Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbst20

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Published online: 26 Oct 2007.

To cite this article: Gisbert Zimmermann (2007): Review on safety of the entomopathogenic fungus Metarhizium anisopliae , Biocontrol Science and Technology, 17:9, 879-920

To link to this article: http://dx.doi.org/10.1080/09583150701593963

Biocontrol Science and Technology, 2007; 17(9): 879–920

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REVIEW

Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*

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(Received 15 March 2007; returned 16 May; accepted 24 July 2007)

Abstract
The entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokin is widely used for biocontrol of pest insects, and many commercial products are on the market or under development. The aim of this review is to summarise all relevant safety data of this fungus, which are necessary for the commercialisation and registration process. The review contains the following sections: (1) identity, (2) biological properties (history, natural occurrence and geographical distribution, host range, mode of action, production of metabolites/toxins, effect of environmental factors), (3) methods to determine and quantify residues, (4) fate and behaviour in the environment (mobility and persistence in air, water and soil), (5) effects on non-target organisms (microorganisms, plants, soil organisms, aquatic organisms, predators, parasitoids, honey bees, earth worms, etc.), (6) effects on vertebrates (fish, amphibia, reptiles, and birds), and (7) effects on mammals and human health (allergy, pathogenicity/toxicity). On the basis of the presented knowledge, *M. anisopliae* is considered to be safe with minimal risks to vertebrates, humans and the environment.

Keywords: Metarhizium anisopliae, *occurrence, host range, toxins, environmental fate, safety, side-effects*

Introduction

For about 130 years, entomopathogenic fungi and especially *Metarhizium anisopliae* (Metschn.) Sorokin, have been used for biocontrol of pest insects. Besides the two other entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii, M. anisopliae* is one of the most widely used fungus and mycoinsecticide throughout the world, mainly as an inundative control agent.

First investigations on the safety and the effect of *M. anisopliae* against mammals were conducted by Schaerffenberg (1968). Later, various aspects of the safety of *M. anisopliae* and other microbial control agents to man, other vertebrates, beneficial

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ISSN 0958-3157 print/ISSN 1360-0478 online
DOI: 10.1080/09583150701593963

and non-target organisms and also to crops were published by many authors (Steinhaus 1957; Müller-Kögler 1965; Schaerffenberg 1968; Heimpel 1971; Ignoffo 1973; Austwick 1980; Burges 1981; Hall et al. 1982; Goettel et al. 1990, 2001; Laird et al. 1990; Saik et al. 1990; Siegel & Shadduck 1990; Zimmermann 1993; Cook et al. 1996; Goettel & Jaronski 1997; Vestergaard et al. 2003). A biopesticide fact sheet and a technical document on various safety issues of *M. anisopliae* strain F52 (029056) and *M. anisopliae* strain ESF1 (129056) are published by the US Environmental Protection Agency (EPA) at www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_029056.htm and 129056.htm, respectively. During the last 25 years, several mycoinsecticides based on *M. anisopliae* have been commercialised and registered in various countries. Some of these are still on the market, while others have disappeared. Generally, commercialisation requires a registration of the product, i.e. the producer has to submit a dossier which should include all data on safety and other relevant publications. The aim of this paper is to summarise our knowledge on safety issues and related ecological aspects of *M. anisopliae* and its varieties in order to present a basis (1) for discussion and eventually further testing, (2) for companies which are interested in the production, registration and commercialisation and (3) for decision-makers within the regulatory authorities.

The content of this paper generally follows the EU guidelines for the registration of microorganisms (Annexes IIB and IIIB to Directive 91/414/EEC). As general safety aspects are already presented in the different sections of a previous review paper on *Beauveria bassiana* and *Beauveria brongniartii* (Zimmermann 2007), this compilation on safety of *M. anisopliae* is restricted only to the relevant literature dealing with this fungus.

Identity of *Metarhizium* spp.

The species *M. anisopliae* was originally described by Metschnikoff (1879) as *Entomophthora anisopliae* and later transferred to the new genus *Metarhizium* by Sorokin (1883) (Table I). The first revision of the genus *Metarhizum* was conducted by Tulloch (1976). Because of orthographic correctness, she suggested to write the genus name with only one 'r' instead of the original spelling with two 'r's. The dominant taxonomic characters are the morphological features of the sporulating structures. The genus is defined on the basis of the arrangement of the phialides bearing chains and columns of dry and generally green, cylindrical or slightly ovoid conidia. The columns are formed by aggregation of the conidial chains. Tulloch accepted *M. anisopliae* and *Metarhizium flavoviride* as the only two species in the genus and distinguished two forms of *M. anisopliae* based on the conidial size: (1) the short-spored form *M. anisopliae* (Metsch.) Sorok. var. *anisopliae*, with conidia of about 5–8 μm long and (2) the long-spored form *M. anisopliae* (Metsch.) Sorok. var. *major* (Johnston), with conidia usually between 10 and 14/16 μm long, which was later transformed to *M. anisopliae* var. *majus* (Johnson) Tulloch by Rombach et al. (1986). *Metarhizium flavoviride* was first described by Gams and Rozsypal (1973). It differed from *M. anisopliae* by its wider and greyish to yellow green conidia. These major species were also listed by Humber (1997). Later, Rombach et al. (1986, 1987) presented a synoptic key to the accepted species of *Metarhizium*, including the following taxa: *M. album* Petch, *M. brunneum* Petch, *M. anisopliae* (Metschn.) Sorokin *var. anisopliae*, *M. anisopliae* (Metschn.) Sorokin v*ar. majus* (Johnston) Tulloch,

Table I. Main species of the genus *Metarhizium* and their synonyms (Bischoff et al. 2006; CABI Bioscience et al. 2007).

Species	Synonyms
M. album Petch (1931)	–
M. anisopliae (Metschn.) Sorokin (1883)	*Entomophthora anisopliae* Metschn. (1879) *Isaria destructor* Metschn. *Isaria anisopliae* (Metschn.) Pettit (1895) *M. anisopliae* (Metschn.) Sorokin (1883) f. *anisopliae* *M. anisopliae* f. *minor* Johnst. (1915) *M. anisopliae* (Metschn.) Sorokin var. *anisopliae* Tulloch (1976) *M. anisopliae* var. *majus* (Johnst.) Tulloch (as '*major*') (1976) *Oospora destructor* (Metschn.) Delacr. (1893) *Penicillium anisopliae* (Metschn.) Vuill. (1904)
M. anisopliae var. *acridum* Driver and Milner (2000)	*M. flavoviride*
M. brunneum Petch (1935)	–
M. cylindrosporae Chen and Guo (1986)	New name: *Nomuraea cylindrosporae* (Chen and Guo) Tzean et al. (1993)
M. flavoviride Gams and Rozsypal (1973)	*M. flavoviride* var. *flavoviride* Gams and Rozsypal (1986)
M. flavoviride var. *minus* Rombach et al. (1986)	–
M. flavoviride var. *pemphigi* Driver and Milner (2000)	–
M. frigidum Bischoff et al. (2006)	*M. anisopliae* var. *frigidum* Rath et al. (1995)
M. guizhouense Chen and Guo (1986)	–

M. flavoviride Gams and Rozsypal *var. flavoviride* and *M. flavoviride* Gams and Rozsypal *var. minus* Rombach, Humber and Roberts.

On the basis of the colour of colonies and various morphological features, some additional taxa have been described in China such as *M. cylindrosporae* Chen and Guo, *M. guizhouense* Chen and Guo, *M. pingshaense* Chen and Guo (Guo et al. 1986) and *M. taii* Liang and Liu together with its teleomorph *Cordyceps taii* Liang and Liu (Liang et al. 1991). However, it is not clear whether these taxa are accepted as separate *Metarhizium* species.

During the past years, isolates of the species *M. anisopliae* have been characterised by various molecular and other techniques. RAPD markers were used to study the genetic diversity of *M. anisopliae* isolates from several insect hosts and various sugar cane areas in Australia, and a high genetic diversity was observed amongst the 31 isolates tested (Fegan et al. 1993). Arbitrarily primed PCR markers were used to study genetic relationships among *M. anisopliae* isolates from Brazilian soil samples or insects (Tigano-Milani et al. 1995), and Curran et al. (1994) found that rDNA sequence data can be used to resolve evolutionary relationships within *Metarhizium.* Furthermore, the polymerase chain reaction (PCR)-based technology, involving random amplification of polymorphic DNA (RAPD), was used to assess the genomic variability between entomopathogenic fungi, including *M. anisopliae* (Bidochka et al. 1994). At the same time, Rath et al. (1995b) demonstrated that the differentiation of 16 strains of *M. anisopliae* was possible using the API50CH system, which includes the utilisation of 49 carbohydrates. The authors conclude that carbohydrate utilisation is a useful and biologically relevant taxonomic criteria for the separation of *Metarhizium* strains. Also the RFLP analysis of the rDNA gene complex and the mtDNA is an

excellent tool for fingerprinting of *M. anisopliae* isolates. This was documented by Mavridou and Typas (1998) while studying the intraspecific variation of 25 *M. anisopliae* var. *anisopliae* isolates from various insect hosts and geographical origins.

A reassessment of the taxonomy of the genus *Metarhizium* started with Driver et al. (2000) when using sequence data and RAPD patterns from 123 isolates recognised as *M. anisopliae*, *M. flavoviride* or *M. album*. The data support the monopoly of the *M. anisopliae* group and recognise four clades within it. Two correspond with *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*. The other are new varieties based on their distinctive ITS sequence data. They are named *M. anisopliae* var. *lepidiotum* and *M. anisopliae* var. *acridum*. Besides *M. album*, *M. flavoviride* var. *flavoviride* and *M. flavoviride* var. *minus*, three clades represent two new varieties based on ITS sequence data: *M. flavoviride* var. *novazealandicum* and *M. flavoviride* var. *pemphigum*.

Recently, the taxonomy and phylogeography of the genus *Metarhizium* were discussed by Bidochka and Small (2005). According to studies on the population genetics, the authors concluded that (1) an association of *M. anisopliae* genotypes occurs with habitat type in temperate and polar regions, (2) associations of *Metarhizium* genotypes with certain host insect preferences occur in tropical and subtropical regions, (3) *Metarhizium* is actually an assemblage of about 10–15 cryptic species, including species of *M. anisopliae*, *M. flavoviride* and *M. album*, i.e. there are morphologically indistinguishable but phylogenetically distinguishable taxonomic units, and (4) southeastern Asia is the probable origin of the evolution and diversity of *M. anisopliae*. Furthermore, double-stranded RNA (dsRNA) has been found in some isolates of *M. anisopliae* (Leal et al. 1994; Bogo et al. 1996; Bidochka et al. 2000), and genetically related isolates were found to have homologous dsRNA viruses (Bidochka et al. 2000).

For a long time, no teleomorph of the genus *Metarhizium* was found. Therefore, *M. anisopliae* was classified in the Deuteromycota, Hyphomycetes. In 1991, *Cordyceps taii* Liang and Liu was described by Liang et al. (1991), which has an anamorphic stage called *Metarhizium taii*. Later, *Cordyceps brittlebankisoides* was isolated (Liu et al. 2001) and proved to be the teleomorph of *Metarhizium anisopliae* var. *majus* (Liu et al. 2002). Therefore, today fungi of the genus *Metarhizium* Sorokin are classified as Nectriaceae, Hypocreales and Ascomycetes (CABI Bioscience et al. 2007).

Actually, 30 names of *Metarhizium* taxa are listed in the Index Fungorum by CABI Bioscience et al. (2007). The main species and their synonyms are mentioned in Table I. In the following sections, those names of *Metarhizium* spp. mentioned in the original publications are used.

Biological properties

History

The genus *Metarhizium* was first established by Sorokin (1883) for the so-called green muscardine fungus, which was found and earlier described by Metschnikoff (1879) near Odessa (Ukraine) on infected larvae of the wheat cockchafer *Anisoplia austriaca* and, later, on *Cleonus punctiventris*. For this fungus, Metschnikoff first proposed the name *Entomophthora anisopliae*, and later renamed it as *Isaria destructor* (see Table I). The history of the detection, description, first scientific investigations and on the use of *M. anisopliae* in biological control is described in detail by Steinhaus (1949) and later

by Müller-Kögler (1965). Its use as a biocontrol agent against pest insects started just after its detection. Between 1880 and 1890, Metschnikoff and later Krassilstschik undertook several control experiments against various pest insects in the former USSR. Even large quantities of *M. anisopliae* spores were produced in a laboratory near Kiew (55 kg/4 months) which were successfully used for control of *Cleonus punctiventris*. Later (1910–1913), the fungus was used against the froghopper, *Tomaspis varia*, in Trinidad (Steinhaus 1949), and excellent results were reported against the rhinoceros beetle, *Oryctes rhinoceros*, on coconut in the Pacific (Friederichs 1913).

