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Monitoring soil fungal community structures and specific fungal biocontrol strains for ecological effect and fate studies used for risk assessment

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Für Johanna Paulina

Die Früchte der Schlehe spenden Kraft,
verkürzen den Winter mit ihrem Saft.
Die kleinen Blätter wiegen im Wind,
sammeln im Sommer Kraft für das Kind.

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Content

Summary

Safe use of naturally occurring insect pathogens as biological control agents (BCA) of pest insects requires a risk assessment, which analyzes potential harm a BCA may cause to the environment. Risk assessment of a substance intentionally released to the environment is based on two key elements. i) Exposure of the environment to the substance. ii) Occurrence of undesirable effects caused by the substance.

Bacteria and fungi are dominant groups of soil organisms and are involved in soil functions such as nutrient recycling, degradation of xenobiotics or maintenance of soil structure. Microorganisms may therefore represent key determinants of soil functions. If presence of a BCA has an effect on the composition of microbial communities, this could also cause effects on natural soil functions. Furthermore, if these effects were negative and caused harm to an ecosystem, exposure to the BCA would pose a risk.

Monitoring of a BCA in soil or analyses of effects on non-target soil fungi are either time consuming or even impossible by use of established cultivation-dependent microbiological methods. One possible way to overcome these limitations can be the use of molecular genetic methods. The goal of this thesis was to evaluate and adapt molecular genetic methods for their use in risk assessments of BCAs in soil. The approach was based on analyses of a BCA in soil and co-occurring fungal communities, including i) development of a cultivation-independent monitoring tool for BCAs and ii) detection of possible effects of a BCA.

A BCA based on the fungus *Beauveria brongniartii* was chosen as an experimental system for this thesis, because this well established system allows to carry out field experiments. *B. brongniartii* is the most important pathogen of the European cockchafer, *Melolontha melolontha*, infecting both adults and larvae. The BCA consists of fungus colonized barley kernels (FCBK) and is applied to soils infested by *M. melolontha* larvae. *B. brongniartii* has a narrow host range and since the commercial introduction of FCBK in 1991, no negative effect on non-target organisms has been reported. However, there are no known analyses on possible effects of this BCA on soil fungal communities.

Spatial heterogeneity of fungal communities in the field could mask possible effects of a BCA. Therefore, a systematic approach was developed to derive a representative fungal community profile for an entire field. First, heterogeneity of fungal communities was assessed from ten different soil samples derived from a single grassland plot. Second, an increasing number of soil samples was mixed until representative profiles were derived.

For accurate and fast exposure analysis, a genotyping method previously developed to identify *B. brongniartii* isolates, was adapted for cultivation-independent use. Such, genotype-specific identification and also quantification of *B. brongniartii* are now possible directly from soil samples.

Possible effects of the *B. brongniartii* BCA on soil fungal communities were tested using soil microcosms amended with FCBK. The respective soil fungal communities were compared to communities in untreated controls and to communities exposed to dead *M. melolontha* larvae. At ten times the recommended dosage of the FCBK and under controlled conditions, small effects were detected in molecular genetic profiles

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representing changes in the composition of the soil fungal community. However, the high variability among profiles of replicated microcosms were observed and the effects caused by the BCA alone were very small when compared to effects caused by dead larvae.

Molecular genetic analyses were shown suitable tools to asses both exposure to the BCA tested and to assess effects on soil fungal communities. A reliable risk assessment of BCA in soil will further require methods to identify organisms possibly affected by a BCA, i.e. indicator organisms and assessment of their functions in a soil ecosystem.

Zusammenfassung

Die sichere Anwendung natürlich vorkommender Krankheitserreger von Insekten als biologische Schädlingsbekämpfungsmittel (engl. „biological control agent" (BCA)) bedingt eine Risikoanalyse zur Abschätzung möglicher Schäden durch ein BCA in der Umwelt. Die Risikoanalysen von absichtlich in die Umwelt freigesetzten Substanzen basiert dabei auf zwei Kernelementen. i) Exposition der Umwelt gegenüber der Substanz. ii) Nicht erwünschte Effekte der Substanz in ihrer Umwelt.

Bakterien und Pilze machen den Grossteil der Biomasse im Boden aus. Sie beeinflussen z.B. Nährstoffkreisläufe im Boden, wandeln xenobiotische Stoffe um oder sind an Aufbau und Erhalt der Bodenstruktur beteiligt. Damit nehmen Mikroorganismen in Bodenökosystemen Schlüsselfunktionen ein. Sollte ein BCA die Häufigkeit von Mikroorganismen beeinflussen, könnten dadurch natürliche Funktionen des Bodens beeinträchtigt werden. In der Folge wären Schäden am betroffenen Ökosystem möglich, d.h. der untersuchte BCA könnte ein Umweltrisiko darstellen.

Untersuchungen über den Verbleib eines BCA im Boden oder die Analyse von Effekten eines BCA auf Bodenpilze sind mit herkömmlichen mikrobiologischen Methoden sehr zeitaufwändig oder gar nicht möglich. Demgegenüber versprechen molekulargenetische Methoden in diesen Punkten grosse Vorteile. Die vorliegende Arbeit hatte zum Ziel, bereits etablierte molekulargenetische Methoden auf ihre Verwendbarkeit in der Risikoanalyse von BCAs im Boden zu prüfen und gegebenenfalls anzupassen. Insbesondere sollten der Verbleib des ausgebrachten BCA und dessen möglichen Effekte auf Bodenpilze kultivierungsfrei untersucht werden.

Als experimentelles System wurde ein kommerzielles pilzliches Schädlingsbekämpfungsmittel gewählt. Neben Laborversuchen erlaubt dies auch die einfache Durchführung von Feldversuchen. *Beauveria brongniartii* ist der wichtigste Krankheitserreger des Europäischen Maikäfers, *Melolontha melolontha,* und befällt sowohl Adulte wie auch Larvenstadien (Engerlinge). Als BCA wird er in Form von mycelbewachsenen Gerstenkörnern (Pilzkörnern) in Böden mit Engerlingsbefall eingearbeitet. *B. brongniartii* zeichnet sich durch ein enges Wirtsspektrum aus, und seit der kommerziellen Markteinführung der Pilzkörner im Jahr 1991 wurden keinerlei Schäden an natürlichen Populationen von Nicht-Ziel-Organismen berichtet. Es bestehen jedoch keine Untersuchungen über die Auswirkungen dieses BCA auf die Gesellschaften von Bodenpilzen.

Die räumliche Heterogenität von Pilzgesellschaften in einem Feld könnte mögliche Effekte eines BCA maskieren. Deshalb wurde ein systematischer Ansatz entwickelt, um für ein ganzes Feld ein repräsentatives Profil der Pilzgesellschaften erstellen zu können. Dazu wurde zuerst die Heterogenität von zehn Bodenproben aus einer einzigen Wiesenparzelle verglichen. Anschliessend wurde die minimale Anzahl zu mischender Bodenproben bestimmt, bis die individuellen Anteile ausgeglichen waren.

Für eine sowohl exakte wie auch rasche Expositionsanalyse wurde eine bereits früher etablierte Methode zur Identifizierung von *B. brongniartii* Isolaten angepasst.

Zusammenfassung

Damit lässt sich *B. brongniartii* nun kultivierungsunabhängig quantifizieren und *B. brongniartii* Genotypen lassen sich kultivierungsunabhängig ansprechen.

Um mögliche Effekte auf die Zusammensetzung von Pilzgesellschaften im Boden zu untersuchen, wurden im Rahmen dieser Arbeit Kleinökosysteme verwendet. Mit Pilzkörnern behandelte Ansätze wurden einerseits mit BCA-freien Kontrollen, andererseits mit toten Engerlingen verglichen. Die Resultate zeigten bei zehnfach überhöhten Aufwandmengen schwache Effekte in der Zusammensetzung der molekulargenetisch untersuchten Pilzgesellschaften. Dabei zeigten sich in den Wiederholung, trotz kontrollierter Bedingungen, hohe Variabilitäten. Die erfassten Effekte des BCA alleine waren um ein vielfaches geringer als die durch tote Engerlinge verursachten Veränderungen der Pilzgesellschaften.

Molekulargenetische Untersuchungen mikrobieller Gemeinschaften können helfen, mögliche Veränderungen in der Häufigkeitsverteilung von Mikroorganismen im Zusammenhang mit der Anwendung eines BCA zu erfassen. Genotypspezifische, kultivierungsunabhängige Nachweise erleichtern zudem die Zuordnung von Effekten zu einem bestimmten BCA und ermöglichen damit adäquate Expositionsstudien, welche die Beurteilung von Risiken ermöglichen. Weiter verlangen zuverlässige Risikoanalysen von biologischen Schädlingsbekämpfungsmitteln im Boden nach weiteren Methoden, um jene Pilze zu identifizieren, die durch das BCA möglicherweise beeinflusst wurden. Solche Indikatoren haben im Bodenökosystem bestimmte Funktionen. Allfällige Risiken einer BCA Freisetzung lassen sich allerdings erst abschätzen, wenn diese Funktionen ebenfalls bestimmt werden können.

CHAPTER I

General Introduction

Chapter I. General Introduction

I.1.   Biological control agents in pest management

Invertebrate pests may be controlled through intentional release of biological control agents (BCA), which may either be predators, parasitoids or pathogens of the pest organisms (Cook *et al.*, 1996). Dependent on the way of application and the expected duration a BCA remains in the environment, three different strategies can be differentiated (Eilenberg *et al.*, 2001): (1) Classical biological control defines the introduction of a BCA not native to the application area, usually resulting in long-term pest control. (2) Inundative biological control solely depends on the organisms released, and thus its effect is transient as long as the BCA does do not multiply in the targeted environment. (3) Inoculative biological control implies an intended multiplication of the released BCA organisms and typically results in long-term control of a pest.

I.1.1.   Entomopathogens in pest management

Entomopathogenic viruses, bacteria, fungi, protozoa or nematodes specifically attack insects and act as natural population regulators (Steinhaus, 1963). Attempts to use entomopathogens in biological, i.e. microbial control programs (Steinhaus, 1956) date back to the late 19th century, but the overwhelming success of chemical pesticides stopped several biocontrol programs after World War II (Lord, 2005). However, with emerging problems caused by pesticide-resistances in insects or pesticide residues in human food, a series of new biocontrol research programs were initiated in the 1970ies (Keller and Brenner, 2005; NRC, 1996; Robinson *et al.*, 1980). As a result, understanding of pest-disease interactions improved and also numbers of successful applications of entomopathogens as BCA increased (Butt *et al.*, 2001; Gerhardson, 2002; Lacey *et al.*, 2001).

Currently four bacterial species are recorded as entomopathogens in the US (EPA, 2007a) and three species in Europe (European Union, 2007). Of these, with dozens of registration entries, *Bacillus thuringensis* spp. and its related products containing

or expressing insecticidal proteins currently account for the largest segment of entomopathogenic BCA (EPA, 2007b; European Union, 2007). From the over 700 species of entomopathogenic fungi described (Butt, 2002; Hajek and Leger, 1994), four species are registered as BCA in the US (EPA, 2007a), of which *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* are also recorded in Europe (European Union, 2007). The Oomycete *Lagendium giganteum* is only listed as entomopathogen by US-EPA (EPA, 2007b) and *Beauveria brongniartii* is only listed in Annex I of European Directive 1991/414/EEC (European Union, 2007).

I.1.2. Entomopathogenic fungi as Biological Control Agents

Most entomopathogenic fungi belong to the orders of the Hyphomycetes or Entomophthorales (Butt et al., 2001). Hyphomycetes were the first described to cause death in insects. In 1835 Agostino Bassi first suggested that a microorganism caused the "white muscardine" disease in silkworm. The causative fungus was later named Beauveria bassiana in his honor (Steinhaus, 1963). In 1879 Metchnikoff attempted to control the wheat cockchafer using the "green muscardine", *Metharizium anisopliae* (Steinhaus, 1963). This fungus has been registered as a commercial product against grasshoppers, more than hundred years after Metchnikoff's first trials (Milner and Hunter, 2001). A different type of "white muscardine" attacks the European cockchafer, *Melolontha melolontha*, forming dense white layers on the insect's cadaver (Fig. 1.1). Dufour (1894) for the first time described a host's population collapse, i.e. an epizootic in *M. melolontha*, caused by an entomopathogenic fungus. The causative agent, the 'white muscardine', was identified as *B. tenella*, later named *B. brongniartii* (De Hoog, 1972). Hyphomycetes typically induce epizootics in populations of soil

Fig. 1.1: *Beauveria brongniartii*, a 'white muscardine' fungus, growing on a cadaver of an European cockchafer, *Melolontha melolontha*

dwelling insects (Keller and Zimmermann, 1989). Epizootics on foliar insects are typically caused by fungi of the order Entomophthorales, naturally regulating insect populations (Evans, 1989; Lacey et al., 2001). Most Entomophthorales are obligate biotrophs and cannot live saprotrophically or on artificial media. This makes it difficult to rear these fungi in large quantities as necessary when applied as a BCA (Pell et al., 2001). Short survival time of asexual spores, i.e. conidia, of Entomophthorales may further cause difficulties to artificially augment populations of these insect pathogenic fungi in the environment. These may be reasons why to date none of the about 230 Entomophthorales species described has been registered as a BCA, neither in the US nor in Europe (EPA, 2007b; European Union, 2007). Because most Hyphomycetes can easily be grown on artificial media, they offer inexpensive mass production (Papagianni, 2004). Currently, available fungal BCA products belong to the Hyphomycetes genera *Beauveria*, *Metarhizium*, *Verticillium*, *Paecilomyces* and *Trichoderma* (EPA, 2007a; European Union, 2007). Of these, the two fungal species *B. brongniartii* and *M. anisopliae* are applied as BCAs below ground: In Europe, the *B. brongniartii* based products BeauveriaSchweizer[TM] (Switzerland), Engerlingsgspilz[TM] (Switzerland), MELOCONT[TM]-Pilzgerste (Austria and Italy) and Betel[TM] (France) are applied against the European cockchafer, *Melolontha melolontha* (Fig. 1.2). All these BCA products are based on cereal grains overgrown with fungal conidio spores (Keller *et al.*, 2002). In Japan, Biolisa-Kamikiri[TM], containing *B. brongniartii* is registered to control scarabidaen garden- and forest-pest beetles (Higuchi et al., 1997). In Australia the commercial product BioGreen[TM], based on *M. anisopliae*, is registered for use against the red-headed pasture cockchafer (Rath et al., 1995).
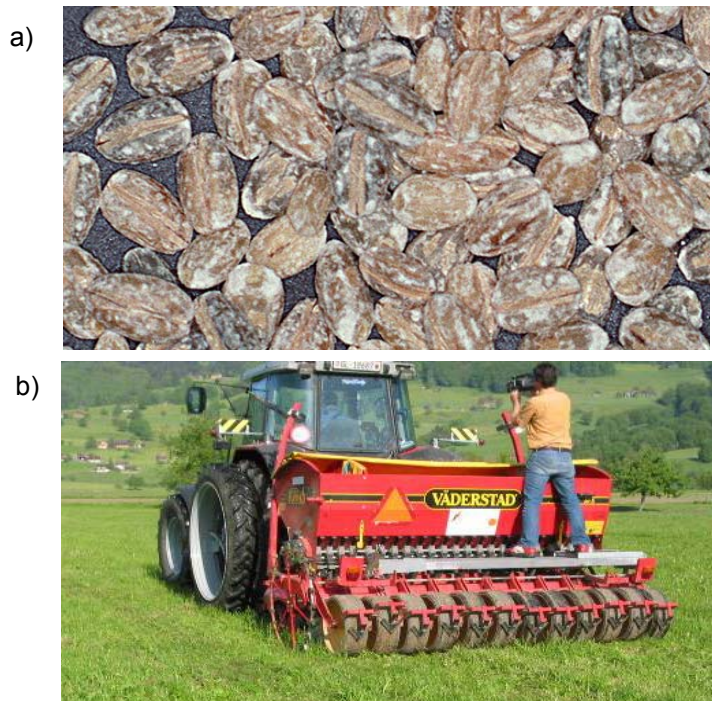
Fig. 1.2: Pealed barley kernels overgrown with the fungal BCA *B. brongniartii* (a), and seeding machinery used to apply the BCA product (b). (Photo a) by S. Keller)

I.2. Control of the European cockchafer with *B. brongniartii*

I.2.1. Agronomical damages caused by *M. melolontha*

Larvae of the European cockchafer, *M. melolontha* (Fig. 1.3, left), are a serious pest to permanent grasslands. After beetles' flight and egg deposition in May, larvae hatch within four to six weeks. Depending on the length of the vegetation period, larvae remain between two to three years in the soil, heavily feeding on plant roots. During the third, respectively fourth year of infestation larvae pupate and adult beetles overwinter in soil until flight (Schmid, 2004). The most severe damages to plants (Fig. 1.3, right) occur in the second year of infestation (Fornallaz, 1992). Threshold levels for economic damages are reached at 20 larvae m^{-2} in grassland or 2 larvae m^{-2} in orchards (Horber, 1954). However, larval population densities can reach mean values of more than 80 larvae m^{-2} (Horber, 1954). Since in Switzerland chemical insecticide applications such as Lindane, DDT or Aldrin to control *M. melolontha* in grasslands were banned in the 1970ies, an alternative approach had to be developed (Keller et al., 1997).

