1-1	Nr. observations	Ref.
ARSEF 1092/297 Ma23, Me1, V245/304/275	A			1.3 ± 2.1		8	(Wang et al., 2003), (Amiri-Besheli et al., 2000), (Skrobek et al., 2008)
ARSEF 1092/297 Ma23, Me1, V245/304	B			1.2 ± 2.2		9	(Wang et al., 2003), (Amiri-Besheli et al., 2000), (Skrobek et al., 2008)
ARSEF 1092/297 Ma23, Me1, V245/304/275	E				0.7 ± 1.7	9	(Wang et al., 2003), (Amiri-Besheli et al., 2000), (Skrobek et al., 2008)

1.4.1.1.2 Beauveria spp.

Table 27. Quantities of secondary metabolites produced by *Beauveria* species.

Species	Strain	Metabolite		100-1000	10-100	1-10	0.1-1	<0.1	Ref.
bassiana	N.D.	oosporein	mg/L	200					(Vining, 1962)
tenella	Hl522	oosporein	mg/L	120-810[7]	26-58[7]				(El Basyouni et al., 1968)
brongniartii	IMBSt 95041	oosporein	mg/L	270					(Strasser et al., 2011b)[1]
brongniartii	IMBSt 95041	oosporein	mg/L	524.9					(Michelitsch et al., 2004)[2]
brongniartii	IMBST 95041	oosporein	mg/kg grain			3.27			(Strasser et al., 2011b)
brongniartii	IMBST 95041	tenellin	mg/L and mg/kg grain					0	(Strasser et al., 2011b)
bassiana		tenellin	mg/L	308[8]					(Leete and Kowanko, 1975)
brongniartii	IMBST 95041	bassianin	mg/L and					0	(Strasser et al., 2011b)

Species	Strain	Metabolite		100-1000	10-100	1-10	0.1-1	<0.1	Ref.
			mg/kg grain						
brongniartii		bassianin	mg/L	148					(El Basyouni et al., 1968)
brongniartii	IMBST 95041	beauvericin	mg/L and mg/kg grain					0	(Strasser et al., 2011b)
bassiana		beauvericin	mg/L					0.00057[3]	(Safavi, 2013)
bassiana		beauvericin	mg/L					0.00618[4]	(Safavi, 2013)
bassiana		beauvericin	mg/L					0.00136[5]	(Safavi, 2013)
bassiana		beauvericin	mg/L					0.00001[6]	(Safavi, 2013)

1: HPLC
2: Differential pulse polarographic measurements.
3: culture filtrate from Potato Dextrose Broth (PDB) substrate
4: mycelia from PDB
5: *In vivo* conidial extracts (conidia from sporulated cadavers)
6: *In vitro* conidial extracts (conidia from artificial medium)
7: quantities depend on supplements
8: phenylalanine was added as the precursor

Table 28. Quantities of secondary metabolites produced by strains of *Beauveria brongniartii* in hosts (µg/insect).

Strain	Metabolite	100-1000	10-100	1-10	0.1-1	Nr. observations	Ref.
W 4574	oosporein				0.23	1	(Strasser et al., 2011b)
W 4574	beauvericin				0	1	(Strasser et al., 2011b)
W 4574	bassianin				0	1	(Strasser et al., 2011b)
W 4574	tenellin				0	1	(Strasser et al., 2011b)

1.4.1.1.3 Isaria spp. (formerly Paecilomyces)

Table 29. Quantities of metabolites produced by different *Paecilomyces* species.

Metabolite	number	Species	1000-10000	100-1000	10-100	1-10	0-1	mg/kg mg/L	Ref.
Beauvericin	B	*tenuipes*				1.2		mg/L	(Nilanonta et al., 2002)
		fumosoroseus					0	mg/L	(Asaff et al., 2005)
						1.6		mg/kg	(Bernandini et al., 1975)
Beauverolide		*fumosoroseus*					0	mg/L	(Asaff et al., 2005)
Cyclosporin	A	*farinosus*					0	mg/kg	(Jegorov et al., 1990)
		fumosoroseus					0	mg/kg	(Jegorov et al., 1990)

Metabolite	number	Species	1000-10000	100-1000	10-100	1-10	0-1	mg/kg mg/L	Ref.
Dipicolinic acid		*fumosoroseus*			41 ± 41			mg/L	(Asaff et al., 2006)
	B	*farinosus*			15.7			mg/L	(Lang et al., 2005b)
Militarinone	A	*militaris*					0.00068	mg/L	(Schmidt et al., 2002)
Oxalic acid		*fumosoroseus*	1992 ± 114					mg/L	(Asaff et al., 2006)
Paecilomycine	A	*tenuipes*		663				mg/kg	(Kikuchi et al., 2004b)
	B	*tenuipes*			21.4			mg/kg	(Kikuchi et al., 2004b)
	C	*tenuipes*			54.1			mg/kg	(Kikuchi et al., 2004b)
Paeciloquinone	A	*carneus*					0	mg/L	(Petersen et al., 1995)
				950				mg/L	(Petersen et al., 1995)
	B	*carneus*	8400					mg/L	(Petersen et al., 1995)
			8750					mg/L	(Petersen et al., 1995)
	C	*carneus*					0	mg/L	(Petersen et al., 1995)
					54			mg/L	(Petersen et al., 1995)
	D	*carneus*					0	mg/L	(Petersen et al., 1995)
				740				mg/L	(Petersen et al., 1995)
	E	*carneus*					0	mg/L	(Petersen et al., 1995)
			1140					mg/L	(Petersen et al., 1995)
	F	*carneus*					0	mg/L	(Petersen et al., 1995)
				530				mg/L	(Petersen et al., 1995)
Paecilosetin		*farinosus*			36.6			mg/L	(Lang et al., 2005b)
Paecilotoxin		*lilacinus*[1]					0	mg/L	(Khan et al., 2003)
Versiconol		*carneus*					0	mg/L	(Petersen et al., 1995)
			1490					mg/L	(Petersen et al., 1995)

1. Current name is *Purpureocillium lilacinum*. The generic name refers to the purple conidia produced by the fungus

Table 29 shows that *Paecilomyces spp.* produce many SMs. Largest quantities of more than 1000 mg/L are produced in the liquid media for oxalic acid, paeciloquinone and versiconol.

1.4.1.1.4 Lecanicillium muscarium

Lecanicillium muscarium strain Ve6 was included in Annex I to Directive 91/414/EEC on 1 May 2009. *L. muscarium* produces 23,24,25,26,27-Pentanorlanost-8-ene-3beta,22-diol (Strasser et al., 2011).

Extracts from laboratory-scale cultures of strain Ve6 grown under liquid conditions contained destruxins A, B and E. According to the EFSA conclusions (EFSA Journal 2010; 8(1): 1446) *L. muscarium* is not competitive in the environment unless the host species are present, it is not pathogenic to humans, nor toxic or infective.

1.4.1.2 Quantities of secondary metabolites produced by fungi other than entomopathogens

1.4.1.2.1 Gliocladium virens

Gliocladium virens produced detectable levels of gliotoxin when grown with an alginate-wheat bran food base system in peat moss-vermiculite, soil-less medium (PV medium) (Lumsden et al., 1992a).

Gliotoxin was not detectable, and biocontrol effectiveness was greatly reduced when the fungus was grown in a medium containing bark ash (charcoal). Gliotoxin was detected in several media, all with natural microbiota but amended with 0.1% alginate prill containing *G. virens* (w/v).

- PV medium (0.42 µg/cm^3),
- composted mineral soil (0.36 µg /cm^3),
- clay soil (0.20 µg /cm^3),
- sandy soil (0.02 µg /cm^3)

At a rate 0.4% amendment, the amount of gliotoxin detected was quadrupled when compared to the 0.1% rate. Gliotoxin was detected 4-5 cm away from the point source of *G. virens* growing from a single bran-alginate prill in PV medium and appeared to be associated with the mycelium of the advancing margins of the colony.

1.4.1.3 Quantities of secondary metabolites produced by bacterial strains

Table 30 shows that the production of antibiotics by introduced bacterial strains in plant-associated environments vary from 5 ng to 1000 µg per gram of soil or plant tissue (Raaijmakers and Mazzola, 2012).

Table 30. In situ antibiotic production by introduced bacteria [modified from Raaijmakers and Mazzola (2012) and references therein].

Antibiotic	Introduced species/strain	Habitat	DAI[1]	Bacterial density (CFU g^{-1})	Amount detected (µg g^{-1})
Pyrrolnitrin	*Burkholderia cepia* B37w	Potato tubers	7	9.5×10^7	$\geq 4.9 \times 10^{-3}$
2,4-DAPG[2]	*Pseudomonas fluorescens* CHA0	Wheat roots	21	$1.1\text{-}1.2 \times 10^8$	0.94-1.36
	P. fluorescens Q2-87	Wheat roots	11	$2.9\text{-}6.4 \times 10^7$	0.47-2.10

	P. fluorescens Q8rl-96	Wheat roots	21	4.2 x 10^{7}	0.25
	P. fluorescens Q8rl-96	Wheat roots	4	1.0-2.0 x 10^{9}	1.65-2.77
Pyluteorin	*P. fluorescens*	Wheat roots	21	6 x 10^{8}	0.03-0.05
PCA[3]	*P. fluorescens* CHA0	Wheat roots	18	1.6-14.3 x 10^{7}	0.04-0.37
	Pseudomonas chlororaphis 30-84	Wheat roots	18	2.9-4.6 x 10^{8}	0.13-0.58
	P. fluorescens 2-79	Wheat roots	35	1.8-4.1 x 10^{7}	0.16-0.17
Herbicolin A	*Erwinia herbicola B247*	Wheat roots	4	1.1-4.6 x 10^{8}	0.8-1.2
Viscosinamide	*P. fluorescens* DR54	Sugar beet seeds	2	9.5 x 10^{7} (seed^{-1})	0.65 (seed^{-1})
Tensin	*P. fluorescens* 96.578	Sugar beet seeds	2	9.5 x 10^{7} (seed^{-1})	0.29 (seed^{-1})
Amphisin	*P. fluorescens DSS73*	Sugar beet seeds	2	9.5 x 10^{7} (seed^{-1})	0.36 (seed^{-1})
Iturin	*Bacillus subtilis* RB14-C	Soil	14	1.3 x 10^{8}	0.49
	B. subtilis QST713	Cucumber roots	15	7.1 x 10^{6}	7
	B. subtilis QST713	Cucumber roots	29	1 x 10^{6}	1000 (view Figure 6)
	B. subtilis QST713	Cucumber roots	43	2.0 x 10^{5}	180
Surfactin	*Bacillus subtilis* RB14-C	Soil	14	1.3 x 10^{8}	4.36
	B. subtilis QST713	Cucumber roots	15	7.1 x 10^{6}	9
	B. subtilis QST713	Cucumber roots	29	1 x 10^{6}	60
	B. subtilis QST713	Cucumber roots	43	2.0 x 10^{5}	30
Fengycins	*B. subtilis* GA1	Apple	5	9.5 x 10^{7}	1.5

1: days after introuction of the bacterial strain
2: 2,4-diacetylphloroglucinol
3: Phenzine-1-carboylic acid

Figure 8 shows the iturin production by *Bacillus subtilis* in potting media (Kinsella et al., 2009).

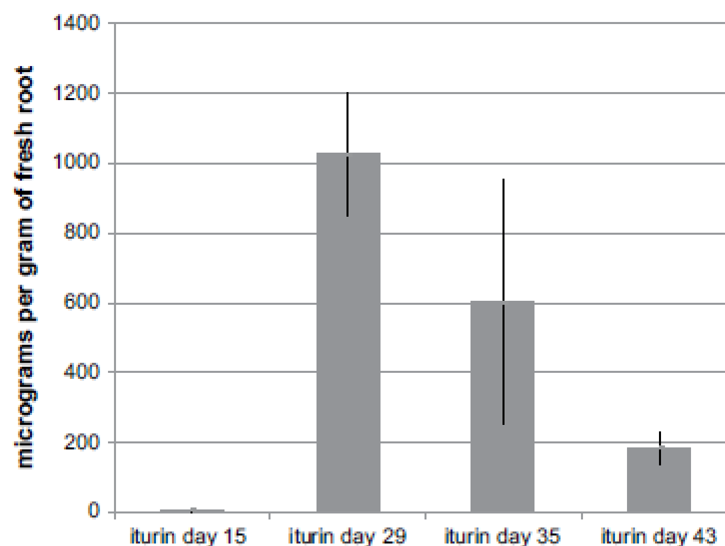

Figure 8. Iturin A concentration in rhizosphere extracts of 15–43 d old cucumber roots grown in *Bacillus subtilis*-inoculated Metro-Mix 360 potting media. Error bars represent ± one standard deviation for n = 28 (Day 15), 5 (Days 29 and 35) and 13 (Day 43).

1.4.2 Plant pathogenic fungi producing the same secondary metabolites

According to Frisvad et al. (2008) "It is not unusual that different fungal species have one or more secondary metabolites in common. Cytochalasin D is one such metabolites". Many SMs are produced by phylogenetically widely different fungi (Cole and Schweikert, 2003). Phylogenetically unrelated species may have arrived at the same SMs independently because of the selective advantage of these bioactive compounds.

Other, naturally occurring fungi could occupy the same environment and produce metabolites that they have in common with biocontrol agents at even higher quantities. Table 31 shows that *Chaetomium aureum* is able to produce quantities of oosporein in liquid media up to 5 times higher than *Beauveria* species. Likewise, *Fusarium* species produce beauvericin in quantities 450 to 2000 times higher than *Paecilomyces fumosoroseus.* Other fungal species could occupy the same environment and may produce even higher quantities of the SM. Thus, the table is an illustration of how background levels could be a confounding factor. In this event, an untreated control should be included in the residue test to correct for any naturally occurring SMs found in or on plants.

This is in line with the report of the OECD/KemI Workshop (Sweden) on SMs produced by pathogens- page 53: "We must recall that the phytopathogenic microorganisms that we are aiming to control also produce secondary metabolites and toxins, which are tolerated at low levels in feed and food. We should not be more restrictive on a biological control agent than we are for the pathogenic microorganisms it controls."

These low levels are indeed given in The Commission Regulation (EC) No 1881/2006 The Commission Regulation (EC) No 1881/2006 of 19 December 2006. In this Regulation maximum levels are set for contaminants in food. Only for some mycotoxins maximum levels are set. These levels are derived from monitoring studies and are based on feasibility: Lower maximum levels would not be practical, as a large part of the crop would have to be destroyed. Council Regulation 315/93/EEC of 8 February 1993 laying down Community procedures for contaminants in food says: contaminant levels shall be kept as low as can reasonably be achieved following recommended good working practices. This is the ALARA principle.

Maximum levels are set for different foodstuffs (different sorts of nuts, cereals, wheat, maize, rice milk, spices, coffee, wine, juices, bread, baby food, etc. according to the ALARA principle. The range of these maximum levels is given in the list below. Maximum levels for baby food are not included in the list below as maximum levels for baby food are always extremely low.

- Aflatoxin (B_1): 2-12 µg/kg;
- Aflatoxin (sum of B_1 , B_2 , G_1 and G_2): 4-15 µg/kg;
- Ochratoxin A: 2-80 µg/kg;
- Patulin: 10 -50 µg/kg;
- Deoxynivalenol: 500-1750 µg/kg;
- Zearalenone: 50-400 µg/kg;
- Fumonisins: 800-4000 µg/kg;
- Citrinin: 2000 µg/kg

The Commission Regulation (EC) No 1881/2006 does not (yet) contain MLs for the SMs given in Table 31.

Table 31. Quantities of secondary metabolites also produced in plant pathogenic fungi (destruxin B, beauvericin and gliotoxin are considered to by mycotoxins, see Section 8.2.1. in the Working document).

Metabolite	Family	Species	mg/kg mg/L	>1000	100-1000	10-100	1-10	Ref.	Also produced in mBCA species:	Compared with production in entomopathogen[1]
Destruxin B	*Alternaria*	*A. brassicae*	mg/kg				43	(Buchwaldt and Jensen, 1991)	*M. anisopliae*	60 ± 123 mg/kg
Destruxin B2	*Alternaria*	*A. brassicae*	mg/kg				1.8	(Buchwaldt and Jensen, 1991)	*M. anisopliae*	6 ± 9 mg/kg
Beauvericin	*Fusarium*	*F. avenaceum*	mg/kg			35 ± 49		(Logrieco et al., 2002)	*B. bassiana*	0-0.00618 mg/L

Metabolite	Family	Species	mg/kg mg/L	>1000	100-1000	10-100	1-10	Ref.	Also produced in mBCA species:	Compared with production in entomopathogen[1]
Beauvericin	*Fusarium*	*F. spec*	mg/kg		720			(Fotso et al., 2002)	*P. fumosoroseus*	1.6 mg/kg
Beauvericin	*Fusarium*	*F. oxysporum*	mg/kg	3200				(Logrieco et al., 1998)		
Beauvericin	*Fusarium*	*F. proliferatum*	mg/kg	1100				(Plattner and Nelson, 1994)		
Beauvericin	*Polyporus*	*P. sulphureus*	mg/kg	3364				(Deol et al., 1978)		
Destruxin	*Ophiospaerella*	*O. herpotrichia*	mg/kg				3.6	(Venkatasubbaiah et al., 1994)		
Oosporein	*Chaetomium*	*C. aureum*	mg/L		1000			(Taniguchi et al., 1984)	*Beauveria* spp.	200-278 mg/L
Oosporein	*Verticillium*	*V. psalliotae*	mg/L		125			(Nagaoka et al., 2004)	*Beauveria* spp.	200-278 mg/L
Cytochalasin D	*Coriolus*	*C. vernicipes*						(Cole and Schweikert, 2003)	*M. anisopliae*	n.d.[2]
Cytochalasin D	*Zygosporium*	*Z. manonii*						(Cole and Schweikert, 2003)		
Cytochalasin D	*Engleromyces*	*E. goetzii*						(Cole and Schweikert, 2003)		
Cytochalasin D	*Hypoxylon*	*H. terricola*						(Cole and Schweikert, 2003)		
Gliotoxin	*Aspergillus*								*Gliocladium, Trichoderma* spp.	
NG-391, NG-393 (= 7-desmethyl analogues of fusarin C and (8Z)-fusarin C)	*Fusarium*	*F. spec.*						(Krasnoff et al., 2006)	*Beauveria* spp.	60, 200, 420, 2150 mg/kg NG-391 30, 200, 290, 1930 mg/kg NG-393

1: production in pathogenic fungus is compared to an entomopathogen (data derived from Background document 1.4.4.1. Dimensions within comparison are always the same (mg/kg or mg/L) except for beauvericin produced by *Beauveria bassiana*.
2: no quantities determined by Aldridge (1967).

Following paragraph shows that production of some SMs occurs in different subclasses and families.

In fungi
Some SMs are produced by fungal families that belong to one or more different classes/subclasses within the ascomycetes. This could be demonstrated for destruxin A, B and E, desmethyl destruxin B, cyclosporin, and bassianolide and trichothecenes:

Destruxins A, B and E are produced within the Ascomycetes
Class Sordariomycetes
subclass Sordariomycetidae,
family Clavicipitaceae by species within the genus of *Metarhizium,*
family Cordycipitaceae by species within the genus of *Lecanicillium,*
Class Dothideomycetes
Subclass Dothideomycetidae
family Incertae sedis by species within the genus of *Verticillium,*
subclass Pleosporomycetidae
family Pleosporaceae by species within the genus of *Alternaria (A. brassicae (*destruxin B)).

Desmethyl destruxin B is produced within the Ascomycetes
Class Sordariomycetes
subclass Sordariomycetidae,
family Clavicipitaceae by (one) species within the genus of *Metarhizium (M. anisopliae),*
Class Dothideomycetes
subclass Pleosporomycetidae
family Pleosporaceae by (one) species within the genus of *Alternaria (A. brassicae).*

Cyclosporin is produced within the Ascomycetes
Class Sordariomycetes
subclass Hypocreomycetidae,
family Ophiocordycipitaceae by species within the genus of *Tolypocladium*,
family Incertae sedis by species within the genus of *Verticillium*.

Bassianolide is produced within the Ascomycetes
Class Sordariomycetes
subclass Hypocreomycetidae,
family Incertae sedis by species within the genus of *Verticillium (V. albo-atrum),*
subclass Sordariomycetidae,
family Cordycipitaceae by species within the genus of *Beauveria.*

Trichothecenes are produced within the Ascomycetes
Class Sordariomycetes
subclass Hypocreomycetidae
family Hypocreaceae by species within the genus of *Trichoderma,*
family Nectriaceae by species within the genus of *Fusarium*.

As an exception, Beauvericin is produced in two different phyla:

Beauvericin is produced within Ascomycetes
subclass Hypocreomycetidae,
family Cordycipitaceae by species within the genus of *Beauveria,*
family Nectriaceae by species within the genus of *Fusarium,*

family Clavicipitaceae by species within the genus of *Isaria,*
and Basidiomycota
class Agaricomycetes
family Polyporaceae by *Polyporus sulphureus*

In bacteria
Pyrrolnitrin is produced within the Proteobacteria
class Betaprotiobacteria,
family Burkholderiaceae by (one) species within the genus of *Burkholderia (B. cepia),*
class Gammarotiobacteria,
family Pseudomonadaceaea by species within the genus of *Pseudomonas*.

Conclusions
Entomopathogenic fungi
The SMs listed in tables 1-15 were obtained from a few selected sources and are therefore not exhaustive. Many other SMs may be present under specific conditions and are not measured as they occur too briefly or in too low quantities or have not been discovered yet with the current techniques.

1. Secondary metabolite production depends on both abiotic and biotic factors and may vary both temporally and spatially. The production of SMs in submerged fermentation may give a vast range of SMs, but the range depends on the media used and the growing conditions.
2. It depends on the techniques that are employed in the preparation of the MCPA whether any metabolites that may have accumulated in the growing medium, are still present in the MPCA. This subject is touched upon in Background document 2. Therefore, the SM analyses in submerged fermentation do not reflect the range and quantities of SMs of the MPCAs.
3. In entomopathogenic fungi, the ranges of SMs produced within the class differ to a great extent.
4. In growth media entomopathogenic fungi show the largest possible range of metabolites production. *In vivo,* on the contrary, *Metarhizium* and *Beauveria* spp. produce only a few SMs (Table 26 and Table 28). Therefore, it does not seem realistic to use quantities produced *in vitro* for risk assessment purposes unless these SMs remain present in the MPCP.
5. Studies in growth media may not identify SMs produced after infecting the host. However, they are useful to identify other possible SMs that could be produced during the manufacturing process of the formulated product (before interaction with the target host). Knowledge about the range and quantities of SMs present in growth media are valid to evaluate the risks of direct contact with sprays containing SMs. Chapter 3 will also address this issue.
6. Secondary metabolites given in the tables are restricted to one particular phylum and within that phylum to one or more classes/subclasses. Beauvericin seems to be the only exception. Beauvericin is produced in several families within the Ascomycetes but has also been identified in one family within the Basidiomycetes.