In 1926 and 1929, the pathogenesis of *M. anisopliae* infections in insects, the silkworm *Bombyx mori* and the European corn borer, *Ostrinia nubilalis*, was reported (see Steinhaus 1949). A comprehensive review on practical use of *M. anisopliae* for biological control is compiled by Müller-Kögler (1965). He presented experiments of this fungus against the following pest insects: *Scotinophara lurida*, *Aeneolamia flavilatera*, *Agriotes obscurus* and *A. sputator*, *Cleonus punctiventris*, *Oryctes rhinoceros*, various scarabs, *Popillia japonica*, *Alissonotum impressicolle*, *Ostrinia* (*Pyrausta*) *nubilalis*, *Agrotis segetum* and *Euxoa* spp. Later, Ferron (1981) summarised the data on the development of *M. anisopliae* as a mycopesticide and on its practical use for biocontrol of pest insects mainly in Brazil. A production unit provided about 100 kg daily of so-called 'Metaquino'. From 1972 to 1978, the area treated against spittlebugs, such as *Mahanarva postica*, increased from 500 to about 50 000 ha (Ferron 1981).

Today, *M. anisopliae* is one of the most important entomopathogenic fungus with actual or intended use against many pest insects. The current target pests are termites, locusts and grasshoppers or cockroaches, spittlebugs and other hemipterans, noctuids, soil dwelling pest insects, such as various scarab species and curculionids, greenhouse pests, such as white flies or thrips, as well as mosquitoes and even ticks. A list on the present mycopesticides based on *M. anisopliae* registered or under commercial

Table II. Mycopesticides of *Metarhizium anisopliae* registered or under commercial development (Butt et al. 2001; Wraight et al. 2001; Copping 2004; Kabaluk & Gazdik 2005; Zimmermann 2005).

Product/Trade name	Company/Producer	Country/Origin
BioBlast	EcoScience	USA
Bio-Cane Granules	Becker-Underwood	Australia
Bio-Catch-M	Stanes	India
Bio-Green Granules	Becker-Underwood	Australia
Bio-Magic	Stanes	India
BioPath	EcoScience	USA
Cobican	Probioagro	Venezuela
Gran Met-P	Kwizda/Agrifutur	Austria/Italy
Green Guard SC	Becker-Underwood	Australia
Green Guard ULV	Becker-Underwood	Australia
Green Muscle	CABI Bioscience/NPP	UK/France
Metaquino	–	Brazil
Metarhizium Schweizer	Lbu (formerly Eric Schweizer Seeds)	Switzerland
Metathripol	ICIPE	Kenya
Muchwatox (proposed)	ICIPE	Kenya
Pacer	SOM Phytopharma	India
Taenure Granular Bioinsecticide	Novozymes Biologicals (formerly Earth BioSciences)	USA
TAE-001 Technical Bioinsecticide	Novozymes Biologicals (formerly Earth BioSciences)	USA
Tick-Ex EC	Novozymes Biologicals (formerly Earth BioSciences)	USA
Tick-Ex G	Novozymes Biologicals (formerly Earth BioSciences)	USA

development is presented in Table II (Zimmermann 1993, 2005; Butt et al. 2001; Wraight et al. 2001; Copping 2004; Kabaluk & Gazdik 2005).

Natural occurrence and geographical distribution

Metarhizium anisopliae has a worldwide distribution from the arctic to the tropics on insects as well as in the soil. In the compendium of soil fungi, Domsch et al. (1980) listed the following countries and habitats: Nepal, New Zealand, New Caledonia, the Bahamas, the USA, Canada, Northern Ireland, Italy, Turkey, the former USSR and Zaire. Non-insect habitats include cysts of the nematodes *Heterodera schachtii* and *Globodera rostochiensis.* Furthermore, Domsch et al. (1980) mention that *M. anisopliae* was isolated from corn fields and forest soils in Canada, banana soils in Honduras, bean- and cornfields in Brazil, grassland soils in New Zealand, wheatfields in Germany, forest soils after burning, muck soil, organic detritus in unpolluted streams, river sediments, a mangrove swamp, lead mine, spoil heaps, birds' roosts and from healthy strawberry roots.

An actual list on the natural occurrence of *M. anisopliae* in soil in different countries is presented in Table III. In most cases, the fungus has been isolated from soil using the so-called *Galleria* bait method (Zimmermann 1986) or by special selective media (see Methods to determine and quantify residues). Additionally, as documented in the scientific literature about *M. anisopliae,* this species has also been isolated in many European countries, such as UK, France or Austria, in Australia and in a range of Asian, African, Central and South American countries.

Host range

Metarhizium anisopliae occurs on a wide range of insect hosts; however, the host range is more restricted than that of *B. bassiana*. The most comprehensive list of host insects was presented by Veen (1968) with records of 204 naturally infected insect species from seven orders (Table IV). Most of the listed host insects of *M. anisopliae* belong to the Coleoptera and especially to soil-dwelling pests insects including over 70 scarab species. In a report on arthropod hosts of entomogenous fungi in Britain, Leatherdale (1970) listed *M. anisopliae* only on two families of Coleoptera, i.e. Elateridae and Curculionidae (*Corymbites cupreus*, *Agriotes lineatus* or *A. obscurus*, *A. sputator*, *Sitona lepidus*) and on one Dipteran (*Lonchaea palposa*). According to Goettel et al. (1990) the host range of *M. anisopliae* contains Symphyla, Orthoptera, Dermaptera, Isoptera, Homoptera, Heteroptera, Diptera, Coleoptera, Hymenoptera, Siphonaptera and Lepidoptera, as well as some nontarget hosts belonging to Malacostrata (Amphipoda), Acari, Ephemeroptera, Dermaptera, Heteroptera, Diptera, Coleoptera, Hymenoptera and Lepidoptera.

While *M. anisopliae* as a species has a wider host range, certain strains and genotypes are more restricted (Ferron et al. 1972; Rombach et al. 1986; Bidochka & Small 2005). Furthermore, isolates are also more specific under field conditions compared to laboratory studies (Jaronski et al. 2003). Some genetic groups of *Metarhizium* from tropical and subtropical environments, especially strains of *M. anisopliae* var. *majus*, *M. flavoviride* and *M. album* show some host-insect preferences and are reported to be specific to Coleoptera, Orthoptera and Hemiptera, respectively (Rombach et al. 1986; Bidochka & Small 2005). Ferron et al. (1972) found that from eight strains of *M. anispliae* (six strains var. *majus*, two strains var.

Table III. Examples of natural occurrence of *Metarhizium anisopliae* in the soil of different countries.

Location	Characteristics	References
Canada	In 266 soil samples from 86 locations the most abundant species was *M. anisopliae* (357 isolates)	Bidochka et al. (1998)
Czech Republic, South Bohemia; (arable soil)	From 146 soil samples, 80 strains of *M. anisopliae* were isolated; no differences in soils between arable fields, conventional and organic farms	Landa et al. (2002)
Finland	From 590 soil samples, *M. anisoliae* was isolated from 15.6%	Vänninen (1996)
Germany	In 100 soil samples from different locations and soil types, *M. anisopliae* was found in 42% and was the most frequently found species	Kleespies et al. (1989)
Italy (south)	From 188 soil samples, *M. anisopliae* was isolated once	Tarasco et al. (1997)
Macquarie Islands	In 163 subantarctic soil samples, six contained *M. anisopliae*	Roddam & Rath (1997)
Nepal	Analysis of soils from three different regions showed that *M. anisopliae* is common in about 50% of the samples	Dhoj & Keller (2003)
New Zealand	*M. anisopliae* was found in all habitat soils	Barker & Barker (1998)
Norway (northern parts)	Significantly higher occurrence of entomopathogenic fungi, including *M. anisopliae*, in soils from arable fields of organically managed farms compared to conventionally managed ones	Klingen et al. (2002a)
Panama (tropical forest)	An abundance of *M. anisopliae* var. *anisopliae* was found in soil near colonies of leaf-cutting ants	Hughes et al. (2004)
Poland	*M. anisopliae* was isolated from all soil types: Hop plantations, arable fields and mid-forest meadows; *M. anisopliae* dominated in light loamy sand	Mietkiewski et al. (1994, 1995, 1996); Tkaczuk & Mietkiewski (1996)
	M. anisopliae was the dominant species isolated from all soil samples in apple and plum orchards	Sapieha-Waszkiewicz et al. (2003)
Spain (Alicante)	*M. anisopliae* was found in 6.4% of soils from 61 sites	Asensio et al. (2003)
Switzerland	*M. anisopliae* was found in all soil samples; soils from arable land had less *M. anisopliae* than soils from meadows	Keller & Schweizer (2001)
	Soil samples from 82 fields were analysed: 96% of all fields contained *M. anisopliae*	Keller et al. (2003)
USA	Soil from 105 sites in 21 orchards mainly contained *M. anisopliae*	Shapiro-Ilan et al. (2003)

anisopliae), tested against nine species of Coleoptera (Scarabaeidae), most of the insect species were susceptible only to that fungus strain isolated from insects of the same species. Species of *Oryctes* are susceptible only to all *Metarhizium* strains isolated from *Oryctes* species. On the other hand, isolates from soil were found to be highly virulent against specific pest insects, e.g. to the legume flower thrips, *Megalurothrips sjostedti* (Ekesi et al. 1998), or to the pod bug *Clavigralla tomentosicollis* (Ekesi 1999).

Table IV. Host insects of *M. anisopliae* according to Veen (1968).

Order	Family	No of species
Orthoptera	Acrididae, Gryllotalpidae	11
Dermaptera	Forficulidae	1
Hemiptera	Cercopidae, Cicididae, Coccidae, Delphacidae, Flatidae, Miridae, Pentatomidae	21
Diptera	Asilidae, Chironomidae, Tipulidae, Trypetidae	4
Hymenoptera	Ichneumonidae, Pamphiliidae, Scoliidae, Tiphiidae	6
Lepidoptera	Aegeriidae, Agrotidae, Artiidae, Bombycidae, Brassolidae, Crambidae, Eusomidae, Galleriidae, Hepialidae, Notodontidae, Phycitidae, Pyraustidae, Saturniidae, Thaumetopoeidae	27
Coleoptera	Byturidae, Carabidae, Cerambicidae, Chrysomelidae, Coccinellidae, Curculionidae, Elateridae, Lampyridae, Scarabaeidae, Scolytidae, Tenebrionidae	134

Mode of action

The infection process of *M. anisopliae* is similar to other entomopathogenic fungi, i.e. the infection pathway consists on the following steps: (1) attachment of the spore to the cuticle, (2) germination and formation of appressoria, (3) penetration through the cuticle, (4) overcoming of the host response and immune defence reactions of the host, (5) spreading within the host by formation of hyphal bodies or blastospores, i.e. yeast like cells, and (6) outgrowing the dead host and production of new conidia. As there is extensive literature on the different steps of the infection process, only a short overview is given here (for further information see Boucias & Pendland 1998). A comprehensive overview on biochemical as well as physico-chemical aspects of disease development, the genetics and molecular mechanisms is presented by St. Leger (1993), Hajek and St. Leger (1994), St. Leger and Bidochka (1996) and Khachatourians (1998).

Generally, the fungus penetrates its host insects percutaneously, i.e. through the outer integument, especially the intersegmental folds, like joints between segments or around the mouthparts, although infections via the buccal cavity in beetles, the siphon tip in mosquito larvae or the gut have been reported. The attachment is due to the hydrophobicity of the conidia as well as the cuticular surface. Germination and successful infection depends on a number of factors, e.g. susceptible host and host stage or various environmental factors such as temperature and humidity. Germination may also be influenced by certain cuticular lipids on the insects, such as short-chain fatty acids, aldehydes, wax esters, ketones and alcohols which may possess antimicrobial activity. However, the cuticle may also be coated with substances that are important for fungal recognition, like free amino acids or peptides, and may trigger

the attachment and germination. Generally, germination of *M. anisopliae* conidia takes place within the first 20 h after contact.