Fig. 1.3: Third instar larvae of *Melolontha melolontha*, the European cockchafer (left) and damage of *M. melolontha* larvae in a hay meadow (right). (Photo to the right by Ch. Schweizer)

I.2.2. Biology and distribution of *B. brongniartii*

B. brongniartii is the most important pathogen of *M. melolontha* (Fig. 1.4). This hyphomycetous fungus is highly host-specific and almost exclusively infects *M. melolontha* and *M. hippocastanei* under natural conditions in Central Europe (Aregger, 1992; Zimmermann, 2007). A survey of 82 fields in Switzerland revealed

the occurrence of *B. brongniartii* to be limited to fields where *M. melolontha* was present or had recently disappeared. (Keller *et al.*, 2003). Introduction of *B. brongniartii* spores to control *M. melolontha* at infested sites has been discussed since the first description of the fungus (Ferron, 1967). However, application of *Beauveria* spores posed difficulties, e.g. due to their sensitivity to sunlight causing their inactivation within a few hours, or due to the

Fig. I.4: *B. brongniartii* mycelium emerging from the cadaver of an infected *M. melolontha* larva in the third instar. (Photo by Ch. Schweizer)

uncertainty where the female adults would lay their eggs (Ferron, 1967; Gardner *et al.,* 1977; Horber, 1954). Following systematic research in reproduction and dispersal of this entomopathogenous fungus (Ferron, 1978; Fornallaz, 1992), two different strategies for the application of *B. brongniartii* spores were tested. In 1985 and 1988 suspensions of spores derived from the haemolymphe of diseased *M. melolontha* larvae, referred to as blastospores, were sprayed on swarming *M. melolontha* beetles. Infected female beetles carried the fungus spores to the breeding sites, infecting eggs or freshly hatched larvae. However, this approach was very laborious and its success was not predictable, varying between complete control and failure (Keller *et al.,* 1997). Alternatively, barley kernels were inoculated with spores collected from dead hosts, i.e. conidiospores (Fig. 1.2 a). These fungus colonized barley kernels (FCBK) allowed for an effective application of infectious material of *B. brongniartii* into *M. melolontha* infested fields without the risk of inactivation through sunlight. Furthermore, when applied in spring one year after flight, *B. brongniartii* spores are dispersed by cockchafer larvae, causing epizootics in the *M. melolontha* population (Aregger, 1992; Keller, 2004; Keller *et al.*, 1997; Matzke, 2000).

I.2.3. Monitoring of *B. brongniartii* populations

Analyses of both naturally occurring *B. brongniartii* populations as well as intentionally applied *B. brongniartii* BCA genotypes are based on the isolation of fungal colonies from soil samples. Total *B. brongniartii* quantities can be assessed from cultivation on a selective medium (Strasser *et al.*, 1996). Subsequently,

genotype-specific identification is based on the analysis of single *B. brongniartii* colonies using highly polymorphic molecular genetic markers. Simple sequence repeats (SSR; Tautz, 1989) revealed to be suitable markers for this purpose and analysis of six different SSR markers (Enkerli *et al.*, 2001) discriminates naturally occurring strains of *B. brongniartii* in Switzerland with over 90% certainty (Enkerli *et al.*, 2004). Monitoring of *B. brongniartii* BCA genotypes using the six SSR markers allowed to assess its establishment within a natural *B. brongniartii* population consisting of diverse genotypes (Enkerli *et al.*, 2005b). Furthermore, monitoring also revealed persistence of the BCA within the applied field plots and a very limited spread into adjacent, untreated plots (Enkerli *et al.*, 2005a).

I.2.4. Non-target effects of *B. brongniartii*

Effects on epigeal arthropods

Even though *B. brongniartii* is considered to have a narrow ecological host range, both Vestergaard *et al.* (2003) as well as Zimmermann (2007) reported in their comprehensive reviews on studies revealing infections in different hosts from other geographical areas. As an example, the report by Leatherdale (1970) listed numerous hosts of *B. brongniartii* mostly collected by T. Petch in Britain during the 1930ies and 1940ies. His catalogue included insects of the orders Heteroptera (Pentatomidae), Lepidoptera (*Hepialus lupulinus*), Coleoptera (Coccinellidae, Chrysomelidae, *Plateumaris braccata*, *Galerucella tenella*, *Strophosomus* spp.), Hymenoptera (Formicidae, *Vespula* spp.) and spiders, however no information on the frequency of infestation of a specific host wer given.

In a series of laboratory tests, Vestergaard (2002) showed that *B. brongniartii* can infest non-target ground beetles (Coleoptera, Carabidae) with frequencies up to 20% if these insects were dipped in suspensions of 10^7 *B. brongniartii* conidiospores ml^{-1}. Out of 20 different ground beetle species four were infected, namely *Clivina fossor, Harpalus affini, Nebria brevicollis* and *Pterostichus versicolor.* In a different experiment, larvae of *Poecilus versicolor*, a predatory ground beetle species, were fed with *B. brongniartii* infested cadavers of *M. melolontha* larvae. A control group of *P. versicolor* larvae was fed with healthy *M. melolontha* larvae. Results showed no significant differences between infested and control group, neither in mortality nor in

development time (Traugott *et al.,* 2005). Honey bees (*Apis mellifera*) dipped into suspensions of either 1.5 x 10^6 or 1.5 x 10^7 *B. brongniartii* conidiospores ml^{-1} neither showed reduced survival time nor showed signs of infection (Vestergaard *et al.,* 2003). These data confirmed previous field observations, which indicated that *B. brongniartii* had no negative effects on *A. mellifera* (Wallner, 1988).

Under environmental conditions, either no or very low numbers of infested non-target insects were observed, dependent on the mode of application of *B. brongniartii*. In two large field tests *B. brongniartii* blastospore suspensions were sprayed from helicopters on adult *M. melolontha* beetles swarming along forest edges (Keller, 1989; Keller, 1992). The tests successfully increased average infection rates from 21% natural infection to a total of 87% infected adult cockchafers in the first trial and from 79% to 96% in the second trial (Keller *et al.,* 1997). To determine the infection rate of non-target arthropods, over 10'000 insects and spiders were collected after the blastospore application. Dependent on time and location, between 0% and 12% of non-target arthropods were infected, with an average of 1.1 % (Baltensweiler and Cerutti, 1986). Infections were determined after incubation in the laboratory. Therefore, the time span from spraying until incubation may have had a significant effect, i.e. under natural conditions, *B. brongniartii* spores adhering to the insects may have been inactivated by sun light. Baltensweiler and Cerutti (1986) thus have assumed the figure of 12% infection rate at one collection site to be an artifact caused by incubation very shortly after blastospore application. Independent from the collection time, all infected insects were found on twigs of bushes or trees, but no infections occurred on ground-dwelling insects of the test areas (Baltensweiler and Cerutti, 1986).

Compared to the aerial application of blastospores, subsurface application of conidiospores using FCBK were shown to act faster, to be easier to apply and more convenient to stock the BCA (Keller, 2004). The FCBK BCA was registered between 1991 and 1999 in Switzerland and other European countries (Keller, 2000; Strasser, 2000). Since the commercial launch of the FCBK, over 1200 ha grassland have been treated in Switzerland. However, there are no reports on epigeal arthropods infested through subsurface application of *B. brongniartii* (S. Keller, pers. comm.).

Effects on soil dwelling insects

Fungivorous collembolans and insectivorous mites were tested for their feeding behavior in presence of *B. brongniartii* spores (Dromph and Vestergaard, 2002; Zimmermann, 2007). If adults of the three collembolan species *Folsomia fimetaria, Proisotoma minuta, Hypogastrura assimilis* were offered solid substrates with 10^1 to 10^7 conidia g^{-1} agar, all three species preferred the highest concentration. Direct inoculation of the three collembolan test species by dipping individuals into a suspension of 10^7 *B. brongniartii* conidiospores ml^{-1} showed no increase in mortality. However, if the same three species were exposed to wet *Sphagnum* enriched with 10^8 conidia g^{-1}, mortality increased in *F. fimetaria* (Dromph and Vestergaard, 2002). This treatment seemed to be an unnatural situation and in a subsequent study, Dromph (2003) showed, that the same collembolan species were even effective vectors for the distribution of *B. brongniartii* conidiospores. When exposed to sphagnum inoculated with 10^7 conidia g^{-1} for 24 hours, the quantities of spores transported were shown to be sufficient to infect a susceptible host, *Tenebrio molitor* (Dromph, 2003). Individual collembolans can move up to 40 cm per day through soil (Bengtsson *et al.*, 1994) and may thus directly influence the epizootiology of *B. brongniartii*, increasing success of the applied *B. brongniartii* BCA (Dromph, 2003). Mites were also found to transport *B. brongniartii* spores, possibly also playing a role as vectors for the dispersion of *B. brongniartii* in soils (Zimmermann, 2007).

Effects on earthworms

Tests on earthworms revealed no negative effects both in the laboratory (Aregger-Zavadil, 1992; Hozzank *et al.*, 2003) or in the field (Hozzank *et al.*, 2003). Instead, earthworms have been considered to play an active role in the short-distance dispersal of *B. brongniartii*. Earthworm channels were shown to be preferentially colonized by *B. brongniartii* hyphae (Callot *et al.*, 1996). Earthworm faeces support saprotrophic growth of the fungus (Keller and Zimmermann, 1989) and *B. brongniartii* spores can be dispersed by the movements of earthworms (Hozzank *et al.*, 2003).

Effects on microorganisms

Little is known about interactions of *B. brongniartii* with microorganisms. Zimmermann (2007) hypothesized that long lasting evolutionary coexistence possibly

resulted in antagonistic or inhibitory processes from *B. brongniartii* towards other microorganisms or *vice versa*. Antagonistic effects of *B. brongniartii* against other fungi were indeed recorded under greenhouse conditions for *Pythium ultimum*, *P. debaryanum* and *Septoria* (*Leptosphaeria*) *nodorum*, while *P. irregulare*, *Phoma betae*, *Phoma exigua* var. *foveata* and *Rhizoctonia solani* showed resistance (Vesely and Koubova, 1994). *B. brongniartii* also strongly reduced growth of *Fomes (Heterobasidium) annosus* (Laine and Nuorteva, 1970). Zimmermann (2007) recorded one report on the hyper-parasitic fungus *Syspastospora parasitical*, also known as *Melanospora parasitica* attacking *B. brongniartii*. Whether these negative interactions take effect in natural fungal communities of grasslands has not yet been reported.

For different *B. brongniartii* strains naturally occurring within a field, Enkerli *et al.* (2005b) have observed coexistence. *B. brongniartii* strains intentionally introduced to existing indigenous populations of this fungus, established and persisted for up to 14 years (Enkerli *et al.*, 2004). However, no data are available on whether the composition of indigenous *B. brongniartii* populations changed after introduction of *B. brongniartii*, or on changes in other soil fungal populations. Analyses of effects on indigenous fungal populations are necessary to further address these questions.

I.3. Risk assessment in pest control

I.3.1. Pest management in agriculture

Development of high-yield crop varieties and crop protection strategies allowed an immense increase in food production during the second half of the twentieth century. For example, grain yield increased from 0.6 to 2.2 x 10^9 metric tons in the period between 1950 and 2006 (Food and Agriculture Organization of the United Nations (FAO), 2007). In 2004 the amount spent on pesticides reached over 30 billion US $, i.e. about 20 times more than in the early 1960ies (Oerke, 2006). This demonstrates the economical importance of pest control and implies the necessity to produce enough food for the ever increasing world population.

Discovery of the insecticidal activity of chlorinated hydrocarbons like DDT and Lindane in the late 1930ies marked the beginning of pest control using synthetic

chemicals (World Health Organization, 1979). High effectiveness against numerous pests, low costs in production and low toxicity to humans made these chemicals very attractive for use in agriculture (Hassal, 1990a). Use of this type of chemical control agents (CCA), however, was also correlated with ecological hazards. Unspecific killing of beneficial insects and development of insect resistance affected self-regulating processes in ecosystems and reduced the effectiveness of the applied CCAs (Carson, 1963). The tendency of chlorinated hydrocarbons to accumulate in organisms and to be transferred through the food chain, caused for example a decline of birds of prey, due to thin-shelled eggs and reduced fertility (Ratcliffe, 1967). Improved understanding of this and other ecological mechanisms led to the development of less persistent CCAs with enhanced degradability, a goal achieved in the mid 1980ies (Barik, 1984; Kapustka *et al.*, 1996). With a paradigm shift from pest elimination to pest management in the 1970ies, development and use of biological control agents also became encouraged (Butt *et al.*, 2001; Cook, 1993; NRC, 1996). With the development of environmental risk assessments and regulations for registration of pesticides, national and international regulatory bodies today aim to protect environmental health while allowing for efficient pest control (Marrs and Ballantyne, 2004).