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2 IDENTIFICATION OF MPCA AND SECONDARY METABOLITE

2.1 Question

Identification of MPCA known or suspected to contain SMs. This may involve surveying the industry for specific knowledge not present in the public domain.

2.2 Relevance of the question

Risk assessment of the SMs may be necessary when these are present in relevant amounts. In the risk methodology the exact material containing the supposed SMs has not been described. It was agreed by workshop members of the OECD meeting that the MPCA should be considered. A well-chosen downstream processing method will avoid that SMs will be present in the MPCA or will result in minimal levels of metabolites (Alabouvette et al., 2012). This can be achieved by selecting the downstream processing method which enables the most efficient separation of the viable propagules from the growing medium (Alabouvette et al., 2012).

A range of information and data must be considered in the risk assessment methodology, such as the presence of metabolites in the MPCA and MPCP. If SMs are not present in the MPCA and MPCP or in very low quantities a risk assessment on SMs is not necessary.

It must be realised that this chapter is focused on entomopathogenic fungi. However, for some MPCP, other than those containing entomopathogenic fungi, the presence of SMs in the MPCP may be desired for product performance.

2.3 Questionnaire

In the risk assessment scheme it seems to be relevant to know whether the MPCA contains SMs or not.

It is assumed that the manufacturing process will determine whether the MPCA will contain either no SMs, small, intermediate or high amounts of SMs.

A questionnaire was sent out among the members of the IBMA. The goal of the questionnaire was to make an overview of the relation between manufacturing processes, species (and strain) used and the presence of SMs in the MPCA. Knowing which manufacturing processes yield very little SMs would offer possibilities of reducing and simplifying the data requirements for the MPCA (and MPCP). On the other hand, the MPCA could contain intermediate or high amounts of SMs on purpose. In that case data requirements could also be reduced and simplified if these SMs are not of concern. A summarizing (Table 32) would give more insight in the relation between the possible types of products (WG, GR, etc.) and the presence of SMs. This information could be used in the design of a decision tree. If a product does not contain relevant amount of SMs, the exposure routes can be limited.

Results

Five companies sent back the questionnaires. This was considered to be insufficient material to be able to generalize the information in Table 32. Extra efforts to search the literature were not further performed as these were too time consuming within this project.

Table 32. Summary of the output of the questionnaire.

Abbreviation Type of product[1]	Active substance [entomopathogenic fungus/ other types of fungi/bacteria	Techniques used to reduce metabolites?	Metabolites present in MPCA?	Metabolites present in the formulation?	Quantities present
WP					
GR					
RB					
WG					
CP					

SC					
OF					
OD					

Abbreviations are explained below

Explanations of the product abbreviations used in Table 32

Following list of formulation types derives from CropLife International (CropLife International, 2002) a catalogue of pesticide formulation types and international coding system. entences in italics are additions made by De Faria and Wraight (2007).

WP **Wettable powder**. A powder formulation to be applied as a suspension after dispersion in water. *In our understanding, WP formulations must be ready-to-use. Thus, products such as hydrophobic technical materials that do not include additives that render them miscible in water (such as surfactants or clays) would not fall in this category.*

GR **Granule**. A free-flowing solid formulation of a defined granule size range ready for use. *Although technical concentrates comprising microbe-colonized granular substrates may resemble and function as granular formulations, the term granule generally refers to more elaborated formulations with particles of controlled and uniform size and with the active ingredient strongly adhered to or incorporated into the granule. Thus, fungus-colonized cereal grains are not included under this definition*.

RB **Bait** (ready for use). A formulation designed to attract and be eaten by the target pests. *This definition is generally applicable to mycopesticides; however, because most fungal pathogens infect their hosts via direct penetration of the cuticle, ingestion may be of little consequence, and baits may therefore be based on attractants other than food.*

WG **Water dispersible granule**. A formulation consisting of granules to be applied after disintegration and dispersion in water.

CP **Contact powder.** Insecticidal formulation in powder form for direct application. *Free-flowing powders suitable for dusting are termed dustable powders (DP) under the CropLife International coding system. However, other than a few early preparations produced by nonprofit organizations, we did not identify any mycoinsecticides formulated specifically for broadcast application as dusts. Thus, we have categorized all powders that do not fit any of the previously mentioned formulation types as contact powders.*

SC **Suspension concentrate** (=flowable concentrate). A stable suspension of active ingredient(s) in water, intended for dilution with water before use.

OF **Oil miscible flowable concentrate** (=oil miscible suspension). A stable suspension of active ingredient(s) in a fluid intended for dilution in an organic liquid before use.

ULV **Ultra-low volume (ULV) suspension (SU).** A suspension ready for use through ULV equipment.

OD **Oil dispersion.** A stable suspension of active ingredient(s) in a water-immiscible fluid, which may contain other dissolved active ingredient(s), intended for dilution in water before use. *In practice, oil dispersions contain emulsifiers to render the mixture miscible in water for spraying (T. S. Woods, personal communication). The word 'stable' in this and other of the abovementioned formulations indicates that the active ingredient does not settle out to a non resuspendable cake during storage (T. S. Woods, personal communication). Here, we consider the definition to include suspensions that tend to settle, but which are designed to be readily resuspendable by the user via manual agitation. Oil dispersions of entomopathogenic fungi have*

been referred to most commonly in the literature as emulsifiable suspensions or emulsifiable oil suspensions and identified by the abbreviation ES. However, under the Croplife International code, the abbreviation ES refers to emulsions for seed treatments

2.4 References

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3 BIOLOGY IN RELATION TO SECONDARY METABOLITE PRODUCTION

3.1 Research question to be answered

A consideration of microorganisms' biology as it relates to SM production (including considering when SMs are formed, e.g. during production or after application, and where, e.g. within a host).
This item should answer the following questions

1. What are the pathways for SM production and what precursors are necessary in the medium?
2. What are the different stadia in the infection process in the target organism?
3. SM production linked to specific fungal stadia. At what point in its lifecycle does the organism produce SMs?

3.2 Introduction

The exact location of the production of SMs is of crucial importance in the risk assessment. It determines whether non-target organisms will be exposed to these SMs or not.

In a worst case risk assessment SMs two possible sources of metabolite production need to be considered.
1) SMs can be present in the MPCP as remnants of the fermentation process or
2) (Toxic) SMs are produced *in vivo*.

In this present question the production of the SMs within the life cycle of the microorganism (option 2) will be investigated using information from the literature.

3.3 Results

3.3.1 Pathways and precursors

Secondary metabolites originate as derivatives from various intermediates in primary metabolism (Vey et al., 2001b). Table 33 shows which pathways are present for SM production and the precursors that are being used in these pathways.

Table 33. Examples of secondary metabolites derived from different pathways and precursors.

Precursor	Pathway	Secondary metabolites	Representative organisms
Sugars		Muscarine	*Amanita muscaria*
		Kojic Acid	*Aspergillus oryzae*
Aromatic amino acids (e.g. phenylalanine, histidine, tryptophan, tyrosine)	Shikimic acid		Bacteria and several eukaryotic organisms such as ascomycetes and basidiomycetes fungi (*Aspergillus nidulans, Neurospora crassa, Cryptococcus neoformans*), apicomplexans, and plants
Aliphatic amino acids (e.g. glycine, alanine, valine, leucine, isoleucine)	Various, including non-ribosomal peptide synthesis (NRPS)	Penicillins	*Penicillium chrysogenum, P. notatum.*
		Fusaric acid	*Fusarium* spp.
		Ergot alkaloids	*Claviceps, Neotyphodium*
		Lysergic acid	*Claviceps pupurea*
		Sporidesmin	*Pithomyces chartarum*
		Cyclosporin	*Tolypocladium inflatum, T.* spp.
		Beauvericin	*Beauveria bassiana, Isaria, Fusarium*
		Oosporein	*Beauveria brongniartii*
		Enniatins	*Verticillium hemipterigenum, Fusarium oxisporum, F.* spp.
		Destruxins	*Metarhizium anisopliae*
		Efrapeptins	*Tolypocladium* spp.
		Peptaibols	*Trichoderma, Hypocrea*
		tripeptide d-(L-a-aminoadipyl)-	*Aspergillus nidulans* *Penicillium chrysogenum*

Precursor	Pathway	Secondary metabolites	Representative organisms
		L-cysteinyl-D-valine (ACV)	*Acremonium chrysogenum*
		Ergotpeptides	*Claviceps purpurea*
		Alamethicin	*Trichoderma viride*
		Cyclopeptin	*Penicillium cyclopium*
		HC-toxin	*Cochliobolus carbonum*
		Tentoxin	*Alternaria alternata*
		Ferrichrome	*Aspergillus quadricinctus*
		Echinocandin	*Aspergillus nidulans*
Organic acids	TCA cycle	Rubratoxin	*Penicillium rubrum*
		Itaconic (=methylenesuccinic) acid	*Aspergillus* spp.
Fatty acids	Lipid metabolism	Polyacetylenes	Basidomycota fruitbodies and hyphae e.g. agrocybin from *Agrocybe perfecta*
Acetyl-CoA	Claissen-type acyl condensation pathway[1]	Patulin	*Penicillium patulum*
		Usnic acid	(many lichens)
		Ochratoxins	*Aspergillus ochraceus*
		Griseofulvin	*Penicillium griseofulvum*
		Aflatoxins	*Aspergillus flavus, A. parasiticus*
		Radicicol	*Pochonia chlamydosporia*
Acetyl CoA	Isoprenoid	Trichothecenes (e.g. T-2, deoxynivalenol)	*Fusarium* spp, *Trichothecium, Trichoderma, Cephalosporium, Stachybotrys* spp.
		Fusicoccin	*Fusicoccum amygdali*
		Several sex hormones: sirenin, trisporic acids, oogoniol, antheridiol	*Mucor* spp., *Achlya* spp.
		Cephalosporins (β-lactam antibiotics)	*Cephalosporium* and related fungi
		Viridin (furanosteroidal antibiotic)	*Trichoderma (Gliocladium) virens*
		Wortmannin (furanosteroidal IP3 kinase inhibitor)	*Talaromyces (Penicillium) wortmannii*

1: in this pathway polyketides are synthesized by enzyme complexes called Polyketide synthases (PKS)

Studies that are found in the literature use a particular fermentation broth to determine the possible range of SMs produced by the microorganism *in vitro*.

Table 33 shows that the entomopathogens (that will be primarily dealt with in this initial search), all have aliphatic amino acids (e.g. glycine, alanine, valine, leucine, isoleucine) as the precursor of their SMs. In the literature, it is found that a multitude of liquid media are in fact modifications based on a limited number of basic media, such as Czapek Dox Broth. The synthesis of active antibiotics can be obtained by including specific organic and fatty acids in the growth medium. A modification can be made by, for instance, adding 0.5% bacto-peptone. These modifications are one explanation for the variety in the range of SMs that are observed. The options in the pathway seem to depend on the availability of the precursors and the affinity of enzymes produced by the entomopathogen for these precursors.

As opposed to tests performed in such media, very few studies are available on the SMs produced *in vivo*. Strasser et al. (2000a) only found oosporein and biassianin in cadavers of *Melolontha melolontha* infected with *B. brongniartii.* Destruxin A, B and E were the only toxins found in diseased insects infected with *M. anisopliae.*

It is therefore assumed that the range of SMs may be broader in fermentation broths than the range of SMs that is actually found in the insect cadavers. A possible explanation could be that more nutrients (precursors of the SMs) are available in the fermentation broths than in the living insect. Another explanation is that the still living insect is able to detoxify SMs (Skrobek et al., 2008).

3.3.2 Life cycle of entomopathogenic fungi

Some reviews are available that describe the infection process (Ortiz-Urquiza and Keyhani, 2013, Shahid et al., 2012, Pedrini et al., 2007, Santi et al., 2010, Charnley, 2003). The general processes in pathogenic fungi are outlined by Boomsma et al. (2014) in the figure below.

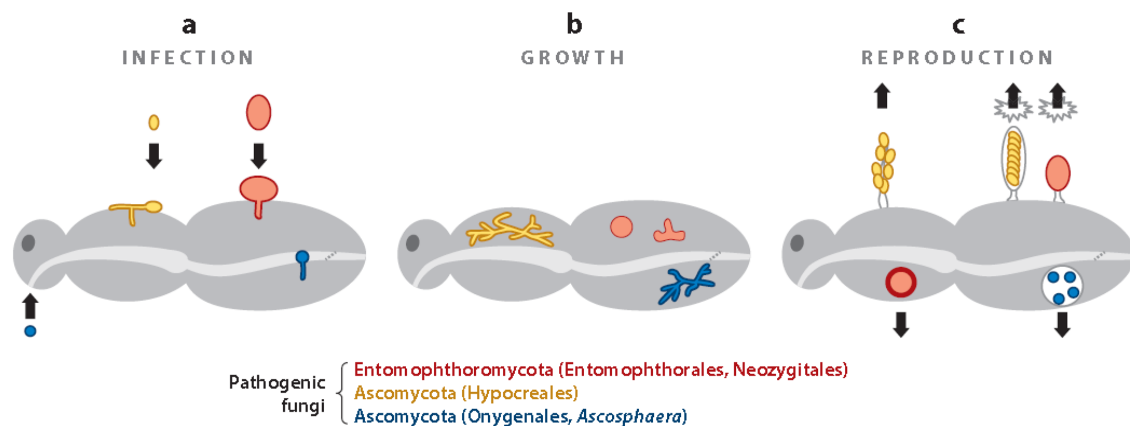

Figure 9. Diagrams illustrating the ways in which pathogenic fungi infect arthropod hosts by asexual or sexual spores, proliferate, and disperse (Boomsma et al., 2014).

Hosts are represented by outlines and a gut with a dotted line indicating that the hindgut of bee larvae is closed. Sizes of colored symbols are approximately proportional to mass but are drawn much larger than they are relative to host size.

(a) Infection: Entomophthoralean fungi infect mainly by large, sticky conidia penetrating the cuticle directly, and hypocrealean fungi infect by small conidia that produce appressorial structures. Ascosphaera spores are also small, enter orally, and infect through the gut epithelium.

(b) Growth: Most Hypocreales and Onygenales proliferate through hyphal growth, and most entomophthoralean fungi proliferate through protoplasts without cell walls.

(c) Reproduction: Asexual conidia of entomophthoralean fungi and sexual ascospores of the Hypocreales are primarily forcibly discharged from the surface of cadavers (upward arrows at right), whereas (very large and thick-walled) sexual resting spores of entomophthoralean fungi and asexual conidia of Hypocreales are passively released (upward and downward arrows at left). Ascosphaera produces only sexual spores that are passively released (downward arrow at right).

The virulence of fungal entomopathogens involves five steps (Shahid et al., 2012): Adhesion, germination, differentiation, penetration and sporulation. Each step is influenced by a range of integrated intrinsic and external factors, which ultimately determine the pathogenicity. Each of these steps is described below.

3.3.2.1 Adhesion

The virulence of an entomopathogenic fungus is recognized first by adhesion to an insect body. A successful infection is achieved by the attachment or adhesion of spores to the host. Adhesion is necessary and normally achieved through the secretion of mucilage. However, enzymes, lectins, as well as hydrophobic and electrostatic forces also play a role (Boucias et al., 1988). Lectins, a kind of carbohydrate binding glycoproteins could be involved in binding between conidia and the insect cuticle.

Also glyceraldehyde-3-phosphate dehydrogenase of the entomopathogenic fungus *M. anisopliae plays a role in the* cell-surface localization and host adhesion (Broetto et al., 2010).

Figure 10 shows that in the adhesion stage both the host and the fungus have specific strategies to either stop the fungus from penetrating or to circumvent the host's defense mechanisms.

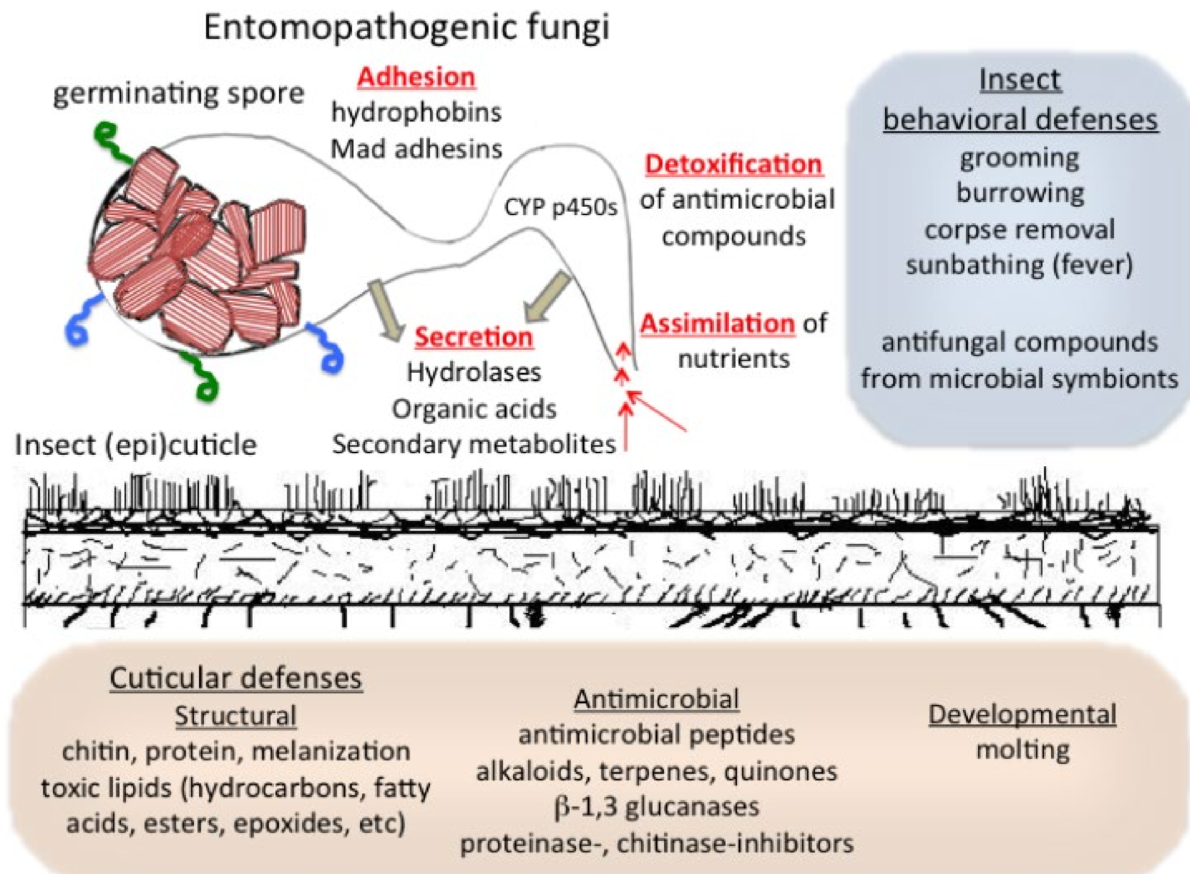

Figure 10. Overview of surface interactions between entomopathogenic fungi and the insect cuticle and host behaviors. (Ortiz-Urquiza and Keyhani, 2013).

3.3.2.2 Germination

During the course of insect infection, *M. anisopliae* and *B.bassiana* encounter different carbon sources. Proteins, hydrocarbons, fatty acids and lipids may be found on the surfaces of insect cuticle, the fat body, haemolymph and within insect haemocytes (Jarrold et al., 2007). These compounds present on the host cuticle stimulate and fuel the germination. Successful germination requires the assimilation of utilizable nutrients.

Figure 11 shows major stages in the infection cycle of entomopathogenic *Metarhizium* spp.

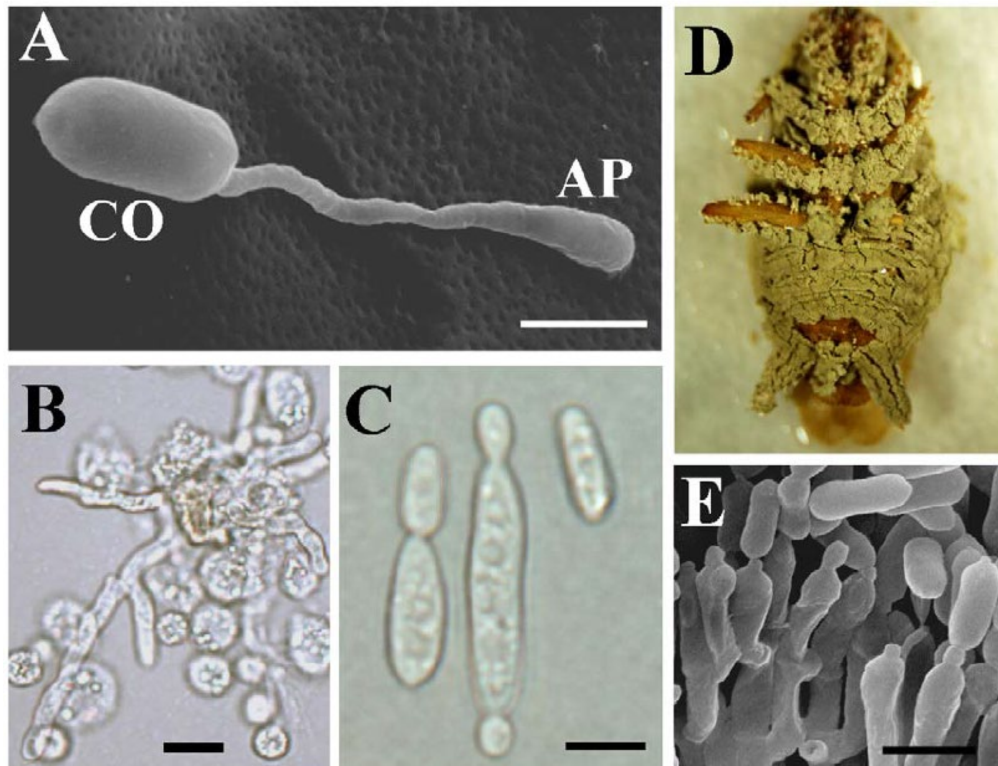

Figure 11. Major stages in the infection cycle of entomopathogenic *Metarhizium* spp.. (A) A germinating conidium producing an appressorium. (B) Mycelia attacked by hemocytes after cuticular penetration. (C) Budding yeast-type cells (blastospores) produced by the fungus to facilitate dispersal in insect hemocoel. (D) Cadaver showing emerging hyphae producing conidia (E). CO, conidium; AP, appressorium. Bar, 5 mm (Gao et al., 2011).

3.3.2.3 Penetration

Most terrestrial pathogens are known to penetrate directly, rarely via wounds, sense organs or spiracles. The penetration process is considered to be a combination of enzymatic and mechanical forces. The exact mechanism for entry is usually specific to the species.