Before penetration, germinated conidia of *M. anisopliae* produce an appressorium, which then forms an infection peg and a penetration plate. The penetration process is mechnical, aided by the production of several enzymes, including proteases, chitinases and lipases. As the cuticular chitin is covered with proteins and lipids, proteases and esterases are released first by the fungus followed by chitinases. Over the years, *M. anisopliae* has been used to study pathogenicity and to identify genes which are involved in appressorium formation and the penetration process. In *M. anisopliae*, a group of cuticle-degrading enzymes were detected which are produced both in culture and during the infection process. This group contains subtilisin-like proteinases, metalloproteases, trypsin, chymotrypsin, aminopeptidases, dipeptidyl peptidases and chitinases. Much attention has been directed to an endoprotease called Pr1, which is a chymoelastase-like protease with high relevance in virulence studies. A table on the characteristics of enzymes produced by *M. anisopliae* including their substrate specificity and probable functions is presented by Boucias and Pendland (1998).

The penetration of the cuticle layers and the beginning of the host invasion by the fungus is accompanied by several host response activities, e.g. by production of phenoloxidases, the formation of certain hemocytes and a melanisation process. The host defence mechanisms and the reactions of the penetrating hyphae are a complex process containing different interactions (see Vilcinskas & Götz 1999).

After successful penetration of the host insect, the fungus produces blastospores or hyphal bodies, which are distributed passively in the hemolymph, enabling the fungus to invade other tissues of the host insect by extensive vegetative growth. During the invasion of the whole insect body, nutrients in the hemolymph and the fat body are depleted. This is followed by the death of the insect and the end of the pathogenic process. During the invasion of the host insect by *M. anisopliae*, a wide range of secondary metabolites or toxins are produced (see Production of metabolites/toxins).

The incubation period depends on the host, the host stage, the temperature and the virulence of the fungus strain. In aphids it may take 3–4 days, in white grubs 2–4 weeks. After the host death and under humid conditions, the fungus starts its saprophytic growth out of the body. Conidia are produced outside of the cadaver. Under very dry conditions, the fungus may also persist in the hyphal stage inside the cadaver or, e.g. in locusts in Africa, where conidia is produced inside the body.

Production of metabolites/toxins

Generally, fungi produce a wide variety of biologically active compounds, mostly as products of the secondary metabolism. In entomopathogenic fungi, the wide terminology associated with toxic fungal metabolites and the various definitions of 'toxin' was discussed by Roberts (1981). These metabolites are mainly acting as pathogenicity determinants by improving the infection and colonisation of the host organism or as antibiotics to suppress other microorganisms thus improving their own survival (Vey et al. 2001). Concerning registration, the questions, however, are when and under which conditions these toxic metabolites are produced, and do they represent a hazard to human or environmental safety? Most of the papers presented below deal with purified toxins, such as destruxin A, B or E, or with crude extracts of

spores, mycelium or culture filtrates of *M. anisopliae*. The question still remains whether these toxins are also produced by the fungus after application in the field and if so, at which amount or under which conditions? In a study on risk assessment of fungal metabolites, Strasser et al. (2000) stated that the quantities produced by *M. anisopliae* and others *in vivo* are usually much less than those secreted in nutrient rich liquid media, thus they pose a minimal risk.

One of the first comprehensive overviews on toxins of entomopathogenic fungi was presented by Roberts (1981). In the genus *Metarhizium*, he listed destruxins (six types) and cytochalasins (C and D) as toxic metabolites. Actually, destruxins, cytochalasin C and swainsonine (not swainsinone) are listed as the main metabolites produced by *M. anisopliae* in culture or *in vivo* (Strasser et al. 2000; Vey et al. 2001). However, recently some new insecticidal antibiotics, hydroxyfungerins A and B, were isolated from culture broth of *Metarhizium* sp. FKI-1079 (Uchida et al. 2005), which showed an inhibitory activity against brine shrimps, *Artemia salina* (Uchida et al. 2005), and two new mutagenic metabolites, NG-391 and NG-393, were identified from the fermentation extract of *M. anisopliae* (Krasnoff et al. 2006). These compounds are 7-desmethyl analogues of fusarin C and (8Z)-fusarin C, mutagenic toxins of *Fusarium* species contaminating corn which exhibit mutagenic activity in the *Salmonella* mutagenicity test. The main activities of destruxins, cytochalasins and swainsonine are described in detail.

Destruxins. The destruxins are the most important metabolites/toxins produced by *M. anisopliae* and were isolated more than 40 years ago (Kodaira 1961). Chemically, destruxins are cyclic hexadepsipeptides containing five amino acids, i.e. β-alanine, alanine, valine, isoleucine and proline, and an α-hydroxy acid. In 1981, Païs et al. (1981) isolated and identified 14 depsipeptides from a culture medium of a strain of *M. anisopliae*. Five were identified as the known destruxins A, B, C, D and desmethyldestruxin B. The structures of the new compounds were named destruxin E, A_1, A_2, B_1, B_2, C_2, D_1, D_2, and E_1. The differences observed are in the peptide chain, i.e. pipecolic acid instead of proline or valine instead of isoleucine. While Strasser et al. (2000) and Vey et al. (2001) mentioned that more than 28 different destruxins have been isolated and described, Pedras et al. (2002) listed 35 destruxins belonging to the A series (9), B series (10), C series (3), D series (3), E series (7), F series (1) and new series (2 pseudodestruxins). Recently, Liu et al. (2004) mentioned that more than 35 different destruxins have been characterised.

Destruxins are not only produced by *M. anisopliae*. Destruxin A4 and A5 and homodestruxin B were also isolated from an undescribed species of the entomopathogenic fungus *Aschersonia* sp. (Krasnoff et al. 1996), and destruxin B was found in *Beauveria felina* (Kim et al. 2002). Furthermore, several destruxins were isolated from the three plant pathogenic fungi *Alternaria brassicae*, *Trichothecium roseum* and *Ophiosphaerella herpotricha* (see Vey et al. 2001), while the pseudodestruxins A and B are produced by the coprophilous fungus *Nigrosabulum globosum* (Che et al. 2001). Destruxin B isolated from *A. brassicae*, both *in vitro* and *in planta*, is the major toxin involved in the pathogenic process of this fungal pathogen attacking *Brassica* species. The most comprehensive overview on synthesis, biosynthesis, biotransformation and biological activity of destruxins was published by Pedras et al. (2002).

Production of destruxins in M. anisopliae. Generally, the production of metabolites depends on the species, the strain and specific environmental and nutritional

conditions. However, the preservation method may also have an impact on the production of secondary metabolites (Ryan et al. 2003). In most strains of *M. anisopliae* tested, changes in the secondary metabolite profiles occurred after relatively short storage periods, irrespective of the preservation method used.

Destruxins from *M. anisopliae* were isolated mainly from complex liquid culture media. The production of destruxins *in vivo* was first demonstrated by Suzuki et al. (1971). In silkworm larvae, *Bombyx mori*, the destruxin content amounted to 240 ng per larva, which usually is much less than the quantities produced by this fungus in nutrient rich liquid media (Strasser et al. 2000). For example, Amiri-Besheli et al. (2000) showed that the destruxin content of one *Galleria mellonella* larva infected with *M. anisopliae* V245 was 0.44 μg destruxin A and 0.5 μg destruxin B on the day of death. In contrast, one litre of liquid culture of the same strain contained up to 12 mg destruxin A and 4 mg destruxin B (Wang et al. 2004).

With respect to safety considerations, but also to the screening for virulent strains with a high insecticidal activity, several studies were conducted to evaluate the production of destruxins by different *M. anisopliae* varieties and strains under various conditions. In 1986, Lin and Roberts (1986) demonstrated that *M. anisopliae* var. *major* is also able to produce destruxins. Five strains were found to produce destruxin A, but the amount produced was only 1.1–2.4% of that obtained from a *M. anisopliae* var. *anisopliae* isolate. An inter- and intra-specific variation in the destruxin production was also detected by Amiri-Besheli et al. (2000). Strains of *M. anisopliae* var. *anisopliae* produced different amounts of destruxin A, B and E. *M. anisopliae* var. *majus*, *M. flavoviride* and *M. album* had different destruxin profiles with destruxin A predominating. Some low toxin producers were also virulent, suggesting that destruxins are not the only pathogenicity determinants.

The production of destruxins of *M. anisopliae* in solid culture was also studied. Liu and Tzeng (1999) found that their strain produced 2.9 and 227 mg kg^{-1} substrate destruxin A and B, respectively, after 2 weeks fermentation on a rice/bran/husk medium at 71% water content with a water activity of 0.921. Wang et al. (2004) stated that the *M. anisopliae* strains V245 and V275 did not produce destruxins in large-scale fermenter cultures or solid Czapek Dox agar. Toxin production could be noticed when the fungus was grown on rice. With increasing peptone in the medium, the amount of destruxin A, B and E also increased. The loss of the ability to produce destruxins was noticed (Wang et al. 2003). A spontaneous subtilisin pr1A and pr1B gene-deficient mutant of strain *M. anisopliae* V275 lost the ability to produce destruxins, both *in vitro* and *in vivo*. The analysis revealed that the mutant lost a conditionally dispensible (CD) chromosome. It is concluded that the toxin synthetase genes of *M. anisopliae* are located on this CD chromosome.

Activities of destruxins. Destruxins have a variety of biological activities, which are described in detail by Roberts (1981), Strasser et al. (2000), Vey et al. (2001) and Pedras et al. (2002). In the following, the most well-documented and important activities of destruxins are summarised:

(1) Effects on insects and the relation to virulence.
(2) Effects on different cells and cell lines.
(3) Effects on vertebrates.
(4) Interactions with plants.

(1) The insecticidal activity of destruxins was tested against many insect species. According to Pedras et al. (2002), studies were conducted against the following arthropod species: *Bemisia argentifolii*, *Bombyx mori*, *Brevicoryne brassicae*, *Cetonia aurata*, *Choristoneura fumiferana*, *Coptotermes formosanus*, *Culex pipiens*, *Delia antigena*, *Drosophila melanogaster*, *Empoasca vitis*, *Epilachna sparsa*, *Galleria mellonella*, *Heliothis virescens*, *Manduca sexta*, *Musca domestica*, *Myzus persicae*, *Oryctes rhinoceros*, *Otiorhynchus sulcatus*, *Phaedon cochlearia*, *Plutella xylostella*, *Rhagoletis pomonella*, *Rhopalosiphum padi* and *Schistocerca gregaria*. It was found that insects vary considerably in their susceptibility to destruxins when it is introduced by intrahaemocoelic injection. In Lepidoptera, the symptoms are an immediate tetanus, and at high dosages a titanic paralysis. Destruxin E seems to be the most potent destruxin with insecticidal activity. For example, repellent and aphicidal properties were observed together with differences in the susceptibility of aphid species (Robert & Riba 1989). *Metarhizium persicae* is sensitive to this molecule which kills 50% of the individuals feeding on a leaf on which destruxin had been deposited at a rate of 0.4 $\mu g\,cm^{-2}$. Feeding by the cabbage aphid, *B. brassicae*, is decreased by 8.8 ppm of destruxin E in the sap, whereas *R. padi* is resistant to inhibition of feeding activity. A contact insecticidal activity of destruxin E was demonstrated by Poprawski et al. (1994). Nymphs of *Empoasca vitis* were susceptible to destruxin E applied by spraying on potato leaves or directly on insects. First instars of *Pieris brassicae* and *Agrotis segetum* were exposed to crude extract of destruxins per os. Again, destruxin E was the most potent against *P. brassicae*, while destruxin A was least active. *Agrotis segetum* larvae were only weakly susceptible to destruxins (Thomsen & Eilenberg 2000). In other experiments, antifeedant properties of low doses of destruxins A, B and E were noticed (Amiri et al. 1999). Treatment of cabbage leaf discs with destruxins significantly reduced feeding by larvae of *Plutella xylostella* and *Phaedon cochlearia.* LC_{50} values for destruxin A4 and A5 isolated from *Aschersonia* sp. in an insecticidal assay against *Drosophila melanogaster* were estimated at 41 and 52 ppm, respectively. Homodestruxin B showed no activity at 400 ppm in the same experiment (Krasnoff et al. 1996).