I.3.2. Environmental risk assessment

In the US, risk assessment was first formulated by the National Research Council (NRC, 1983) as a process to assess risks possibly associated with exposure of humans to toxic substances. This approach involved five steps: problem formulation (what chemical agent needs to be tested under which circumstances?), hazard identification (does the agent cause a particular adverse effect?), dose-response assessment (what is the relationship between dose and effect in humans?), exposure assessment (what exposures are experienced under different conditions?) and risk characterization (what is the estimated extend of the adverse effect in a given population?). The US Environmental Protection Agency (EPA; 1992; 1998) adapted the NRC approach and formulated an environmental risk assessment suitable to describe undesirable human-induced changes in the environment. Such changes may be caused by any type of an agent, i.e. any biological, chemical or physical factor occurring in an environment. The EPA combined the two aspects of hazard

identification and dose-response assessment within the term 'ecological effect' and formulated an ecological risk assessment based on four steps (EPA, 1998): (1) Problem formulation: identification of an agent and the circumstances, e.g. which plant treatment practices need to be tested in which environment. (2) Effect analysis: identification of possible negative effects of an agent in the environment, e.g. unintended killing of non-target insects. (3) Exposure analysis: identification and quantification of the spatial and temporal distribution of an agent, e.g. time and concentration an insecticide persists in the environment. (4) The final process of risk characterization combines results of effect and exposure analyses to evaluate the likelihood of undesirable changes which result from exposure to an agent (EPA, 1998).

Analogously, the European Community formulated four elements of risk assessment, called hazard identification, hazard characterization, exposure assessment and risk characterization (European Union, 2000). The European concept was formulated for the characterization of risks to human as well as to environmental health and resembles both the risk concepts of the NRC (1983), as well as the EPA (1998): the European "hazard identification" includes the identification of a risk source (analogous to the EPA problem formulation) as well as its adverse effects (analogous to the EPA effect analysis). Hazard characterization focuses on the quantification of adverse effects (analogous to the NRC dose-response assessment). Exposure assessment is concerned with levels and duration of exposure to a risk source (analogous to the EPA exposure analysis). Risk characterization is formulated as the estimation of the probability of occurrence and severity of adverse effects, based on the characterizations of both exposure and hazard (European Union, 2000).
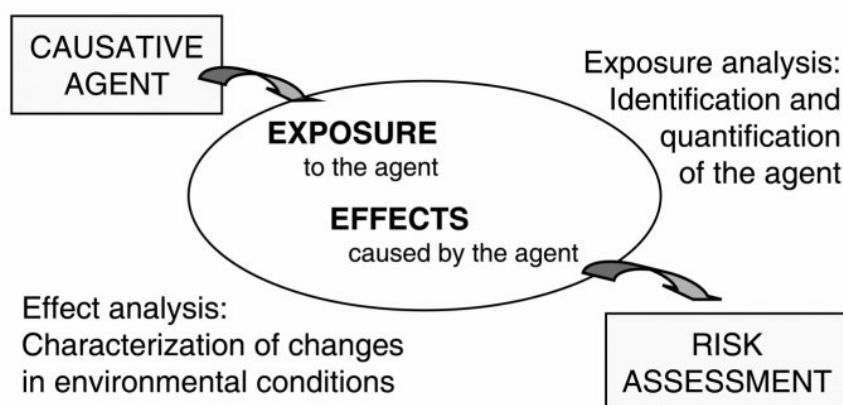
Fig. 1.5 Exposure and effect analyses are part of an environmental risk assessment for any agent released to the environment.

Independent of details in the different procedures, exposure- and effect-analyses are core elements of risk assessments (Fig. 1.5). Based on these two measures, ecosystem health is not at risk, unless an agent (1) has the ability to cause an adverse effect, and (2) occurs long enough and at a sufficient quantity to cause an identifiable adverse effect (EPA, 1998). Therefore, the process of risk characterization should allow to identify levels of exposure without adverse effects to the environmental health (European Union, 2000).

I.3.3.   Regulations for registration of biocontrol agents

EPA (1978) defined "any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest" to be a pesticide. Thus, also biological control agents were assigned pesticides, a view adopted by the EU where BCAs were assigned to plant protection products (European Union, 1991). The general concepts of risk assessment may therefore apply both for chemical as well as biological control agents. However, due to their different mode of action and their ability to multiply in nature, BCAs are today regulated separately. Data requirements for microbial pesticides are listed by US EPA rule 40 CFR part 158 (EPA, 2007b) and by EU directive 2005/25 (European Union, 2005). Because of differences in regulations for the use of BCAs in various EU member states, a harmonization process was initiated in 1991 and formulated in EU directive 1991/414/EEC (European Union, 1991). The follow-up EU-directive 2001/36/EC gave expanded details on identification, distribution and ecological effects of microbial plant protection products (MPPP). Recently, EU-directive 2005/25/EC (European Union, 2005) further specified, how dossiers of MPPPs have to be evaluated in regard to directive 2001/36. During the harmonization process, BCAs already in use within the EU are further permitted until final decision. However, since in May 2000 directive 1991/414/EEC (European Union, 1991) came into force, all MPPPs previously registered in any of the member states were included in Annex I of directive 1991/414/EEC, in order to be evaluated for EU-wide registration (Hussey and Bell, 2004).

With regard to effect and exposure analyses, requirements for microbial pesticides are similar in the EU and the US, and also correspond with regulations in Canada or Japan (Hunt *et al.*, 2007; OECD, 2003). However, the different regulatory agencies

focus on various steps in the decision process and ask for different types of tests (Mensink and Scheepmaker, 2007). For example, US EPA rule 40 CFR regulates tests on mammals, birds, fish, bees and plants as well as studies on the fate of BCAs in aquatic and terrestrial ecosystems, but EPA does not ask for specific tests to assess effects on microorganisms (EPA, 2007b). In this regard, the European council directive 2005/25/EC is concerned about possible effects of microorganisms, based on the assumption that microorganisms may become part of an ecosystem. The respective paragraph says: "*No authorization shall be granted if it can be expected that the microorganism and/or its possible relevant metabolites/toxins will persist in the environment in concentrations considerably higher than the natural background levels, taking into account repeated applications over the years, unless a robust risk assessment indicates that the risks from accumulated plateau concentrations are acceptable*" (European Union, 2005). However, this formulation may be of little help as long as natural variability in background populations are unknown (Strauch *et al.*, 2007) and the regulation does not define how to determine a background level or to what extent this has to be exceeded to be inacceptable. In conclusion, specific regulations to assess potential risks to non-target soil microorganisms are missing (Kiewnick, 2004).

In order to solve uncertainties in BCA evaluation, various initiatives have been launched with the goal of improving risk assessments of BCAs (Hokkanen *et al.*, 2002; IOBC/wprs, 2008; REBECA, 2008) and several schemes have been proposed (Mensink and Scheepmaker, 2007; Strauch *et al.*, 2006; van Lenteren *et al.*, 2006). However, all of these approaches focus on non-target effects on epigeal organisms and do not readily address effects on soil microorganisms.

I.3.4. Analyses of exposure and effects of biocontrol agents

Monitoring fate and persistence of biocontrol agents

Exposure of non-target organisms largely depends on the persistence of a control agent in the environment and therefore, CCAs are being developed with short residual times in soil (Marrs and Ballantyne, 2004). Inoculatively applied BCAs however, need to multiply and to reach densities large enough to control a target population (Paulitz, 2000). Persistence of such BCAs is therefore not a disadvantage,

but a prerequisite to effectively control pests and BCA monitoring helps in timely re-application if BCA numbers were too low for effective control (Eilenberg *et al.*, 2001).

Strain-specific monitoring of a BCA allows for accurate exposure analysis, i.e. determination of spread, persistence and quantity of a specific BCA strain even within native populations of the same species, which is a prerequisite to ascribe an environmental effect to a specific agent. Furthermore, strain-specific monitoring also allows to identify whether successful pest control was achieved by the applied BCA strain or possibly by a native strain of the same species (Enkerli *et al.*, 2004).

Analyzing effects on non-target soil microorganisms

Microorganisms mediate many soil functions influencing soil physical, chemical and biological characteristics (Griffiths *et al.*, 2006; Johnson *et al.*, 2002; Ritz and Young, 2004; van der Heijden *et al.*, 1998). As part of the soil food web, microorganisms interact with higher trophic levels and thus changes on any of these levels may have impacts on the composition of microbial assemblages in soil (Cole *et al.*, 2004; de Boer *et al.*, 2003; de Ruiter *et al.*, 1995; Mikola and Setala, 1998; Mulder *et al.*, 2003). Therefore, changes in the composition of microorganism assemblages may either be direct or indirect effects and their analyses may thus serve as indicators for any changes in the soil food web.

Bacteria and fungi are the dominant groups of soil organisms both with regard to biomass as well as species numbers (Brookes *et al.*, 1982). Dominance of either group may depend on the ecosystem: Bacteria usually dominate in ecosystems with frequent disturbance of the soil structure, such as intensively managed agro-ecosystems (Bardgett *et al.*, 2005; Bittman *et al.*, 2005), while fungi often dominate in undisturbed low-input ecosystems such as upland grasslands or set-aside land (Bardgett and McAlister, 1999; Donnison *et al.*, 2000; Ingham *et al.*, 1989; Klingen *et al.*, 2002). In order to assess possible effects of BCAs on non-target soil microorganisms, analyses of bacterial communities may be preferable in intensively managed systems, while in low-input grassland ecosystems fungal communities may preferentially be investigated.

I.4. Soil fungal communities

I.4.1. Characteristics of soil fungal communities

Assemblages of individuals of the same species are described as populations and an assemblage of diverse species occupying the same environment at the same time constitute a community (Abercombie *et al.*, 1957; Hawksworth and Mueller, 2005). Scale is not defined, as populations or communities within larger systems may contain several smaller ones (Cooke and Rayner, 1984a). The composition of a community of soil fungi may be influenced by environmental factors such as availability of substrates, other microorganisms, soil animals, wildlife or soil management practices. Typically, habitat specificity of environmental factors select for specific fungal species and fungal communities often show habitat-characteristic compositions (Carlile *et al.*, 2001; Lilleskov, 2005). For example, natural grassland ecosystems may harbor highly diverse fungal communities, possibly containing rare species (Watling, 2005), especially if they are nutrient-deficient (Golovko and Ellanska, 2005). In intensively managed fields, repeated tillage may permanently reduce numbers of filamentous fungi, which are sensitive to physical disturbance (Stromberger, 2005). Even under constant management regimes, fungal communities are not of constant composition. The usual seasonal cycles of environmental factors such as resource availability result in habitat-specific succession-cycles in community composition and specific individuals may not always be detectable (Bardgett *et al.*, 1999; Ponge, 2005). Assessment of a community therefore represents its constitution just at a specific spatial and temporal point. Therefore, repeated samplings are required to understand the functioning of an ecosystem and to develop a long-term perspective on possible effects (Walker, 1992).

I.4.2. Methods for analyzing soil fungal communities

The information obtained on the composition or structure of a fungal community considerably depends on the fungal structures investigated and the analysis methods used (Carlile *et al.*, 2001). The main fungal growth form, the hyphal mycelium, typically shows morphological differences on high taxonomic levels such as among

phyla or orders, but is morphologically highly uniform among lower taxonomic levels, i.e. among different species of an order (Hawksworth, 1995; Kimbrough, 1994). Identification based on sexual spores and spore-bearing sporophores may be the most accurate morphological classification traits, but many fungi do not reproduce sexually. Mitosporic fungi, lacking sexually produced spores, may often be described by their asexual spores, but to a lesser precision (Bridge *et al.*, 2005). Analyses of a fungus' distribution based on the spore distribution in soil may be misleading, because spore distribution often does not correlate with the extend of vegetative parts of a fungus (Gardes and Bruns, 1996; Zhou and Hogetsu, 2002). For these reasons, morphological descriptions of fungal communities may be limited (Schmidt and Lodge, 2005).

Cultivation allows to detect any fungal entity that can grow and form a colony. This may be either sexual or asexual spores, yeast cells or pieces of hyphae. However, availability of suitable growth media often restrict detection of species present in a community at low numbers (Carlile *et al.*, 2001). Taking into account that co-occurring fungi may have different abilities to metabolize specific substrates (Cooke and Rayner, 1984a), parallel cultivation of a sample on multiple substrates may increase the number of culturable species. The method of community level substrate utilization (CLSU) takes advantage of this approach by incubating environmental samples on 95 different substrates in order to derive a substrate dependent community pattern (Dobranic and Zak, 1999). Fungal CLSU have been shown to be sensitive indicators of fungal functional diversity that allow for example to discriminate between fungal communities at different elevations within a desert ecosystem (Sobek and Zak, 2003). However, the method did not allow to discriminate between communities from different grassland types in Britain (Grayston *et al.*, 2004). Criticism of the method have been raised, e.g. because the fungi that grow on the individual substrates may be fast growing types being able to grow on a single substrate and that fungi which require more than one substrate may not be detectable. Thus, it is unlikely that CLSU provide a comprehensive view of community structures (Bidartondo and Gardes, 2005).

Molecular genetic profiling methods exploit sequence differences of marker genes and can be used to analyze fungal community structures. Resulting fungal community profiles are anonymous "fingerprints" of the communities analyzed, i.e. individual community members are not taxonomically identified (Anderson and

Cairney, 2004; Viaud *et al.*, 2000). Polymerase chain reaction (PCR; Mullis and Faloona, 1987) can be applied to amplify marker genes directly from environmental samples. Thus, fungal community profiling does not require cultivation of the fungal targets (Gardes and Bruns, 1991; Smit *et al.*, 1999; Vainio and Hantula, 2000) and molecular genetic profiling methods are therefore not biased by differences in culturability of different fungal taxa within a community (Bridge, 2002; Guarro *et al.*, 1999). However, fungal community profiles can be influenced by PCR biases and artifacts (Hartmann *et al.*, 2007; Suzuki and Giovannoni, 1996; Von Wintzingerode *et al.*, 1997).

The ribosomal genes, i.e. ribosomal DNA sequences, have often been used in fungal community profiling (Bidartondo and Gardes, 2005). These sequences comprise both highly conserved domains and variable sequences (Berbee and Taylor, 1993; Pringle *et al.*, 2000; Tehler *et al.*, 2000; Van de Peer and De Wachter, 1997; Woese, 2000). This allows on one side to design fungal-specific PCR primers and on the other side resolution of fungal community compositions on various hierarchical levels from phylum to strain (Gardes and Bruns, 1993; Sequerra *et al.*, 1997; Smit *et al.*, 1999; Vainio and Hantula, 2000; White T.J., 1990). The different profiling methods that have been developed to analyze fungal communities can be divided into three main categories. The first category is based on size differences of PCR products. These can be due to natural size variability of amplicons as exploited in ribosomal intergenic spacer analysis (RISA; Fisher and Triplett, 1999; RISA; Ranjard *et al.*, 2001) or due to different amplicon sizes after restriction digestion as exploited in ribosomal fragment length polymorphism (RFLP; Horton and Bruns, 2001; Viaud *et al.*, 2000) and terminal-RFLP (TRFLP; Blackwood *et al.*, 2003a; TRFLP; Liu *et al.*, 1997) analyses. The second category of profiling methods is based on differences in melting behaviour of amplified DNA sequences. In denaturing gradient gel electrophoresis (DGGE) DNA fragments of the same length are separated through a linear increasing chemical gradient (Brodie *et al.*, 2003; Muyzer *et al.*, 1993), while in temperature gradient gel electrophoresis (TGGE) fragments are separated by a temperature gradient (Heuer *et al.*, 1997; Smit *et al.*, 1999). The third category of profiling methods is based on different conformations of single stranded DNA sequences. Single-stranded conformational polymorphism (SSCP) analyses of PCR products of the same lengths result in profiles composed of multiple bands that migrate differently under non-denaturing conditions (Grosch *et al.*, 2006; Peters *et*

al., 2000; Saiki *et al.*, 1988). Generally, SSCP is highly sensitive to differences between highly similar sequences (He *et al.*, 2005; Jansa *et al.*, 2003), but results revealed a limited resolution of complex communities (Zinger *et al.*, 2008). Generally, profiles derived by DGGE, TGGE and SSCP may be useful to differentiate between communities of low diversity, while T-RFLP and RISA profiling may be most convenient to analyze complex communities (Anderson and Cairney, 2004; Brodie *et al.*, 2003; Thies, 2007).