A range of cuticle-degrading enzymes are produced during penetration into the host (Gillespie et al., 1998). The most important enzymes secreted by entomopathogenic fungi are lipases, proteases and chitinases, which are produced sequentially, reflecting the order of the substrates they encounter. Proteases have been shown to play a key role in the penetration process and a wide range has been identified, including trypsin, chymotrypsin, elastases, collagenase and chymoelastase.

Some secondary metabolites may also be produced. These also play a role in the defense against competing microbes (Ortiz-Urquiza and Keyhani, 2013; Santi et al., 2010; Charnley, 2003).

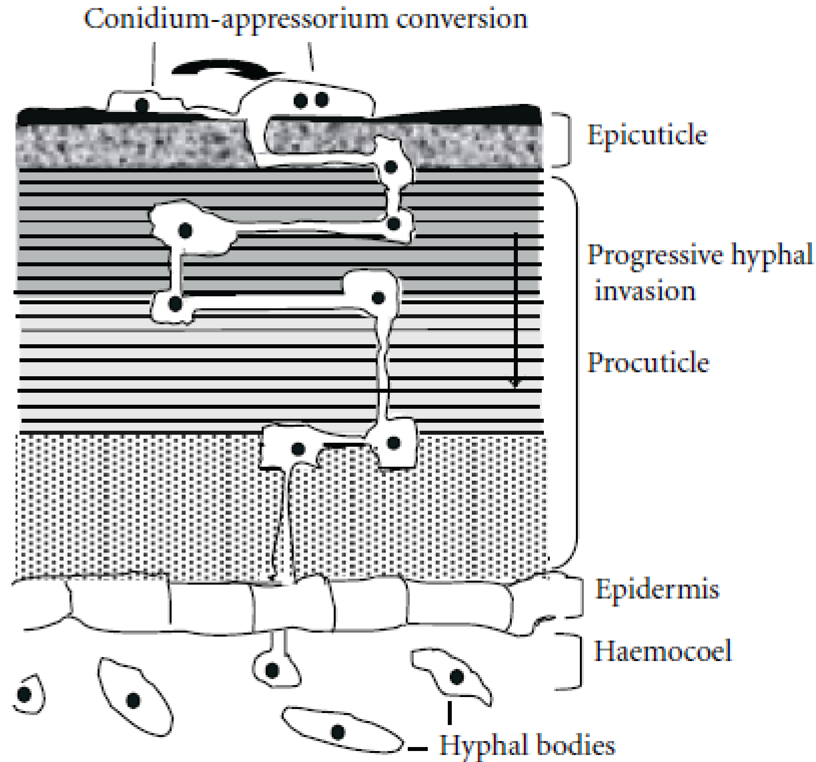

Figure 12. Depiction of the infection process in *Beauveria bassiana*: structure of the insect cuticle and mode of penetration of fungal hyphae. Formation of the appressorium from the conidia helps in cuticle invasion and subsequent hyphal penetration to the haemocoel. Tissue invasion by hyphae and proliferation of hyphal bodies leads to insect death (Sandhu et al., 2012).

3.3.2.4 Proliferation

Figure 9 shows different types of proliferation for three different fungi infecting insects, the entomophtoralean fungi and the Hypocreales and Onygenales. (Note that no examples of MPCAs from the entomophtoralean fungi and Onygenales have been included in Background document Section 1). The Hypocreales and Onygenales proliferate through hyphal growth, while most entomophthoralean fungi proliferate through protoplasts without cell walls. The proliferation of fungal cells in the host will lead to its death.

Some species of hypocrealean entomopathogens produce SMs within their insect hosts that are postulated to help the fungus outcompete opportunists during the saprotrophic phase of insect utilization (Strasser et al., 2000a; Strasser et al., 2000b).

3.3.2.5 Sporulation

Frisvad et al (2008) mention that some SMs are only produced during sporulation.
For instance, Krassnoff et al. (2007) isolated the cyclic heptapeptides, serinocyclins A and B from the conidia of the entomopathogenic fungus *M. anisopliae.*

Conidia of *M. anisopliae* strains V245 and V275 from insect cadavers contain destruxin A, B and E in maximum amounts of 80 µg per 10^{10} spores (Skrobek et al., 2008). Quantities depend on the strain and the insect species. Destruxin B and E for instance were not extracted from *Galleria mellonella.* This study indicated that the destruxins were probably transported from the infected cadavers into the developing conidia.

Summary of entomopathogens:

- In the life cycle of the entomopathogen several stages can be identified: During adhesion enzymes and lectins play an important role.
- During germination no SMs are being produced. Instead the process uses nutrients such as fatty acids, fatty acid esters, glucose, amino acids and peptides.
- During penetration enzymes such as lipases, proteases and chitinases are produced. Some SMs may also be produced. These also play a role in the defense against competing microbes.
- During proliferation SMs are produced in the insect.
- During sporulation SMs seem to be transported from the insect into the spores. It has not been found in the literature so far, whether these metabolites are being released again during adhesion, in a new cycle, but this seems very likely.

Conclusions on entomopathogens

- Metabolites of entomopathogens are not produced on the surface of the insect cuticle.
- Greatest metabolite production takes place in the insect host during proliferation of the fungus in the hemolymph.
- Smaller amount of metabolites can be released during penetration.
- Expression and secretion of SMs is controlled by various genetic and cellular regulatory mechanisms. Precursors need to be present in a pathway to initiate the production of metabolites. Also, there is evidence that genes are differently expressed in the different stages of *B. bassiana* (aerial conidia, in vitro blastospores and submerged conidia).
-

3.3.3 Life cycle of some important fungal genera (other than entomopathogens)

Vinale et al. (2014) write that fungal SMs with a direct antibiotic activity against plant pathogens have been mainly isolated from biocontrol strains of the genus *Trichoderma* spp.

3.3.3.1 Trichoderma

Many excellent reviews have been produced on *Trichoderma* spp.. This paragraph is foremost based on the reviews of Keswani et al. (2014), Reino et al. (2008), Vinale et al. (2008), Vinale et al. (2012) and Vinale et al. (2014).

Trichoderma (teleomorph *Hypocrea*) is a genus of asexual fungi found in the soils of all climatic zones. *Trichoderma* spp. are a secondary opportunistic invader, a fast growing fungus, a strong spore producer, a source of cell wall degrading enzymes (cellulases, chitinases, glucanases, etc.) and an important producer of SMs (Vinale et al., 2014).

It has been observed by electron microscopy that the hyphae of the biocontrol agent penetrate the root cortex but the colonisation by *Trichoderma* spp. are stopped, probably by the deposition of callose barriers by the surrounding plant tissues (Vinale et al., 2012)

According to references mentioned in Keswani et al. (2014), *Trichoderma* spp. constitutively[3] secretes a variety of lytic enzymes to detect the presence of a competent fungus by sensing their degraded cell wall components (see Figure 13). It is of great importance to this Background document that no reports are available on the constitutive secretion of antimicrobial SMs (while potential competitors are absent). For *Trichoderma* spp. this means that *in vivo,* the production of SMs needs to be triggered upon sensing of the pathogen.

[3] a gene that is transcribed continually compared to a facultative gene which is only transcribed as needed

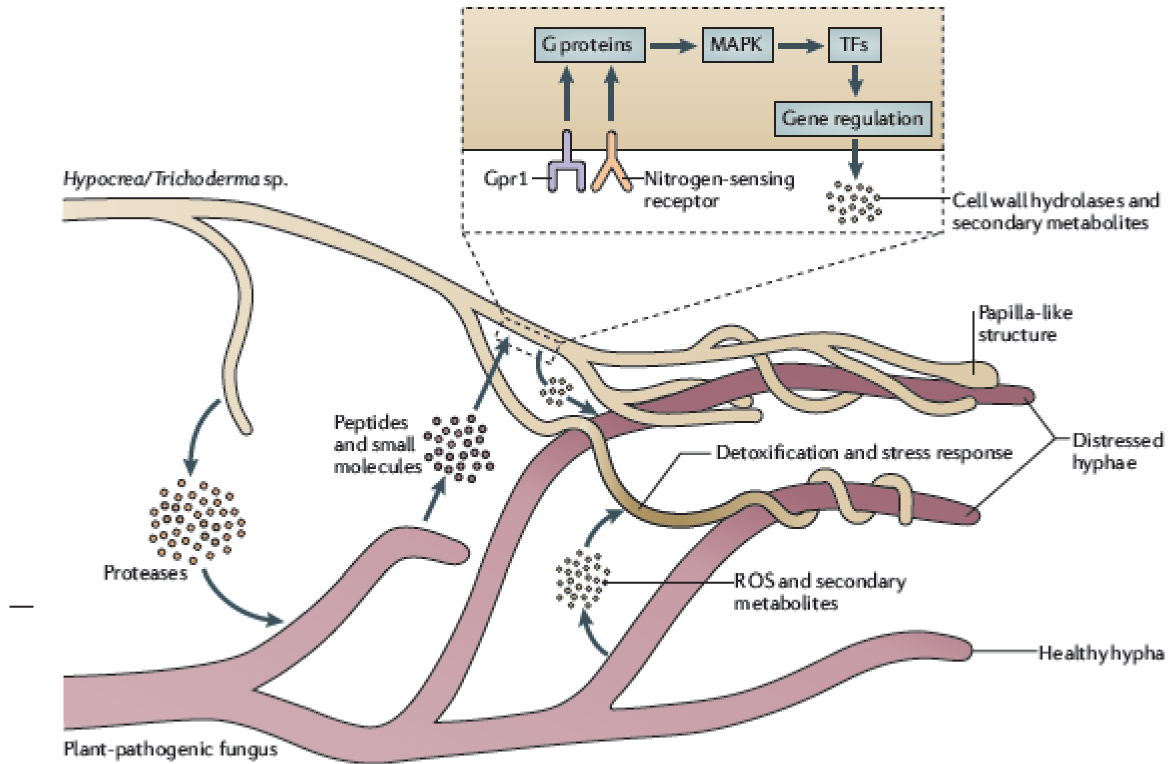

Figure 13. Mycoparasitism of Hypocrea/*Trichoderma* spp. within the soil community. Hypocrea/*Trichoderma* spp. recognise a plant-pathogenic fungus (a prey) via small molecules that are released by the pathogen; some of these molecules may be peptides that are released by the action of proteases secreted by the Hypocrea/*Trichoderma* sp. before contact. These molecules may bind to G protein-coupled receptors (such as Gpr1) or nitrogen-sensing receptors on the surface of the Hypocrea/*Trichoderma* spp. hyphae, thereby eliciting a signaling cascade comprising G proteins and mitogen-activated protein kinases (MAPKs), which may ultimately modulate the activities of as-yet-unknown transcription factors (TFs). These factors then enhance the constitutive expression of genes that encode enzymes for the biosynthesis of SMs and for cell wall lysis. Lectins from the pathogenic fungus and proteins harbouring cellulose-binding modules from hyphae of Hypocrea/*Trichoderma* spp. may collaborate in the attachment of the predator to the prey. At the same time, the plant-pathogenic prey responds by forming SMs and reactive oxygen species (ROS) that elicit a stress response and detoxification in Hypocrea/*Trichoderma* spp. (Druzhinina et al., 2011).

In Figure 14 an attempt has been made to outline the potential roles of SMs in diverse commercial applications.

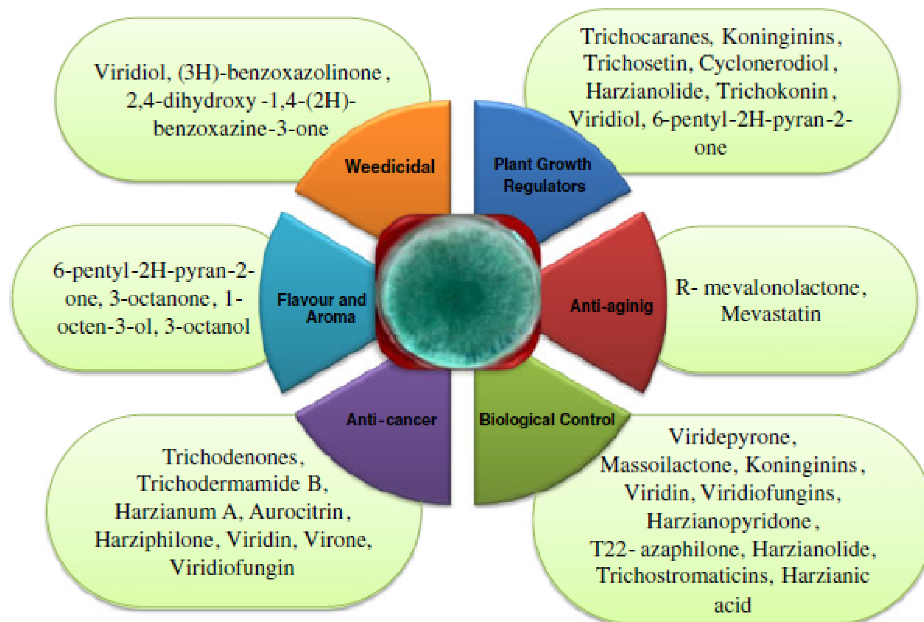

Figure 14. Multi-various role of *Trichoderma* spp. secondary metabolites (note: weedicidal should be herbicidal).

It is clear that there is a multitude of SMs being produced and that one or more roles as pictured in Figure 14 can be attributed to each of them.

The review of Keswani et al (2014) provides a detailed list of SMs produced by various *Trichoderma* species. The information is too extensive to include in the table of Background document 1. In this Background document the focus will be on SMs which play a role in biological control mechanism. The review of Reino et al. (2008) describes 186 SMs. According to these authors, the different metabolites exhibiting antifungal activity can be classified as two main types:

1. Low molecular weight and volatile metabolites
   These represent simple aromatic compounds, some polyketides such as pyrones and the butenolides, volatile terpenes, and the isocyane metabolites. All of these are relatively non-polar substances with a significant vapor pressure. These ‘‘volatile organic compounds’’ in the soil environment would be expected to travel over distance through systems and thus enhance the status of one organism by affecting the physiology of competitor organisms.
2. High molecular weight polar metabolites
   These SMs may have a direct activity on the interactions between *Trichoderma* spp. and their antagonists. Peptaibols represent an important group which are a family of antimicrobial peptides synthesized by non-ribosomal peptide synthetases and contain a high proportion of 2-amino-isobutyric acid. Todate, 317 peptaibols have been identified and this number is still increasing. The majority of them are produced by *Trichoderma* spp. strains. Examples of peptaibols in *Trichoderma* spp. are listed in (Reino et al., 2008). They include trichotoxin A40, trichovirins II, trichorovins, trichodecenins I, II, trichocellins, polysporins A-D, trichosporin B-V, paracelsin, saturnisporins, trichokonins, trikoningin, atroviridins, trichorzianines, trichokindins, harzianins, trichorozins (n.b. more peptaibols are given in this paper).

Vinale et al. (2014) write that elite strains usually produce only a few main SMs. The quality and the quantity of SMs synthesized depend on:
- the compound considered;
- the species and the strain;
- the occurrence of other microorganisms;

- the equilibrium among elicited biosynthesis and biotransformation rate;
- the growth conditions;
- further, in some cases, the biocontrol agent was able to modulate the production of toxic SMs according to the presence or the absence of the target pathogen.

Two important *Trichoderma* spp. biocontrol strains T22 and T39 were shown to only produce three major metabolites in *in vitro* plate tests where each of the biocontrol strains were grown together with the pathogens *Rhizoctonia solani, Pythium ultimum* and *Gaeumannomyces graminis* var. *tritici* (Vinale et al., 2006). The T22/*R. solani* EtOAc extract showed the presence of T22azaphilone.

Similarly, the T39/*R. solani* interaction showed the presence of T39butenolide and harzianopyridone, but not harzianolide. T22 and T39 strains did not produce 6-n-pentyl-6H-pyran-2-one, the most characterized and important of the *Trichoderma* antifungal metabolites (Vinale et al., 2006).

Trichothecenes (Trichodermin, Trichodermol, Harzianum A, (2Z,4E,6E)-octa-2,4,6-trienoic acid) and gliotoxin are produced by *T. viride, T. polysporum, T. sporulosum, T. reesei.*

Trichodermin and gliotoxin (also produced by *Alternaria* spp*., Aspergillus fumigatus, Penicillium* spp*.)* are known mycotoxins, listed in Section 8.2.1 of the Working document. These metabolites should be considered in the risk assessment of *Trichoderma* spp.

No information is yet available on the production of SMs on treated plants. It may be possible to discriminate these SMs from all other compounds (including plant metabolites) using LC-MS or LC-MS/MS (or GC-MS for volatile compounds.

Summary for *Trichoderma* spp.:
- *In vivo,* the production of SMs needs to be triggered upon sensing of the pathogen (degraded cell wall components).
- Production of SMs is tightly regulated and always in close contact with stressed hyphae of the pathogen.
- A multitude of SMs are produced, and one or more roles can be dedicated to each of them (biocontrol, herbicidal, plant growth regulator, therapeutic).
- Each strain used for biocontrol purposes only produces a few main SMs.
- Trichothecenes (Trichodermin, Trichodermol, Harzianum A, (2Z,4E,6E)-octa-2,4,6-trienoic acid) and gliotoxin are produced by *T. viride, T. polysporum, T. sporulosum, T. reesei.*
- Trichodermin and gliotoxin (also produced by *Alternaria* spp*., Aspergillus fumigatus, Penicillium* spp*.)* are known mycotoxins, listed in Section 8.2.1 of the Working document. These metabolites should be considered in the risk assessment of *Trichoderma* spp..
- No information is yet available on the production of SMs on treated plants.

Conclusion for *Trichoderma* spp.:
Although a multitude of SMs are produced in *Trichoderma* spp. only a few are produced in biocontrol strains. Production of trichodermin and gliotoxin needs attention in the risk assessment. SM production is a tightly regulated process in which SMs are produced in reaction to molecules that are released by stressed hyphae of the parasitized pathogen. SM quantities are just high enough to be toxic for the pathogen. These quantities are not likely to affect other non-target organisms.

3.3.4 Life cycle of some important bacterial genera

Raaijmakers and Mazolla (2012) extensively reviewed soil- and plant-associated bacteria, with a focus on antibiotics produced by beneficial bacteria used in biological control of plant pathogens and on antibiotics products produced by plant-pathogenic bacteria. They refer to studies that show that the genomes of several well-studied bacterial species indicate that the number of orphan pathways (i.e. pathways expected to be presented based on orphan/cryptic antibiotic gene clusters, producing yet bacteria.

Important biocontrol species such as *Pseudomonas*, *Burkholderia, Streptomyces* and *Bacillus* are closely associated with soil (pathogenic) fungi, as described by Haq et al. (2014). Figure 15 shows the major steps in the colonization process.

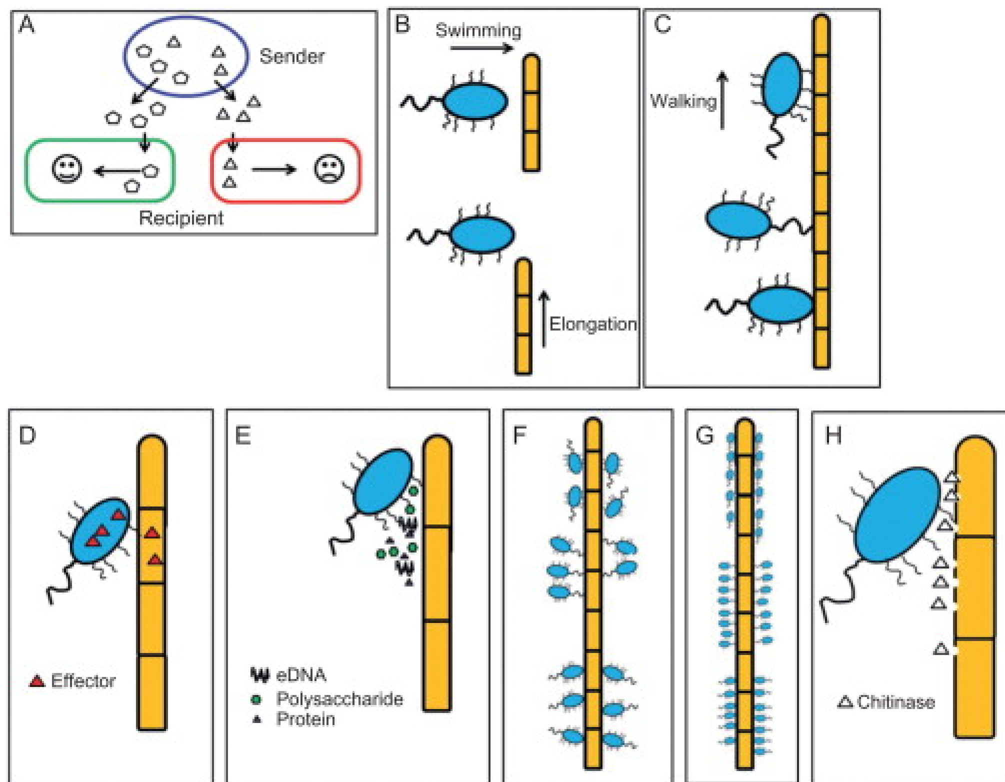

Figure 15. The process of bacterial–fungal interactions. (A) Cell-to-cell contact-independent interaction. (B–H) Cell-to-cell contact-dependent interaction; (B) approximation; (C) recognition and attachment; (D) effector injection; (E) EPS (extracellular polymeric substance) alteration; (F) bacterial growth; (G) biofilm formation; (H) cell wall degradation (Haq et al. 2014).

In step A the two processes are shown. In the first is the recipient is a fungus with plant growth stimulating capacities, in the second the recipient is a pathogenic fungus. In step A there is no physical contact between the bacterium and the fungus. The perception of the fungus by the bacterium is presumed to be effectuated by the release of signaling molecules. According to Haq et al. (2014) these can be quorum-sensing molecules (See Box 1 for an explanation of quorum sensing), antibiotic-like substances, SMs, as well as volatile organic compounds (VOCs). The recipient cells of the pathogenic fungus react to the signal molecules by growth inhibition.

BOX 1 Quorum sensing.

Quorum sensing has been studied well among biocontrol bacteria. From Miller and Bassler (2001): Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. Gram-positive and Gram-negative bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation.

In the following paragraphs the genera *Bacillus, Pseudomonas, Burkholderia, Serratia, Streptomyces, Agrobacterium, Erwinia and Xanthomonas* are briefly reviewed for SM production in relation to their life cycle.

3.3.4.1 Bacillus spp.

This review excludes *B. thruingiensis* which have a distinct and different mode of action that other *Bacillus* Spp, used in biological control.

Several biocontrol *Bacillus* spp. are indicated in table 46 of Background document 1. These are *B. amyloliquefaciens* strain FZB42, *B. subtilis* (strains AU195 and QST 713), *B. licheniformis* strain ATCC 14580 and *B. pumilus*.