There always was a discussion on the correlation between the destruxin production and the virulence of *M. anisopliae* strains. In infection experiments with purified destruxins and scarabaeid larvae of *Cetonia aurata* and *Oryctes rhinoceros*, Fargues et al. (1985) stated that the role of destruxins produced by *M. anisopliae* depends on the host species and their ability to detoxify these metabolites and on the pathotypes and their capacity to produce toxins in their specific or non-specific host, as demonstrated in *C. aurata* larvae. Samuels et al. (1988) suggested that destruxins may have a 'pathogenic role', when the toxins are active in causing disease, or an 'aggressive role', when they facilitate the establishment of the pathogen. In the black vine weevil, *Otiorhynchus sulcatus*, and the tobacco hornworm, *Manduca sexta*, a significant negative correlation was found between the titer of destruxin production *in vitro* of isolates of *M. anisopliae* var. *anisopliae* and the median lethal time, suggesting a role of destruxins in the virulence of isolates. However, one isolate was highly virulent for *M. sexta*, but did not produce destruxins *in vitro* (Kershaw et al. 1999). For the desert locust, *Schistocerca gregaria*, a strong positive correlation between *in vitro* toxin production and the percentage mortality was observed. From these experiments, it is concluded that in the pathogenesis of *M. anisopliae* against *M. sexta, S. gregaria* and *O. sulcatus*, there is a relationship between the titer of destruxin production *in vitro* and the killing power. These findings are supported by Amiri-Besheli et al. (2000). Most virulent strains also

produced large quantities of destruxins, but some low toxin producers were also virulent, suggesting, that destruxins are not the only pathogenicity determinants.

(2) Various cytopathological effects of destruxins on insect and invertebrate cells and cell lines were described, including the action as ionophores, inducing the formation of pores in cellular membranes, or as mitochondrial ATPase inhibitors, but also causing morphological alterations in midgut cells which is a Ca-dependent process (Dumas et al. 1996). Ultrastructural alterations were observed in insect plasmatocytes and granular haemocytes with a toxic dose of destruxin E, the most active compound. It is suggested that the fungal peptides may intervene during the disease by a true immune-inhibitory effect occurring at doses which do not cause paralysis or any general sign of toxicity (Vey et al. 2002). A comprehensive table on various activities of destruxins is presented by Vey et al. (2001). For example, destruxin A, B and E cause *B. mori* cell lines to contract, become granulated and stop dividing; destruxin E affects invertebrate cells by aggregation of chromatin, deformation of nuclei, degradation of mitochondria and rough endoplasmatic reticulum and impaired functioning of the ribosomes; destruxins inhibit phagocytosis in plasmatocytes *in vitro* and in infected larvae and inhibit synthesis of DNA, RNA and proteins of mouse P388 leukaemic cell lines; destruxins have antiviral effects in insect cells and cause rapid decrease in the transmembrane resting potential.

Recently, the toxicity of individual destruxins (destruxin A, B and E) and the complete crude extract from liquid fungus culture was compared using human and insect cell lines (Skrobek & Butt 2005). All three destruxins had no effect on human leukemic HL60 cells at 500 ppm, while destruxin A caused mortality in the insect cells SF9. In contrast, the crude extract of *M. anisopliae* was cytotoxic to both cell lines. Therefore, crude extracts are recommended by the authors for assessing the risks of metabolites within the registration procedure instead of purified individual destruxins.

(3) Destruxins have been found to be toxic to small mammals. After intraperitoneal injection of destruxin A, the LD_{50} in mice was 1.0–1.35 mg kg^{-1} and of destruxin B, 13.2–16.9 mg kg^{-1}. An antibacterial or antifungal activity of both compounds was not detected (Kodaira 1961). They are less toxic to fish and amphibians, i.e. no lethal or teratogenic effect of the embryos of the fish *Brachydanio rerio* was observed and the acute toxicity of destruxins on the amphibians *Xenopus laevis* and *Rana temporaria* is low (see Vey et al. 2001).

A neutral extract from *M. anisopliae* cultures was evaluated for toxicity and mutagenicity using an aquatic animal bioassay and the Ames test (Genthner et al. 1998). While the average LC_{50} of 24-h-old *Mysidopsis bahia* was 2.41 mg L^{-1}, the purified destruxins alone were not responsible for the toxicity in mysids. The neutral extract was fetotoxic to developing grass shrimp, *Palaemonetes pugio*, and frog, *Xenopus laevis*, embryos. This neutral extract was also toxic to juvenile mosquito fish, *Gambusia affinis*, at an LC_{50} value of 141 mg L^{-1}. Adult females of *G. affinis* survived a 24-h exposure to 200 μg mL^{-1} of the neutral extract.

(4) As mentioned above, destruxins are also produced by the plant pathogen *A. brassicae* during its infestation and colonisation of the host plant. In this case, destruxins were shown to be phytotoxic, which is manifested by chlorosis and necrotic spots on the leaf surface of host and non-host plants (Buchwaldt & Green 1992). A list on the phytotoxicity against a variety of plants, excised leaves, leaf disks, seedlings, pollen grains, protoplasts and cell cultures is given by Pedras et al. (2002). A destruxin B detoxification pathway is present in *Sinapis alba* and was also found in *Camelina*

sativa, *Capsella bursa-pastoris* and *Eruca sativa* (Pedras et al. 2003). *Camelina sativa* and *C. bursa-pastoris* detoxify destruxin B to the phytoalexins camalexins.

Investigations on the efficacy of destruxin E against aphids revealed that this metabolite might be systemic in plants, because the cabbage aphid *B. brassicae* is repelled by cabbage leaves soaked in a solution of destruxin E (Robert & Riba 1989).

Cytochalasins. Up to 1981, 10 cytochalasins produced by a wide variety of fungi were known (Roberts 1981). In the Merck Index, more than 20 cytochalasins isolated from several fungi are mentioned (Budavari 1996). Cytochalasins are characterised by a hydrogenated isoindole ring to which a microcyclic ring is fused. Two cytochalasins, C and D, were isolated from cultures of *M. anisopliae* (see Roberts 1981). Cytochalasin D additionally occurs in the fungi *Zygosporium mansonii* and *Helminthosporium* sp. The acute toxicity of cytochalasin D (LD_{50}) in mice was 1.85 mg kg^{-1} by subcutaneous injection, 36 mg kg^{-1} per os and 10 mg kg^{-1} by intraperitoneal injection (see Roberts 1981).

At present, the major interest in cytochalasins is in medicine and cell biology, especially cytological research. Their activities, uses and applications are documented at http://en.wikipedia.org/wiki/Cytochalasin. Generally, cytochalasins are cell-permeable fungal metabolites that bind to actin filaments, inhibit actin polymerisation and may interfere with various cellular processes.

Swainsonine. The indolizidine alkaloid swainsonine was first discovered in *Swainsona canescens*, a herbaceous legume native to Australia (Colegate et al. 1979). Meanwhile, it has been found also in plant species of *Oxytropis*, *Astragalus*, *Swainsona*, *Sphaerophysa*, *Ipomea* and *Sida*. Swainsonine is believed to be synthesised by the plants or, in species of *Oxytropis* and *Astragalus*, also by an endophytic fungus called *Embellisia* sp. (see Valdez-Barillas 2006). It is the primary toxic compound of a number of *Astragalus* species, which have a worldwide distribution. In North America, certain *Astragalus* species produce the so-called 'poisoning by locoweeds' to livestock and wildlife (see Valdez-Barillas 2006).

Swainsonine synthesis has also been reported in *M. anisopliae* (Patrick et al. 1993; Sim & Perry 1995, 1997) and in the fungus *Rhizoctonia leguminicola* (Harris et al. 1988). Swainsonine is a potent inhibitor of mannosidases. It inhibits glycoprotein processing and acts as an immune modulator. It is used in traditional medicine and has some therapeutic potential in cancer treatment. Because of its pharmaceutical activities, the production of swainsonine by *M. anisopliae* was studied in detail (Tamerler-Yildir et al. 1997; Tamerler et al. 1998; Tamerler & Keshavarz 1999).

Effect of environmental factors (temperature, humidity, solar radiation)

An increased understanding of the ecology of *M. anisopliae* and the impact of environmental factors on this fungus is important not only with respect to the development of successful biocontrol strategies, but also to our knowledge on its fate and persistence in the environment. According to Fuxa (1995), one definition of 'ecology' is the scientific study of the distribution and abundance of organisms. This distribution and abundance in the environment is mostly affected by various abiotic and biotic factors, which influence the entomopathogen itself as well as its host insects and the crops.

With respect to the wide use of *M. anisopliae* as a biocontrol agent, there are numerous studies and compilations on the impact of environmental factors on its efficacy and viability (e.g. Müller-Kögler 1965; Roberts & Campbell 1977; Keller & Zimmermann 1989; Glare 1991; Fuxa 1995). In this section, the most important abiotic factors, i.e. temperature, humidity and solar radiation, affecting growth, efficacy and stability of *M. anisopliae* in the environment are summarised.

Temperature. Temperature can affect the germination and growth as well as the viability of an entomopathogenic fungus in the laboratory as well as in the field. *Metarhizium anisopliae* is a mesophilic fungus with a temperature range generally between 15 and 35°C, and the optimum for germination and growth between 25 and 30°C (see Müller-Kögler 1965; Walstad et al. 1970; Roberts & Campbell 1977; Alves et al. 1984; Hywel-Jones & Gillespie 1990; Welling et al. 1994; Ekesi et al. 1999a; Milner et al. 2003a). Significant differences among isolates in germination and radial growth at constant temperatures were found (Dimbi et al. 2004), i.e. over 80% of conidia germinated at 20, 25 and 30°C after 24h, while 26–67% germinated at 35°C and less than 10% at 15°C. In all isolates, the optimum temperature was 25°C, but also cold-active and heat-tolerant isolates were found. Rath et al. (1995a) reported that *M. anisopliae* DAT F-001 spores are able to germinate at all temperatures from 2 to 25°C. This strain is capable to infect its host insect, the scarab *Adoryphorus couloni*, at temperatures of 10°C or at a fluctuating temperature of 15/5°C. Two isolates of *M. anisopliae* from the subantarctic soils of Macquarie Island germinated within 49 days at 2.5°C, while the remaining two required temperatures of 7.5 and 10°C (Roddam & Rath 1997). Ten out of 32 isolates of *M. anisopliae* mostly obtained from Ontario, Canada, were deemed cold-active because of their ability to grow at 8°C (De Croos & Bidochka 1999). There was no general relationship between latitude and growth rates, however, the authors found that all cold-active isolates were from the more northern sites and no isolate originating below 43.5° latitude showed cold activity. Later, cold-induced proteins could be observed in cold-active isolates grown at 8°C when compared with 25°C (De Croos & Bidochka 2001).

On the other hand, there are isolates of *M. anisopliae*, mostly from tropical regions, which are able to grow at temperatures above 35°C. This is an important aspect for different reasons: (1) possible infection and growth at body temperature of mammals (37°C), (2) effectiveness of the fungus against pest insects such as locusts under tropical and subtropical areas, (3) evaluation of thermotolerance and thermal death point, and (4) stability of conidia or the product under elevated temperatures. Four isolates from Madagascar grew at 36°C and one isolate of *M. flavoviride* even at 38°C (Welling et al. 1994). Of 22 isolates of *M. anisopliae* and 14 isolates of *M. flavoviride*, the majority of the isolates of both species grew between 11 and 32°C; several isolates grew at 8° and 37°C, none at 40°C (Ouedraogo et al. 1997). Hallsworth and Magan (1999) mentioned that the temperature ranges for growth of *M. anisopliae* were 5–40°C with an optimum growth temperature of their strains at 30°C. In this respect, it should be mentioned that fungus-infected locusts, such as *Locusta migratoria*, have the capacity to develop a behavioural fever which inhibits fungal growth of *M. anisopliae* var. *acridum* at elevated temperatures (e.g. Ouedraogo et al. 2003).

In nature, *M. anisopliae* may be exposed to high temperatures, e.g. in tropical crops or mainly in the upper soil layer, where the fungus is primarily found. When the fungus was considered for biocontrol of larvae of *Oryctes rhinoceros* in compost heaps

in the Pacific Islands, temperatures lethal to *M. anisopliae* conidia were already studied (Johnpulle 1938). Conidia germinated after 48 h exposure time to 42°C or 60 min to 48°C. However, exposure of *M. anisopliae* conidia for 5 min in sterile dung extract at temperatures of 55°C and above inhibited germination. According to Walstad et al. (1970), the thermal death point for *M. anisopliae* spores was near 50°C (49°C for 10 min). However, it is well known that heat resistence of fungal spores is closely related to moisture conditions. The median lethal temperature of conidia after 30 min in water was 42°C, at 100% RH 50.5°C, at 75% RH 57.5°C and at 33% RH 68.8°C (Zimmermann 1982).