I.4.3. Molecular genetic fungal community profiles

Accuracy of molecular genetic profiles to represent any fungal community can depend on the DNA extraction method used (Carrigg *et al.*, 2007), the profiling method chosen (Kennedy and Clipson, 2003; Lord *et al.*, 2002) or the selection of PCR primers (Anderson *et al.*, 2003b; Hagn *et al.*, 2003a). Selection of adequate methods and primers provided, community profiling has been shown to sensitively detect effects on soil fungal communities, for example caused by changed management practices of grasslands (Brodie *et al.*, 2003; Klamer and Hedlund, 2004) in response to elevated cupper concentrations in soil (Ranjard *et al.*, 2006) or in response to the addition of soil organic matter in form of compost (Pérez-Piqueres *et al.*, 2006). Difficulties to accurately represent a fungal community of a sampling area can derive from heterogeneous distributions of fungi, causing variability in the community structure across the area. Variability of fungal community profiles derived from different soil samples within one sample area may be larger than variability among differently treated areas (Girvan *et al.*, 2004; Klamer *et al.*, 2002). One approach to reduce such variability may be the collection of spatial replicates to derive averaged profiles from multiple samples (Anderson *et al.*, 2003a; Klamer and Hedlund, 2004; Ranjard *et al.*, 2003). However, averaging highly heterogeneous samples results in large standard deviations that may mask effects possibly caused by a treatment factor (Ranjard *et al.*, 2003). An alternative approach to sensitively detect effects caused by management factors may be the reduction of random heterogeneity using controlled experimental systems (Hajek and Goettel, 2007; Pérez-Piqueres *et al.*, 2006; Timms-Wilson *et al.*, 2004). Small, controlled laboratory ecosystems can be referred to as soil microcosms because they attempt to simulate a portion of the real world (Gillett and Witt, 1979).

I.5. Objectives and outline of the thesis

I.5.1. Objectives

Risk assessment of biocontrol agents evaluates potential harm to humans, animals and the environment. Risk derives from exposure of an organism to an adverse effect and therefore assessment of exposure to a BCA and analysis of effects caused by a BCA are considered core elements in risk assessment (EPA, 1998; European Union, 2000). Assessment of potential risks to the environment therefore requires suitable tools for (1) monitoring of a BCA in the environment and (2) detection of effects on environmental parameters that are connected to potential harm to the environment. Monitoring of a BCA includes its reliable identification and quantification. Detection of effects depends on the type of non-target organisms to be analyzed. If a BCA affects the abundance of fungal populations, analyses of fungal community structures likely reveal the resulting BCA effects.

With the aim to contribute to an improvement in risk analysis of the *B. brongniartii* BCA, this thesis had three objectives:

– Development of a systematic approach to detect variability in soil fungal community structures within a field and to derive a representative fungal community profile for an entire field, based on T-RFLP analyses.

– Development of cultivation-independent genotyping and quantification of *B. brongniartii* in soil using simple sequence repeat (SSR) markers and comparison with an established cultivation-dependent analysis.

– Studying effects of the *B. brongniartii* BCA on communities of non-target fungi under controlled conditions and comparison of these effects with effects caused by an insecticide applied to control *M. melolontha*.

I.5.2. Outline

In chapter one a general introduction is given that places the thesis within the framework of general risk assessment for biocontrol agents. Biological control of entomopathogenic organisms is introduced with a focus on fungal BCAs. The experimental system is introduced and current knowledge on non-target effects of the

B. brongniartii BCA is reviewed. A review section on the development of ecological risk assessment constitutes the framework for discussing the experimental findings of the thesis.

In chapter two a strategy is presented on how variability among fungal community profiles derived from several soil samples obtained from a single field can be adjusted. First, different quantities of soil DNA used for PCR were evaluated for reproducible profiling of one sample. Second, differences among fungal community structures were assessed from ten soil samples derived from one field plot. Third, fungal community profiles derived from sample pools of different complexity were compared to averaged community profiles of all samples. With this approach the smallest pool was determined that allowed to obtain fungal community profiles representative for the entire field plot.

In chapter three development of cultivation-independent analysis of fungal genotypes is described. Analysis of SSR markers allows for strain-specific identification of *B. brongniartii*, which so far required isolation and cultivation of individual strains prior to analysis. Cultivation-independent analysis of a fungal strain requires both species specificity and sensitivity to detect a strain in the environment. Species specificity of SSR detection was verified with a collection of entomopathogenic and typical soil fungal species. Sensitivity was tested using either soil samples spiked with known *B. brongniartii* genotypes or field soil samples containing unknown *B. brongniartii* genotypes. For all analyses genotypes obtained by the new cultivation-independent approach were compared to results from the cultivation-dependent method.

In chapter four the findings of a study comparing effects on fungal communities in response to the *B. brongniartii* BCA and a CCA are presented. Soil microcosms were either amended with the *B. brongniartii* BCA or an alternative CCA alone or in combination with larvae of the target insect *M. melolontha*. Molecular genetic analyses of fungal community structures derived from replicated microcosms were used to compare effects after 7, 42 and 91 days incubation under controlled conditions. Exposure to the *B. brongniartii* BCA was assessed by a cultivation-dependent quantification technique and cultivation-independent quantification based on a single SSR marker specific to *B. brongniartii*.

In chapter five the suitability of the methods developed and data obtained in this thesis are discussed with regard to risk assessment of BCAs. Improvements on strain-specific exposure studies of BCAs as well as potential and limitations of molecular genetic community profiling to study potentially adverse effects of BCAs on soil fungi are analyzed. Perspectives on future applications of the methods developed or evaluated within the frame of this thesis are outlined.

CHAPTER II

Objective criteria to assess representativity of soil fungal community profiles

Kaspar Schwarzenbach, Jürg Enkerli, and Franco Widmer

Chapter II. Objective criteria to assess representativity of soil fungal community profiles

II.1. Abstract

Soil fungal community structures are often highly heterogeneous even among samples taken from small field plots. Sample pooling is widely used in order to overcome this heterogeneity, however, no objective criteria have yet been defined on how to determine the number of samples to be pooled for representatively profiling a field plot. In the present study PCR/RFLP and T-RFLP analysis of fungal 18S rDNA in ten soil samples obtained from a grassland plot of 400 m^2 also revealed this known heterogeneity in fungal community structures. Based on these data a three-step approach to assess representativity of fungal community profiles was established. First, soil DNA quantities needed for robust community profiling were determined. Second, profiles of single or multiple samples were theoretically averaged to test for statistically significant clustering in order to determine the minimal number of samples to be pooled to achieve representativity. Third, DNA extracts of single or multiple samples were pooled prior to profiling in order to test for statistically significant clustering. Analyses revealed robust profiles for 50 ng soil DNA but not for 5 ng. Averaged T-RFLP profiles from five or more soil samples and experimental T-RFLP profiles from pools of seven or more samples formed one significant branch. Theoretical averaging and experimental pooling revealed that five to seven samples have to be pooled for robustly representing the field plot. Our results demonstrate that representativity of soil fungal community profiles can objectively be determined for a field plot with only little deviation between theoretical and experimental approaches. This three-step approach will be of assistance for designing sampling and pooling strategies for comparative analyses of soil fungal communities in ecological studies.

Keywords: Soil fungal community structure; Statistical analysis; Sample pooling; Ribosomal RNA genes; Representative genetic profiling

II.2.   Introduction

Various methods to assess fungal communities in soil have been developed, which have great potential to contribute to a better understanding of the ecological role of fungi in soil habitats. Diversity described as total number and abundance of fungal species may not be determinable in soils due to limitations in taxonomic definitions and methods, thus composition of fungal communities may more generally be described by fungal community structures (Kirk *et al.*, 2004). Ribosomal RNA (rRNA) genes have been shown to be suitable markers to study microbial community structures (Amann and Ludwig, 2000; Bundt *et al.*, 2001; Woese, 1967) and analysis of their terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997) is considered a sensitive approach for comparative community profiling (Marsh, 1999) providing highresolution profiles suitable for statistical analysis (Brodie *et al.*, 2003; Hartmann *et al.*, 2005). Although community-level TRFLP profiling of rRNA genes has been reported to be highly robust for the analysis of bacterial communities (Hartmann *et al.*, 2005; Osborn *et al.*, 2000; Pesaro *et al.*, 2004), robustness of fungal T-RFLP profiles appears to be much more affected by soil sample size (Ranjard *et al.*, 2003), cell lysis efficiency (DeSantis *et al.*, 2005; He *et al.*, 2005; Kirk *et al.*, 2004) or DNA quantity used for PCR (Brodie *et al.*, 2003).

There might be several explanations for the reduced robustness of fungal community profiles.With approximately 105 colony forming units (cfu) per gram soil, fungi are roughly 100 times less abundant than bacteria (Foster, 1988), with fungi–bacteria biomass ratios ranging between 0.22 in a litter rich prairie soil and 0.12 in a tilled soil (Allison *et al.*, 2005). Distribution and density of fungi have been reported to be highly heterogeneous. For example, ectomycorrhizal species typically occur in 10 to 25% of soil samples with volumes of approximately 260 cm^3, as estimated from several studies with an average of 30 samples per hectare (Horton and Bruns, 2001). Generally, the heterogeneity of fungal populations per area has been reported to be higher when compared to bacteria (Horner-Devine *et al.*, 2004).

If fungal community structures of two or multiple sites are compared, within-site heterogeneity may reduce resolution of the analysis (Kasuga *et al.*, 2002). Therefore robust field representative fungal community profiles are needed. Mixed or composite samples have been used to increase the representativity of fungal community

profiling (Anderson *et al.*, 2003a; Hagn *et al.*, 2003b; Klamer and Hedlund, 2004), but some studies still revealed high variability between replicated samples (Girvan *et al.*, 2004; Klamer *et al.*, 2002). This demonstrates the need to individually adjust sampling schemes for representative analyses of soil fungal community structures (Morris *et al.*, 2002). Moreover, different schemes may be needed to either robustly analyze heterogeneities of fungal communities within a field or to generate a mean fungal community profile, representative for the entire field plot.

In the present study we applied PCR/RFLP and T-RFLP profiling of fungal 18S rRNA genes to analyze the fungal community structure of a grassland soil. Our objective was to develop a strategy to generate 1) fungal community profiles representative for individual samples in order to assess fungal heterogeneity and 2) fungal community profiles representative for an entire field.

II.3. Materials and methods

II.3.1. Experimental approach

Pooling of soil samples is often applied to obtain representative samples for a certain field plot or a specific experimental treatment (Anderson *et al.*, 2003a; Gomes *et al.*, 2003; Milling *et al.*, 2004). However, objective criteria for determination of the optimal number of samples to be pooled have not been available. We designed an experimental approach to address exactly this question. For these analyses we applied three different types of sample pooling, which were based on defined mixes of soil samples ('soil-mixes'), mixes of soil DNA extracts ('DNA-mixes'), or calculated averages of T-RFLP data ('TRFLP-averages'). This approach aimed at the establishment of a strategy for efficient determination of the optimal number of soil samples to be pooled in order to obtain representative genetic soil fungal community profiles.

II.3.2. Soil sampling

The study site was a hay-meadow with an eutric cambisol (sandy loam) situated in Central Switzerland, at an elevation of 700 m, with an annual mean temperature of

7.2 °C and annual mean precipitation of 1491 mm (Meteo-Schweiz, Switzerland). In June 2003 an area of 400 m^2 (20×20 m) was sampled at 10 points, which were distributed across three longitudinal transects. At each point two adjacent soil cores were taken using a stainless-steel corer with an internal diameter of 5.5 cm. The 5 to 15 cm depth fractions of the two adjacent cores were pooled (Kessler *et al.*, 2003). Fifty grams from each of the ten fresh samples were bulked and homogenized by sieving (5 mm) to form a 'ten-soil-mix' sample. Samples were stored in plastic bags at 4 °C and processed within 48 h. Dry weights were determined from 10 g fresh soil of each sample dried at 105 °C for 24 h.

II.3.3. Soil DNA extraction, purification and quantification

Nucleic acids were extracted from fresh soil according to Hartmann et al. (2005): 0.6 g fresh soil and 0.75 g glass beads (0.10 mm diameter, B. Braun Biotech International, Melsungen, D) were suspended in 1 ml extraction buffer (0.2% hexadecyltrimethylammonium bromide (CTAB), 0.2 M sodium phosphate buffer pH 8, 0.1 M NaCl and 50 mM EDTA) and extracted using a bead beating procedure with a FastPrep FP120 (Savant Instruments, Farmingdale, NY) at 5.5 m s^{-1} (approx. 6800 rpm) for 40 s. Supernatants were collected and pooled with corresponding supernatants of two further extractions of each soil sample. Extracted DNA was precipitated and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8) at 1 ml g^{-1} dry weight equivalent of extracted soil. Twenty-five microliters of each extract were purified using Extract-II DNA purification columns (Machery and Nagel, Easton, PA). DNA content was quantified fluorometrically (Sandaa, 1998) using Pico Green (Molecular Probes, Eugene, OR) and a luminescence spectrometer LS30 (Perkin Elmer, Wellesley, MA). Herring sperm DNA (Promega, Madison, WI) was used as DNA concentration standard and soil DNA content was expressed as μg DNA g^{-1} soil dry weight. All subsequent metagenomic analyses of single samples were processed in six replicates (a–f).

II.3.4. Mixtures of metagenomic DNA

DNA mixtures were obtained by mixing DNA extracts of three, five, seven, nine or ten samples. For each level of complexity, three sample combinations were randomly

selected and processed in six replicates, except for the 'ten-DNA-mix', containing DNA from all ten samples, which was processed in ten replicates.

II.3.5. PCR amplification

Partial fungal 18S rRNA genes were amplified from 50 ng or 5 ng template DNA respectively, according to Vainio and Hantula (2000) using 5′ 6-FAM (6-carboxyfluorescein) labelled forward primer NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and reverse primer FR1 (5′-AICCATTCAATCGGTANT-3′; I represents inosine) yielding a product of approximately 1650 bp (Vainio and Hantula, 2000). Samples were incubated in a volume of 11 µl in aqueous solution containing 0.6 µg BSA per ng DNA for 45 min at 37 °C to scavenge PCR inhibitory substances (Kreader, 1996; Watson and Blackwell, 2000). After chilling on ice, PCR mixture (containing 0.3 µg BSA) was added, yielding a final volume of 50 µl with concentrations of 1× PCR buffer (Qiagen, Hilden, Germany), 0.2 µM of each primer, 3.5 mM $MgCl_2$, 0.4 mM dNTP (Invitrogen, Carlsbad, CA) and 2 U of HotStar Taq DNA polymerase (Qiagen). Amplifications were performed in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) either with 42 cycles for 50 ng template DNA or 46 cycles for 5 ng template DNA. After initial denaturation for 15 min at 95 °C, cycles with 25 s at 92 °C, 40 s at 53 °C and 3 min at 72 °C were performed, followed by a final elongation for 10 min at 72 °C. The quality of amplification products was verified by gel electrophoresis in 1.5% agarose gels and ethidium bromide staining.