Some *Bacillus* spp. are able to form biofilms at the (root) surface. Biofilms are defined as dense aggregates formed after cell division and proliferation of single bacterial cells attaching to surfaces.

For *Bacillus* spp., lipopeptides can play an important role in surface attachment and biofilm formation. (Raaijmakers et al., 2010). In *Bacillus* spp., quorum sensing plays important roles in the regulation of lipopeptide biosynthesis. For example, the expression of the surfactin genes is associated with increased cell densities and occurs especially in the transition from exponential to stationary growth phase, whereas the biosynthesis of fengycins and iturins usually occurs later in the stationary phase.

The lipopeptides fengicin, surfactin and iturin A are surfactants with a hydrophilic ring of seven amino acids and a long, hydrophobic hydrocarbon tail (Figure 16). The hydrocarbon tail penetrates pathogen cell membranes, while the amino acid end stays in the soil solution. This action creates openings in cell membranes, inhibiting the growth of many pathogens (Kinsella et al., 2009).

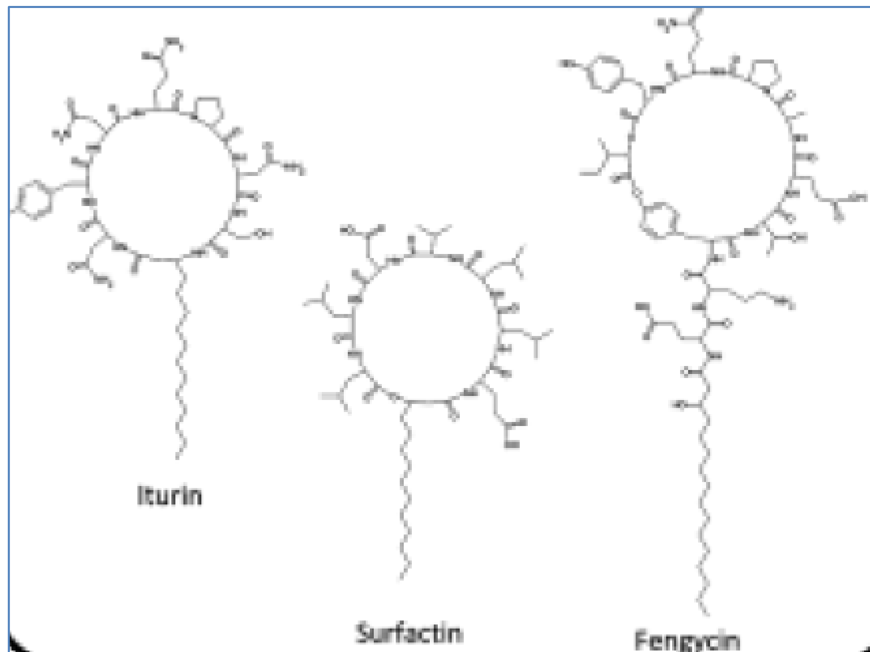

Figure 16. Lipopeptides from *Bacillus* (Raaijmakers et al., 2010).

In order to maintain antibiotic production over the growing season, *B. subtilis* root biofilms must not only be maintained over time, but must continue to spread down the elongating and branching root surface (Kinsella et al., 2009).

Summary for *Bacillus* spp. (excluding *B. thuringiensis)*:

- *Bacillus* spp. are closely associated with soil (pathogenic) fungi.
- *Bacillus* spp. can make biofilms on the surface of roots.
- The expression of the surfactin genes is associated with increased cell densities and occurs especially in the transition from exponential to stationary growth phase.
- Fengycins and iturins usually occur later in the stationary phase.
- The SMs fengycin, iturin and surfactin have a hydrocarbon tail penetrates pathogen cell membranes, while the amino acid end stays in the soil solution. This action creates openings in cell membranes, inhibiting the growth of many pathogens.
- SM production is control by quorum sensing.

Conclusion for *Bacillus* spp.

Bacillus spp. are closely associated with soil (pathogenic) fungi forming biofilms. Metabolite production is mediated by quorum sensing and the mode of action of the SMs produced by *Bacillus* spp. (fengycin, iturin and surfactin) is limited to penetration of cell membranes of the pathogen.

3.3.4.2 Pseudomonas spp.

This group of bacteria is known to produce a diverse array of antifungal compounds and in many cases the production of these compounds has been directly correlated with biocontrol activity. *Pseudomonas* spp. strains can suppress plant pathogens, promote plant growth and induce systemic resistance (ISR). The production of SMs by these bacteria appears to provide a competitive advantage in the colonization of the rhizosphere.

Similar to some *Bacillus* spp., *Pseudomonas* spp. can forms biofilms andsome lipopeptides are considered to play an important role in surface attachment and biofilm formation (Raaijmakers et al., 2010).

The lipopeptides produced by *Pseudomonas* spp. are illustrated in Figure 17.

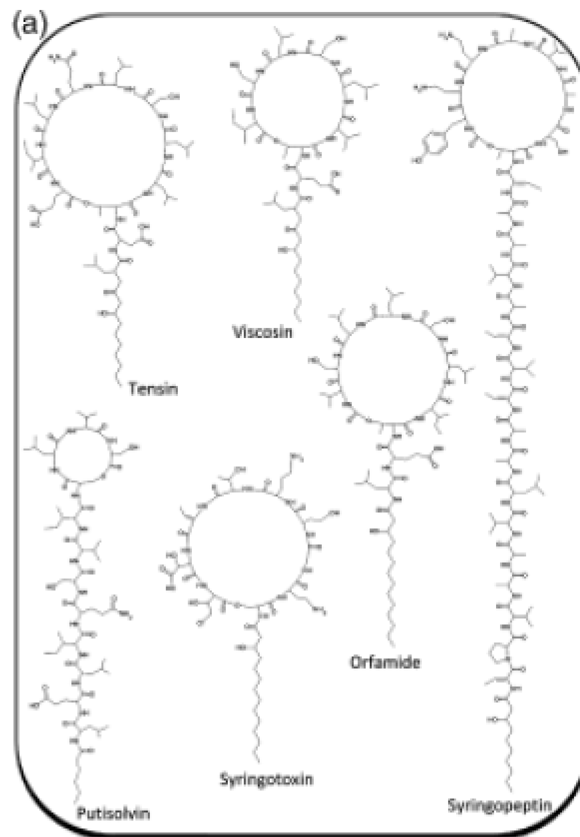

Figure 17. Lipopeptides from *Pseudomonas* (Raaijmakers, 2010).

According to Schnider-Keel et al. (2000) "Certain root-associated strains of fluorescent *Pseudomonas* spp. produce and excrete metabolites that are inhibitory to soil borne plant pathogens. Among these metabolites, 2,4-diacetylphloroglucinol (2,4-DAPG) has received particular attention because of its production by a wide range of pseudomonads used for the biological control of root diseases. 2,4-DAPG is a phenolic compound with broad spectrum antifungal, antibacterial, antihelminthic, and phytotoxic activity."

Summary for *Pseudomonas* spp.:

- *Pseudomonas* spp. areclosely associated with soil (pathogenic) fungi.
- *Pseudomonas* spp. can form biofilms on many surfaces.
- Signaling molecules are used to locate the fungus: these could be metabolites.
- Some lipopeptides such as orfamide, viscosin and tensin play an important role in surface attachment and biofilm formation.
- The production of SMs by *Pseudomonas* spp. appears to provide a competitive advantage in the colonization of the rhizosphere.
- Mode of action of 2,4-DAPG is antifungal, antibacterial, antihelminthic, and phytotoxic.
- SM production is under the control of quorum sensing.

Conclusion for *Pseudomonas* spp.

Pseudomonas spp. can be closely associated with soil (pathogenic) fungi, forming biofilms. Secondary metabolite production is mediated by quorum sensing and the production of SMs by *Pseudomonas* spp. appears to provide a competitive advantage in the colonization of the rhizosphere.

3.3.4.3 Burkholderia spp.

The *Burkholderia* genus, β subdivision of the proteobacteria, comprises more than 40 species that inhabit remarkably diverse ecological niches, as they have been isolated from soil, plant rhizosphere, water, insects, fungus, and hospital environments and from infected humans (Vial et al., 2007). Several *Burkholderia* spp. are opportunistic human pathogens. These species include all *B. cepacia* complex (Bcc) bacteria. *Burkholderia cepacia* was first recovered from sputum cultures of patients with cystic fibrosis in the late 1970s.

Certain species are used as biocontrol agents, while others are efficient in bioremediation and plant growth promotion. Bcc organisms have also been the focus of considerable research by plant pathologists who have shown that some strains are effective biocontrol agents against soilborne , foliar, and post-harvest diseases (see references in Parke and Gurian-Sherman, 2001).

The original species *B. cepacia* has been split into eight genetic species (genomovars) (seeTable 34), including five named species, but taxonomic distinctions have not enabled biological control strains to be clearly distinguished from human pathogenic strains. Several biopesticides (Deny®, Blue Circle®, Intercept®) containing any of three strains of *B. cepacia* (M36, M54, andJ82) were registered in 1992 and 1996 but after reevaluation registration of Deny was cancelled in the USA in 2000 (Parke and Gurian-Sherman, 2001) as a 1.4-kb fragment was associated with some epidemic strains. Strain A396 was registered by EPA in 2014.

It should be stressed that virulence and pathogenicity traits necessary for a Bcc infection of Cystic fibrosis patients have not been identified, and even if they were, differences between clinical and environmental strains may occur at the level of gene regulation, rather than presence or absence of genes (Parke and Gurian-Sherman, 2001).

The use of these products is restricted to seed treatments, in furrow incorporation, or drip irrigation to reduce the likelihood of aerosol production.

Registration of biocontrol species appears to be complex as related *Burkholderia* spp. are human pathogens. Park and Gurian-Sherman (2001) conclude that at the time of their publication "it is not possible to distinguish, with certainty, Bcc strains that are potential human pathogens from those that are not. New registrations will not take place until there is either a robust test to determine that biocontrol strains are not pathogenic to people with CF, or the strain is identified as a species or genomovar that does not contain human pathogens".

Many species of *Burkholderia* have the ability to produce compounds
with antimicrobial activity (see references in Compant et al., (2008)) and can potentially be used as biocontrol agents of:

- phytopathogenic fungi. This was well demonstrated with *B. cenocepacia, B. cepacia, B. ambifaria, B. pyrrocinia, B. vietnamiensis* and *B. phytofirmans* strains towards *Pythium aphanidermatum, Pythium ultimum, Fusarium sp., Phytophthora capsici, Botrytis cinerea* and/or *Rhizoctonia solani.*
- other bacteria, protozoa and nematodes
- plant diseases in many different crops, such as corn, sweet corn, cotton, grapevine, pea, tomato and pepper

According to Compant et al. (2008) the beneficial effects of *Burkholderia* spp. involve:

- diverse mechanisms of action including rhizosphere competence determining their population density on root surface,
- secretion of allelochemicals (toxic chemicals produced by one species that affect a receiving susceptible species), including antibiotics and siderophores,
- competition for nutrients, as well as induced systemic resistance.
- Siderophores produced by several strains of *Burkholderia* spp. are very efficient low-molecular-weight iron-chelating compounds. These compounds are implicated in antibiosis against plant

pathogens through iron sequestration under iron-limiting conditions. Ornibactins are the predominant siderophores produced by *Burkholderia* strains. Several other siderophores are also produced by *Burkholderia* strains such as cepaciacheline and cepabactine.

Table 34. Characteristics of members of the *Burkholderia cepacia* complex (Parke and Gurian-Sherman, 2001).

B. cepacia complex member	Species designation	Reference	Source	Distribution of species among US CF patients with Bcc (%)[a]
Genomovar I	*B. cepacia*	(10, 158, 167)	Plant pathogen (onion); rhizosphere, soil, water, humans (non-CF and CF). Includes type strain	2.6
Genomovar II	*B. multivorans*	(158)	Rhizosphere, humans (non-CF and CF)	37.8
Genomovar III	—	(158)	Hospital environment, humans (non-CF and CF), rhizosphere, soil	50.0[b]
Genomovar IV	*B. stabilis*	(159)	Hospital environment, humans (non-CF and CF)	0.2
Genomovar V	*B. vietnamiensis*	(39)	Rhizosphere, soil, humans (non-CF and CF). Fixes nitrogen	5.1
Genomovar VI	—	(18)	Humans (CF)	2.0
Genomovar VII	*B. ambifaria*	(19)	Rhizosphere, soil, humans (CF). Includes many biocontrol strains	0.7
—	*B. pyrrocinia*	(162)	Soil	0.0

[a]Percent of 606 patients (96). For patients from whom multiple isolates were received, only the first to be confirmed as *B.cepacia* complex was identified. The identity of 1.6% of the isolates was not determined.

[b]46% of genomovar III strains in this study contain the putative marker for transmissibility *esmR*.

Burkholderia spp. secrete a variety of extracellular enzymes with proteolytic, lipolytic, and hemolytic activities. Several strains secrete also toxins, antibiotics, and siderophores (Vial et al., 2007). Many strains of the Bcc produce one or more antibiotics active against a broad range of plant pathogenic fungi. These antibiotics appear, in many cases, to be important for disease suppression.
Cordova-Kreylos et al. (2013) report that “Some of the known toxins produced by *Burkholderia* spp. include:

- toxoflavin (1,6-dimethylpyrimido[5,4-e]-1,2,4-triazine-5,7(1H, 6H)-dione);
- fervenulin (a tautomeric isomer of toxoflavin) with antibacterial, antifungal, and herbicidal activities;
- rhizobitoxin (2-amino-4-(2-amino-3-hydroxypropoxy)-trans-but-3-enoic-acid), which, among other phytotoxic effects, induces foliar chlorosis due to inhibition of cystathione-β-lyase;
- rhizoxin, a macrocyclic polyketide which kills rice seedlings through binding to β-tubulin, resulting in inhibition of the normal cell division cycle. This compound also demonstrates broad antitumor activity in vitro;
- bongkrekic acid, which inhibits adenine nucleotide translocase as well as cell apoptosis;
- rhizonins A and B, hepatotoxic cyclopeptides that were first discovered from a fungal *Rhizopus* sp. but later on were shown to be produced by a bacterial endosymbiont of the genus *Burkholderia;*
- tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), a nonbenzenoid aromatic compound with both phenolic and acidic moieties and proven antimicrobial, antifungal, and insecticidal properties. Tropolone is produced by *B. plantarii*.”

Regulation of secondary metabolite production

Quorum sensing has been studied extensively among Bcc bacteria and it is found in every *Burkholderia* spp. strain investigated.

Summary of *Burkholderia* spp.:

- Registration of biocontrol species appears to be complex as related *Burkholderia* spp. are human pathogens. The use of some products was restricted to seed treatments, in furrow incorporation, or drip irrigation to reduce the likelihood of aerosol production.

- The beneficial effects of *Burkholderia* spp. involve diverse mechanisms of action including rhizosphere competence determining their population density on root surface, secretion of allelochemicals, including antibiotics and siderophores, competition for nutrients, as well as induced systemic resistance.
- Siderophores are implicated in antibiosis against plant pathogens through iron sequestration under iron-limiting conditions. Ornibactins are the predominant siderophores produced by *Burkholderia* strains. Several other siderophores are also produced by *Burkholderia* strains such as cepaciacheline and cepabactine.
- SMs production is regulated by quorum sensing.

Conclusion for *Burkholderia* spp.
The beneficial effects of *Burkholderia* spp. involve diverse mechanisms of action including rhizosphere competence determining their population density on root surface, secretion of allelochemicals, including antibiotics and siderophores, competition for nutrients, as well as induced systemic resistance. The production of SMs is regulated by quorum sensing. As *Burkholderia* biocontrol species are related to *Burkholderia* species known as human pathogens, their registration is complex and is limited to some uses with no exposure to humans.

3.3.4.4 Serratia spp.
The genus *Serratia* is a member of the Enterobacteriaceae and is comprised of a group of bacteria that are related both phenotypically and by DNA sequence. *Serratia* spp. are associated with different habitats such as plants, mammals, water and hospitalized patients.

There is an extensive literature on *Serratia* spp. associated with insects. This topic has been reviewed in detail (see references mentioned in Grimont and Grimont 2006). The insects involved belong to numerous species and genera of the orders Orthoptera (crickets and grasshoppers), Isoptera (termites), Coleoptera (beetles and weevils), Lepidoptera (moths), Hymenoptera (bees and wasps), and Diptera (flies).

<u>Life cycle</u>
Most *Serratia* spp. need to enter the hemocoel of an insect larvae to successfully cause infection.

<u>Secondary metabolites</u>
The insecticidal potency of *Serratia* spp. is caused by a combination of immune resistance, invasive capabilities, and exoenzymatic activities (references in Petersen and Tisa (2013). Proteases, chitinases and LPS are mentioned as a possible virulence factor, but the mode of actions seem to be largely unknown.

The production of toxins is rare among the studied *Serratia* spp. *S. entomophila* and *S. proteamaculans* carry the 155 kb mega-plasmid pADAP (amber disease associated plasmid). This disease is only found in the New Zealand grass grub (*Costelytra zealandica*).

In *Serratia* spp. the SM prodigiosin is only produced by strains of *S. marcescens, S. plymuthica*, and *S. rubidaea* (Grimont and Grimont, 2006). The isolated pigment showed antimicrobial activity against different pathogenic bacteria and fungi (Suryawanshi et al., 2014).

Summary for *Serratia* spp.:
- Only some species *S. marcescens, S. plymuthica*, and *S. rubidaea* produce prodigiosin which has antimicrobial activity against fungi and bacteria.
- Insecticidal potency of *Serratia* spp. is not caused by SMs but by combination of immune resistance, invasive capabilities, and exoenzymatic activities.

Conclusion for *Serratia* spp.:

Only some species *S. marcescens, S. plymuthica*, and *S. rubidaea* produce prodigiosin which has antimicrobial activity against fungi and bacteria. *Serratia* species with insecticidal potency are not known to produce SMs.

3.3.4.5 Streptomyces spp.

Life cycle

According to Muschko et al. (2002), *Streptomyces* spp. are Gram-positive soil bacteria (Actinobacteria = actinomycetes) which are characterized by a complex life cycle. Beginning with an arthrospore, they first form an interconnected dense mycelium within the substrate (MI in Figure 18 and Figure 19).

The growth of the vegetative mycelium eventually slows as a result of nutrient exhaustion and breaks down (MII in Figure 18 and Figure 19). The breakdown of hyphae is also called programmed cell death (PCD). The aerial mycelium develops at the expens e of nutrients released by breakdown of the vegetative hyphae (Challis and Hopwood, 2003). Much of the published data indicate that the most important environmental signal triggering secondary metabolism is nutrient starvation, particularly that of phosphate (Sola-Landa et al. 2003). The signaling networks involved in the regulation of secondary metabolism in *Streptomycetes* have been reviewed by Bibb (2005).

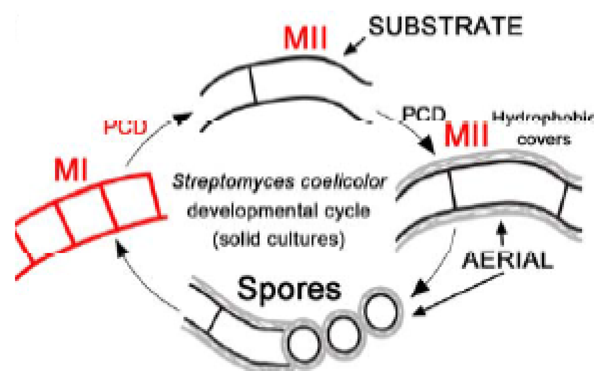

Figure 18. *Streptomyces* spp. developmental cycle in solid cultures. Cell-cycle features of *Streptomyces* spp. development. In red, newly described structures and the proposed nomenclature: MI, first compartmentalized mycelium; MII, second multinucleated mycelium). Classical nomenclature (substrate and aerial mycelium) and hydrophobic layers (in grey) are also indicated. PCD, programmed cell death (Manteca and Sanchez, 2010).

In natural soils MI is the predominant phase which may last for one month (Manteca and Sanchez, 2010).

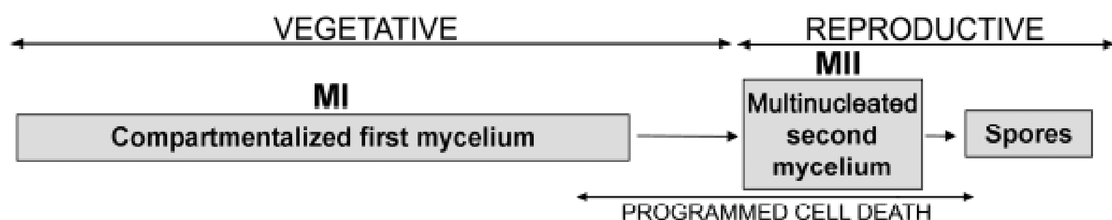

Figure 19. Cell-cycle features of *Streptomyces* spp. growing in natural soils. Mycelial structures (MI, first mycelium; MII, second mycelium), vegetative and reproductive phases and PCD are indicated (Manteca and Sanchez, 2010).

Secondary metabolites

Actinobacteria are thought to produce two-thirds of the total number of antibiotics (Challis and Hopwood, 2003). Metabolites of actinomycetes include medically important compounds such as antibacterials, antifungals, antivirals, anticancer and immunosuppressive drugs, antiparasitic agents, cholesterol-lowering agents and antidiabetic drugs (van Keulen and Dyson, 2014).

Within the actinobacteria, *Streptomyces* spp. accounts for 70-80% of the SMs. During the reproductive phase several metabolites are produced, e.g. alkaloids, glycosides and other bioactive compounds. The most studied group of SMs is the antibiotics. The different groups are illustrated in Figure 20.

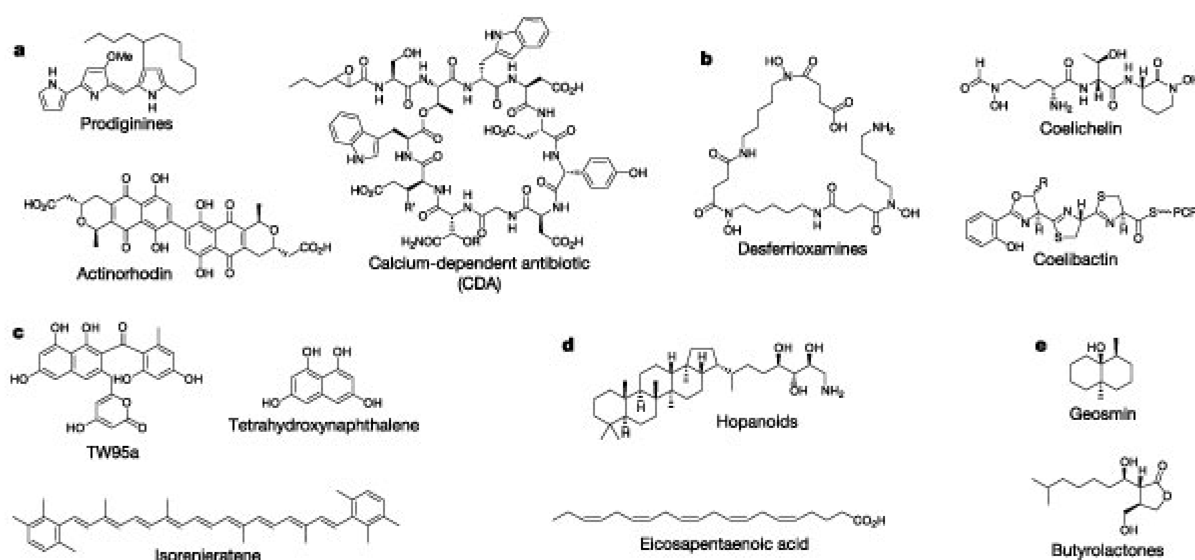

Figure 20. Secondary metabolites known or predicted to be made by *Streptomyces coelicolor* A3(2), grouped according to their putative function: antibiotics (a), siderophores (b), pigments (c), lipids (d) and other molecules (e) (Bentley et al., 2002).