Soil temperatures of up to 65°C have been reported in Niger, West Africa, and of about 50–60°C in corn fields in the USA (Rangel et al. 2005). The authors studied the variability in thermotolerance of 16 strains from *M. anisopliae* var. *anisopliae* and one from *M. anisopliae* var. *acridum* isolated from latitudes 61°N to 54°S. After 12 h of exposure, most of the isolates tolerated 40°C with germination rates of about 90%. After 2 h exposure at 45°C, six isolates showed a germination of 80%, three isolates between 50 and 70% and eight isolates between 0 and 30%. After 8 and 12 h at 45°C, only two isolates pathogenic to grasshoppers still had high germination. In general, isolates from higher latitudes showed a greater heat susceptibility than those from nearer the equator. Dry conidia better tolerated 50°C (Rangel et al. 2005).

Temperature also affects the storability of fungi. For example, the viability of dry *M. anisopliae* conidia decreases with increasing storage temperature from 8 to 25°C and also by exposure to light (Clerk & Madelin 1965). The effect of fluctuating temperature regimes between 20 and 50°C at 13.7% moisture content showed that change in temperature per se had no effect on the survival of conidia of *M. flavoviride* (Hong et al. 1999).

Humidity. Humidity is a very important environmental factor not only affecting the efficacy but also the survival of an entomopathogen. Generally, a high relative humidity (RH) is necessary for germination of *M. anisopliae*. Walstad et al. (1970) found that the best germination occurred at 100% RH. Some germination was also noticed at 92.5% RH but no germination at 85% RH. According to Milner et al. (1997), germination was increasingly delayed at water activities equivalent to 99, 98 and 96% RH and completely inhibited at 94, 92 and 90% RH. In bioassays with termites at RH down to 86%, however, no effect of humidity on pathogenicity was detected. This demonstrates the importance of the microclimate near the germinating conidia. In various *M. anisopliae* strains tested by Hallsworth and Magan (1999), the water activity (a_w) optima for growth ranged between 0.99 and 0.97 on KCl-, glycerol-, and PEG 600-modified media. Recently, Lazzarini et al. (2006) found that at 0.93 a_w, germination was delayed but most isolates germinated within 216 h of incubation. However, no relationship was found between the germination at 0.93 a_w and the activity of these strains in bioassays against *Triatoma infestans* at 98, 75 and 43% RH. In the field, successful infection at very low relative humidities of about 20–30% was observed in desert locusts using oil formulations (Bateman et al. 1993).The RH affects the viability of conidia of *M. anisopliae* after storage at different temperatures (Clerk & Madelin 1965; Roberts & Campbell 1977). In *M. flavoviride* conidia, the optimal moisture content for storage was 4–5%. Dried conidia stored as powder at 10–14°C showed 95% germination, but only up to 27% at 28–32°C. Dried

conidia maintained greater than 90% germination over 128 days with or without silica gel at 10–14°C or –15 to –18°C (Moore et al. 1996a).

Solar radiation. Besides temperature and relative humidity, solar ultraviolet radiation (UV-A and UV-B) is one of the key factors responsible for the efficacy or unreliability of *M. anisopliae* in the field, i.e. it may affect the survival and, thus, plays an important safety issue with respect to residues on treated crops.

There are many studies on the susceptibility of *M. anisopliae* conidia to artificial or natural sunlight with or without sunscreens (Zimmermann 1982; Ignoffo & Garcia 1992; Moore et al. 1993, 1996b; Hunt et al. 1994; Fargues et al. 1996; Alves et al. 1998; Shah et al. 1998; Braga et al. 2001a,b,c; Rangel et al. 2004). All results demonstrate that UV-B (280–320 nm) and UV-A (320–400 nm) are the most detrimental components of natural sunlight which cause inactivation of *M. anisopliae* conidia within hours. Under artificial sunlight, the half-life was 1 h 40 min after 24 h incubation and 2 h 45 min at 48 h incubation, i.e. the germination process after irradiation is impaired (Zimmermann 1982). The survival of conidia of 23 isolates of *M. anisopliae* and 14 of *M. flavoviride*, irradiated with artificial sunlight (295–1100 nm), decreased with increasing exposure, i.e. exposure for 2 h or more was detrimental to all isolates tested (Fargues et al. 1996). According to Braga et al. (2001a), exposure to UV radiation of only 1 h caused a delay of several hours in the germination of surviving *M. anisopliae* conidia. These were unable to germinate during direct exposure to UV-B of simulated sunlight, but the beginning of germination increased the UV tolerance, while it was decreased when the exposure started on the sixth hour of germination (Braga et al. 2001a).

Variability in UV-B tolerance in some species and strains of *Metarhizium* was demonstrated (Fargues et al. 1996; Braga et al. 2001b,c). Fargues et al. (1996) found that *M. flavoviride* was most resistant followed by *M. anisopliae*. Braga et al. (2001b) reported that 4 h exposure to full-spectrum sunlight reduced the relative culturability by approximately 30% for *M. anisopliae* ARSEF 324 and by 100% for ARSEF 23 and 2575. In the laboratory, a 4-h exposure to solar UV-A reduced the relative culturability by 10% for strain ARSEF 324, 40% for ARSEF 23 and 60% for ARSEF 2575. The results also clearly demonstrate the negative effects of the UV-A component on survival and germination of *M. anisopliae* conidia under natural conditions.

The growth substrate on which conidia are produced may also influence their UV-B tolerance and speed of germination (Rangel et al. 2004). Conidia of two *M. anisopliae* isolates produced on insects were significantly more sensitive to UV-B radiation than conidia produced on PDAY. Furthermore, conidia produced on Czapek's and Emerson's YpSs agar media or on rice grains showed a higher tolerance to UV-B and germinated faster than conidia from PDA and PDAY.

Another aspect to consider is the pigmentation of the conidia. Ignoffo and Garcia (1992) reported that entomogenous fungi with dark pigmented conidia were more stable than the lighter pigmented conidia. The half-life of *M. anisopliae* was about 1.5 h, while the black conidia of *Aspergillus niger* were more stable to simulated sunlight (nearly 15 h). However, these findings contradict the results of Fargues et al. (1996) who found that 61% of *B. bassiana* isolates and only 26% of *M. anisopliae* isolates exhibited over 50% survival after 1 h of irradiation.

With respect to a successful use of this fungus as a biocontrol agent against leaf feeding pest insects in the field, the high susceptibility of *M. anisopliae* to natural solar

radiation necessitates the development of formulations including UV protectants. Several investigations have been conducted to develop more UV stable formulations (Moore et al. 1993, 1996b; Hunt et al. 1994; Alves et al. 1998; Shah et al. 1998). Exposure of conidia of *M. flavoviride* in water to UV radiation for 1 h resulted in 4.7% germination after 24 h incubation, compared with 36.5% germination for conidia in oil. The addition of 1% oxybenzone to the oil formulation resulted in 82% germination after 3 h exposure and 48 h incubation, compared with 28% germination without the sunscreen (Moore et al. 1993). However, when the sunscreen oxybenzone (2%) was tested in a field trial in Mali against the grasshopper *Kraussella amabile*, no significant differences between the *M. flavoviride* treatments with or without oxybenzone were noticed (Shah et al. 1998). Based on cage mortality data, the half-life for conidial infectivity on the treated vegetation was 4.3 days. With increasing temperature, oil-formulated conidia of *M. flavoviride* showed an increased damage to UV light (Moore et al. 1996b), and different oils, such as peanut oil or Shellsol plus Ondina, significantly enhanced the conidial tolerance against UV light for up to 6 h of exposure (Alves et al. 1998).

Summarising these results, UV protection using sunscreens may prolong the survival of conidia against solar radiation only for hours rather than days.

Methods to determine and quantify residues

Generally, two methods are used for reisolation of entomopathogenic fungi and especially *M. anisopliae*: (1) selective media and (2) the so-called '*Galleria* bait method' (Zimmermann 1986). Selective media can be used for quantitative studies and for reisolation of the fungus from soil or plant material, while the bait method can only be used for qualitative studies, which give an indication of the presence and activity of the fungus in soil.

Initially, semiselective media used for isolation of soil fungi were also tested for entomopathogenic fungi. These media generally contained glucose, peptone, oxgall, rose Bengal, sodium-propionate, crystal violet, cycloheximide and antibiotics, such as chloramphenicol and streptomycine (Veen & Ferron 1966; Müller-Kögler & Stein 1976; Pereira et al. 1979; Doberski & Tribe 1980; Mohan & Pillai 1982). It was Beilharz et al. (1982) who introduced the fungicide Dodine® (*n*-dodecylguanidine acetate) to their medium based on oatmeal agar for selective isolation of certain soil fungi. This medium proved also to be very useful for isolation of entomopathogenic fungi and especially *M. anisopliae*. Later, several authors found that the combination of Dodine® with cycloheximide at different concentrations was optimal for reisolation of *M. anisopliae* (e.g. Sneh 1991; Liu et al. 1993). The addition of the fungicide Benomyl® to the selective agar also proved to be useful (Chase et al. 1986), and as *M. anisopliae* was shown to be very tolerant to copper, the use of copper-amended media was suggested (Bååth 1991).

Today, different selective media for *M. anisopliae* are in use, including a medium developed for reisolation of *Beauveria brongniartii* (Strasser et al. 1996). Generally, in addition to oatmeal, glucose or peptone, they contain cycloheximide, dodine and antibiotics, such as streptomycine, chloramphenicol and tetracycline, all in various concentrations. Generally, up to about 10^2 conidia or propagules per 1 g of soil can be recovered.

The so-called '*Galleria* bait method' was used in most of the investigations on the natural occurrence of *M. anisopliae* in different soil types, areas or countries (Zimmermann 1986). This technique allows the isolation of *M. anisopliae* and other entomopathogenic fungi from soil samples using larvae of the greater wax moth, *Galleria mellonella*, or of the yellow meal worm, *Tenebrio molitor*, as a bait. A detailed description of a standardised method is given by Zimmermann (1998).

Fate and behaviour in the environment

Mobility and persistence in air

Metarhizium anisopliae is a typical soil-borne fungus with conidia produced in chains and sticking together more or less closely to conidial columns. Thus, it is unlikely, that conidia of this fungus are naturally occurring in the air, and so far, to my knowledge, no reports on *M. anisopliae* as an air-borne fungus are known. Nevertheless, the fungus has been found naturally on leaf feeding and plant sucking insects, and *M. anisopliae* was used within the so-called autodissemination strategy by combination with attractant traps or contamination devices in order to contaminate attracted pest insects which then transmit the fungus to the target pest population (Kaakeh et al. 1996; Klein & Lacey 1999; Maniania 2002; Quesada-Moraga et al. 2004). The persistence of conidia of *M. anisopliae* in the air which is strongly affected by temperature, relative humidity (RH), solar radiation and moisture content of the spores is discussed in Effect of environmental factors.

Mobility and persistence in water

There are different aspects affecting the mobility and persistence of fungal spores in water: (1) water can be used for long-term storage of fungi, (2) water is responsible for migration/percolation of spores into the soil, and (3) water as raindrops is responsible for dispersal of fungal conidia. These aspects will be discussed here. However, to my knowledge, there are no informations on the fate and behaviour of unformulated and formulated *M. anisopliae* conidia when sprayed or drifted onto water surfaces such as lakes. Some effects on aquatic organisms and fish in the laboratory are reported later.

For example, after storage of *M. anisopliae* in a sterile aqueous solution of 0.675% NaCl at 4°C, the fungus survived 1–2 years in the laboratory (Müller-Kögler & Zimmermann 1980). Studies on the germination of *M. anisopliae* conidia in water are summarised by Roberts and Campbell (1977). No germination was observed in sterile water, in sterile, distilled water, in tap water or in 1 and 2% saline. In rain water, no to very low germination was recorded, while in water +2% peptone, a moderate to abundant germination of conidia was noticed. In sterile, deionised water+Tween 80, some germination was noted after 6 weeks at room temperature.