II.3.6. Restriction fragment length polymorphism analysis

Twenty-five microliters of each PCR product were digested using restriction endonuclease HaeIII (Promega). Buffer conditions for restriction digests were adjusted directly in the PCR sample by adding 50 µl of concentration conversion buffer consisting of 10 mM Tris–HCl (pH 7.4), 50 mM NaCl and 13.25 mM $MgCl_2$ (Hartmann et al., 2005). Eight units of HaeIII in 5 µl 1× buffer C (Promega) were added. Digestion was performed for 10 h at 37 °C followed by inactivation of the enzyme at 65 °C for 20 min. Digestion products were purified using Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA) and adjusted to 20 µl with TE buffer. Three microliters of purified digests were separated by electrophoresis in a

12% polyacrylamide gel in 1× TAE buffer (40 mM Tris-EDTA, 1 mM EDTA, pH 8) for 4.5 h at 200 Vand 35 °C (DCode system; Bio-Rad). Two microliters of 1 kb DNA size standard (Invitrogen) were used. Gels were stained for 30 min with SYBR Green (1:5000 in TAE (1×); Molecular Probes) and RFLP profiles were recorded with a GelDoc XRS system and the Quantity One software (Bio-Rad).

II.3.7. Terminal restriction fragment length polymorphism analysis

For T-RFLP analysis 2 µl purified restriction digest were mixed with 12 µl of formamide and 0.2 µl of X-Rhodamine labeled size standard MapMarker1000 (BioVentures, Murfreesboro, TN). Denatured DNA fragments were separated using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) equipped with sixteen 36 cm capillaries filled with POP-4 polymer (Applied Biosystems). Sizes of terminal restriction fragments (T-RFs) were determined as relative migration units (rmu) with the GeneScan 3.7 analysis software (Applied Biosystems). Signal intensities given as relative fluorescent units (rfu) of T-RFs were scored between 60 and 800 rmu using the Genotyper 3.7 NT software (Applied Biosystems).

Because sizing accuracy decreases with increasing sizes of the fragments, T-RFs below 300 rmu were categorized at ±0.5 rmu, between 300 to 400 rmu at ±1 rmu and fragments longer than 400 rmu at ±0.5% of the corresponding fragment length according to suggestions of ABI Switzerland. Because T-RF signal intensities can be important to distinguish samples (Blackwood *et al.*, 2003b) signal threshold was set at 20 rfu. However, if signal intensities of T-RFs belonging to the same length category were all below 100 rfu, they were excluded from the analysis, as differences in signal intensities were not interpretable. If unambiguous determination of signal intensities of a specific T-RF was not possible in all samples, this TRF was excluded from further analysis.

II.3.8. Statistical data analysis

Relative signal intensities within a profile were calculated by dividing signal intensities of each individual T-RF by the sum of all signal intensities in a profile (Blackwood *et al.*, 2003b). This normalization procedure allowed to compare signal intensities among different samples. For multivariate analysis relative signal intensities were *z*-

transformed to average 0 with a standard deviation of 1 in order to give each T-RF the same relative weight. Statistica software package version 6.1 (StatSoft, Tulsa, OK) was used for Ward cluster analysis based on squared Euclidean distances (Blackwood *et al.*, 2003b). Analysis of variance (ANOVA) and discriminant function analysis (DFA) (Statistica, version 6.1) were used to identify T-RFs that significantly discriminated among samples. To compare dendrograms generated by cluster analyses calculated from different numbers of T-RFs, similarities among distance matrices were determined with the NTSYS-pc 2.2 software (Rohlf, 2005) using Mantel test statistics (Mantel, 1967). Additionally, the proportion of means of linkage distances among samples to means of linkage distances among replicates was used to compare degrees of separation of samples and replicates. The larger this proportion, the better the distinction of sample-clusters. All further statistical analyses were exclusively calculated from TRFs discriminating between samples.

Statistical significance of differences between samples was assessed with permutation testing applied on redundancy analysis (RDA) with the Canoco Software (Microcomputer Power, Ithaca, NY) according to ter Braak and Smilauer (2002) using 999 permutations. Classification of samples and mixes were tested using DFA (Statistica, version 6.1), an ordination technique that generates a set of discriminant functions separating a priori defined groups (Hastie *et al.*, 2001). Groups were defined based on replicate T-RFLP data derived from specific groups, i.e. from individual soil samples as well as calculated T-RFLP averages across the soil samples.

II.4. Results

II.4.1. Reproducibility of RFLP and T-RFLP profiles

Metagenomic DNA yield for the ten soil samples from the 400 m^2 grass land plot was between 135 and 250 µg g^{-1} soil dry weight with an average of 180 µg g^{-1}. Thus, 50 ng or 5 ng DNA used for PCR corresponded on the average to 0.27 mg and 0.027 mg soil, respectively. No inhibition of PCR was observed when 50 ng DNA was added to 50 µl reactions (data not shown). Three soil samples were used to test reproducibility and representativity of HaeIII RFLP patterns of fungal 18S rRNA

genes. RFLP patterns of replicated PCR products derived from 50 ng template DNA consistently revealed sample-specific banding patterns (sample 7 shown in Fig. 2.1 a), demonstrating reproducibility of the method. However, RFLP patterns derived from 5 ng template DNA revealed variability among replicates with respect to inconsistent presence of bands, e.g. bands at 155 or 195 bp, or differences in intensities, e.g. bands at 600, 265, 165 or 95 bp (Fig. 2.1 b).

T-RFLP analysis using 50 ng template DNA of three replicates of the same three samples yielded 22 to 30 T-RFs with a mean standard deviation of 0.58 fragments per sample. Fragment lengths ranged from 80 to 717 rmu. Cluster analysis revealed reproducible and significant ($p < 0.05$) distinction of the three soil samples (Fig. 2.2 a). T-RFLP profiling from 5 ng template DNA yielded between 15 and 30 T-RFs with a mean standard deviation of 4.55 fragments per sample. Cluster analysis revealed no distinct cluster for any of the three samples (Fig. 2.2 a) and Monte Carlo permutation
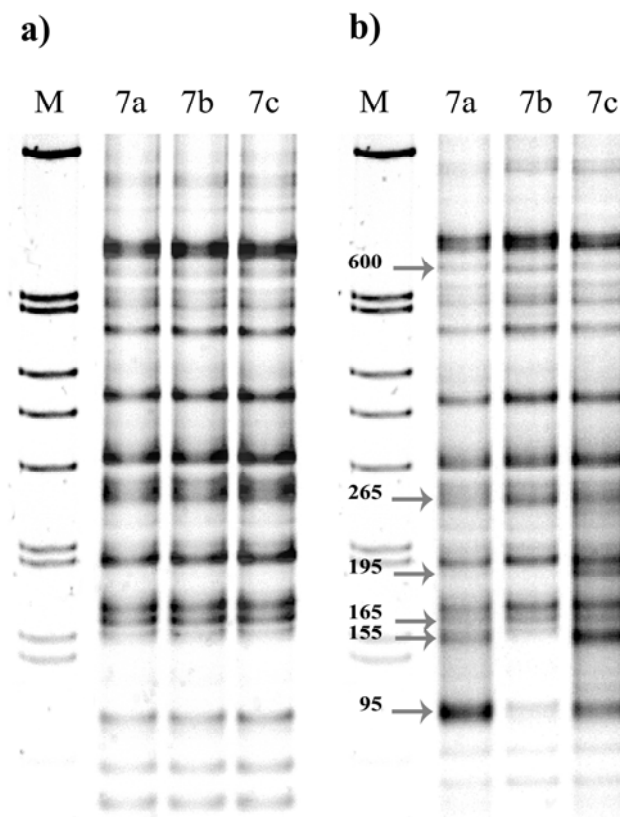
Fig. 2.1: PCR/RFLP patterns obtained from HaeIII digests of fungal 18S rRNA genes amplified from the soil metagenomic DNA of sample seven. PCR was performed on 50 ng a) or 5 ng b) template DNA in triplicates (7a, 7b, 7c). Bands revealing inconsistency or variability among replicated fingerprints are marked with arrows and fragment sizes are indicated. Lane M: 1kb DNA size standard

testing revealed only a significant (p<0.05) distinction of soil sample 2. Based on these results 50 ng soil metagenomic DNA were used for all further PCR analyses.
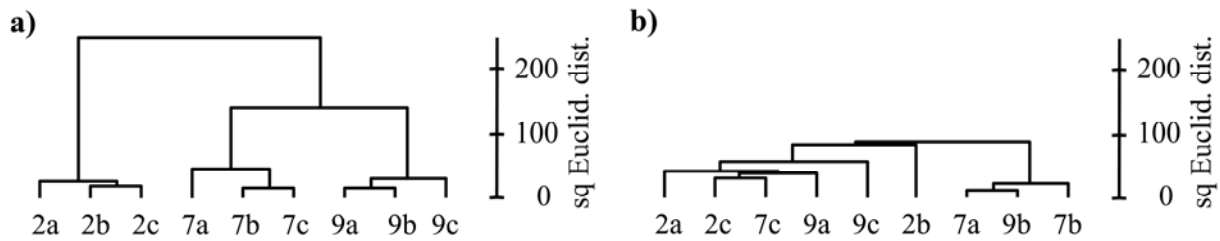
Fig. 2.2 Cluster analysis of T-RFLP data derived from soil fungal 18S rRNA genes using 50 ng a) or 5 ng b) template DNA of three samples (2, 7, 9) processed in three replicates (a, b, c). Dendrograms were inferred using Ward's hierarchical cluster analysis based on squared Euclidean distances.

II.4.2. Selection of T-RFs significantly discriminating between samples

Cluster analysis of fungal community T-RFLP profiles from all ten samples, based on standardized rfu-values of 40 scorable TRFs (80-717 rmu) with relative signal intensities between 5×10^{-4} (T-RF of 327 rmu) and 28.93×10^{-2} (T-RF of 473 rmu), clustered the six profile replicates consistently into ten sample specific groups (data not shown). Distinction of samples may be increased if only the discriminating T-RFs are included in an analysis. ANOVA revealed that with exception of the T-RF at 83 rmu, all T-RFs significantly (p<0.05) discriminated between samples (Table 2.1). DFA identified 20 T-RFs between 80 and 688 rmu with relative signal intensities between 6×10^{-4} (T-RF of 353 rmu) and 9.05×10^{-2} (T-RF of 624 rmu) that significantly (p<0.05) discriminated the ten soil samples (Table 2.1). T-RFs not significantly discriminating soil samples were derived from the entire range of sizes and intensities (Table 2.1). Neither mean signal intensities nor their standard deviations were significantly different (t-test, p<0.05) for the discriminating and non discriminating T-RFs. A dendrogram based on the 20 significantly discriminating T-RFs formed ten distinct clusters (Fig. 2.3), whereas a dendrogram calculated from the 20 not significantly discriminating T-RFs (Table 2.1) only clustered replicates of samples 1, 4, 6, 7 (data not shown). The Mantel test revealed a correlation of 0.54 (p<0.001) between dendrogram matrices derived from 20 significant and 20 nonsignificant T-RFs. Mantel test analyses of dendrogram matrices derived from 40 T-RFs (data not shown) and 20 significantly discriminating T-RFs (Fig. 2.2) showed a correlation of 0.83 (p<0.001), demonstrating consistency of tree topologies. Sample separation was

increased when using only significantly discriminating T-RFs for cluster analysis, as seen by a decrease of mean squared Euclidean distances among replicates from 12.4 to 6.4 and an increase of mean distance among samples from 42.6 to 43.1. This increased the mean distance proportion from 3.4 to 6.7. In order to increase the stringency for assessing the field representativity of T-RFLP profiles, all further analyses were performed with the 20 significantly discriminating T-RFs.
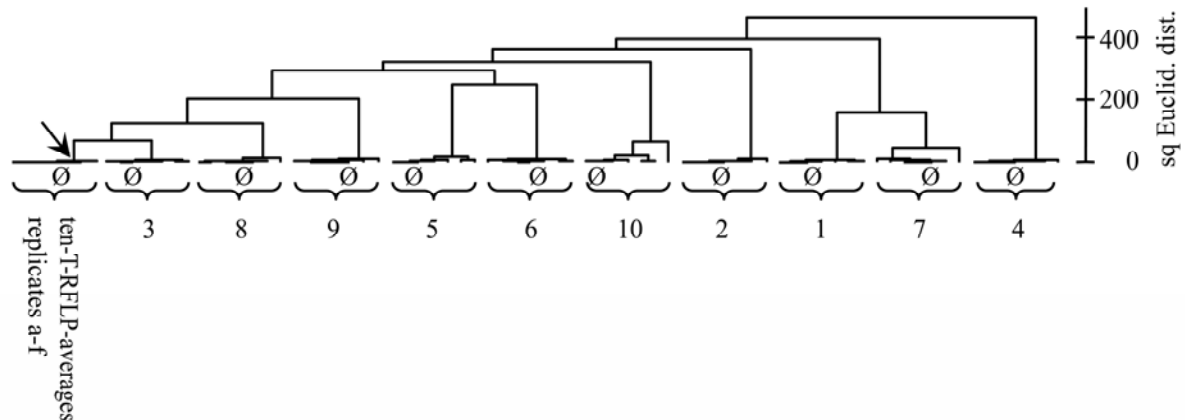
Fig. 2.3: Cluster analysis of T-RFLP data derived from soil fungal 18S rRNA genes from ten soil samples (1 to 10) in 6 replicates from a field plot of 400 m2. Sample averages (Ø) were calculated as means from all six replicates of every sample. The dendrogram was inferred using Ward's method based on squared Euclidean distances calculated from 20 T-RFs significantly discriminating between the ten samples (Table 2.1). 'Ten-T-RFLP-averages' were calculated as mean values of replicates across all ten samples. The arrow indicates the branch of the 'ten-T-RFLP-averages' cluster

II.4.3. Determination of averaged T-RFLP profiles

Mathematically averaged profiles were calculated from all six replicates (a to f) of each sample. These average profiles consistently clustered with corresponding replicates (Ø in Fig. 2.3). For a theoretical field-representative T-RFLP profile, average profiles of all six replicates were calculated across all ten soil samples and labeled 'ten-T-RFLP-averages'. Cluster analysis revealed a separate cluster for the 'ten-T-RFLP-averages' with closest association to soil sample 3 (Fig. 2.3). These eleven groups were used in DFA as explaining variables while T-RFLP profiles were then tested as dependent variables in DFA. The resulting first three canonical axes explained 75% of total variation in the data and significantly discriminated the eleven groups identified by cluster analysis (data not shown).

Table 2.1: Length and relative signal intensities of 40 T-RFs and their significances to discriminate 10 soil samples using analysis of variance (ANOVA) or discriminant function analysis (DFA).