SMs of *Streptomyces* spp. include polyketides, pyrones, peptides, siderophores, γ-butyrolactones, butenolides, furans, terpenoids, fatty acids, oligopyrroles, and deoxysugars (van Keulen and Dyson, 2014). This large number of SMs is likely to be explained by the fact that *Streptomyces* spp. are non-motile; stresses cannot be avoided but must be met (Challis and Hopwood, 2003).

The biocontrol agent *S. melanosporofaciens* strain EF-76 produces geldanamycin, an antibiotic which displays antagonistic activity towards several Gram-positive bacteria and fungi (Clermont et al., 2010). In culture media geldanamycin biosynthesis was only stimulated in the presence of 1 g L^{-1} of chitin. Chitin is known to be a major component of fungi. It can therefore be assumed that production of this metabolite *in vivo* is only possible following contact with pathogenic fungi in the root system or on the canopy.

Products based on *Streptomyces* spp. contain its dried spores and mycelium. According to the Manual of Biocontrol Agents, the mode of action is a combination of factors including root colonisation and competition with the pathogenic fungi, lysis of the cell walls of the pathogenic fungi by extracellular enzymes and production of antifungal metabolites.

Products are either applied as a drench to the soil or as a spray application to the leaves for foliar diseases such as Powdery and Downy Mildew, *Botrytis* spp., *Alternaria* spp. and others.

Summary for *Streptomyces* spp.:
- *Streptomyces* spp. ares non-motile.
- They grow at the expense of pathogenic fungi in the root system and on the canopy.
- ITheyproduce a large range of metabolites but only after nutrient depletion of the source (pathogenic fungi).
- Production of geldanamycin *in vivo* is stimulated by chitin which is a major component of fungi.

Conclusions for *Streptomyces* spp.
Streptomyces spp. are known for the wide range of SMs, possible explained by the fact that they are non-motile. The biocontrol agent *S. melanosporofaciens* strain EF-76 produces geldanamycin, an antibiotic which displays antagonistic activity towards several Gram-positive bacteria and fungi.

3.3.5 Endophytes
Endophytes as a possible source of SMs were not included as a research topic for these background documents and have therefore not been reviewed adequately.

A short search showed that a vast number of wild and crop plant species have been shown to harbour endophytes. Among these are biocontrol species such as *Beauveria, Acremonium, Cladosporium, Clonostachys,* and *Paecilomyces* (Vega et al., 2008). These authors assume that the mode of action involves feeding deterrence or antibiosis after consumption of the plant.

If some fungal entomopathogenic species occur as endophytes and exert their action against insects via the production of metabolites, then their mode of action would be analogous/similar to the mode of action reported for known entomopathogenic fungi. This raises the issue of whether inoculation of agronomically important plants with fungal entomopathogens might create a problem by producing metabolites that could potentially enter the food chain (Vega et al., 2008). This area is not well studied yet and would need further testing. RAFBCA field experiments with some biocontrol species (see Background document 5) did not detect SMs in an array of crops. This suggests that these tested biocontrol species do not live endophytically or produce SMs endophytically below the limit of detection.

Bacterial biocontrol agents in specific plant growth-promoting bacteria (PGPB) are associated with many, if not all, plant species and are commonly present in many environments. Plant growth-promoting rhizobacteria (PGPR) in specific colonize the root surfaces and the closely adhering soil interface, i.e., the rhizosphere (see references in Compant et al. 2005). Some of these PGPR can also enter root interior and establish endophytic populations. According to Compant et al. (2005) many of them are able to traverse the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers, and other organs.

The production of antibiotic/fungitoxic compounds by endophytes can induce disease resistance to the host plants.

Bacteria, such as *Pseudomonas, Bacillus* spp. are also well known endophytes. It is however not clear whether biocontrol species can also live endophytically or that this capacity is limited to specific species.

3.3.6 Gene expression
Several review articles for in-depth coverage of these molecular genetic and genomic topics are available (Schab and Keller, 2008). It is generally accepted that the expression and secretion of SMs is controlled by various genetic and cellular regulatory mechanisms (Hoffmeister and Keller, 2007). This sophisticated regulatory network of SM expression is regarded as a flexible device to meet the various ecological challenges encountered during the lifetime of a fungus (Rohlfs and Churchill, 2011). Different genes and regulatory molecules play a major role in the expression of profiles of SMs. The genes involved in the biosynsthesis of metabolites of *M. anisopliae* are still unknown, with the exception of serinocyclins and NG-391 (see references in Gao et al. 2011).

Genes, such as LaeA, are global regulators of secondary metabolism in *Aspergillus* (Bok and Keller, 2004), and G-protein down-regulates some SMs, but up-regulate others (Tag et al., 2000). These hormone-like compounds and genes are of equal importance in secondary metabolite production in nature and on laboratory media.

As an example, Figure 21 shows that pathways are differently activated by the spore, depending on the host/pathogen combination. Certain genes on the host cuticle are transcribed, and certain proteins can be up-regulated. In the figure the gene STM1 is not transcribed and does not lead to successful germination of the spore. Other genes SF1, SF3 or SF5 may be transcribed, depending on the fungus species. This figure shows that it is important to understand that SM production very much depends on the combination of pathogen and host. If genes are successfully transcribed this leads to the formation of an appressorium (AP).

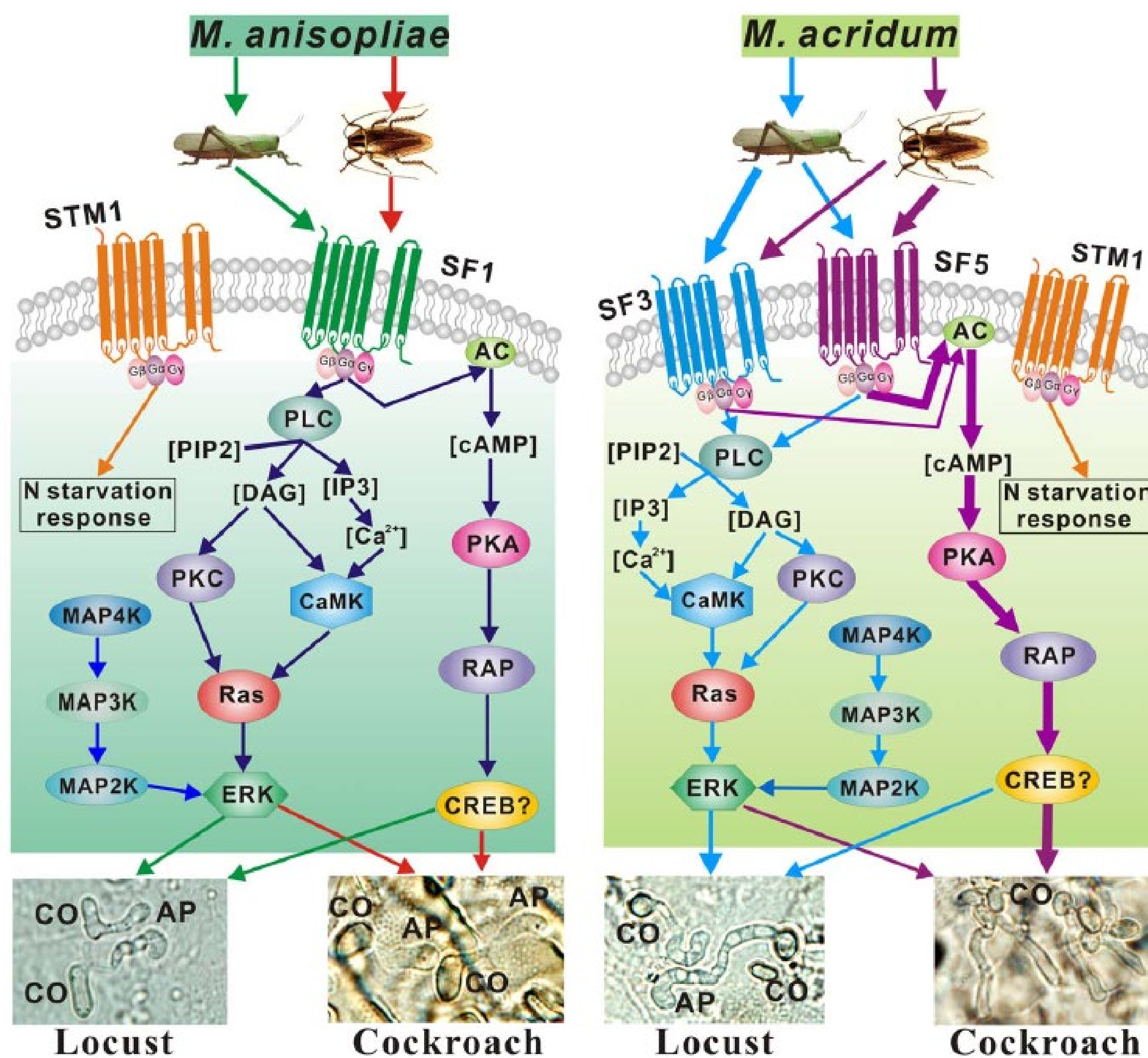

Figure 21. Differentially regulated signaling pathways employed by *Metarhizium anisopliae* and *M. acridum* infecting cockroach and locust cuticles. Both the MAP kinase and cAMP dependent protein kinase A (PKA) pathways were activated by *M. anisopliae* and *M. acridum* infecting cockroach and locust cuticles.

PLC, phosphatidyl inositol-specific phospholipase C;

PIP2, phosphatidylinositol 4,5-bisphosphate;

IP3, inositol 1,4,5-triphosphate;

DAG, diacylglycerol;

PKC, protein kinase C;

CaMK, calcium/calmodulin regulated kinase;

ERK, extracellular signal-regulated protein kinase;

CREB, a basic leucine zipper transcription factor that is a potential cAMP response element-binding protein;

CO, conidium;

AP, appressorium.

Thicker arrows indicate pathways that are more highly expressed by M. acridum on either locust or cockroach cuticles. doi:10.1371/journal.pgen.1001264.g007

3.3.6.1 Secondary metabolism linked to certain events in fungal differentiation

For assessing risks, it is necessary to know the moment in the life cycle of the fungus where the production of SMs occurs. If production is limited to a stage in which non-target organisms are not likely to be exposed, this is of ultimate importance to the risk assessment.

According to Rolphs and Churchill (2011) SMs are either formed *constitutively* (= constant formation at a low rate) or *induced,* (= only expressed in response to contact with host insects*)*. It was hypothesized that the production of constitutively expressed metabolites can be elevated and that the *de novo* synthesis of additional SMs can be stimulated by compounds released at the damaged cuticle of the insect. Such a flexible system would minimize the costs of the SM biosynthesis and maximize its effect. The hypothesis would be that the fungus should prevent the costs of chemical defense when this defense is unnecessary.

Luckner et al (1977) discovered that secondary metabolism is linked to different events in fungal differentiation, especially sporulation.

3.3.6.1.1 Example *Beauveria bassiana*

Cho et al. (2006b) performed an expressed sequence tag (EST) analysis of aerial conidia, *in vitro* blastospores and submerged conidia in order to characterize the *B. bassiana* transcriptome. Analysis of the libraries revealed a robust diversity in expressed transcripts, with the majority of sequenced ESTs unique to each library. These results show that genes are differently expressed in the different stages of *B. bassiana*. Products of gene expression not only include SMs but also enzymes and other proteins.

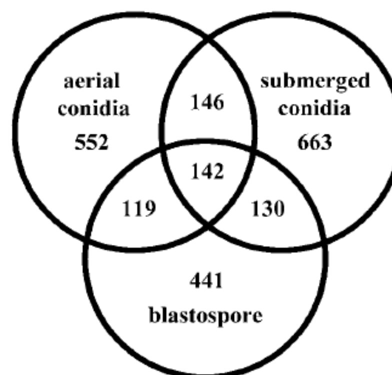

Figure 22. Comparative analysis of sequenced ESTs derived from the *Beauveria bassiana* aerial conidia, blastospore and submerged conidia cDNA libraries: the Venn diagram shows unique and overlapping sets of transcripts between the libraries (Cho et al., 2006b).

Although no specific information could be given on the diversified expression of SMs in conidia, blastospore and submerged conidia, it was clear that the relatively few transcripts (4.9-6%) were shared by the three forms.

The ESTs shared between only the aerial conidia and blastospores accounted for 4.9% (119/2416) of the total unigene set. This common set included a number of ABC-type multidrug transporters, an EST similar to the *Schizosaccharomyces pombe* multidrug resistance transporter conferring brefeldin A resistance (Nagao et al., 1995), and an EST similar to the *Saccharomyces cerevisiae* PDR5 protein that confers resistance to cycloheximide, sporidesmin and other mycotoxins.

Blastospore and submerged conidia libraries uniquely shared 130 transcripts corresponding to 5.4% of the total unigene set. A cell wall b-glucan synthase component was shared, as was a transcript with similarity to the fruit fly, *Drosophila melanogaster*, salivary glue protein Sgs3.

Aerial conidia and submerged conidia shared 146 transcripts (6% of the total), although a number of these transcripts corresponded to housekeeping genes such as ribosomal components, transcription factors and metabolic enzymes.

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4 TOXICITY

4.1 Research question to be answered

Determination of the toxicity of MPCAs identified as containing or producing SMs; again this may involve a literature search. If a potential for toxicity is indicated, the potential for exposure should be evaluated to determine if the SM (especially toxins) induces risks to humans or non-target organisms. In such a case, further information should be sought and provided'

List of tasks:

1. Prepare a table with toxicity values using the sources mentioned above (Data are not necessarily limited to MPCA).
2. Determine sources of exposure (including duration and route of exposure).
   a. Direct contact with metabolites in the sprayed product (metabolites present as a result of accumulation in the fermentation broth, and sprayed along with the active substance, depending on the type of formulation).
   b. Contact with metabolites produced only after contact with the host
   - *Canopy*: metabolites are produced, after application of the product, by micro-organism in contact with pathogenic fungi on the canopy.
   - *Soil:* the same as for pathogenic fungi on canopy.
   - *Insects*: secondary poisoning of birds and other wildlife/predators by eating mycosed insects.
3. Further calculations (comparison of available exposure rates with toxicity data). The main difficulty will be the availability of toxicity tests that have comparable extensions (in mg/L for instance). Also the question should be posed whether or not a safety factor should be used?

4.2 Introduction

Much toxicity data can be found in the literature. Toxicity data needs to be divided according to:

- toxicity to target organism
- toxicity to non-target organisms
  - toxicity to humans
  - toxicity to environmental organisms

4.2.1 Toxicity humans

The EFSA report on human toxicity data (EFSA, 2015) reviews toxin production and the toxicity of metabolites and toxins in plant protection products (PPP). It includes effects caused by several bacterial and fungal species.

A list of studied effects as described in the EFSA review is given below. Only those effects that are caused by SMs [not by toxins (proteins) or metabolite of chemical pesticides] and from which the exposure was are relevant to risk assessment are given in bold. Only the study of Favilla et al. (2006) is relevant for risk assessment of environmental organisms. The results were included in the tables that are given in this Background document.

List of studied effects described in the EFSA report (EFSA, 2015)

- Food poisoning (caused by toxins/proteins *Bacillus* strains such as *B. cereus*),
- Depolarized mammalian plasma and mitochondrial membranes (*B. amyloliquefaciens*),
- Inhibition cancer cell growth (*Bacillus subtilis*)
- Inhibition sperm motility (*Bacillus pumilus*)
- Haemolytic activity (*Bacillus subtilis, licheniformis, pumilus*)
- Hepatoxicity or cytotoxicity *(Bacillus subtilis*)
- Emesis in young children (*Bacillus cereus*)
- Toxic to sperm cells, erythrocytes and Vero cells (*Bacillus licheniformis*)
- Toxic to specific insect pests (toxins/proteins *Bacillus thuringiensis*)
- Reduction fertility and lesions in kidneys, liver and lungs rats (*B. thuringiensis* subsp. *aizawai*)

- Haemolytic, cytotoxic or inhibit protein synthesis (*Lysinibacillus sphaericus*)
- Cytotoxicity against cancer cells (*Streptomyces hypgroscopicus*)
- Toxic to mice (milbemycin oxime produced by *Streptomyces*)
- Production various cytokines in splenocytes (*Aureobasidium pullans*)
- Inhibition cancer cell growth (*Beauveria bassiana*)
- Toxicity to *Artemia salina* and *Daphnia magna* (various fungal species) (Favilla et al. 2006)
- Potent mutagens (*Metarhizium anisopliae*)
- Cytotoxic (crude extracts of *Metarhizium anisopliae)*
- Mouse death *(Metarhizium anisopliae)*
- Inflammatory response in test mice (*Metarhizium anisopliae)*
- Splenomegaly and liver paleness in mice (heat killed conidia of *Isaria fumosorosea*)
- Inhibition boar sperm cell motility (*Trichoderma* species)
- Weak cytotoxic effects against cancer cells (*Trichoderma reesei*)
- Moderate cytotoxic effects (*Trichoderma reesei*)
- Cytotoxic effects against leukaemia cell lines (*Trichoderma harzianum*)

The above list shows that no relevant toxicity data were found in the EFSA document specifically for humans and mammals.

4.2.2 Toxicity environmental organisms

Several roles of metabolites were described by Vey et al. (2001b) for entomopathogenic fungi:

1. Antagonism/competition: to displace competitor or inhibit its development, thus enabling the biocontrol agent to compete for nutrients and space
2. Pathogenesis:
    - to kill and enable pathogen to switch from biotrophic to saprotrophic mode of nutrition.
    - to suppress or disrupt host immune system.
3. Defence: to protect the biocontrol agent against antagonistic microorganisms, microphagous organisms.

Interesting studies have been performed to unravel the working mechanisms of metabolites

- on the hydrolytic activity of a vacuolar type ATPase (Bandani et al., 2001),
- on the inhibition of V-ATPase. Destruxins, especially destruxin B, are inhibitors of V-ATPase (vacuolar H+-translocating ATPase) (Vazquez et al., 2005; Bandani et al., 2001). V-ATPase maintains an acidic environment within lysosomes and vacuoles to help digest ingested materials. Clearly, the suppression of V-ATPase by destruxin B will prevent the insect's cellular phagocytosis from attacking the invading entomopathogenic fungi.
- on target epithelial cells (Dumas et al., 1996), (Muroi et al., 1994)
- on *Locusta migratoria* visceral muscles (Ruiz-Sanchez et al., 2010b) and Malpighian tubules of *Rhodnius prolixus* (Ruiz-Sanchez et al., 2010a).
- on ultrastructural changes in the salivary glands of *Spodoptera litura* (Sowjanya S and Padmaja, 2008)
- on the host's immune system (Chen et al., 2014). Destruxins might be useful weapons used by entomopathogenic fungi to destroy a host's immunity system. However, during the infection process, cells of *M. anisopliae* are attacked instantly by the hemocyte immune system of the insect.

Other activities of SMs are reviewed by Vey et al. (2001b). These types of studies, although essential in understanding how SMs act, were not further reviewed in this Background document; the studies cannot be used for risk assessment purposes as in these experiments there is no link to actual exposure levels.

4.2.3 Toxicity of Metarhizium species

Many toxicity data are available for *Metarhizium*spp.. Although *Metarhizium* spp. produce a wide array of metabolites, such as destruxins, cytochalasins, swainsonine and subtilins only a few, mostly destruxins were tested for their toxicitiy. Destruxins are cyclic hexadepsipeptides produced by certain species of the fungal genera *Metarhizium*, *Alternaria* and *Trichothecium*, which are toxic to a wide range

of invertebrates and plants (Pedras et al., 2002). As the toxicity of a SM depends on the route of exposure, care was taken to be precise about the way of exposure in the following tables.

Table 35 shows some LC50 and LD50 data obtained in *in vitro* or *in vivo* tests. Exposure was either through direct contact or dietary exposure.

Table 35. Toxicity data of *Metarhizium* spp. metabolites.