The effect of water on the percolation of *M. anisopliae* spores in soil will be discussed in Mobility and persistence in soil. It is obvious that water as rain has an impact on the stability and efficacy of fungal spores on plants in the field. Inyang et al. (2000) investigated the effect of simulated rain on the persistence of oil and water formulations of conidia of *M. anisopliae* when applied to oilseed rape foliage. When plants were exposed to simulated rain for 1 h, the mortality of larvae of the mustard beetle, *Phaedon cochleariae*, was reduced by 42, 57 and 51% after treatment with aqueous Tween, Shellsol T or sunflower oil/Shellsol T, respectively.

Mobility and persistence in soil

The mobility of fungal spores in soil is influenced by the soil type, water (rain), soil organisms, and by plant roots. Their persistence depends on various abiotic and biotic factors, such as temperature, moisture/water potential, agrochemicals, soil microorganisms, soil arthropods and plants (Keller & Zimmermann 1989). Mobility and persistence are closely related to microbial adhesion, which is of fundamental significance in the function and interaction between microorganisms. According to Marshall and Bitton (1980), microbial adhesion, i.e. attachment to surfaces, ensures that the microorganisms are not eliminated from their particular ecosystem.

As demonstrated in Natural occurrence and geographical distribution, *M. anisopliae* was isolated often from various soil sites and mostly from the upper 20 cm of the soil layer, which suggests that it is a typical soil-borne fungus.

Mobility. Research on the mobility of *M. anisopliae* in soil was first carried out with the product BIO 1020 (Reinecke et al. 1990). In a laboratory assay, the mortality of the test larvae of *Tenebrio molitor* was examined at different soil depths. After 4 and 8 weeks incubation of the soil columns, the mortality of the larvae below 6 cm was 51 and 41%, respectively, and at 16–18 cm, it was 5 and 22%, respectively. According to the authors, under practical conditions, the movement of conidia into the soil would be less than in the test model. Hartwig and Oehmig (1992) also observed that BIO 1020 cell granules and the produced conidia are not translocated in the soil, i.e. a high efficacy of the product could only be achieved if BIO 1020 is mixed into the soil area where the target pest is located. Zimmermann (1992) found, that in columns (length 30 cm) filled with standard soil and sand, the number of conidia of *M. anisopliae* per g soil decreased rapidly with soil depth after watering. At 10 cm, the spore number was reduced by 10 or 100-fold. Studies on the vertical movement of wet and dry spores of *M. anisopliae* through a 30-cm sand column revealed that less than one spore per 1 mL effluent was found. The results demonstrated that a contamination of groundwater by *M. anisopliae* is very unlikely (Zimmermann 1992). Under field conditions in Finland, most of the spores sprayed on the ground were kept at 0–5 cm in loamy soil, while in humus, they were found in deeper soil layers at 5–15 cm and 15–20 cm (Tyni-Juslin & Vänninen 1990; Vänninen et al. 2000). In a recent paper on the diversity and significance of mould species in Norwegian drinking water, *M. anisopliae* was not listed in contrast to *B. bassiana* and *B. brongniartii* (Hageskal et al. 2006).

As already mentioned, mobility of fungal spores in the soil can also be caused by soil organisms. It was demonstrated that Acari, collembolans, small dipterous and coleopterous larvae are able to transport conidia of *M. anisopliae* passively through soil layers (Zimmermann & Bode 1983). In a laboratory trial, the two mites *Histiogaster anops* and *Macrocheles* sp. were able to transfer spores of *M. anisopliae*. Phoretic mites can act as carriers, however, both mite species were also susceptible to *M. anisopliae* (Schabel 1982). Dromph (2003) found that three collembolans, *Folsomia fimitaria*, *Hypogastrura assimilis* and *Proisotoma minuta,* were able to transmit spores of *M. anisopliae* to a susceptible host, *Tenebrio molitor.* Thus, collembolans are able to transmit and disperse the fungus within the soil, and the transmission is by faecal pellets and by adhesion of spores on their bodies.

Persistence. The persistence of a biocontrol agent can be seen from two different perspectives, i.e. from the demands of a regulator or from those of a producer or biocontrol user. The former prefers a low persistence of the fungus with respect to possible undesirable environmental impacts, while the latter is interested in a long persistence for better and prolonged product efficacy. Results of the following papers demonstrate that conidia of *M. anisopliae* may persist in the soil for several months and even years depending on the strain and the soil conditions.

In the laboratory, the fungus was able to survive up to 6 years after storage in sterile soil at 4°C (Müller-Kögler & Zimmermann 1980). Under field conditions, the persistence of *M. anisopliae* in the upper soil layer is mainly affected by temperature and soil moisture. Soil temperatures of up to 65°C have been reported in Niger, West Africa, and of about 50–60°C in corn fields in the USA (Rangel et al. 2005). This means that conidia of *M. anisopliae* in solar-exposed soil are subjected to high and lethal temperatures. In studies on the thermotolerance of 16 isolates of *M. anisopliae* var. *anisopliae* and one of *M. anisopliae* var. *acridum*, Rangel et al. (2005) found that most of the isolates tolerated 40°C after 12 h of exposure; after 8 h and 12 h at 45°C, however, only two isolates pathogenic to grasshoppers still had high germination.

Investigations on the effect of soil temperature and moisture on the survival and infectivity of *M. anisopliae* to tephritid fruit fly puparia demonstrated that the mortality of puparia at 20–30°C was highest at a water potential of –0.1 and –0.01 mega Pascal (MPA) and lowest at –0.0055 and –0.0035 MPA (Ekesi et al. 2003).

Further studies reveal that the persistence of conidia is also strain dependent (Fargues & Robert 1985; Vänninen et al. 2000; Milner et al. 2003b). For example, while *M. anisopliae* No 32 was substantially degraded after 6 months incubation (70–80% dry weight loss), *M. anisopliae* No 51 remained at the initial level after 21 months (Fargues & Robert 1985).

In Finnish experiments, conidia of *M. anisopliae* and *B. bassiana* were spread on test areas as water suspensions at a rate of 10^{10} spores m^{-2} (Tyni-Juslin & Vänninen 1990). After one year, the mean counts were about 34% for *M. anisopliae* and only 0.2% for *B. bassiana* of the originally spread spore amount, indicating that *M. anisopliae* is more stable. Later, Vänninen et al. (2000) found that *M. anisopliae* was still infective in soil 3 years post-application.

The viability in soil of the *M. anisopliae* product BIO 1020 was tested with respect to a long-term effect against the black vine weevil, *Otiorhynchus sulcatus* (Hartwig & Oehmig 1992). After 4 weeks at 20°C, the number of conidia in soil declined to about 50% of the number at the beginning of the experiment. Nevertheless, *O. sulcatus* could still be controlled completely up to the 42nd week. In a field experiment starting in May, Hartwig and Oehmig (1992) further found that BIO 1020 retained its efficacy of 100% until October, while in February, the number of conidia declined below an adequate level of efficacy.

In a long-term experiment in Australia, the field persistence in sugar cane soil of two isolates of *M. anisopliae* and four formulations was tested for 3.5 years (Milner et al. 2003b). After 3 years post-application, the 'BioCane' formulation of rice still provided some level of infection of the target pest insect in the soil. A monthly decay rate was observed and only a small proportion of conidia survived the 3.5 years at all sites and all formulations. One isolate persisted better than the other and rainfall or soil type had negligible effects on the persistence.

The behaviour of a transformed strain of *M. anisopliae* ARSEF 1080, carrying the *Aequorea victoria* green fluorescent protein (gfp) gene alone (GMa) and additional protease genes (Pr1) (GPMa), was investigated in the soil under field conditions (Hu & St. Leger 2002). The study confirmed the utility of gfp for monitoring *M. anisopliae* strains in field populations and found that recombinant fungi were genetically stable over one year under these conditions. Results on the survival of the transgenic strains showed that in nonrhizosphere soil, the GMa decreased from 10^5 propagules/g at 0–2 cm to 10^3 per g after several months, while the densities of GMa remained at 10^5 propagules/g in the inner rhizosphere, demonstrating that rhizospheric soils are a potential reservoir for *M. anisopliae*.

Effects on non-target organisms

Effects on other microorganisms

Metarhizium anisopliae has antagonistic properties against some phytopathogenic fungi, but is also suppressed by several mycoparasites. The fungus was shown to be antagonistic to two strains of *Ophistoma ulmi* (=*Ceratocystis ulmi*), the cause of Dutch elm disease (Gemma et al. 1984). Under greenhouse conditions and in the field, a strain of *M. anisopliae* was also effectively used against *Phoma betae*, the blackleg of beet (Roberti et al. 1993). No phytotoxic effects on seedlings were observed, and *in vitro*, a clear inhibition zone between the two fungi was noted. Recently, the compatibility of *M. anisopliae* and other entomopathogenic fungi with the mycoparasites *Clonostachys* spp. (formerly *Gliocladium* spp.), *Trichoderma harzianum* and *Lecanicillium lecanii* was investigated (Krauss et al. 2004). *In vitro* host-range tests showed that *M. anisopliae* was highly susceptible to all mycoparasites tested. However, coapplication of mycoparasites with the entomopathogen did not affect their biocontrol efficacy *in vivo*.

Investigations on the behaviour of a transformed strain of *M. anisopliae* ARSEF 1080 in the soil under field conditions showed that the deployment of the transgenic strain did not depress the culturable indigenous fungal microflora (Hu & St. Leger 2002).

Effects on plants

So far, no phytopathogenic or phytotoxic effects of *M. anisopliae*, either on leaves or plant roots, are known. For example, no reaction of the plants was noticed after treatment of strawberry plants or root sensitive azalea with a high conidial suspension of *M. anisopliae* (Zimmermann 1981). Furthermore, no negative effects on the root development and plant growth were observed after application of the former *M. anisopliae* product BIO 1020 to the soil of various ornamental or nursery plants (Stenzel 1992). In some cases, even a plant growth promoting activity was noted. However, there is also some evidence that roots and root exudates of certain plant species may have a negative effect on the activity of *M. anisopliae*. In greenhouse experiments on biocontrol of the black vine weevil, *Otiorhynchus sulcatus*, with *M. anisopliae*, Zimmermann (1984) noticed that the fungus always was less effective in cyclamen. Later, an inhibitory effect of cyclamen metabolites was verified *in vitro*

(Zimmermann, unpubl.). Similar findings were supported by Ekesi et al. (2000), while studying the susceptibility of *Megalurothrips sjostedti* to *M. anisopliae* when reared on susceptible, tolerant and moderately resistant varieties of cowpea. The mortality was significantly higher on the moderately resistant variety compared to the susceptible and tolerant varieties, suggesting the existence of anti-fungal substances in the latter two varieties, or the thrips feeding on resistant varieties are more stressed and so more susceptible to infection.

Epicuticular waxes or plant volatiles may have positive or negative effects on the activity and infectivity of *M. anisopliae*. The epicuticular waxes of some crucifers, like oilseed rape or Chinese cabbage, contain a mixture of stimulatory and inhibitory compounds. Plant extracts stimulated germination and formulation of conidia in leachates or leaf extracts increased the virulence of *M. anisopliae* (Inyang et al. 1999a). Oilseed rape and other Brassicaceae contain glucosinolates, some of which are hydrolysed to volatile isothiocyanates when plant tissues are disrupted. In their study, Inyang et al. (1999b) showed that isothiocyanates inhibited both germination and subsequent growth of *M. anisopliae in vitro* and reduced its ability to infect insects. In a profound study on interactions between brassicaceous plants, isothiocyanates and *M. anisopliae*, it was shown that 100 parts per million of 2-phenylethyl isothiocyanate completely inhibited the fungus, however, no fungal inhibition was found when using a more realistic fungus/plant/soil microcosm (Klingen et al. 2002b). Purified cucurbitacin E glycoside from watermelons did not inhibit the growth of *M. anisopliae* (Martin & Schroder 2000).

Destruxin B is a major metabolite produced not only by *M. anisopliae* but also by the phytopathogen *Alternaria brassicae*. Buchwaldt and Green (1992) could demonstrate that destruxin B causes necrotic and chlorotic symptoms both on host and non-host plants. But *Brassica* species were most sensitive to the toxin. However, there are no observations on phytotoxic reactions in plants after application of *M. anisopliae*, which demonstrate that destruxin B is produced only after successful infection of the plant by *A. brassicae*.