T-RF length [rmu]	Relative signal intensities x100 [$\bar{x}$] [± SD]	ANOVA		DFA	
		F-value	p-class	F-value	p-class
302	0.81 ±2.46	2488.60	***	359.03	***
315	3.27 ±2.78	290.48	***	22.62	***
238	0.96 ±2.91	1083.15	***	21.29	***
688	0.12 ±0.20	69.88	***	13.53	***
164	0.15 ±0.36	114.19	***	12.99	***
312	6.30 ±4.54	190.35	***	11.09	***
632	2.11 ±4.00	531.71	***	10.31	***
80	1.89 ±4.88	382.55	***	9.15	***
246	2.36 ±1.88	52.95	***	8.31	***
314	0.60 ±0.82	35.00	***	7.72	***
207	1.04 ±0.65	13.91	***	7.54	***
261	0.68 ±1.21	157.61	***	6.89	***
499	0.51 ±0.37	65.58	***	5.62	***
260	2.67 ±1.77	25.69	***	5.61	***
664	2.69 ±1.68	86.04	***	5.22	***
345	1.95 ±0.84	37.24	***	4.37	***
353	0.06 ±0.18	111.45	***	3.98	**
477	2.35 ±0.92	8.53	***	3.80	**
624	9.05 ±1.55	20.08	***	3.37	*
210	0.58 ±0.34	6.88	***	2.63	*
267	0.29 ±0.47	25.38	***	2.19	n.s.
327	0.05 ±0.15	77.46	***	2.05	n.s.
643	1.91 ±0.69	12.95	***	1.94	n.s.
628	0.82 ±0.99	27.79	***	1.50	n.s.
257	2.98 ±1.30	28.04	***	1.41	n.s.
271	7.52 ±2.33	31.42	***	1.23	n.s.
162	0.32 ±0.42	25.51	***	1.15	n.s.
323	1.04 ±1.13	48.06	***	1.15	n.s.
717	1.01 ±0.19	3.77	**	1.11	n.s.
203	0.83 ±0.53	18.74	***	1.10	n.s.
215	2.13 ±0.98	29.84	***	1.04	n.s.
264	1.41 ±0.64	9.98	***	1.03	n.s.
348	0.30 ±0.40	35.86	***	0.96	n.s.
682	2.62 ±0.51	12.67	***	0.86	n.s.
490	0.55 ±0.28	11.74	***	0.82	n.s.
473	28.93 ±5.46	66.80	***	0.76	n.s.
83	0.35 ±0.75	2.00	n.s.	0.64	n.s.
647	3.23 ±0.75	9.65	***	0.60	n.s.
357	1.15 ±0.57	57.97	***	0.60	n.s.
158	2.41 ±0.86	5.26	***	0.44	n.s.

Analyses were based on 6 replicate DNA extracts.

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s. not significant.

II.4.4. Comparison of theoretical and experimental means of profiles

Cluster analysis of T-RFLP profiles of 'ten-soil-mixes' or of 'ten-DNA-mixes' together with the ten soil samples revealed that each mix clustered at the branch indicated with the arrow in Fig. 2.3 and dendrogram topology remained identical to the one in Fig. 2.3 (data not shown). When all three composite samples were combined with the ten soil samples in a cluster analysis they defined by the 'ten-T-RFLP-averages' and indicated with the arrow in Fig. 2.3 (Fig. 2.4, arrow). DFA of all three composite samples together with the ten soil samples resulted in ten independent sample groups and three overlapping groups of the pools (data not shown). The branch consistently formed by the three pool-types of all ten soil samples (arrows in Fig. 2.3 and Fig. 2.4) was therefore defined as representative for the field plot. For determining the minimal number of samples required for representing the field plot, the 'ten-T-RFLP-averages' were used as reference in the following analyses.

Fig. 2.4: Cluster analysis as shown in Fig. 2.3, however including 'ten-DNA-mixes' and 'ten-soil-mixes'. The arrow indicates the branch containing the mixed samples as shown in Fig. 2.3 The black triangle represents the remaining part of the dendrogram shown in Fig. 2.3**.**

II.4.5. Determination of minimal pool sizes for representative profiling

T-RFLP profiles derived from pools of decreasing complexity, i.e. from nine, seven, five or three samples, were used to test whether subsets of the ten soil samples clustered with the 'ten-TRFLP- averages', i.e. whether they were representative for the field plot. For each level of complexity, three randomized sample combinations were selected and each processed in six replicates. Replicates of nine, seven, or five 'T-RFLP-averages' consistently clustered with 'ten-T-RFLP-averages' at the branch indicated with the arrow in Fig. 2.3 (Table 2.2). For 'T-RFLPaverages' from three samples only 44% clustered with the 'ten-T-RFLP-averages'. T-RFLP profiles from nine and seven 'DNA-mixes' consistently clustered with 'ten-T-RFLP-averages'. Only 66% of the 'five-DNA-mixes' and 40% of the 'three-DNA-mixes' clustered with 'ten-T-

RFLP-averages' profiles (Table 2.2). DFA assigned all replicates from nine, seven and five 'T-RFLP-averages' to the 'ten-T-RFLP-averages'. Only 88% of 'three-T-RFLP-averages' were also assigned to 'ten-TRFLP-averages' (Table 2.2). For DNA-mixes of seven or nine samples all replicates were assigned to 'ten-T-RFLP-averages' while only 73% of 'five-DNA-mixes' and 53% of 'three-DNAmixes' were classified to the 'ten-T-RFLP-averages' (Table 2.2).

Table 2.2: Percentages of calculated 'T-RFLP-averages' and experimental T-RFLP of 'DNA-mixtures' clustering with the 'ten-T-RFLP-averages' defined in Fig. 2.3 or grouping with the 'ten-T-RFLP-averages' in a discriminant function analysis.

number of pooled samples	cluster analysis		discriminant function analysis	
	T-RFLP averages[a]	DNA-mixes[b]	T-RFLP averages[a]	DNA mixes[b]
10	100	100	100	100
9	100	100	100	100
7	100	100	100	100
5	100	66	100	73
3	44	40	88	53

[a] number of replicates was 18

[b] number of replicates was 10 for 'ten-DNA-mixes', 15 for 'three-' and 'five-DNA-mixes', 16 and 17 for 'seven- and 'nine-DNA-mixes' respectively

II.5. Discussion

A generally applicable three-step approach was developed to assess representativity of soil fungal community profiles. Fungal PCR/RFLP and T-RFLP were based on widely used fungi specific PCR primers (Vainio and Hantula, 2000) and highly reproducible when derived from 50 ng soil metagenomic DNA. Fungal community structures were significantly different among ten soil samples from a 400 m^2 grassland field plot. Cluster analysis as well as discriminant function analysis consistently revealed that averages of five or pools of seven samples were sufficient to generate a representative profile for this field plot.

II.5.1. DNA quantity and reproducibility of community profiles

PCR inhibition caused by coextracted substances such as humic acids is one reason to use small quantities of template DNA. For this study we choose PCR template quantities of 5 ng and 50 ng soil DNA, which have been used for analyses of rhizosphere (Milling *et al.*, 2004) and bulk soil metagenomic DNA (Anderson *et al.*, 2003a; Ranjard *et al.*, 2003; Viaud *et al.*, 2000). T-RFLP analyses resulted in 15 to T-RFs per sample. These figures were in the same range as reported for comparable studies on fungal community structures of grasslands (Brodie *et al.*, 2003; Edel-Hermann *et al.*, 2004; Klamer and Hedlund, 2004). RFLP as well as T-RFLP derived from 50 ng template DNA revealed high reproducibility as illustrated by sample characteristic RFLP banding patterns and consistent clustering of T-RFLP replicates resulting in significant separation of samples. In contrast, RFLP patterns derived from 5 ng template DNA were not reproducible and corresponding T-RFLP profiles revealed inconsistent clustering (Fig. 2.2). This high reproducibility of restriction fragment analyses derived from 50 ng template DNA indicated that neither primer binding biases (Suzuki and Giovannoni, 1996) or stochastic PCR artifacts like nucleotide misincorporation (Eckert and Kunkel, 1991) nor chimera formation (Kanagawa, 2003) were limiting factors in these analyses. Variability in T-RFLP profiles derived from 5 ng template DNA may have originated from stochastic dilution of specific target sequences in individual PCR reactions or from PCR artifacts related to the large cycle numbers required. Required template DNA quantities may also depend on the profiling method. For example, Brodie (2003) have found 10 ng of template DNA to be sufficient for reproducible TRFLP profiling, but to be insufficient for denaturing gradient gel electrophoresis (DGGE). Improvements in DNA purification have allowed to increase template DNA quantities (Watson and Blackwell, 2000), e.g. up to 200 ng (Kennedy *et al.*, 2005b). Reproducibility of fungal genetic profiling may also depend on the quantity of extracted soil (Ranjard *et al.*, 2003) and on the DNA extraction method applied as these strongly influence both quality and quantity of extracted soil metagenomic DNA (Bürgmann *et al.*, 2001; Martin-Laurent *et al.*, 2001). Whether 50 ng template DNA is generally sufficient for soil fungal community profiling is unknown and needs to be tested on a case by case base.

II.5.2. Discriminative T-RFs for improved sample separation

T-RFs common to and evenly occurring in all samples may mask differences between samples. In an earlier study, ANOVA has been used for the detection of indicator T-RFs for bacteria associated with heavy metal contaminated soils (Hartmann *et al.*, 2005). In the present study ANOVA revealed all but the T-RF of 83 rmu as significantly (p<0.05) discriminating between ten soil samples taken across a 400 m^2 area. While ANOVA calculates the partial contribution of all T-RFs on the overall separation in one analysis, DFA stepwise adds, or likewise, removes variables from the dataset and calculates the separation of groups until no further improvement is achieved (Gill, 2001; Leotta, 2004). This process excludes common T-RFs, reduces the number of variables under analysis and thus improves the validity of multivariate analyses (Kourtev *et al.*, 2002). This is illustrated by our results where DFA revealed only 20 significantly discriminating T-RFs (Table 2.1). The analyses of profiles, which exclusively contain T-RFs discriminating between samples emphasize differences between samples and thus represent a more stringent approach for defining representative profiles. Therefore, in this study we discarded 20 of the measured T-RFs and calculated reference profiles exclusively from the 20 T-RFs, which discriminated significantly between the ten soil samples.

II.5.3. Representative reference profiles

Pooling of PCR products has been applied to increase representativity of molecular genetic profiles (Blackwood *et al.*, 2003b; Hunt *et al.*, 2004; Von Wintzingerode *et al.*, 1997). Instead of mixing PCR products, multiple T-RFLP profiles were averaged in the present study. When the average was calculated from replicated T-RFLP profiles of a sample, it was shown representative for this sample. In analogy, we averaged profiles of all ten soil samples to generate a profile representing the entire field plot (Fig. 2.3, arrow). T-RFLP profiles derived from mixes of the ten soil samples or of the ten DNA extracts, associated in distinct clusters under the same branch (Fig. 2.4, arrow). The small differences among these three clusters may be explained with differences in sample preparation. However, data demonstrate that effects of different preparations of these 'ten-mixes' were minor if compared to differences among individual soil samples. These results also demonstrated comparability of

DNA extracts obtained from soil samples mixed prior to extraction to DNA pools prepared from extracts of individual soil samples. This information was used to define the branch that was considered representative for the field plot (indicated by the arrows in Figs. II.3 and II.4). There have been several studies, which indicated reduced resolution caused by within-site variability of fungal communities. Klamer (2002) analyzed fungal communities based on internal transcribed spacer (ITS) analysis of DNA extracted from one sample of 0.25 g soil per replicate plot. Heterogeneity among 8 replicated plots has been found to be as high as heterogeneity between controls and treatments. Girvan (2004) assessed both fungal and bacterial community structures by DGGE profiling from mixed samples derived from 20 soil cores collected over an area of 1.2 ha. They detected effects of a pesticide treatment on bacterial communities, but heterogeneity of fungal communities interfered with the detection of differences between treated and control plots. The approach presented here will be of assistance to assess profiling robustness and to define the sample numbers required for representative profiles.

II.5.4. Optimized sample pool size for representative fungal community profiling

The initial collection of ten soil samples allowed for efficient assessment of spatial heterogeneity of fungal community structures within the field plot of 400 m2 and to define its representative fungal community profile. If the averages of TRFLP profiles derived from individual samples were calculated, averages of five were sufficient to yield a representative profile of the community structure. If pooling was performed at the DNA level, it was necessary to combine at least seven DNA extracts to obtain a representative profile. As all averages of five T-RFLP profiles or mixes of seven DNA extracts consistently clustered at the same branch (Table 2.2), differences in T-RF composition among the ten samples were considered quantitative and stochastic. However, in cases of non-stochastic distributions, e.g. due to gradient forming influences, or restriction of T-RFs to particular samples, the number of samples in a pool might not be reduced without loss of representativity. In such cases, a representative sample pool may not be obtained and analyses of changes in community structures may be difficult. Even though not performed in this study, we assume that the same strategy will be applicable to soil mixes of different

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complexities in order to determine the number of soil samples to be pooled for representative genetic profiling. However, this would be laborious and possibly not feasibly performed for many studies. The use of 'T-RFLP-averages' and 'DNA-mixes' as performed in the present study represents a feasible approach in order to assess representativity of soil fungal community profiles.

II.5.5. Conclusions

In this study we established a three-step approach to assess representativity of soil fungal community profiles. Optimal template DNA quantities for robust profiling, theoretical reference profiles, and experimental sample pools were determined for assessing representativity. For many studies determination of theoretical reference profiles based on 'TRFLP- averages', may be sufficient to feasibly assess representativity. If required also 'DNA-mixes' or even 'soil-mixes' could be used. Our strategy will be advantageous to reliably separate stochastic within-site heterogeneity of fungal T-RF genotypes from between-site heterogeneity and will allow to improve resolution in effect studies.

II.6. Acknowledgements

CHAPTER III

Cultivation-independent analysis of fungal genotypes in soil by using simple sequence repeat markers

Kaspar Schwarzenbach, Franco Widmer, and Jürg Enkerli

Chapter III. Cultivation-independent analysis of fungal genotypes in soil by using simple sequence repeat markers

III.1. Abstract

Cultivation-independent analyses of fungi are used for community profiling as well as identification of specifics trains in environmental samples. The objective of the present study was to adapt genotyping based on simple sequence repeat (SSR) marker detection for use in cultivation-independent monitoring of fungal species or strains in bulk soil DNA. As a model system, a fungal biocontrol agent (BCA) based on *Beauveria brongniartii*, for which six SSR markers have been developed, was used. Species specificity of SSR detection was verified with 15 fungal species. Real-time PCR was used to adjust for different detection sensitivities of the six SSR markers as well as for different template quantities. The limit for reliable detection per PCR assay was below 2 pg target DNA, corresponding to an estimated 45 genome copies of *B. brongniartii.* The cultivation-independent approach was compared to cultivatio- dependent SSR analysis with soil samples from a *B. brongniartii* BCA-treated field plot. Results of the cultivatio- independent method were consistent with cultivation-dependent genotyping and allowed for unambiguous identification and differentiation of the applied as well as indigenous strains in the samples. Due to the larger quantities of soil used for cultivation-dependent analysis, its sensitivity was higher, but cultivation-independent SSR genotyping was much faster. Therefore, cultivation-independent monitoring of *B. brongniartii* based on multiple SSR markers represents a rapid and strain-specific approach. This strategy may also be applicable to other fungal species or strains for which SSR markers have been developed.