Secondary metabolite	Test organism	Endpoint	In vivo/ vitro	Test system	Level of effect	Ref.
AQUATIC						
Destruxin A	*Artemia salina*	LC50	In vivo	bioassay	2.92 µg/mL, 36 h 9.78 µg/mL, 24 h	(Favilla, 2006)
Destruxin A	*Daphnia magna*	LC50	In vivo	bioassay	0.16 µg/mL, 36 h 0.20 µg/mL, 24 h	(Favilla, 2006)
Crude extract V245	*Daphnia magna*	LC50	In vitro	Wells in test plate	0.30 µg/mL, 24 h	(Skrobek et al., 2006)
Crude extract V275	*Daphnia magna*	LC50	In vitro	Wells in test plate	0.04 µg/mL, 48 h 0.06 µg/mL, 24 h	(Skrobek et al., 2006)
TERRESTRIAL						
Mammals						
Destruxin A	Mice	LD50	In vivo	intraperitoneally	1–1.35 mg/kg	(Kodaira, 1961)
Destruxin B	Mice	LD50	In vivo	intraperitoneally,	13.2-16.9 mg/kg	(Kodaira, 1961)
Insects						
Destruxin A	Silkworm larvae	LC50	In vivo		0.015–0.030 mg/g, 24 h	(Kodaira, 1961)
Destruxin A	*Plutella xylostella* (Black diamond moth)	LC50	In vivo	leaf-dip assay contact assay	30 µg/mL, 4 d 56 µg/mL, 4 d	(Amiri et al., 1999)
Destruxin A	*Phaedon cochleariae* (mustard leaf beetle)	LC50	In vivo	leaf-dip assay contact assay	79 µg/mL, 4 d 87 µg/mL , 4 d	(Amiri et al., 1999)
Destruxin B	*Bemisia tabaci*	LC50	In vivo		96.5 µg/mL, 5 d	(Hu, 2009)
Destruxin B	*Phaedon cochleariae*	LC50	In vivo	leaf-dip assay contact assay	27 µg/mL, 4 d >500 µg/mL, 4 d	(Amiri et al., 1999)
Destruxin B	*Plutella xylostella*	LC50	In vivo	leaf-dip assay contact assay	25 µg/mL, 4 d 376 µg/mL ,4 d	(Amiri et al., 1999)
Destruxin E	*Phaedon cochleariae*	LC50	In vivo	leaf-dip assay contact assay	58 µg/mL, 4 d 50 µg/mL, 4 d	(Amiri et al., 1999)
Destruxin E	*Plutella xylostella*	LC50	In vivo	leaf-dip assay contact assay	17 µg/mL, 4 d 53 µg/mL, 4 d	(Amiri et al., 1999)
Crude extract V275	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	>500 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin A	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	5.2 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin B	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	>500 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin E	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	>500 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin A+B	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	12 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin A+E	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	9 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin B+E	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	> 500 µg/mL, 24 h	(Skrobek and Butt, 2005)
Destruxin A+B+E	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	8 µg/mL, 24 h	(Skrobek and Butt, 2005)
Fraction 1[1]	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	120 µg/mL, 24 h	(Skrobek and Butt, 2005)

Secondary metabolite	Test organism	Endpoint	In vivo/ vitro	Test system	Level of effect	Ref.
Fraction 2[1]	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	240 µg/mL, 24 h	(Skrobek and Butt, 2005)
Fraction 3[1]	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	> 500 µg/mL, 24 h	(Skrobek and Butt, 2005)
Fraction 4[1]	*Spodoptera frugiperda*	LC50	In vitro	SP9 cell line	110 µg/mL, 24 h	(Skrobek and Butt, 2005)

1: The crude extract batches were separated into four fractions with decreasing polarity (Skrobek et al., 2006)

Table 35 shows that SP6 cell line of *S. frugiperda* is most sensitive to destruxin A (5.2 µg/mL, 24 h). Other combinations of destruxins or fraction of the crude extract did not cause lower LC50 values. Skrobeb and Butt (2005) however suggest the use of different cell lines as the response of other types of cell lines can be different, as was shown for HL60 human leukemic cells. Unlike SF9 cells, these cell lines were more sensitive to the crude extract of *M. anisopliae* than to the pure destruxins (data not shown in Table 35).

According to Dudley et al. (2004), destruxin E is the most bioactive destruxin metabolite in tested insects. This is corroborated by Dumas et al. (1994) who wrote that in comparative studies, destruxin E often proves to be the most toxic of the destruxin family, probably because of its reactive epoxide group. The toxicity of destruxin E however seems to vary with the species: Housefly maggot indeed were more sensitive to destruxin E than to destruxin A or B. *Galleria* larvae were more sensitive to destruxin E than *Musca domestica* (see references in Vey et al. 2001a).

The study with target insects, *P. cochleariae* and *P. xylostella,* shows the lowest LC50 values varying from 0.017 to 0.376 µg/mL, depending on the type of destruxin and the type of assay. In general, leaf dip assays cause higher toxicity rates than contact assays.

4.2.4 Toxicity of Beauveria species

Beauveria spp. produce an array of SMs that play specific roles in antibiosis and pathogenicity (Cho et al., 2006a):

- Oosporein, a red dibenzoquinone, which can oxidize proteins and amino acids (El Basyouni and Vining 1966; Kucera and Samsináková, 1968; Seger et al., 2005). Oosporein displays antibiotic activity towards Gram-positive (but not Gram-negative) bacteria, and appears to cause avian gout in chickens and turkeys (Pegram et al., 1982a).
- Bassianin and tenellin, yellow pigments that affect membrane ATPases.
- Various antimicrobial hexadepsipeptides known as beauvericins.
- Small molecular mass organic compounds, such as oxalic acid, that play important roles in pathogenesis (Isaka et al., 2005; Jeffs and Khachatourians, 1997; Kagamizono et al., 1995; Kirkland et al., 2005; Takahashi et al., 1998).

Table 36. Toxicity data for *Beauveria* spp. or from other fungi-producing the same secondary metabolites.

Fungal MPCA	Secondary metabolite	Test organism	End-point	In vivo/ vitro	Test system	Level of effect	Ref.
AQUATIC							
Pure compound	oosporein	*Artemia salina*	LC50	In vivo	Bioassay	1.0 µg/mL	(Mao et al., 2010)
Pure compound	oosporein	*Artemia salina*	LC50	In vivo	Bioassay	Not toxic at 61.2 µg/mL	(Favilla, 2006)
Pure compound	oosporein	*Daphnia magna*	LC50	In vivo	Bioassay	68.4 µg/mL, 24 h	(Favilla, 2006)

Fungal MPCA	Secondary metabolite	Test organism	End-point	In vivo/ vitro	Test system	Level of effect	Ref.
						5.84 µg/mL, 24 h	
Fusarium subglutinans	beauvericin	*Artemia salina*	LD50	In vivo	Bioassay	2.8 µg/mL	(Moretti et al., 1995)
Fusarium subglutinans	beauvericin	*Mysidopsis bahia*	LD50	In vivo	Bioassay	560 µg/mL	(Moretti et al., 1995)
TERRESTRIAL							
Mammals							
Verticillium psalliotae	oosporein	Mice	LD50	In vivo	Feed	No effect at 7 mg/kg	(Wainwright et al., 1986)
Birds							
Beauveria spp.	oosporein	Turkeys, 0-3 weeks old	LC50	In vivo	Feed	1500 µg/g feed	(Pegram et al., 1982b)
Beauveria spp.	oosporein	1-d old male chickens	LD50	In vivo	Feed	6.12 mg/kg	(Manning and Wyatt, 1984)
Metarhizium anisopliae var. *acridum*	Mycosed[2] grasshoppers	Pheasants 9-25 d	mortality	In vivo	5-8 d post-inoculated grasshoppers	No mortality or any other effects	(Johnson et al., 2002)
Beauveria bassiana	Mycosed[2] grasshoppers	Pheasants 9-25 d	mortality	In vivo	5-8 d post-inoculated grasshoppers	No mortality or any other effects	(Johnson et al., 2002)
Metarhizium flavoviride	Mycosed grasshoppers[3] containing metabolites	Ring-necked pheasants	mortality; pathological effects	In vivo	7-12 d post-inoculated grasshoppers	No mortality or any other effects	(Smits et al., 1999)
Chaetomium trilaterale	oosporein	Broiler chickens	pathological effects	In vivo	Feed	No effect at 100 µg/g feed; At 200 µg/g feed: Mucosal necrosis, proventicular, hepatic and renal inflammation	(Pegram and Wyatt, 1981)
Chaetomium trilaterale	oosporein	Broiler chickens	mortality	In vivo	Feed	0, 13, 30, 57, and 95% for the 0, 200, 300, 400, and 600 µg/g levels	(Pegram and Wyatt, 1981)
Reptiles							
Metarhizium anisopliae var. *acridum*	Mycosed locust nymphs containing metabolites	*Acanthodactylus dumerili*	mortality; signs of toxicity; food consumption	In vivo	Fresh 4-d post-inoculated nymphs = field rate	No mortality No signs of toxicity No reduction	(Peveling and Demba, 2003)
Insects							
B. bassiana	beauvericin	*Galleria mellonella*	mortality	In vivo	injected	Depending on the toxin soure and concentration Maximally 48.8%[1]	(Safavi, 2013)

1: actual concentration of beauvericin /µL was unclear
2: 1-1.5x10^5 spores/insect
3: 5x10^4 to 1.2x10^5 spores/insect

4.2.5 Toxicity of Paecilomyces species

Table 37. Toxicity data of *Paecilomyces* spp. secondary metabolites.

Fungal MPCA	metabolite	Test organism	Endpoint	In vivo/ vitro	Test system	Level of effect	Ref.
AQUATIC							
P. fumosoroseus	Dipicolinic acid (DPA)	*A. salina*	LD50			44.5 mg/L	(Asaff et al., 2005)
TERRESTRIAL							
mammals							
P. fumosoroseus	bassianin, bassianolide, tenellin,	Mice	Acute toxicity	n.r.	n.r.	n.r.	(EFSA, 2013a)
Insects							
P. fumosoroseus	bassianin, bassianolide, tenellin,	*Poecilus versicolor,* Bee, *Diadegma semiclausum*, Silkworm larvae	Acute toxicity, Reduced longevity, Reduction cocoon production			13 ppm	(EFSA, 2013a)
P. fumosoroseus	dipicolinic acid (DPA)	*Bemisa tabaci* *B. argentifolii*	mortality			32.3% mortality at 41 mg/L (ctrl mortality 16.3%)	(Asaff et al., 2005)
P. fumosoroseus	dipicolinic acid (DPA)	*Calliphora erythrocephala*	LD50	In vivo	Topical application	42 µg/fly	(Claydon and Grove, 1982)
P. fumosoroseus	dipicolinate dimethyl ester	*Bemisa tabaci*				No effect	(Asaff et al., 2005)
P. fumosoroseus	dipicolinate dimethyl ester	*Calliphora erythrocephala*	LD50	In vivo	Topical application	No effect at 50 µg/fly	(Claydon and Grove, 1982)

n.r.= not reported

4.2.6 Toxicity of Lecanicillium species

No information was given in the EFSA report on microbial organisms (EFSA, 2013b).

4.2.7 Toxicity of Isaria species

The EFSA conclusions on *Isaria fumosorosea* strain Apopka 97 (EFSA, 2013b) mention that the RMS considered that beauverolides are of no concern on the basis of an Ames test (negative). However the list of endpoints mentions that further data may be required pending on the identification/quantification of toxins/SMs. EFSA considered that the information provided on beauverolides was not sufficient.

An acute oral mouse toxicity study (LD_{50} > 5000 mg/kg bw) was performed with a mixture of beauverolides from strain Apopka 97.

4.2.8 Toxicity of Aschersonia aleyrodes species

No information was given in the EFSA report on microbial organisms (EFSA, 2013a).

4.2.9 Toxicity of Nomuraea species

No information was given in the EFSA report on microbial organisms (EFSA, 2013a).

4.3 Other fungi

4.3.1 Toxicity Trichoderma species

The genus *Trichoderma* includes species that can cause opportunistic infections in humans. These species belong to the *brevicompactum* clade. This clade is however not closely related to the species that have biological control applications.

Nielsen (2005) reported that mycotoxins from *Trichoderma* spp. include the cytotoxic and immunosuppressive trichothecenes (trichodermin, harzianum A), gliotoxin, and ribosome-inactivating proteins. Other bioactive metabolite groups are peptides and peptaibols, volatile pyrones and lactones, isonitriles, and various low-molecular weight compounds.

Some toxicity data for *Trichoderma* spp, SMs are given in Table 38. No useful toxicity data on trichodermin, harzianum A and gliotoxin produced by *Trichoderma spp.* were found by the author.

Table 38. Toxicity data of *Trichoderma* spp. secondary metabolites for several groups of non-target organisms.

Secondary metabolite	species	Known non-target	Observed non-target effect/dose	Reference
Invertebrates				
Alamethicin(s)	*T. viride*	*Artemia salina*	24-h LC50 = 10.4 µg/mL[5] 36-h LC50 = 3.84 µg/mL[5]	Ref. in (Favilla, 2006)
Alamethicin(s)	*T. viride*	*Daphnia magna*	24-h LC50 = 2.3 µg/mL[5] 36-h LC50 = 1.9 µg/mL[5]	Ref. in (Favilla, 2006)
Harzianum A[4]		There are no good GLP studies of the toxicity of harzianum A.		(Degenkolb et al., 2008a)
Paracelsin(s)	*T. spp.*	*Artemia salina*	24-h LC50 = 40.8 µg/mL[5] 36-h LC50 = 18.6 µg/mL[5]	Ref. in (Favilla, 2006)
Paracelsin(s)	*T. spp.*	*Daphnia magna*	24-h LC50 = 14.8 µg/mL[5] 36-h LC50 = 10.8 µg/mL[5]	Ref. in (Favilla, 2006)
Mollusca				
Alamethicin and different groups of peptaibols	*T. longibrachiatum*	*Crassostrea gigas* embryo's	EC50 ranging from 10 to 64 nM	(Poirier et al., 2007)
Mammals				
Paracelsin(s)	*T. spp.*	Mice, intraperitoneally	LD50 = 5 mg/kg	Ref. in (Favilla, 2006)
Alamethicin(s)	*T. viride*	Mice, oral	LC50 = 80 mg/kg	Ref. in (Favilla, 2006)
Antiamoebin, trichotoxin A, Aibellin, Ampullosporn, trichofumin		Oral administration to rodents and ruminants (cattle and sheep)		References in (Degenkolb et al., 2008b)
Trichodermin[4]		There are no good GLP studies of the toxicity of trichodermin		(Degenkolb et al., 2008a)
No toxicity information				
Trichostromaticin(s)[1]		No information found		(EFSA, 2013a)
Trichotoxin(s)[1]		No information found		(EFSA, 2013a)

Secondary metabolite	species	Known non-target	Observed non-target effect/dose	Reference
6-pentyl-2H-pyran-2-one[2]	*T.* spec *T. harzianum*	No information found		(EFSA, 2013a)
6-pentyl-alpha-pyrone[2]	*T.* spec *T. harzianum*	No information found		(EFSA, 2013a)
Viridiofungin A[3]	*T. viride* *T. harzianum*	No information found		(EFSA, 2013a)
Plants				
Trichodermin	*T. viride*	Plants: bean and corn		(Cutler and LeFiles, 1978)

1: category within the peptaibiols (peptabiotics). These are known to destroy the cell-wall structure of plant pathogens or inhibiting the protein and DNA synthesis in the cells of the pathogens thus blocking their growth. An extensive review on these linear and cyclic peptide antibiotics has been made by Degenkolb and Brückner (Degenkolb and Brückner, 2008)
2: Volatiles active against fungi and nematodes, mode of action not fully understood
3: member of amino alkyl citrate antibiotics, broad spectrum fungicidal activity by inhibiting fungal growth through the inhibition of squalene synthase
4: trichothecene-type mycotoxin
5: An alternative explanation of the toxicity might be that the batch of the alamethicin standard used (Sigma-Aldrich, product number A-4665) may have been contaminated with the trichothecene mycotoxin harzianum A that is produced by the strain of Trichoderma cf. brevicompactum used for alamethicin fermentations (Degenkolb et al., 2008b).

Cited from the website of IAQM (http://www.iaqm.com/trichothecene.html). An email has been sent at 18-3-2015 to IAQM asking for a list of references.

Modes of action of trichothecenes.
The mechanisms by which trichothecenes produce toxicity are varied, and their relative importance in producing illness is not fully understood (Coulombe, 1993). They include the following:

- Inhibition of protein synthesis, thought to be the most important effect (Ueno, 1983); (Ueno, 1984); (Tutelyan and Kravchenko, 1981).
- Inhibition of DNA synthesis (Thompson and Wannemacher, 1984), which might contribute to their radiomimetic properties.
- Impairment of ribosome function (Coulombe, 1993); (Tutelyan and Kravchenko, 1981).
- Inhibition of mitochondrial protein synthesis (Pace *et al.*, 1988).
- Induction of reparable single strand breaks in DNA.
- Immunosuppression, allowing secondary and opportunistic bacterial infections and possibly delayed hypersensitivity; (Ueno, 1983); Yarom et al., 1984; Jagadeesan *et al.,* 1982).

Trichothecenes react readily with thiol groups at low concentrations and inhibit thiol enzymes (e.g., creatine kinase, lactate dehydrogenase) (Tutelyan and Kravchenko, 1981; Ueno, 1984). Unlike the aflatoxins that require metabolic activation, the trichothecenes are directly toxic without activation, as is suggested by their prompt effects on the gastrointestinal mucosa with epithelial cell necrosis (Busby and Wogan, 1979).

The following toxicity data were also obtained from the IAQM website (http://www.iaqm.com/trichothecene.html). The full references were not provided on this website.

From these data, it is not clear which SMs of the trichothecene group, or which combinations of trichothecene metabolites were involved in these studies. This is an important omission since only a few trichothecenes are produced by *Trichoderma* spp. An important conclusion that can be made from these data is that toxicity of trichothecenes is high.

Skin exposures
5-50 ng in liquid Min. erythema dose (guinea pig, rat), Ueno/Wannemacher et al. (1983)
209 ng/cm2 in liquid Min. erythema dose (monkey), Wannemacher/Bunner et al. (1983)
1 µg/cm2 Irritation (guinea pig, rabbit), Fairhurst et al. (1987)
2 µg Vesication, skin injury Bunner (1983)
0.25 mg/kg Severe illness, diarrhea (monkey), Bunner et al. (1983)

1.5 mg/kg in DMSO LD50 (rat; mean time to death, 19 hr.), Wannemacher (1983)
4.2 mg/kg in methanol LD50 (guin pigs; time to death 190 hr.), Wannemacher et al. (1983)

Eye
1 µg Detectable corneal injury, USAMRIID (1983)
2 µg Severe corneal injury, conjunctivitis, Bunner (1983)

Respiratory
0.24 mg/kg (abs) Mouse LD50, Creasia et al. (1987)
0.05 mg/kg (abs) Rat LD50, Bunner et al. (1985)
0.6-2.0 mg/kg (abs) Guinea pig LD50, Wieser (1997, p. 661)
5,479 mg-min/m3 Guinea pig LD50 AD, Little (1986)
200-1,800 mg-min/m3 Estimated LCT50, U.S. Army (1990)

Systemic Toxicity
500 µg/kg Estimated human LD50, U.S. Army (1990)
470 µg/kg intram. Rat LD50, Bunner et al. (1985)
1.17 mg/kg Rat LD50, Bunner et al. (1985)
650 µg/kg intram. Monkey LD20, Cosgriff et al. (1986)
790 µg/kg intraven. Monkey LD50, AD Little (1986)
850 µg/kg intram. Rat LD50, Chan and Gentry (1984)
111 mg/kgintram. Rabbit LD50, Chan and Gentry (1984)

Oral Toxicity
0.1-0.2 mg/kg Swine, emesis, Busby and Wogan (79)
0.1-1.0 mg/kg Swine, diarrhea, Ueno (1983a)
2.29 mg/kg Rat LD50, Bunner et al. (1985)
3.06 mg/kg Guinea pig LD50, AD Little (1986)
1.0 mg/kg Male monkey LD100, Rukimi, Prasad, and Rao (1980)
1 5 mg/kg Estimated human LD50, U.S. Army (1990)

Following table is copied from (Franz, 1997). The trichothecenes produced by *Trichoderma* spp. (trichodermin, harzianum A) were not included.

Table 39 Relative acute parenteral toxicity of the most abundant trichothecene mycotoxins.

Trichothecenes Tested	Mammals Tested							
	Mouse	Rat	Guinea Pig	Rabbit	Cat	Dog	Pig	Monkey
	LD_{50} (mg/kg)							
T-2 Toxin	5.2 (IV)	0.9 (IV)	1.0 (IV)	1.0 (IM)	< 0.5 (SC)	—	1.2 (IV)	0.8 (IM)
HT-2 Toxin	9.0 (IP)	—	—	—	—	—	—	—
4,15-Diacetoxy-scripenol (DAS)	12.0 (IV)	1.3 (IV)	—	1.0 (IV)	—	1.1 (IV)	0.38 (IV)	—
Nivalenol	6.3 (IV)	—	—	—	—	—	—	—
Deoxynivalenol (DON)	43 (SC)	—	—	—	—	—	—	—
Verrucarin A	1.5 (IV)	0.8 (IV)	—	0.54 (IV)	—	—	—	—
Roridin A	1.0 (IV)	—	—	—	—	—	—	—
Satratoxin H	1.0 (IP)	—	—	—	—	—	—	—

Routes of administration of the mycotoxin: IV: intravenous; IM: intramuscular; SC: subcutaneous; IP: intraperitoneal
—: Not determined
Data sources: (1) Ueno Y. Trichothecene mycotoxins: Mycology, chemistry, and toxicology. *Adv Nut Res*. 1989;3:301–353. (2) Wannemacher RW Jr, Bunner DL, Neufeld HA. Toxicity of trichothecenes and other related mycotoxins in laboratory animals. In: Smith JE, Henderson RS, eds. *Mycotoxins and Animal Foods*. Boca Raton, Fla: CRC Press; 1991: 499–552. (3) Sharma RP, Kim Y-W. Trichothecenes. In: Sharma RP, Salunkhe DK, eds. *Mycotoxins and Phytoalexins*. Boca Raton, Fla: CRC Press; 1991: 339–359. (4) Jarvis BB. Macrocyclic trichothecenes. In: Sharma RP, Salunkhe DK, eds. *Mycotoxins and Phytoalexins*. Boca Raton, Fla: CRC Press; 1991: 361–421.

4.3.2 Toxicity of Gliocladium species

Toxicity values of metabolites produced by *Gliocladium* are given in Table 40.

Table 40. Secondary etabolites of *Gliocladium* spp. fungal biocontrol agents (Favilla, 2006).

Metabolite	Producing BCA(s)	Chemical nature	Toxicity data	References
Antiamoebin(s)	*Gliocladium catenulatum*[a]	Polypeptide	Antiprotozoal-anthelmintic	Thirumalachar (1968)
			Human erythrocytes, $CC_{50} = 125$ µM	Brückner et al. (1984)
Gliotoxin	*Gliocladium virens*[a]	Epidithiodiketo-piperazine	Antibacterial, antifungal	Johnson et al. (1943)
			Mice, intraperitoneally, $LD_{50} = 25$ mg/kg	Johnson et al. (1943)
			Lepidopteran SF-9 cell line, $CC_{50} = 4$ µM	Fornelli et al. (2004)
			Human lung carcinoma A549 cell line, $IC_{50} = 0.3$ µM	Kreja and Seidel (2002)
			Human HL-60 cell line, $CC_{50} = 100$ µM	Macchia et al. (2003)
			Human U-937 cell line, $CC_{50} = 200$ µM	Macchia et al. (2003)
			Artemia salina, LC_{50} at 16 h = 3.5 µg/ml	Harwig and Scott (1971)

4.3.3 Toxicity of Stagonospora species

Toxicity values of metabolites produced by *Stagonospora* species are given in Table 41.

Table 41. Secondary metabolites of *Stagonospora* spp. fungal biocontrol agents (Favilla, 2006).

Metabolite	Producing BCA(s)	Chemical nature	Toxicity data	References
Elsinochrome A	*Stagonospora* spp.[c]	Perylenequinone	Monkey embryo R366.4 cell line, CC_{50} at 5 min = 100 μM	Ma et al. (2003)
			Human Hce-8693 cell line, CC_{50} at 5 min = 100 μM	Ma et al. (2003)

Alamethicin (ALA), paracelsin (PCS) and antiamoebin (AAM), belong to the family of peptaibols. Peptaibols are short-chain linear peptides, typically made of 15–20 residues, which are produced by a number of fungal genera, including *Trichoderma, Gliocladium, Acremonium, Paecilomyces*, and *Emericellopsis* (Degenkolb et al., 2003). Toxicity data are given in Table 42 and Table 43. All experiments were performed with purified SMs.