Effects on soil organisms

As *M. anisopliae* is a typical and widespread soil-borne fungus, its impact on soil organisms, such as mites or collembolans, has to be evaluated. In a laboratory experiment, Schabel (1982) demonstrated that the two phoretic mites *Histiogaster anops* and *Macrocheles* sp. were able to transfer spores of *M. anisopliae*, however, both mite species were also susceptible to *M. anisopliae*.

The effect of *M. anisopliae* on collembolans was also investigated in other tests. Experiments with BIO 1020 revealed that collembolans were not affected (Reinecke et al. 1990), and Broza et al. (2001) found that *Folsomia candida* was not susceptible. The collembollans consumed and inactivated the insect pathogen without any harmful effects. Adults of the three collembolan species *Folsomia fimetaria*, *Hypogastrura assimilis* and *Proisotoma minuta* were dipped in 1×10^7 conidia mL^{-1} of *M. anisopliae* and *F. fimetaria* in 1×10^8 conidia mL^{-1} (Dromph & Vestergaard 2002). No mortality was observed. Yet, incubating adult *F. fimetaria* and *P. minuta* for 14 days at 20°C in sphagnum containing 1×10^8 conidia g^{-1} wet weight resulted in an increased mortality of 42% compared to 17% in the control. The results demonstrated

that *M. anisopliae* obviously has a low virulence against these collembolans. Dromph (2003) found that collembolans, *F. fimitaria*, *H. assimils* and *P. minuta*, are able to transmit spores of *M. anisopliae* to a susceptible host, *Tenebrio molitor*. The author mentions that ingestion of spores by *P. minuta* and *F. fimetaria* significantly reduces the viability of conidia of *M. anisopliae* compared to conidia ingested by *H. assimilis*.

Effects on aquatic organisms

Developing embryos of the grass shrimp *Palaemonetes pugio*, a ubiquitous estuarine decapode living along the eastern and southern coasts of the USA, were exposed to conidia of *M. anisopliae* ARSEF 1080 (Genthner et al. 1997, 1998). The responses at about 1×10^4, 1×10^5 and 1×10^6 conidia mL^{-1} were variable, however, dead embryos and larvae with visible growth of *M. anisopliae* were observed in all experiments (Genthner et al. 1997). Significant adverse effects were noticed at 1×10^5 and 1×10^6 conidia mL^{-1} (Genthner et al. 1998). The authors found that conidia produced on homogenised corn earworm were more virulent than those produced on glucose-yeast extract-basal salts agar medium, and the higher virulence was correlated with an increase in the N-Acetyl-glucosaminidase activity (Genthner et al. 1997). The question remains, whether these findings are strain-specific, and whether the conidia concentrations used in the laboratory are reached under natural conditions on water areas in the field. Additional investigations are necessary.

A neutral extract from *M. anisopliae* ARSEF 2575 cultures was tested for toxicity and mutagenicity using aquatic animal bioassays and the Ames test (Genthner et al. 1998). The average LC_{50} of the neutral extract conducted with 24-h-old *Mysidopsis bahia* was 2.41 mg L^{-1}. The neutral extract was fetotoxic to developing *P. pugio*. Eye spot abnormalities were observed (Genthner et al. 1998). No adverse effects were observed on nymphs of mayfly, *Ulmerophlebia* sp., in laboratory tests of *M. anisopliae* var. *acridum* (1.3×10^6 conidia mL^{-1}), while the same dose caused 100% mortality in the cladoceran, *Ceriodaphnia dubia* within 48 h. At 6.7×10^3 conidia mL^{-1}, there was only 5% mortality after 192 h (Milner et al. 2002b). Based on the level of conidia entering the water during application, the authors conclude that the fungus is very unlikely to pose any hazard to aquatic organisms.

Effects on predators, parasitoids, honey bees, earth worms and other non-target arthropods

There is a large number of studies dealing with possible side effects of *M. anisopliae* on non-target organisms in the laboratory and in the field, especially on predators, parasitoids, honey bees and earthworms (Table V). The subject is already summarised in several, general reviews (e.g. Goettel et al. 1990; Hokkanen et al. 2003; Vestergaard et al. 2003). Vestergaard et al. (2003) stated that *M. anisopliae* var. *anisopliae* can infect a wide range of insects in laboratory experiments. Effects on some non-target organisms also appeared when the fungus was applied in the field, however, lower effects occurred in the field than in the laboratory. An interesting report on the relevance of regulatory requirements for ecotoxicological assessment of microbial insecticides is also published by Jaronski et al. (2003). The authors present data which document that results from the laboratory are not useful in predicting risks under field use. Furthermore, possible side effects must always be seen in connection with the intended use of *M. anisopliae,* i.e. application in the soil, in glasshouses or in the field on certain plants or crops.

Table V. Examples of side-effects of *M. anisopliae* (*M.a.;* strains and formulations) on non-target organisms.

Non-target organism	Strain/Formulation	Lab./Field tests (LF)	Results/Observations	References
Apis mellifera	–	F	*M.a.* was used in field trials for control of *Varroa destructor* It was harmless to adult bees or brood, and colony development was not affected	Kanga et al. (2003)
Apis mellifera	–	F	There was no evidence of any adverse effect on the honey bee colonies	Butt et al. (1998)
Apis mellifera	–	F	*M.a.* conidia were applied in bee hives: low mortality and no noticeable effect on behaviour, larvae and colony characteristics	Alves et al. (1996)
Apis mellifera	V208 and V245	L	Bees were less susceptible at low doses, but at 1×10^{10} conidia mL^{-1} almost all bees died	Butt et al. (1994)
Apis mellifera	'Bio-Catch M'	–	Non-toxic	Copping (2004)
Apis mellifera	*M.a.* var. *acridum* (Green Muscle)	–	No adverse effects in orientation tests	Copping (2004)
Apis mellifera	BIO 1020	L	In orientating tests honey bees were not susceptible	Reinecke et al. (1990)
Apoanagyrus (=*Epidinocarsis*) *lopezi*	*M.a.* var. *acridum* (Green Muscle)	L/F	First exp.: 24% reduction in longevity and 16% mycosis. Second exp.: no significant effect on mortality	Stolz et al. (2002)
Bombus terrestris	–	L/F	*M.a.* is able to infect bumblebees; it appears that there are no risks if the fungus is incorporated into soil or sprayed onto plants that are not attractive to bumblebees	Hokkanen et al. (2003)
Chrysoperla kolthoffi	–	L	Treated females showed lengthening in preoviposition and reduced daily and total fecundity	Ventura et al. (2000)
Earthworms	BIO 1020	L	Earthworms were not influenced	Reinecke et al. (1990)
Earthworms	*M.a.* var. *acridum* (Green Muscle)	–	LC_{50} (14 days) >1000 mg kg^{-1} soil	Copping (2004)
Earthworms: *Aporrectodea caliginosa*	M.a. 39	L	No effect on the hatching rate of cocoons	Nuutinen et al. (1991)
Earthworms: *Lumbricus terrestris*	Barley grains with *M.a.*	L	No effect	Hozzank et al. (2003)
Epigeal arthropod scavengers	IMI 330189	F	Scavenging rates remained high in fungus treated plots in contrast to the fenitrothion plot	Arthurs et al. (2003)
Hippodamia convergens, Acheta domesticus, Oncopeltus fasciatus	ESCI, Bio-Blast	L	Significant mortality in *H. convergens* and *A. domesticus*, marginal effects on *O. fasciatus*	Ginsberg et al. (2002)

Table V (*Continued*)

Non-target organism	Strain/Formulation	Lab./Field tests (LF)	Results/Observations	References
Neoseiulus idaeus, Clavigralla tomentosicollis, Orius albidipennis	*M.a.* var. *acridum*	L	No sporulation was observed on any of the species tested; evidence for narrow host range	Attignon & Peveling (1999)
Non-target arthropods (Carabidae, Tenebrionidae, Formicidae, Ephydridae)	*M.a.* var. *acridum*	F	None of the taxa proved susceptible; median effect <25%	Peveling et al. (1999)
Non-target arthropods in a spruce stand	BIO 1020		BIO 1020 caused no severe negative effects on non-target arthropods of soil	Wernicke & Funke (1995)
Non-target beetle communities	Strain SP 3 and SP 9	F	SP 3 had distinct effects; SP 9 no detectable effects	Ivie et al. (2002)
Non-target invertebrates	DAT F-001	F	Incorporation of *M.a.* into soil did not reduce the number of non-target invertebrates	Rath et al. (1995c)
Non-target organisms	–	F	No adverse effect on populations of nontarget organisms	Ekesi et al. (1999b)
Phanerotoma sp.	*M.a.* var. *acridum* (Green Muscle)	L/F	Not susceptible	Stolz et al. (2002)
Phradis morionellus, Diospilus capito	2 strains	L/F	*P. morionellus* is less affected than the target pest; in *D. capito* the infection rate was higher	Husberg & Hokkanen (2001)
Pimelia senegalensis, Trachyderma hispida, Bracon hebetor, Apoanagyrus lopezi	Strain IMI 330189 and others	L	No infection in *P. senegalensis* and *T. hispida*; 100% mortality in the parasitoids *B. hebetor* and *A. lopezi*	Danfa & Van Der Valk (1999)
Prorops nasuta	3 isolates	L	*M.a.* strain 4 caused the lowest infection level	De La Rosa et al. (2000)
Spalangia cameroni	–	L	Female parasitoids were moderately susceptible; total fecundity was not different from uninfected ones	Nielsen et al. (2005)
Trichopsidea oestracea	Myco-insecticide	L	Parasitized grasshoppers were less susceptible; *M.a.* did not infect the parasitic nemestrinid larvae	Milner et al. (2002a)

Effects on vertebrates (fish, amphibia, reptiles and birds)

Fish

First experiments on the side effects of *M. anisopliae* against fish were carried out by Roberts (1976). Conidia applied to water showed no difference in mortality of *Epiplatys bifasciatus* compared to untreated fish. Several adverse effects were observed in embryos and the newly hatched larvae of the inland silverside fish *Menidia beryllina*; such as transitory effects on the heart, rupture of the chorion and fungal growth on mandibles of larvae (Genthner & Middaugh 1995). However, the responses were highly variable. Genthner et al. (1998) found, that the neutral extract of *M. anisopliae* was toxic to juvenile mosquito fish, *Gambusia affinis*, at an LC_{50} value of 141 mg L^{-1}. But no mortalities or adverse effects were observed in adult *Gambusia affinis* after 3 months, when fed a diet partially composed of a freeze-dried *M. anisopliae* culture. Significant mortalities were obtained when embryos of inland silverside fish, *Menidia beryllina*, were exposed to 1×10^6 conidia mL^{-1} of *M. anisopliae* ARSEF 1080. Laboratory tests of *M. anisopliae* var. *acridum* at 1.3×10^6 conidia mL^{-1} had no adverse effects on 8-week-old fry of the rainbow fish, *Melanotaenia duboulayi* (Milner et al. 2002b).

Amphibia

A fungal suspension of 1×10^9 spores of *M. anisopliae*, representing 2.95×10^{12} for a 70-kg human, was fed to the leopard frog, *Rana pipiens*. No mortality or recovery was recorded in any of the tissues. The viscera were free of fungal elements. Viability of spores was established in faecal washings of the pellets (Donovan-Peluso et al. 1980). In a special design, Genthner et al. (1998) studied the toxicity and mutagenicity of *M. anisopliae* using aquatic animal bioassays and the Ames test. The neutral extract of *M. anisopliae* cultures was fetotoxic to the frog, *Xenopus laevis*, embryos, however, exposure of frog embryos to *M. anisopliae* conidiospores did not cause significant mortalities or malformations.

Reptiles

Austwick (1980) mentions that *M. anisopliae* has been found in lesions in crocodilia and that it was experimentally possible to infect reptiles (lizards and terrapins). *M. anisopliae* var. *acridum* (isolate IMI 330189, Green Muscle®) was tested to the fringe-toed lizard, *Acanthodactylus dumerili*, in Mauretania, West Africa, using inhalation, oral exposure and feeding of mycosed locusts of *Schistocerca gregaria*. From the results it is concluded that no risks due to the fungus are anticipated at recommended field application rates. In contrast, a high toxicity of the insecticide Fipronil® to lizards was noted (Peveling & Demba 2003).