III.2. Introduction

Characterization and monitoring of fungi in the environment are important aspects for many research questions in fungal biology and ecology. These include, for example,

characterization of fungal population structures (Breuillin *et al.*, 2006; Stukenbrock *et al.*, 2006), investigations of fungal functions in ecosystems (Naef *et al.*, 2006) or natural occurrence of specified fungal groups, e.g., entomopathogenic fungi (Meyling and Eilenberg, 2006), and studies of survival, spread, and persistence of fungal strains released to the environment (Bidochka, 2001; Enkerli *et al.*, 2004). Traditionally, identification and characterization of fungi has relied on cultivation, followed by morphological (Keller and Bidochka, 1998), biochemical (Nahar *et al.*, 2004; Ratcliff *et al.*, 2006), or molecular (Enkerli *et al.*, 2001) analyses. However, the cultivation step required makes these approaches both laborious and time-consuming.

Cultivation-independent molecular genetic detection of fungal populations directly in DNA extracted from complex environmental samples could reduce the time and cost for monitoring and analysis (Cairney, 2005). Such analyses have successfully been applied to fungal community profiling and are highly valuable for analyzing population structures at various phylogenetic levels (Anderson and Cairney, 2004). Typically, specific PCR primers target conserved regions in phylogenetic markers, like the small as well as the large subunit of rRNA genes or their internal transcribed spacer regions. However, limited resolution often does not allow for identification of particular strains based on these markers (Anderson and Cairney, 2004). For identification of specific fungal groups in complex environmental samples, specific primers have been designed within variable regions of marker genes, such as the internal transcribed spacer region (Atkins *et al.*, 2005; Entz *et al.*, 2005) or sequence-characterized amplified regions (Castrillo *et al.*, 2003; Dauch *et al.*, 2003; Dodd *et al.*, 2004). Because specificity of a single marker may be limited to particular ecosystems or a range of tested strains, the use of multiple markers would improve reliability and resolution of such analyses (Hermosa *et al.*, 2001; Zhou *et al.*, 2001).

Multilocus simple sequence repeat (SSR) genotyping is a commonly used technique for characterization of cultivated fungi based on PCR amplification of multiple markers (loci). The polymorphic character of SSRs produces highly discriminating fingerprints that often allow characterization of fungi at a strain level (Barres *et al.*, 2006; Dalleau-Clouet *et al.*, 2005; Enkerli *et al.*, 2004). Several fungal SSR markers have been reported to be species specific (Barres *et al.*, 2006 ; Prospero *et al.*, 2004; Zhou *et al.*, 2001), and therefore multilocus SSR genotyping may be a promising

option for cultivation- independent detection of fungal strains in soil samples (Cairney, 2005). However, it is important to notice that detection sensitivities of individual SSR markers can be different (Bustin, 2004; Gobbin *et al.*, 2003). Thus, in cultivation-independent analyses of environmental templates, SSR-specific detection sensitivities would have to be adjusted for reliable multilocus genotyping.

The filamentous ascomycete *Beauveria brongniartii* is a naturally occurring soil fungus and pathogen of the European cockchafer (*Melolontha melolontha*), a pest in permanent grasslands and orchards (Keller *et al.*, 2003). Since 1991, *B. brongniartii* has been available as a commercial biocontrol agent (BCA) to control soil-dwelling larvae of *M. melolontha* (Kessler *et al.*, 2004). A cultivation-monitoring approach based on six polymorphic SSR markers has been developed (Enkerli *et al.*, 2001) and has successfully been used to characterize natural soil populations of *B. brongniartii* and to monitor applied BCA strains (Enkerli *et al.*, 2004).

The objective of the present study was to develop a cultivation-independent approach for SSR genotyping of *B. brongniartii* strains in soil. For this purpose, the six *B. brongniartii* SSR primer pairs were tested for species specificity and performance in bulk soil DNA extracts. Sensitivities for detecting the different SSR loci were determined, and differences were adjusted by adapted PCR conditions. A grassland plot treated with a commercially available *B. brongniartii* BCA strain was used as a model system to compare efficiencies and sensitivities of the established cultivation-dependent and the new cultivation-independent SSR genotyping approaches.

III.3. Material and methods

III.3.1. Fungal reference strains

Fifteen reference strains, including typical soil fungal species, close relatives of *B. brongniartii*, and other entomopathogenic fungi, were obtained from several culture collections (Table 3.1). Reference strains were grown at 22°C for 3 weeks on Difco modified Sabouraud agar supplemented with Difco yeast extract (Becton Dickinson, Sparks, MD) (Papierok and Hajek, 1997). Egg yolk (17%) was added to the medium for growing the Entomophthorales strains (Papierok and Hajek, 1997).

Table 3.1: Detection specificity of six *B. brongniartii* SSR markers tested on a collection of 15 fungal reference strains, including ubiquitous soil fungal species, close relatives of *B. brongniartii,* and other entomopathogenic fungi.

Species	Order[a]	Family[a]	Strain collection accession no.[b]	Presence or absence of SSR locus[c]:					
				Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
Beauveria brongniartii	*Hypocreales*	*Clavicipitaceae*	DSMZ 15205	+	+	+	+	+	+
Beauveria bassiana	*Hypocreales*	*Clavicipitaceae*	ARSEF 5066	-	-	-	-	-	+[d]
Hirsutella thompsonii	*Hypocreales*	*Clavicipitaceae*	ARSEF 2800	-	-	-	-	-	-
Trichoderma harzianum	*Hypocreales*	*Hypocreaceae*	DSMZ 63059	-	-	-	-	-	-
Tolypocladium cylindrosporum	*Hypocreales*	*Incerta sedis*	ARSEF 2777	-	-	-	-	-	-
Verticillium lecanii	*Hypocreales*	*Incerta sedis*	ARSEF 1102	-	-	-	-	-	-
Metarhizium anisopliae	*Hypocreales*	*Nectriaceae*	ARSEF 1066	-	-	-	-	-	-
Aspergillus parasiticus	*Eurotiales*	*Trichocomaceae*	ARSEF 5470	-	-	-	-	-	-
Paecilomyces fumosoroseus	*Eurotiales*	*Trichocomaceae*	ARSEF 1645	-	-	-	-	-	-
Penicillium chrysogenum	*Eurotiales*	*Trichocomaceae*	ACW 931	-	-	-	-	-	-
Conidiobolus coronatus	*Entomophthorales*	*Ancylistaceae*	IESR 04:427	-	-	-	-	-	-
Entomophthora culicis	*Entomophthorales*	*Entomorphtoraceae*	ARSEF 387	-	-	-	-	-	-
Erynia rhizospora	*Entomophthorales*	*Entomorphtoraceae*	ARSEF 1440	-	-	-	-	-	-
Zoophthora radicans	*Entomophthorales*	*Entomorphtoraceae*	ARSEF 388	-	-	-	-	-	-
Mucor hiemalis	*Mucorales*	*Mucoraceae*	ACW 922	-	-	-	-	-	-

[a] According to CABI Bioscience Database.

[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ARSEF, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; ACW: Agroscope Changins-Wädenswil, Changins, Switzerland; IESR, Collection of Microorganisms, Institute of Environment and Sustainability Research, Staffordshire University, United Kingdom.

[c] Presence (+) or absence (-) of characteristic SSR marker signal.

[d] Length was 163 bp and different from *B. brongniartii*, which was 172bp.

III.3.2. Field application of the *B. brongniartii* BCA strain and soil sampling

Field experiments were carried out in an *M. melolontha*-infested hay meadow with a humus-rich eutric cambisol. Two adjacent plots of 400 m^2 (20 by 20 m) each were either treated with the commercially available *B. brongniartii* BCA product Beauveria-Schweizer (E. Schweizer Seeds Ltd., Thun, Switzerland) or left as an untreated control. The BCA product consisting of barley kernels overgrown with *B. brongniartii* strain DSMZ 15205 (BCA strain) was applied once in spring 2002 in quantities of 40 to 50 kg ha^{-1} (Kessler *et al.*, 2003). In September 2004, five soil samples from the treated plot (T1 to T5) and from the untreated control (C1 to C5) were collected. At each of the evenly distributed sampling points, two adjacent soil cores were taken using a stainless-steel corer with an internal diameter of 5.5 cm. The 5- to 15-cm-depth fractions of adjacent cores were pooled (Kessler *et al.*, 2003) and stored at 4°C until use (see below).

III.3.3. *B. brongniartii* density and field isolates.

Within 2 weeks after sampling, *B. brongniartii* density in each soil sample was determined. Twenty grams of soil was mixed with 100 ml of 4 mM tetra-sodiumpyrophosphate (Na$_4$P$_2$O$_7$ - 10H$_2$O) and suspended at room temperature for 2 h at 110 rpm (Kessler *et al.*, 2004). After sedimentation for 15 s, 100-µl aliquots of the supernatant were plated in triplicate on solid selective medium (SM) (Strasser *et al.*, 1996). After incubation for 14 days at 22°C, densities of *B. brongniartii* were determined as numbers of CFU per gram soil (dry weight). *B. brongniartii* isolates were obtained from single colonies randomly picked from SM plates, transferred to solid complete medium (Riba and Ravelojoana, 1984), and maintained at 22°C.

III.3.4. Extraction of genomic DNA

Mycelia for DNA extraction were produced by inoculation of 80 ml liquid complete medium with conidia collected from plates and growth for 2 to 6 days at 20°C at 120 rpm. Mycelia were harvested by filtration as described by Enkerli et al. (Enkerli *et al.*, 2001). Genomic DNA was extracted from lyophilized mycelium by using a DNeasy plant mini kit (QIAGEN, Hilden, Germany) and quantified by gel electrophoresis using

a GelDoc XRS (Bio-Rad Laboratories, Hercules, CA) gel imaging system with Quantity One analysis software (Bio-Rad Laboratories) and a high-mass DNA ladder (Promega, Madison, WI) as the standard. The suitability of DNA for PCR was tested by amplifying the 18S rRNA gene with universal primers SSU-uni-b-for (5'-TGCCAGCMGCCGCGGTA-3') (modified from reference, Giovannoni *et al.*, 1988) and SSU-uni-b-rev (5'-GACGGGCGGTGTGTRCAA-3') (Bundt *et al.*, 2001). PCR was performed on an iCycler (Bio-Rad Laboratories) in volumes of 25 µl containing 20 ng DNA, 1 U HotStart *Taq* polymerase (QIAGEN), 1× PCR buffer (QIAGEN), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 0.4 mM deoxynucleoside triphosphate, and 0.6 mg ml^{-1} bovine serum albumin (BSA). Cycling conditions consisted of a 15-min initial denaturation at 95°C and 30 PCR cycles of 25 s at 92°C, 40 s at 53°C, and 3 min at 72°C, followed by a final extension for 10 min at 72°C. The quality of amplification products was confirmed by gel electrophoresis in 1.5% agarose gels and ethidium bromide staining.

III.3.5. Analysis of SSR markers in fungal genomic DNA

SSR marker detection for the six SSR loci Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, and Bb8D6 from *B. brongniartii* was performed according to the method of Enkerli *et al.* (Enkerli *et al.*, 2001). Reaction volumes of 25 µl contained 20 ng genomic template DNA, 12.5 µl iQSYBR green supermix (Bio-Rad Laboratories), 0.2 µM of fluorescently labelled forward primer, 0.2 µM of unlabeled reverse primer, and 0.6 mg ml^{-1} BSA. Cycling conditions consisted of a 3-min initial denaturation at 95°C and 36 PCR cycles of 40 s at 92°C, 40 s at 58°C, and 30 s at 72°C, followed by a final extension of 10 min at 72°C.

Detection sensitivities for the six SSR markers were compared based on cycle threshold (C_T) values of each primer pair determined from 2 pg genomic DNA of the BCA strain running real-time PCR with the same conditions as described above, except that 45 PCR cycles were applied. Real-time PCR data were analyzed using an iCycler iQ real-time PCR detection system and software v3.1 (Bio-Rad Laboratories). For comparison of detection sensitivities of the six loci, differences in C_T values were expressed as relative C_T ($C_{T\text{-rel}}$) values, calculated as quotients between C_T values of the most efficiently amplified SSR locus, Bb4H9 ($C_{T\text{-4H9}}$), and C_T values of each of the other five SSR loci.

PCR products were analyzed for SSR sizes and the presence of SSR characteristic stutter peak patterns on an ABI Prism 3100 genetic analyzer with 36-cm capillaries and POP-4 polymer (Applied Biosystems, Foster City, CA). For that purpose, 20 µl PCR product was purified (Montage PCR cleanup kit; Millipore, Bedford, MA), resuspended in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and further diluted 1 to 10. Two microliters of the diluted product was used for analysis. GeneScan ROX400 (Applied Biosystems) was used as an internal size standard, and signals were analyzed using GeneScan v3.7 and Genotyper v3.7 NT analysis software (Applied Biosystems).

III.3.6. SSR analysis in bulk soil DNA

Nucleic acids were extracted within 48 h after collection of soil samples. Six hundred milligrams fresh soil was extracted three consecutive times by using a bead-beating procedure, and bulk soil DNA of each sample was pooled and suspended in TE buffer at 1 ml g^{-1} (dry weight equivalent) of extracted soil (Hartmann *et al.*, 2005). Twenty-five microliters of each extract was purified using NucleoSpin Extract-II DNA purification columns (Macherey & Nagel, Easton, PA) and quantified fluorometrically with PicoGreen (Molecular Probes, Eugene, OR) according to the method of Hartmann et al. (Hartmann *et al.*, 2005).

The potential to amplify the six *B. brongniartii* SSR markers from bulk soil DNA was assessed with 50 ng *B. brongniartii*-free bulk soil DNA from sample C1 spiked with 2 pg of genomic DNA of the BCA strain (spiked-soil DNA). Prior to PCR, soil DNA was incubated with 30 µg BSA in a volume of 12 µl for 45 min at 37°C in order to scavenge PCR inhibitory substances present in the soil DNA extract (Kreader, 1996). C_T and $C_{T\text{-rel}}$ of spiked-soil DNA were determined for each SSR locus as described above for genomic DNA of the BCA strain.

The potential to detect multiple genotypes of *B. brongniartii* in bulk soil DNA was tested using 50 ng of *B. brongniartii*-free bulk soil DNA from sample C1 spiked with various combinations of 20, 2, or 0.2 pg genomic DNA from three different genotypes (I, II, and III) of *B. brongniartii* (Table 3.2).

Variable template quantities in soil samples as well as differences in locus specific detection sensitivities were accounted for by the use of adapted amplification cycle

(C_a) numbers. The C_a values were determined in a three-step process for each of the 10 field soil samples and each locus. First, $C_{T\text{-}4H9}$ was determined experimentally in duplicate from each of the 10 field soil samples. Second, C_T values of loci Bb1F4, Bb2A3, Bb2F8, Bb5F4, and Bb8D6 were calculated as products of averaged $C_{T\text{-}4H9}$ and corresponding $C_{T\text{-rel}}$ values derived from spiked-soil DNA. Third, obtained C_T values were grouped in classes of adapted cycle numbers according to the following rules: $C_{a\text{-}28}$ for C_T of <30, $C_{a\text{-}32}$ for $30 \leq C_T < 34$; $C_{a\text{-}36}$ for $34 \leq C_T < 38$; and $C_{a\text{-}40}$ for C_T of ≥38. Therefore, PCR was run at 28, 32, 36, or 40 cycles according to *Ca* and amplification products were analyzed for SSR sizes and characteristics as described for genomic DNA of the BCA strain.

Table 3.2: SSR fingerprints of the three *B. brongniartii* strains

Genotype	Allele length (bp) of SSR locus:					
	Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
I[a]	242	118	172	171	208	172
II[b]	245	118	205	202	171	172
III[b]	235	124	208	153	205	172

[a] Genotype of the applied BCA strain

[b] Genotypes of two indigenous *B. brongniartii* isolates from the experimental field.