Cited from Favilla (2006) "Possible use of invertebrate bioassays for testing the toxigenicity of BCAs and for the preliminary evaluation of the general toxicity of their metabolites. In fact, BCA strains are able to synthesize a plethora of bioactive metabolites with different structures and modes of action (Taylor, 1986), some of which may be still unknown. Moreover these metabolites may have additive or synergistic toxic effects. Therefore, 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 organisms characterized by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites. Based on this approach, *A. salina* or *D. magna* bioassays could be used by the industry for inexpensive and rapid screening of new candidate BCAs at an early stage of development. If appreciable toxicity is found, it might be considered not to proceed with the development of a BCA before any toxicity testing is carried out in appropriate vertebrate models. Based on the findings presented herein, *D. magna* appears to be a more sensitive and reproducible invertebrate model for testing BCA toxins than *A. salina*, and therefore it may be preferable for regulatory purposes".

Table 42. LC50 values of fungal BCA toxins to brine shrimps (Favilla, 2006).

LC_{50} of fungal BCA toxins to brine shrimps (*A. salina*)

Compound	MW	Exposure (h)	LC_{50} (95% confidence) (μM)	Slope	LC_{50} (μg/ml)
Alamethicin	1960	24	5.32 (4.19–6.87)	1.697	10.43
		36	1.96 (1.53–2.46)	1.678	3.84
Paracelsin	1921	24	21.26 (16.53–29.58)	1.603	40.84
		36	9.66 (7.63–12.73)	1.675	18.56
Antiamoebin	1671	24	19.79 (15.49–27.31)	1.954	33.07
		36	8.25 (6.14–11.67)	1.806	13.79
Gliotoxin	326	24	39.49 (27.96–61.90)	1.990	12.87
		36	20.44 (17.28–24.44)	1.874	6.66
Destruxin A	578	24	16.92 (13.40–21.67)	1.597	9.78
		36	5.05 (3.87–6.61)	2.459	2.92
Oosporein	306	24	ND[a]	–	ND[a]
		36	ND[a]	–	ND[a]
Elsinochrome A	544	24	20.18 (18.81–21.64)	3.705	10.98
		36	15.26[b]		8.30[b]

[a] Not determined. The maximal concentration tested (200 μM = 61.2 μg/ml) was not toxic at 24 h and gave 32.58 ± 1.6% mortality at 36 h.
[b] Inferred from dose–mortality curve (see text).

Table 43. LC50 values of fungal BCA toxins to water flea (Favilla, 2006).

LC_{50} of fungal BCA toxins to water flea (*D. magna*)

Compound	MW	Exposure (h)	LC_{50} (95% confidence) (μM)	Slope	LC_{50} (μg/ml)
Alamethicin	1960	24	1.19 (1.08–1.30)	4.854	2.33
		36	0.99 (0.84–1.15)	3.753	1.94
Paracelsin	1921	24	7.70 (6.83–8.77)	4.516	14.79
		36	5.60 (4.72–6.77)	3.933	10.76
Antiamoebin	1671	24	14.61 (11.96–19.33)	3.246	24.41
		36	7.17 (6.43–8.02)	3.613	11.98
Gliotoxin	326	24	0.85 (0.67–1.06)	2.104	0.28
		36	0.50 (0.39–0.62)	3.535	0.16
Destruxin A	578	24	0.35 (0.31–0.38)	9.103	0.20
		36	0.27 (0.25–0.29)	9.472	0.16
Oosporein	306	24	223.54 (166.30–357.93)	1.985	68.40
		36	19.10 (11.51–27.40)	2.745	5.84
Elsinochrome A	544	24	0.53 (0.42–0.69)	3.978	0.29
		36	0.40 (0.33–0.49)	4.620	0.22

4.4 Bacteria

4.4.1 Toxicity of Streptomyces species

Table 44 shows that only few data are available on the toxicity of secondary metabolites produced by *Streptomyces* spp.

Table 44. Toxicity data of *Streptomyces* spp. secondary metabolites.

Secondary metabolite	species	Known non-target	Observed non-target effect/dose	Reference
Prodigiosin	*S. griseoviridis*	*Alteromonas* sp. *Gallionella* sp. (target bacteria on ships; antifouling purpose) *Artemia parthenogenetica*	MIC = 6.75 μg/mL, MBC = 12.5 μg/mL LD50 = 50 μg/mL	(Priya et al., 2013)
Viridogrisein Dechlororoseophilin Actinobolin Roseophilin Coronamycin Griseoviridin	*S. griseoviridis*		No information available	(EFSA, 2013a)
Geldanamycin	*S. hygroscopicus* var. *geldanus* and others	mammals	weak antibiotic activity potent antitumor activity	http://geldanamycin.info /index.htm

4.4.2 Toxicity of Pseudomonas species

Table 45 shows that only few data on the toxicity of SMs produced by *Pseudomonas* species were found in the EFSA document (EFSA, 2013).

Table 45. Toxicity data of *Pseudomonas* spp. secondary metabolites.

Secondary metabolite	species	Known non-target	Observed non-target effect/dose	Reference
Viscosinamide	*P. fluorescens*	*Hartmanella vermiformis* (microorganism)	Growth inhibition at 1.9 ng/mL	(EFSA, 2013a)

4.4.3 *Toxicity of Bacillus species*

Table 46 shows that no information was found on the toxicity of SMs produced by *Bacillus* species in the EFSA document (EFSA, 2013).
It is further acknowledged that *Bacillus* spp. is a very wide genus a it would need o lot of effort to cover them in this section. Table 46 is therefore certainly incomplete.

Table 46. Toxicity data of *Bacillus* spp. secondary metabolites.

Secondary metabolite	species	Known non-target	Observed non-target effect/dose	Reference
Iturin A, Surfactin, Fengycin, Bacillomycin, Mycosubtilin, Zwittermicin, Difficin, Amphomycin, Azalomycin, Arhtrobactin, Valinomycin, Bacillibactin Plantazolicin, Macrolactin, Bacillaene, Bacilysin, chlorotenaine	*B. amyloliquefaciens, B. subtilis, B. pumilus, B. cereus*		No information found	(EFSA, 2013a)
enterotoxins	*B. cereus*			
surfactin	*B. subtilus*	rat		(Hwang et al., 2009)

4.4.4 *Toxicity of other bacterial species*

No toxicological data have yet been found in the literature for *Agrobacterium, Erwinia, Serratia* and *Xanthomonas* spp. For *Burkholderia* spp. insecticidal and miticidal activities have been described by Cordova-Kreylos et al. (2013).

4.5 Relation between exposure and toxicity

Many studies presented in the tables above have been performed in a range of concentrations to establish the LC50 of a particular SM for a particular species. In the studies with *Metarizhium anisopliae,* the target insects *Plutella cochleariae* and *P. xylostella,* show the lowest LC50 values vary from 0.017 to 0.376 µg/mL, depending on the type of destruxin and the type of assay. In general, leaf dip assays cause higher toxicity rates than contact assays.

The question is how results of metabolite toxicity studies can be used for risk assessment purposes.
All SM toxicity studies presented in the tables mimic spray applications of a product, assuming that spray applications will contain SMs. For products with entomopathogenic fungi this may not be true as the MPCA can be free of SMs when using certain manufacturing techniques (such as sieving the spores). It should be realized that concentrations of SMs that are formed in the host do not have any relation with concentrations of the same metabolites in the sprayed product.

In case SMs are also formed during the manufacturing process and remain present in the end product, the question needs to be posed whether amounts of SMs would become a problem in the risk assessment. The different types of exposure that are identified are summarized below:

Exposure operator/worker/bystander

In this Background document no data have been included for operator/worker/bystander as these were not available in the main literature searched. The conclusions below mainly focus on the environmental

risk assessment, however, many scenarios are also relevant for humans, and can be used in the human risk assessment.

Exposure non-target organisms

Direct contact with SMs in the sprayed product (SMs present as a result of accumulation in the fermentation broth, and sprayed along with the active substance, depending on the type of formulation). Exposure may not be of concern for entomopathogenic fungi when concentrations of SMs are low in the MPCP and/or and degrade rapidly (either on the leaf surface or in the soil). The applicant should be able to present analytical details on the presence of SMs in the MPCP. A worst case calculation can be performed in case toxicity endpoints are available (see Box 2). An LC50 value for a target species is particularly useful as the target species is a sensitive species per definition.

Box 2. Calculation of acceptable concentrations of secondary metabolites in end product of an entomopathogenic fungus

For this calculation, the lowest LC50 value for a sensitive target insect *Plutella xylostella* is used, i.e. of 17 µg destruxin E/mL (see Table 35 in Background Document 4).

In the field:

In order to obtain these effects the sprayed MPCA should contain at least 17 mg destruxin E/L. For this calculation, it is assumed that the spray solution is obtained by mixing 1 L of product in 1000 L of water.
In order to have a concentration of 17 mg destruxin E/L, the liquid formulation itself should contain 17,000 mg destruxin E/L. This can be compared with the concentrations of destruxin E as indicated by the applicant. Table 25 in Background Document 1 gives a value of 97 ± 146 mg destruxin E/L in the production medium. This calculation shows that for the most sensitive target insect, contact or feeding mortalities are not expected to occur as a result of the presence of SMs in field applications when the MPCP contains 97 mg of destruxin E. In this example the levels of destruxin E in the application solution of the field application is 175 times lower than the lowest LC50 value for the most sensitive target insect.

Off-field

For the off-field area, decreasing exposure/hectarage should be considered as only a small percentage of the application rate is transported out of field via drift. If the off-field area is vegetated the vegetative structure should lead to a further dilution of drifted material (Barrett et al., 1994).

Conclusion deriving from Box 2:

Studies with a NOEL as the endpoint could be used to perform a similar calculation to consider sublethal effects. These studies were not available.

It is advised to minimize the quantities of toxic metabolites in the final product to below sublethal levels in sensitive hosts.

For synthetic plant protection products ESCORT 2 acknowledges that transient in-field effects could occur. These in-field effects are acceptable if a *potential for recovery* can be demonstrated, i.e. if aging of test item on plant matrices sufficiently reduces toxicity to a level at which recovery of NTA populations can take place.

End products containing herbicidal MPCAs could contain SMs in the high possible concentrations as the SMs in the MPCP are part of the mode of action. The mode of action of these SMs may be restricted to interfering with the plant metabolism.

This should be taken into consideration when effect levels on non-target organisms are not available.

Exposure to secondary metabolites produced *in vivo* (= in contact with pathogen) in different compartments

- *Insect:* SMs are produced, after application of the product, by entomopathogenic fungi.
- *Canopy*: SMs are produced, after application of the product, by micro-organisms in contact with pathogenic fungi on the canopy. This concerns fungal and bacterial control agents such as *Pseudomonas*, *Bacillus*, *Trichoderma* spp.
- *Soil:* metabolites are produced, after application of the product, by micro-organisms in contact with pathogenic fungi in the soil. This concerns fungal and bacterial control agents such as *Bacillus, Pseudomonas, Burkholderia* and *Streptomyces* spp. which live in the rhizosphere. Any SMs that will be formed are formed in this micro-niche. In bacteria, production of SMs is regulated by quorum sensing upon reaching a certain population density (see Background document 3). In general, the soil is an extremely versatile ecosystem and effects on soil micro-organisms, if occurring, are transient.

- Effects on soil micro-organisms by chemical agents are more pronounced than those from biological control agents (Scheepmaker and Kassteele, 2011). In this meta-analysis the effects were always transient. It can be stated that if effects on soil micro-organisms are not observed further studies of effects of SMs on soil micro-organisms is not deemed necessary.
- Effects of secondary metabolites on earthworms and soil-dwelling arthropods also need to be considered after exposure to soil. Endpoints for these organisms were not found in the literature so far, which indicates that there has not been an incentive to study the effects of SMs on these organisms. Apparently, there was no urgent reason to study the effects of SMs on these organisms.

- *Secondary poisoning birds/mammals/amphibians/reptiles*
  - Consumption of diseased/deceased insects: Feeding experiments with *Beauveria* spp.- or *Metarhizium* spp.- mycosed locusts performed by Johnson et al. (2002) and Smits et al. (1999) showed that at realistic field rates, no toxic effects are seen in pheasants. However, in the EFSA review of *M. anisopliae* F52, EFSA set a data gap for the risk to small insectivorous birds. Pheasants were thought to be too big to be used in risk assessment.

    No effects at field rate were demonstrated for the reptile *Acanthodactylus dumerili* (Peveling and Demba, 2003). It is important to mention that the mycosed insects were readily eaten by the birds and reptiles, but the test animals were probably not offered any choice situation. It should be kept in mind that several other oosporein producers such as the pathogenic fungus *Chaetomium aureum* produce considerably higher quantities (Table 31 in Background document 1) compared to oosporein-producing strains of *Beauveria* spp.
  - Consumption of mycosed granules:
    Table 27 in Background document 1 on the quantities of SMs produced by *Beauveria* spp., shows that only 3.27 µg oosporein/g grain is produced by *B. brongniartii.* Levels of 200 µg oosporein/g grain produced by *Chaetomium trilaterale* (Background document 4, Table 36) caused effects in broiler chickens whereas a lower concentration of 100 µg oosporein/g grain did not cause any effects. Using these toxicity data it can be calculated that one chicken can consume 30.6 g of mycosed grain (100/3.27) without any apparent effects. A consumption of 61.2 g (200/3.27) would give effects of mucosal necrosis, proventicular, hepatic and renal inflammation.

4.6 References

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5 DEGRADATION IN THE ENVIRONMENT

5.1 Research question to be answered

Review the literature with respect to degradation in the environment, considering the stability of the SM and how this may affect potential effects on target and non-target organisms.

5.2 Relevance of the question

The persistence of the SM in the environment is of great importance in the risk assessment. If it can be adequately demonstrated that the relevant SM is not persistent outside the microorganisms, fewer (if any) data requirements are needed to conduct a risk assessment. Like any other substance, the fate of SMs is influenced by several factors, depending on the exact location theSMs are found.

For risk assessment purposes it is important to discern between the possible location of the SMs.Possible locations of the SM that are of interest are:

- the submerged medium;
- the hemolymph of infected insects;
- the hemolymph post mortem;
- the plant;
- the vial (extracted and stored)

At each of these locations several factors may play a role in the degradation of the SMs:

- the intrinsic stability;
- edaphic factors (soil texture, organic matter content, pH);
- biotic factors (micro-organisms);
- detoxification induced by the immune system of the host.

5.3 Results

Following subsections present an overview of degradation of SMs at different locations.

5.3.1 Persistence secondary metabolites entomopathogenic fungi

5.3.1.1 Submerged medium

Production of SMs is influenced by several parameters such as strain type, nutrients compositions of the culture medium and cultivation conditions. Figure 23 was prepared using data from Amiri-Besheli *et al.* (2000) and shows that peaks of destruxin production differ among destruxin A, B and E for quantities and time of production. Very similar production is found by Wang *et al.* (2004) as shown in Figure 24.

The causes of the decline of SMs in the medium could be a combination of intrinsic (un)stability and degradation by enzymes. Also, the decline indicates the existence of metabolic pathways. As the presence of some metabolic products can be very short-lived, they may not be identified in the study.

It is not useful to speculate on the persistence of SMs using data from submerged cultivations as they are not representative of the persistence *in vivo.* It does however show that during submerged cultivation metabolites are not stable. The actual level of SMs is the result of an increase of newly produced metabolites and a decrease as the result of degradation/further metabolisation.

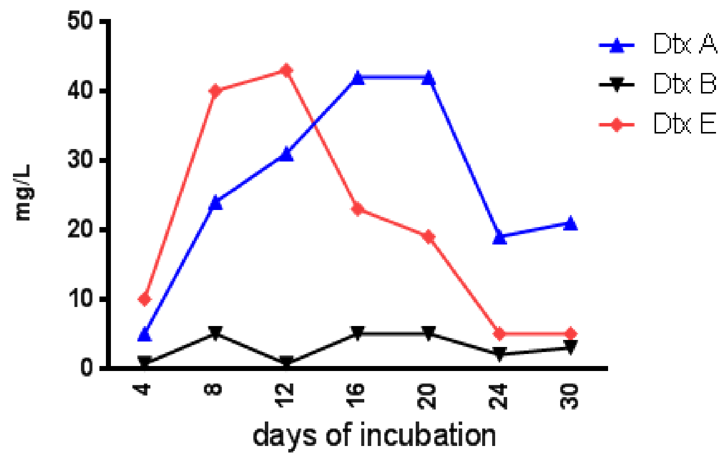

Figure 23. Time course for production of destruxins by *Metarhizium. anisopliae* V245 in liquid medium (using data from Amiri-Besheli et al., 2000).

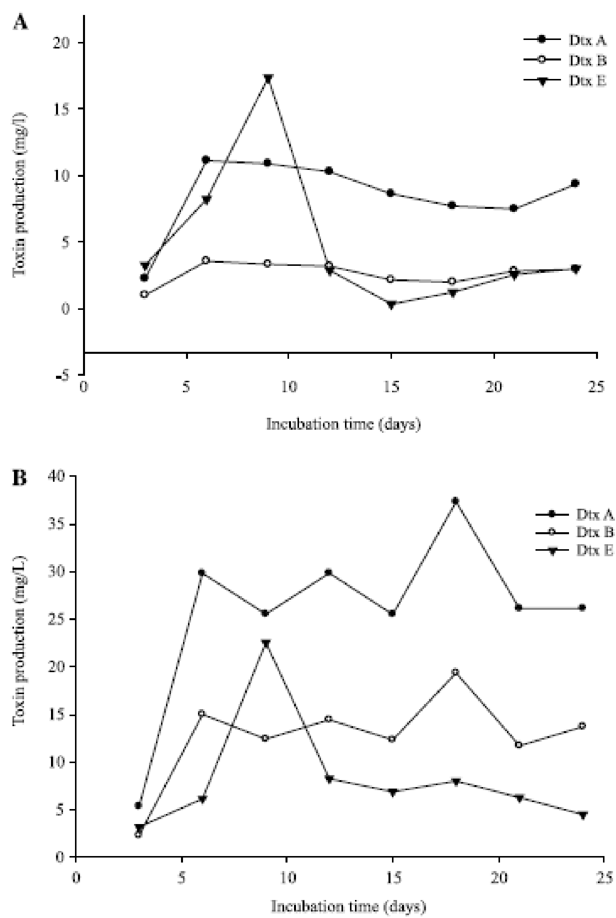

Figure 24. Toxin production dynamics of strains V245 (A) and V275 (B) in Czapek Dox liquid medium (Wang et al., 2004).

5.3.1.2 *Hemolymph of infected, still living insects*

Penetration of the entomopathogen into the host hemolymph has shown to induce a strong immune response (Dubovskiy et al., 2013). They also showed that antimicrobial peptides (AMP) and immunity related genes in larvae of *Galleria mellonella* were upregulated by the presence of *B. bassiana* and *M. anisopliae.*

Figure 25 shows the metabolic pathway of destruxin E in *G. mellonella* larvae as proposed by Hubert et al. (1999). Mass spectrometric analysis of the destruxin E in hemolymph extracts shows that metabolic by-products appear in the hemolymph of *G. mellonella* larvae within 10 minutes to 24 hours after incubation. The resulting metabolic by-products show lower activity than destruxin E (Liu and Tzeng, 2012).

Skrobek et al. (2008) showed that destruxin production was absent in infected, but still living *Galleria* larvae using *M. anisopliae,* strain V275. Low levels of destruxin A and B were produced by strain V245 (1.3 and 0.6 µg/insect 4 days after inoculation, destruxin A and B, respectively). The larvae died after 4-4.5 days. The same experiment in *Tenebrio* larvae showed that both strains produced destruxin A and B in almost similar quantities (strain V245: 2.2 and 1.3 µg/insect 6 days after inoculation, destruxin A and B, respectively; strain V275: 2.2 and 1.1 µg/insect 6 days after inoculation, destruxin A and B, respectively). Insects died after 7 days (both strains).

Metabolite	m/Z	Duration
DE (*30*)	593	0 min
DEdiol (*35*)	610	10 min~24 h
DEdiolP (DEdiolS) (*43/44*)	690	1 ~ 24 h
DESCys (*45*)	713	10 min~24 h
DESG (*46*)	899	10 min~24 h

Figure 25. The proposed metabolic pathway of destruxin E in G. mellonella larvae (Liu and Tzeng, 2012).

It can be concluded that the ability of the entomopathogenic fungus to produce SMs depends on various host-pathogen interactions, e.g., genotype, host physiology, immunological reactions, detoxification processes, etc.

5.3.1.3 Hemolymph post mortem

In the figures below the levels of destruxin A, B and E are given after infection of *G. mellonella* with *M. anisopliae* strains V275[4] (= strain F52) and V245, modified from Skrobek et al. (2008). Data are presented for *G. mellonella*. Destruxin levels declined marginally slower in *Tenebrio.* Data were therefore only given for *G. mellonella*.

Experiments were performed under laboratory, greenhouse and field conditions.
Laboratory experiments were performed at 26 °C in the dark. Destruxin levels in mycosed cadavers were assessed from the day of death until 10 days post mortem (Figure 26 and Figure 27).
In greenhouse experiments (Figure 28), dead insects were buried in four types of soil (sterilized or not). Cadavers and the surrounding soil were sampled on days 0, 5, 10 and 21 after burying the cadavers.
In field experiments (Figure 29), cadavers were buried 10 cm deep in the field (sandy loam). Samples were taken on days 0, 5, 10, 21 and 28 after burying the cadavers.

Some representative data were brought into figures below, in order to show the most prominent results. Figure 26 and Figure 27 show the decrease of destruxins in cadavers of *G. mellonella* infected by *M. anisopliae* V245. Data of *M. anisopliae* V275 were quite similar and were not presented in these figures. The figures show a strong decrease in destruxin levels, especially destruxins B and E, within the first 72 h of host death indicating intrinsic instability and the possible involvement of host enzymes in destruxin detoxification (Skrobek et al. 2008). In Figure 27 the conidia were removed from the cadavers before extraction of the destruxins. Clearly, this action resulted in fewer destruxins 3 days post mortem. According to the authors this show that destruxins are present in infected insects at death but are quickly transported into conidia during sporulation.