Birds

Birds may become exposed to *M. anisopliae* either directly, by consuming conidia deposited on their food, or secondarily, by consuming infected insects. Avian safety studies were conducted with the Japanese quail by Wasti et al. (1980). The test birds were allowed to consume spore suspensions of *M. anisopliae*. The total number of spores consumed was 4.9×10^{10}/bird. There was no mortality or abnormal behaviour in the experimental birds. Positive recoveries of *M. anisopliae* on plates streaked with

faecal washings were noticed. *Metarhizium anisopliae* was recovered from heart and lung smears of two test birds. However, careful histological examination showed no evidence of spores or hyphae in these tissues. In an orientating test, the *M. anisopliae* product BIO 1020 showed no adverse effects on quails (Reinecke et al. 1990). Ring-necked pheasants (*Phasianus colchicus*) were exposed to one of three diets; spore-coated feed, infected insects, or untreated feed of *M. flavoviride* (actual name *M. anisopliae* var. *acridum*), a biocontrol agent for locusts in Africa (Smits et al. 1999). Consumption resulted in neither pathological changes, or significant changes in weight, growth rate, behaviour or mortalities. Histological examination of organs indicated no changes compared to normal tissues. In another experiment with male and female ring-necked pheasants and *M. anisopliae* var. *acridum*, results of per os challenge from fungus-infected food showed no significant differences between the challenge and the control groups, and histopathological changes were generally undetectable (Johnson et al. 2002).

Effects on mammals and human health

Within the frame of the development of biocontrol products based on *M. anisopliae* and its worldwide use in agriculture and forestry, extensive safety studies on mammals have been carried out with this fungus. Reviews are presented by Ignoffo (1973), Saik et al. (1990), Siegel and Shadduck (1990), Burges (1981), Zimmermann (1993), Goettel and Jaronski (1997), Goettel et al. (2001) and Vestergaard et al. (2003). As already mentioned, a biopesticide fact sheet and a technical document on various safety issues of *M. anisopliae* strain F52 (029056) and *M. anisopliae* strain ESF1 (129056) are published by the US Environmental Protection Agency (EPA) at www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_029056.htm and 129056.htm, respectively. In the following section, the actual knowledge on *M. anisopliae* regarding allergenicity, pathogenicity or toxicity to mammals and humans is summarised.

Allergenicity

In contrast to *B. bassiana*, there are only a few records on the allergenicity of *M. anisopliae* and, to my knowledge, no findings on the natural occurrence of *M. anisopliae* in the air. This may be explained by the relatively large conidia sticking together in chains which therefore are not easily dispersed in the air.

Investigations on the allergenic potential of *M. anisopliae* were made mainly in the laboratory. First inhalation experiments of *M. anisopliae* conidia were conducted in rats, guinea pigs and mice (Schaerffenberg 1968; Shadduck et al. 1982; El-Kadi et al. 1983). No allergic reactions were observed. Further investigations, however, demonstrated that *M. anisopliae* has an allergenic potential. In a series of studies, Ward et al. (1998, 2000a,b) carried out crude allergenic extract inoculations, obtained from a *M. anisopliae* strain, into Balb/c mice and demonstrated that this extract contains components that induce immunological, inflammatory, and histopathological responses, which are characteristic of allergy. Allergic fractions of a specific crude extract of *M. anisopliae* were also obtained by Barbieri et al. (2005), and *M. anisopliae* was found to have the ability of increasing an allergic response to an allergen in mice and, thus, may also worsen allergy in susceptible individuals (Instanes et al. 2006).

In humans, a severe dermal hyperallergic response caused by an isolate of *M. anisopliae* var. *acridum* was reported by Goettel et al. (2001), and due to the wide use of *M. anisopliae* for biocontrol of various sugar cane pests in Brazil, several persons showing asthmatic symptoms due to this fungus were detected (see Barbieri et al. 2005). On the other hand, no allergic effects on the research or manufacturing staff, the formulators or on field workers have been observed when working with mycoinsecticides based on various strains of *M. anisopliae* var. *anisopliae* (BIO 1020, strain FI-1045, strains ICIPE 30 and 69) and *M. anisopliae* var. *acridum* (strains IMI 330189 and FI-985) (Copping 2004).

Pathogenicity/Toxicity

Natural occurrence. Safety data on *M. anisopliae* were summarised by Siegel and Shadduck (1990) and Zimmermann (1993). Until that time, *M. anisopliae* has never been reported as infecting mammals or humans. However, during the past years, some cases of human and mammalian infections have been described, but none was associated with the use of *M. anisopliae* as a biocontrol agent. The first well documented case of mammalian infection by *M. anisopliae* var. *anisopliae* was described by Muir et al. (1998). An invasive mycotic rhinitis was diagnosed in a cat with a 4-month history of nasal discharge and subcutaneous swelling of the nasal bridge. The infection was treated with orally administered itraconazole.

In humans, there are six reported cases of disease caused by *M. anisopliae*. *M. anisopliae* var. *anisopliae* was isolated for the first time from the eye of a Colombian male as the aetiological agent of keratomycosis (Cepero de Garcia et al. 1997). A topical natamycin treatment was successful. The first reported human case of possible disseminated infection with *M. anisopliae* var. *anisopliae* was reported by Burgner et al. (1998). A 9-year-old, immunosuppressed boy with a 5-year history of acute leukaemia was under chemotherapy throughout this period. *Metarhizium anisopliae* was isolated from three separate sites. Despite antifungal treatment with amphotericin and 5-flucytosine, the patient eventually died. By *in vitro* antifungal susceptibility testing it was found that this strain of *M. anisopliae* was resistant to itraconazole, fluconazole, ketoconazole and 5-flucytosine, but sensitive to amphotericin B. Two other cases of human infections were described by Revankar et al. (1999), a 36-year-old male with frontal and ethmoid sinusitis and a 76-year-old female. All isolates were resistant to amphotericin B, 5-flucytosine and fluconazole. Itraconazole and newer azole compounds were more active. One case of fungal keratitis due to *M. anisopliae* was described in a 36-year-old female in the United States (Jani et al. 2001), and recently, recurrent disseminated skin lesions caused by *M. anisopliae* were reported in an adult patient with acute myelogenous leukaemia (Osorio et al. 2007). The lesions were treated with voriconazole with prompt resolution.

Experiments. First experiments on mammalian safety of *M. anisopliae* were conducted about 40 years ago by Schaerffenberg (1968). These tests included injection, inhalation and feeding tests against adult white rats and did not show any toxic or pathogenic reactions. In another test, conidia of *M. anisopliae* from oat kernels containing 10% spores were fed to 10 white mice and four 3-week-old guinea pigs for 28 days. After histological examination, no loss of weight, a normal behaviour and no abnormalities in tissues could be observed (Latch 1976). Later, Shadduck et al.

(1982) conducted experiments with rats, mice and guinea pigs. No animals died after injection of, or exposure to *M. anisopliae*. There was no evidence of ocular irritation or spore germination in tissues. *Metarhizium anisopliae* was recovered from the stomach, lung and spleen after 2 weeks of exposure of mice to dusts but not at the end of week 3. The authors concluded that there was no evidence of human or mammalian pathogenicity of *M. anisopliae*.

El-Kadi et al. (1983) reported safety tests with guinea pigs and white mice. *Metarhizium anisopliae* was administered to the test animals by ingestion, inhalation and cutaneous and subcutaneous application. Anatomical and histopathological examination revealed that *M. anisopliae* was neither toxic nor pathogenic to test animals. Subcutaneous tests demonstrated that the conidia remained viable for at least one month in the body tissue. Viable spores were eliminated with the faeces. In other acute and chronic infection tests to rats, mice and guinea pigs including histopathological studies, strain *M. anisopliae* 83 proved to be safe (Fan et al. 1990). Furthermore, rats, mice, and rabbits treated with *M. anisopliae* in the laboratory by inhalation, orally, by injection or topical administration had no signs of infection or illness (Siegel & Shadduck 1990). Viable conidia were recovered from the spleens of rats for as long as 18 days after i.p. injection. *Metarhizium anisopliae* was recovered from the stomach, spleen and lungs of mice for as long as 14 d after inhalation of conidia. However, recovery declined and disappeared from the lungs after 14 days. In another test with albino rats treated with a fungal spore suspension both orally and parenterally, all animals were normal in appearance and behaviour throughout the 21 days after administration. No symptoms of toxicity or death were observed in rats, and blood analyses were normal (Jevanand & Kannan 1995). A *M. anisopliae* isolate from spittlebugs in Mexico was tested for oral acute intragastric pathogenicity and toxicity in CD-1 mice (Toriello et al. 2006). No pathogenic or toxic effects were noted at the end of the study.

Careful safety tests were also carried out for the commercialisation and registration of *M. anisopliae* as BIO 1020 (Reinecke et al. 1990). The acute oral LD_{50} in rats was $>2000\ mg\ kg^{-1}$ (max. applicable amount); the acute dermal LD_{50} in rats was $>2000\ mg\ kg^{-1}$ (max. applicable amount). No irritation of rabbit skin and slight irritation of eyes were observed.

Mycopesticides based on various strains of *M. anisopliae* var. *anisopliae* (BIO 1020, strains FI-1045, ICIPE 30 and 69) and *M. anisopliae* var. *acridum* (strains IMI 330189 and FI-985) showed no adverse toxicological effects on research or manufacturing staff, on formulators or on field workers (Copping 2004). In *M. anisopliae* var. *acridum* (product 'Green Muscle'), the acute oral LD_{50} in rats was $>2000\ mg\ kg^{-1}$ and the acute dermal LD_{50} was also $>2000\ mg\ kg^{-1}$. After inhalation, the acute pulmonary toxicity/infectivity LC_{50} in rats was $>4850\ mg\ m^{-3}$. No irritation of skin and eyes of rabbits was observed.

In contrast to these negative findings on safety of *M. anisopliae* to mammals, Goettel and Jaronski (1997) reported that the company Mycotech observed extreme toxicity to mice after pulmonary (intranasal) challenge by an isolate of *M. anisopliae* var. *anisopliae* and another of *M. flavoviride* (actual name *M. anisopliae* var. *acridum*). Intraperitoneal administration of other *M. anisopliae* var. *anisopliae* isolates did not cause any pathological effects.

Conclusions

Since the isolation and identification of *M. anisopliae* nearly 130 years ago, this fungus is considered one of the most important entomopathogenic fungi used for biocontrol of many pest insects throughout the world. As a consequence, a large amount of data on the biological characteristics of *M. anisopliae*, the impact of environmental factors, on side effects and on various safety issues have been published and are presented within this review. The safety data presented are from various *M. anisopliae* strains and varieties from many countries and continents. Based on this information, the following conclusions can be drawn: (1) *M. anisopliae* is a typical soil-borne fungus and consists of several genotypes which have a worldwide distribution from the arctic to the tropics. (2) It has a large host range as a species, however, strains and certain genotypes generally are more specific. (3) It produces a variety of metabolites/toxins, mainly destruxins, which have diverse biological activities. However, no toxic effects due to these metabolites have been reported when *M. anisopliae* was used for biocontrol purposes. (4) There is little information on the fate and behaviour of *M. anisopliae* in and on water surfaces. In contrast, there are many papers dealing with mobility and persistence in soil, i.e. conidia adhere in the upper 20 cm and may persist up to 3–4 years with monthly decaying rates. (5) *Metarhizium anisopliae* has no effect on plants, and only minor effects on soil organisms, but some pathogenic effects against a decapode and a cladoceran in water, which needs further study. The fungus was tested against a wide range of non-target organisms. The results should be considered in context. Normally, the effects in the field are lower than those in the laboratory. (6) In some fish, especially embryos and reptiles, pathogenic effects were reported, which were not associated with the use of this fungus. No adverse effects were observed in birds. (7) *Metarhizium anisopliae* has some allergenic potential, i.e. certain precautions are necessary to avoid dermal contact or extensive inhalation of conidia. In recent years, some natural infections in mammals and humans have been reported. In contrast, experiments on mammalian safety were negative, and no adverse effects on the manufacturing staff or applicators were noticed. Based on this knowledge, we can conclude that *M. anisopliae* is safe with minimal risks. With respect to the commercialisation and registration of future isolates of *M. anisopliae,* the question is, which regulations and tests are further necessary in order to provide the user and consumer with a safe biocontrol product.

Acknowledgements

The author thanks Dr Mark Goettel, Lethbridge Research Centre, Canada, and two unknown reviewers for kindly reading the manuscript and providing helpful suggestions.

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