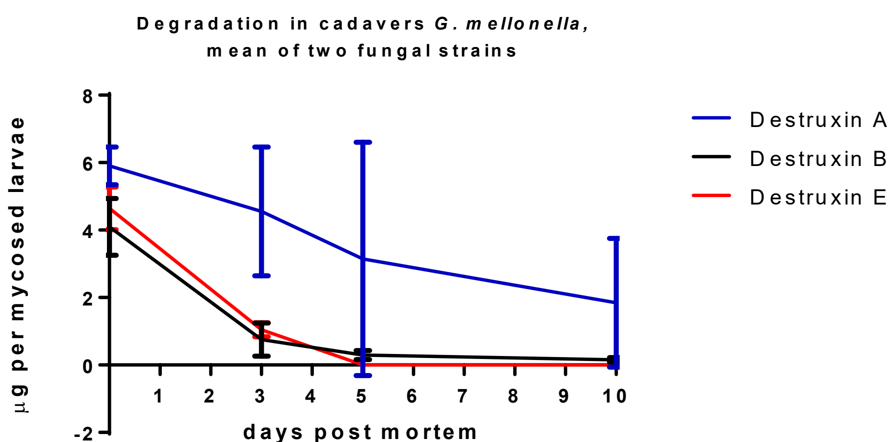

Figure 26. Time course for production of destruxins by *Metarhizium anisopliae* V245 in G. mellonella cadavers (modified from Skrobek et al. 2008).

[4] Strain BIPESCO 5 and F52 are closely related strains but probably not identical. Strain F52 corresponds to V275

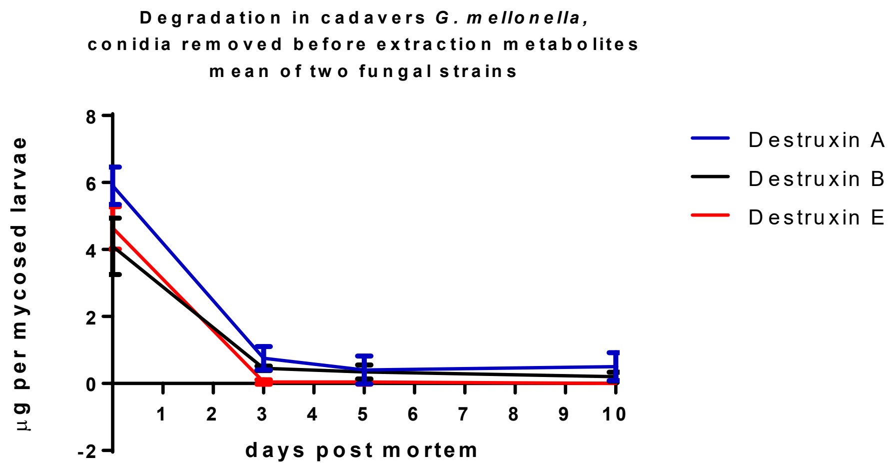

Figure 27. Time course for production of destruxins by *M. anisopliae* V245 in *G. mellonella* cadavers, after conidia being removed (modified from Skrobek et al. 2008).

Figure 28 and Figure 29 show the decrease of destruxins A, B and E in cadavers of G. mellonella in non-sterilized soil under greenhouse and field conditions, respectively. Data on strain V275 and *Tenebrio molitor* were not presented as these were quite similar.

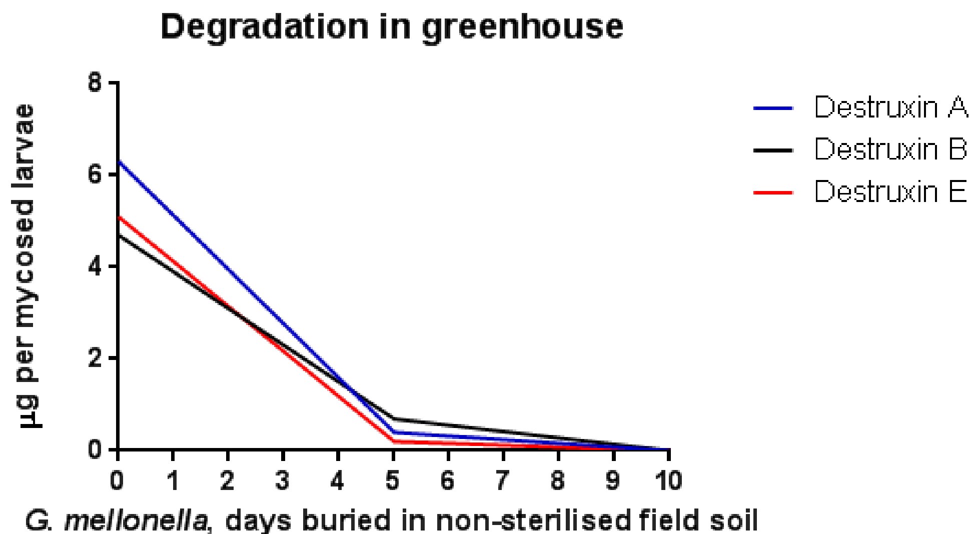

Figure 28. Levels of destruxins A, B and E by *M. anisopliae* V245 in G. mellonella cadavers, from the greenhouse (modified from Skrobek et al. 2008).

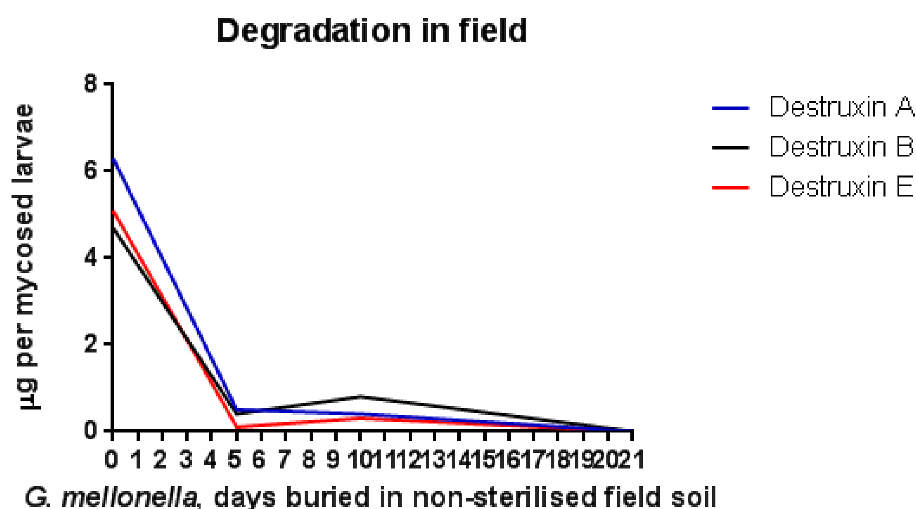

Figure 29. Levels of destruxins A, B and E by M. anisopliae V245 in G. mellonella cadavers measured in the field (modified from Skrobek et al. 2008).

These experiments showed that levels of destruxins decreased rapidly within 5 days. In field trials where temperatures were lower, the decline of destruxins was slower, i.e., destruxins were still present in buried cadavers after 10 days but not in cadavers from the greenhouse.

5.3.1.4 The plant

In the RAFBCA project several field (e.g. potato, maize and radish) and glasshouse trials (tomato, cucumber) were conducted under commercial conditions. Studies were performed to monitor major SMs in the environment to see if they enter the food chain.

Fungal applications were at 5x, 10x and 100x the recommended rate. Harvested plant material was analysed for the major SMs. These trials all showed that no metabolites were found in the plant material (source: powerpoint presentation RAFBCA main findings; it is foreseen that these data are still going to be published). Some examples:

Beauveria brongniartii for cockchafer control in field crops (potato, barley, maize, carrot, lettuce).
At application rates of 50 and 250 kg/ha (1x and 5x recommended rate) oosporein was not detected in the plant material but only in the target insect.

Stagonospora convolvuli for bindweed control in strawberries.
At application rates of 10x the recommended rate elsinochrome A was not detected in strawberries or target weed. Further, the fungus did not grow on the fruits.

Gliocladium catenulatum and *G. roseum* for control of seedling blight and root rot (*Phytium, Rhizoctonia)* in lettuce and cucumber.
At application rates of 10x the recommended rate gliotoxin was not detected in any of the material analysed.

Metarhizium anisopliae for the control of *Othiorhynchus sulcatus* in cyclamen, polyanthus, potato and radish (addendum to DAR (European Commission, 2011)).
RAFBCA experiments showed that at applications rates of 10^{10} conidia of *M. anisopliae* V275 per liter growth substrate, destruxins A, B and E could not be detected in extracts from cyclamen or polyanthus roots.

Moreover, destruxins A, B and E could also not be detected in potato or radish treated with a 10-fold dose of *M. anisopliae* BIPESCO 5/F52 as soil application or in cucumber treated with a 10-fold dose of *M. anisopliae* BIPESCO 5/F52 as soil application plus foliar application.

Destruxins are stable on inert glass surfaces but degrade on plant surfaces. Only 40 % destruxin A and 20 % destruxin B was recovered from tomato leaves, 14 days post-incubation under glasshouse conditions. (RAFBCA WP5 conclusions).

Other trials were performed with:
Trichoderma harzianum – controls fungal diseases
Verticillium lecanii – controls insect pests

All trials showed that the selected main SMs that were analysed were not detected in any plant material. It was concluded that metabolites of selected fungal BCAs do not enter the food chain.

5.3.1.4.1 Other examples, RAFBCA WP5 unpublished results

Environmental conditions (substrate, temperature, humidity and pH) were shown to influence the stability of fungal metabolites. These results have not been published to date. Some examples are listed below:

- Destruxins are denatured by boiling while oosporein has a half-life of 0.3 days at 53 °C.
- Destruxin E is unstable at low RH and cannot be recovered after 4 weeks at 8% RH.
- Oosporein degrades quickly under moderately alkaline conditions (half-life is 12 days at 23°C, pH 8) but is more stable under moderately acidic conditions (half life is 74 days at 23 °C, pH 6).
- Elsinochrome A is not degraded by boiling or cooking but is highly unstable when exposed to daylight. It has a half-life of 14 h on sunny days (8 - 10 hours of sunshine/day) and of 99 h when exposed to daylight on cloudy days (0 - 2 hours of sunshine/day). Photo-degraded elsinochrome A was not toxic to the highly sensitive test organism *Trichoderma atroviride* P1.

Discussion

Unfortunately, these trials have not been published and insight in the data is not possible, except for *M. anisopliae* in the addendum to the DAR **(**European Commission, 2011). The results presented in the powerpoint presentation show that the main metabolites that were analysed were not found in the plant material.
Assuming that the target pest was present at the time of treatment, it can be concluded that SMs were not present in the plant material or at levels lower than the detection limit. It is however not possible to make conclusions on degradation.

5.3.1.5 Extracted an stored in vials

In a study by Dudley et al. (2004) the stability of destruxin E was determined in crude extracts and from purified destruxin E under different temperature regimes. Destruxin E was chosen as it was considered to be the most bioactive destruxin of *M. anisopliae*.

Destruxin E was prepared from crude extracts of liquid media grown with *M. anisopliae* strains V275 and V245. Destruxin E was also purified from the crude extract.

The material was prepared in brown high-performance liquid chromatography (HPLC) vials (Fisher) in triplicate and

1. Stored at -20, 4, 25, and 37 °C.
2. Stored in an unheated greenhouse which fluctuated in temperature (range 10–50 °C between March and September).

Purified and crude metabolites were retrieved from the above treatments at 1-month intervals.

Figure 30 shows that destruxin E is degraded gradually, but with increasing degradation rates at higher temperatures.

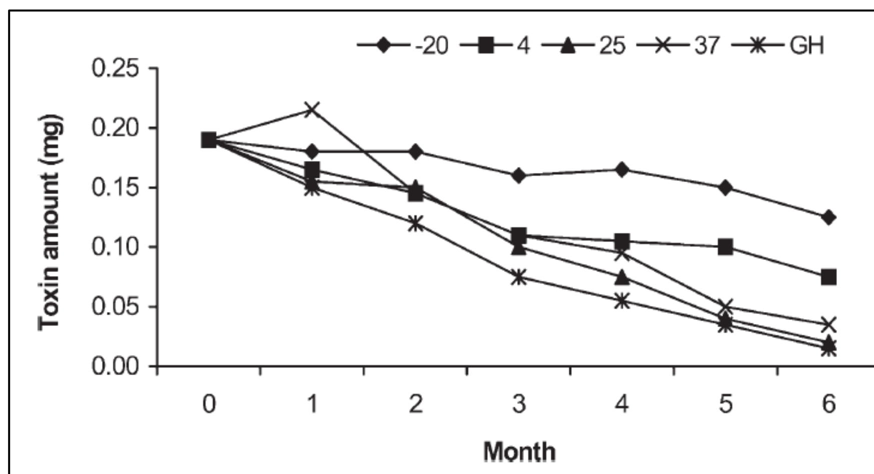

Figure 30. Breakdown dynamics of the purified destruxin E at different temperatures (Dudley et al., 2004). GH = Greenhouse.

Table 47 compares the degradation rates between purified destruxin E and destruxin E in crude extracts. It shows that the degradation of purified destruxin E and destruxin E in the crude extract are more or less the same.

Table 47. Breakdown ratio (%) of *Metarhizium anisopliae* destruxin E after storage under different conditions for 6 months.

Temperature of storage	Purified dtx E	Dtx E from V275 crude extract	Dtx E from V245 crude extract
−20°C	34.21 ± 2.36	35.25 ± 3.65	35.90 ± 2.65
4°C	60.53 ± 5.65	67.63 ± 6.35	66.67 ± 4.68
25°C	89.47 ± 7.12	87.05 ± 7.65	79.49 ± 3.72
37°C	81.58 ± 3.65	88.49 ± 4.64	74.36 ± 5.97
GH	92.11 ± 6.23	89.21 ± 4.51	82.05 ± 6.31

*, GH, greenhouse; Values are mean ± SD.

Extra information on destruxins A and B was provided in the addendum to the DAR (EU, 2005a). The stability of the destruxins during storage - dry and greenhouse conditions - was temperature-dependent; half-lives decreased with higher temperatures. Half-lives were 9.6, 7.7, and 1.9 months at 25ºC for destruxin A, B and E, respectively under dry storage conditions. These half-lives were similar to those found under greenhouse conditions (Information on destruxin E was published by Dudley et al. (2004) as described above).

5.3.2 Persistence of secondary metabolites produced by fungal biocontrol species other than entomopathogenic fungi

Relatively few information is available on the fate of SMs in the environment. Some information retrieved in the literature is given below.

5.3.2.1 Trichoderma viride

Figure 31 shows that gliotoxin was inactivated more rapidly in nonsterile than in sterile soil, which suggests microbiological degradation of the compound (Jefferys, 1952). The soil pH influenced the degradation rate: buffer solutions of gliotoxin were highly stable at pH 3.4 and 4.9. Above pH 4.9 inactivation took place more rapidly at higher pH values.

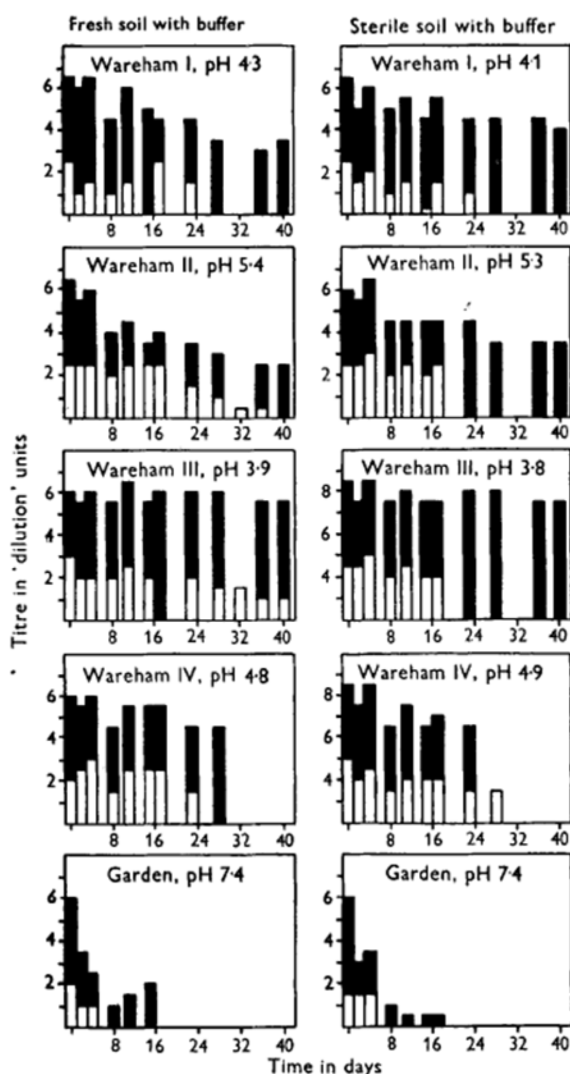

Figure 31. Fungistatice activity of solutions of gliotoxin (Jefferys, 1952).

In Table 48 Evans and Gottlieb (1955) showed that in inoculated sterilized soil gliotoxin accumulated to maximally 3.13 µg/g soil sample after 30 days but in nonsterilized soil gliotoxin only accumulated to 0.13 µg/g soil. Increases coincided with the colonization of *Trichoderma viride* in the soil and it was assumed that gliotoxin was produced by *T. viride*. The lack of accumulation in nonsterilized soil was ascribed to (a)

nonproduction of the antibiotic, (b) chemical instability of gliotoxin or (c) degradation by other soil microorganisms. A decrease of toxins was observed between 30 and 70 days after inoculation.

Table 48. Production of gliotoxin by *Trichoderma viride* in sterilized and nonsterilized soils (Evans and Gottlieb, 1955).

Time After Inoculation	Inoculated Soil		Uninoculated Soil	
	Sterilized	Nonsterilized	Sterilized	Nonsterilized
days	µg./g.	µg./g.	µg./g.	µg./g.
0	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00
4	0.12	0.12	0.00	0.00
6	1.25	0.00	0.00	0.13
10	2.00	0.00	0.00	0.00
14	2.50	0.00	0.00	0.00
30	3.13	0.13	0.00	0.00
60	0.63	0.00	0.00	0.00
70	0.25	0.00	0.00	0.00

Trichothecene mycotoxin compounds

T-2 is one of the more stable toxins, retaining its bioactivity even when heated to high temperatures (Franz, 1997). When maintained as either crystalline powders or liquid solutions, trichothecene mycotoxin compounds are stable when exposed to air, light, or both (see references in Franz, 1997). Moreover, these mycotoxins are not inactivated by autoclaving but require heating at 900°F for 10 minutes or 500°F for 30 minutes for complete inactivation (Franz, 1997).

5.3.2.2 Chaetomium globosum

Secondary metabolites produced by *Chaetomium globosum* strains QM 103a, NRL 6929 and ATCC 58917 used for apple scab biocontrol, were extracted and the sterile culture extracts were stored (Boudreau and Andrews, 1987). Activity of the strain QM 103a did not change significantly in time. When stored in a buffer of varying pH, activity was destroyed after 1 day at pH 11. Activity remained highest at pH 7.0 and a decline of approximately 50% was observed at pH 5.5 and 8.8.

Pronounced reduction of activity was observed after drying/aeration of the extract.

Interestingly Boudreau and Andrews (1987) showed convincing evidence that passive seepage of SMs from ascospores is the main source of SMs on the leaf surface. Passage of antibiotic substances, already present in the ascospores, is likely as biocontrol activity was present in case of ungerminated spores.

The authors suggested that toxicity may be lost through dilution of antibiotic by diffusion across or in the leaf, through degradation of the antibiotic, or both.

5.3.3 Stability of secondary metabolites in bacterial biocontrol species

5.3.3.1 Pseudomonas spp.

Pyrrolnitrin is considered to be the lead metabolite produced by *Pseudomonas* spp. Pyrrolnitrin is active against a wide range of Deuteromycetes, Ascomycete and Basidiomycete fungi. Pyrrolnitrin was found to be unsuitable for use as an agricultural fungicide since it is highly labile in sunlight (Ligon et al., 2000). There are other (unknown) SMs from *Pseudomonas* for which no data are available on degradation.

5.3.3.2 Streptomyces spp.

Geldanamycin was mentioned to be highly persistent in the EFSA report. A reference was however not provided. A reference is not yet found in the literature.

Table 49. Metabolites of *Streptomyces*.

Secondary metabolite	species	Observed compartment		Reference
geldanamycin	*Streptomyces* spec.	soil	High persistence and high mobility	(EFSA, 2013a) no specific reference cited herein

5.3.3.3 Other bacterial species

Information on persistence of SMs is scarce and/or difficult to locate in the literature.

5.4 Conclusions on stability

5.4.1 Entomopathogenic fungi

Stability of SMs was reviewed for different compartments. In short the following conclusions can be made:

1. Submerged media
   - Although SM concentrations in submerged media are not representative of degradation *in vivo* it is clear that SM concentrations are unstable and that the actual level of SMs is the result of an increase of newly produced metabolites and a decrease of SM concentrations as the result of degradation/further metabolisation.
2. Hemolymph of infected, still living insects
   - Relatively low production of metabolites in infected larvae that are still alive.
   - In studies with *Metarhizium* sp. that focused on the detection of destruxins, only destruxins A and B were detected. Quantities produced depend on the host species. Strain V275 did not produce metabolites in *Galleria mellonella.* Destruxin E was not detected in larvae (*Galleria* and *Tenebrio* spp.) that were still alive.
   - It is assumed that insects that are still alive are able to remove destruxins from the hemolymph through structural modifications or abiotic processes.
3. Hemolymph post mortem
   - Levels of destruxins had decreased to <1 µg/insect on day 5. The decline of destruxins B and E was greater than the decline of destruxin A.
   - The RAFBCA team suggested that when the insect host is dead the fungus switches from a parasitic to a saprophytic mode, and their results indicate that destruxin production stopped at that stage and that the destruxins still present in the cadaver were degraded.
   - The rapid disappearance from the cadavers was assumed to be due to the presence of hydrolytic enzymes in the hemocoel.
   - Destruxins are likely transported into conidia during sporulation.
4. Crops
   - In the RAFBCA studies several crop/biocontrol agent combination were studied for presence of SMs in the plant material. It can be concluded that metabolites were not present in the plant material or at levels lower than the detection limit.
5. Intrinsic stability
   - The tests were performed on inert substrate (glass), hence detoxification by the plant itself could not have occurred. Under different conditions (substrate, temperature, humidity and pH) SMs were found to be unstable. As experiments were performed with purified toxins, there was no interaction with other compounds, e.g. no breakdown by enzymes, organic acids, etc. DT50 values determined in these types of experiments are probably worst case (higher than in natural situations).

5.4.2 Other fungi

Information is available on the stability of gliotoxin produced by *Trichoderma viride.* Gliotoxin stability was influenced by the pH. Gliotoxin was below the limit of detection in nonsterilized soil.

Only when maintained as either crystalline powders or liquid solutions, trichothecene mycotoxin compounds (some of them are produced by *Trichoderma* spp. are stable when exposed to air, light, or both.

5.4.3 Bacteria

Very little information on degradation of SMs produced by bacteria was found.
Pyrrolnitrin produced by *Pseudomonas* species is highly unstable in sunlight.

Information on geldanamycin produced by *Streptomyces* spp. could not be verified.

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