III.4. Results

III.4.1. Specificity of *B. brongniartii* SSR marker detection

SSR analysis of the 15 reference strains revealed specific PCR amplifications from *B. brongniartii* with typical SSRs for all six loci Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, and Bb8D6 (Fig. 3.1) a for locus Bb2F8 and Table 3.1). From *Beauveria bassiana*, one typical SSR product (163 bp) was obtained for locus Bb8D6 (Table 3.1). For the other loci tested, PCR products with lengths of 191 bp (locus Bb1F4), 203 bp (locus Bb4H9), and 133 bp (locus Bb5F4) were obtained from *B. bassiana*,
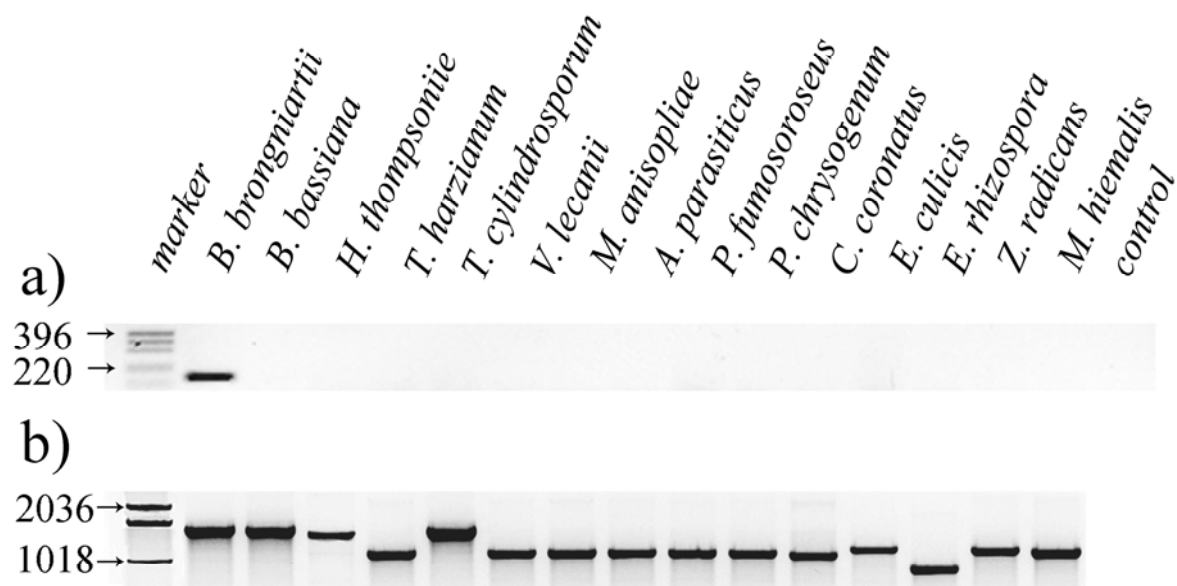
Fig. 3.1: Gel electrophoretic analyses of PCR products derived from the 15 fungal reference strains specified in Table 1. (a) The band at about 200 bp represents the PCR product for SSR locus Bb2F8 separated in 1.5% agarose. (b) The bands between 1000 and 1500 bp represent PCR products obtained with the universal primer pair targeting the small-subunit rRNA gene separated in 1.5% agarose. Marker, 1-kb DNA ladder (Promega).

and for locus Bb1F4 a product of 191 bp was obtained from *Trichoderma harzianum*; however, analyses revealed no SSR characteristic stutter patterns for these PCR products and they were therefore considered unspecific (data not shown). DNA of all reference strains was suitable for PCR as revealed by positive-control PCR using universal primers for the small-subunit rRNA gene (Fig. 3.1b).

III.4.2. Sensitivity of cultivation-independent SSR detection

The PCR amplification efficiency for each of the six SSR loci was determined for 2 pg genomic DNA of the BCA strain and resulted in CT values ranging from 28.83 ± 1.86 (locus Bb4H9) to 34.57 ± 0.65 (locus Bb2A3) (Table 3.3). PCR detection in 50 ng bulk soil DNA from *Beauveria*-free sample C1 that was spiked with 2 pg genomic DNA of the BCA strain (spiked-soil DNA) resulted in values not significantly different ($P < 0.05$, two-sided Student's t test) from the results with pure genomic DNA (Table 3.3). The C_T values for these analyses ranged from 28.53 ± 0.68 (locus Bb8D6) to 34.35 ± 1.09 (locus Bb2A3). $C_{T\text{-rel}}$ values (with reference to locus Bb4H9) ranged from 1.02 (locus Bb8D6) to 1.20 (locus Bb2A3) for genomic DNA from the BCA strain and from 0.99 (locus Bb8D6) to 1.20 (locus Bb2A3) for spiked-soil DNA (Table 3.3).

III.4.3. Simultaneous detection of multiple genotypes in bulk soil DNA.

The sensitivity for detecting multiple genotypes of *B. brongniartii* in bulk soil DNA was tested again using 50 ng *Beauveria*-free bulk soil DNA extract of sample C1 spiked with different quantities of genomic DNA from three different genotypes of *B. brongniartii* (Tables III.2 and III.4). All three genotypes had a common allele at locus Bb8D6, and genotypes I and II were identical at locus Bb2A3 (Table 2).

Marker detection sensitivities for this experiment were adjusted according to the total quantities of spiked genomic DNA and according to the relative efficiencies of the six loci according to *CT*-rel (Table 3.3). For mixtures containing a minimum of 6 pg spiked genomic template DNA (Table 3.4, mixtures a to c), cycle numbers for the more efficiently amplifying lociBb4H9, Bb5F4, and Bb8D6 were set to 28 cycles and for the less efficiently amplifying loci Bb1F4, Bb2A3, and Bb2F8 to 32cycles. For mixtures with less than 6 pg spiked genomic DNA (Table 3.4, mixtures d and e), the more efficiently amplifying loci were processed at 36 PCR cycles while the less efficiently amplifying loci were processed at 40 PCR cycles.

The unambiguous assignment of fingerprints to individual genotypes was possible for spiked genomic DNA quantities of 2 pg or 20 pg (Table 3.4, mixtures a to d). Analysis of samples with 0.2 pg spiked genomic DNA of a specific genotype yielded incomplete corresponding fingerprints, and thus no genotype could be assigned.

In mixture b, containing 0.2 pg DNA of genotype I and 20 pg DNA each of genotypes II and III, genotype I-specific alleles at loci Bb1F4 and Bb5F4 were detected, but those of loci Bb2F8 and Bb4H9 were not detected. In mixture d, containing 0.2 pg spiked genomic DNA of genotypes II and III, genotype II alleles that are shared with genotype I (locus Bb2A3) or common to all three genotypes (locusBb8D6) were detected. In mixture e, containing 0.6 pg DNA, i.e., 0.2 pg of each of the three genotypes, the shared allele at locus Bb8D6 and the genotype I-specific allele at locus Bb4H9 were detected (Table 3.4).

Table 3.3: C_T values for PCR amplification of six *B. brongniartii* loci and corresponding C_T-rel values

Value measured	Template DNA	Value[a] for SSR locus:					
		Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6
C_T	Genomic DNA from BCA strain	31.93 ± 0.75	34.57 ± 0.65	31.07 ± 1.07	28.83 ± 1.86	30.97 ± 1.31	29.30 ± 1.74
	Spiked-soil DNA	32.05 ± 0.84	34.35 ± 1.09	31.10 ± 0.75	28.58 ± 0.22	29.75 ± 0.59	28.53 ± 0.68
$C_{T\text{-rel}}$	Genomic DNA from BCA-strain	1.11 ± 0.04	1.20 ± 0.05	1.08 ± 0.06	1.00 ± 0.00	1.08 ± 0.07	1.02 ± 0.08
	Spiked-soil DNA	1.12 ± 0.02	1.20 ± 0.04	1.09 ± 0.02	1.00 ± 0.00	1.04 ± 0.02	0.99 ± 0.02

[a] $C_{T\text{-rel}}$ values were determined with reference to locus Bb4H9 derived either from 2 pg genomic DNA of the BCA strain (results are means of three replications) or from 50 ng bulk soil DNA spiked with 2 pg genomic DNA of the BCA strain (spiked-soil DNA) (results are means of four replications).

Table 3.4: Detection of genotype-specific alleles of six SSR loci in 50 ng *B. brongniartii*-free bulk soil DNA spiked with mixtures of different quantities of three different genotypes of *B. brongniartii*

Spiked genotype[a]	Template quantity[b] (pg)	Mixture	Presence (+) or absence (-) of alleles at locus:						Retrieved genotype[a]
			Bb1F4	Bb2A3[c]	Bb2F8	Bb4H9	Bb5F4	Bb8D6[d]	
I	2	a	+	+	+	+	+	+	I
II	20	a	+	+	+	+	+	+	II
III	20	a	+	+	+	+	+	+	III
I	0.2	b	+	+	-	-	+	+	UD[e]
II	20	b	+	+	+	+	+	+	II
III	20	b	+	+	+	+	+	+	III
I	2	c	+	+	+	+	+	+	I
II	2	c	+	+	+	+	+	+	II
III	2	c	+	+	+	+	+	+	III
I	2	d	+	+	+	+	+	+	I
II	0.2	d	-	+	-	-	-	+	UD
III	0.2	d	-	-	-	-	-	+	UD
I	0.2	e	-	-	-	+	-	+	UD
II	0.2	e	-	-	-	-	-	+	UD
III	0.2	e	-	-	-	-	-	+	UD

a According to Table 3.2.

b Based on genome size of *B. bassiana* (Pfeifer and Khachatourians, 1993), 2 pg of *B. brongniartii* genomic DNA corresponds to 45 genome copies.

c No discrimination of genotypes I and II possible (Table 3.2).

d No discrimination of the three genotypes possible (Table 3.2).

e UD, undefined.

III.4.4. Cultivation-dependent analyses of B. brongniartii field populations

Plating on SM revealed the presence of *B. brongniartii* in all five soil samples (T1 to T5) from the field plot treated with the BCA strain (Table 3.5). The mean *B. brongniartii* density was 98,484 CFU g^{-1} (dry weight), with a minimum of 9,405 CFU g^{-1} (dry weight) (sample T3) and a maximum of 229,266 CFU g^{-1} (dry weight) (sample T2). From the control plot, two samples were free of *B. brongniartii*, while two samples contained *B. brongniartii* at low densities (261 and 816 CFU g^{-1} [dry weight]) and one sample contained *B. brongniartii* at the highest density observed, i.e., 724,441 CFU g^{-1} (dry weight). Genotypes of 24 isolates from the plating experiment were characterized by analysis of the six *B. brongniartii* SSR loci (Table 3.5). Three different genotypes were identified (Table 3.2). Genotype I of the applied BCA strain as well as indigenous genotype II was detected for 14 *B. brongniartii* isolates randomly selected from the treated plot (Table 3.5). A different indigenous genotype (genotype III) was found exclusively in 10 isolates from the control plot (Table 3.5). Genotypes I and II displayed the same allele at locus Bb2A3, while all three genotypes had a common allele at locus Bb8D6 (Table 3.2).

III.4.5. Cultivation-independent analysis of *B. brongniartii* field populations

DNA extraction from the 10 field soil samples yielded 261 to 299 µg DNA g^{-1} (dry weight) for the treated plot and 287 to 350 µg DNA g^{-1} (dry weight) for the control plot. Mean quantities of 276 µg DNA g^{-1} (dry weight) for the treated plot and 300 µg DNA g^{-1} (dry weight) for the untreated plot were not significantly different ($P < 0.05$, two-sided Student's *t* test).

Relative differences of SSR detection sensitivities ($C_{T\text{-rel}}$) derived from bulk soil DNA spiked with genomic DNA (spiked-soil DNA) (Table 3.3) were confirmed with field soil samples. Correlation coefficients between experimentally determined C_T values and C_T values calculated as products of $C_{T\text{-4H9}}$ and corresponding $C_{T\text{-rel}}$ from spiked-soil DNA were $r = 0.99$ for soil sample T2, with high *B. brongniartii* plate counts, and $r = 0.93$ for soil sample T3, with 24-times-lower plate counts.

Table 3.5: Monitoring of *B. brongniartii* in a BCA treated plot (samples T1-T5) and in a control plot (samples C1-C5).

Soil sample	Cultivation-dependent analyses[i]			Cultivation-independent analyses[h]						
	CFU g^{-1} soil (dry wt)	No. of isolates genotyped	Genotype[a]	Detection of SSR locus:						Genotype[a]
				Bb1F4	Bb2A3	Bb2F8	Bb4H9	Bb5F4	Bb8D6	
T1	14,383	NA[b]	NA	+ (36)	+ (40)	+ (36)	+ (32)	+ (36)	+ (32)	I
T2	229,266	4	I	+ (36)	+ (36)	+ (32)	+ (32)	+ (32)	+ (32)	I
T3	9405	2	I	+ (36)	+ (40)	+ (36)	+ (36)	+ (36)	+ (36)	I
T4	118,463	NA	NA	+ (32)	+ (36)	+ (32)	+ (28)	+ (28)	+ (28)	I
T5	120,904	8	II	+ (32)	+ (36)	+ (32)	+ (32)	+ (32)	+ (32)	II
C1	0	0	ND[c]	- (40)	- (40)	- (40)	- (40)[d]	- (40)[d]	- (40)[d]	ND
C2	724,441	8	III	+ (28)	+ (32)	+ (28)	+ (28)	+ (28)	+ (28)	III
C3	261	1	III	+ (40)	- (40)	- (40)	+ (40)[d]	+ (40)[d]	+ (40)[d]	UDC[e]
C4	0	0	ND	- (40)	- (40)	- (40)	+[f] (40)[d]	- (40)[d]	+ (40)[d]	UD[g]
C5	816	1	III	- (40)	- (40)	- (40)	- (40)[d]	- (40)[d]	- (40)[d]	ND

[a] According to Table 3.2.

[b] NA, not analyzed.

[c] ND, not detected.

[d] Analysis performed at C_a but repeated with 40 PCR cycles.

[e] UDC, undefined but consistent with genotype III.

[f] Allele of 215 bp, different from all three genotypes I, II, III.

[g] UD, undefined

[h] The presence (+) or absence (-) of SSR alleles and resulting genotypes was determined with cultivation-independent analyses of 50 ng bulk soil DNA of each sample. Numbers in parentheses are the numbers of cycles to which Ca values were set.

[I] Total CFU and genotypes of 24 selected isolates were determined with cultivation-dependent analyses.

Values for C_{T-4H9} varied between 26.4 and 35.2 among 8 of the 10 soil samples, with a maximum difference of 1.1 cycles for duplicate analyses. Based on these results, C_a values were set to 28, 32, 36, or 40 cycles (Tables III.3 and III.5). If amplification at C_a did not allow for detection of a specific locus, PCR was repeated with 40 cycles (Table 3.5). All six loci were detected in the five samples from the treated plot. *B. brongniartii* genotype I, i.e., the applied BCA strain, was detected in four of the five soil samples, and genotype II was detected in sample T5 (Table 3.5). Among the samples from the control plot, only sample C2 yielded PCR products for each SSR locus. The resulting fingerprint corresponded to genotype III. Even though alleles corresponding to four loci of genotype III were detected in soil sample C3, the fingerprint remained incomplete and no genotype was assigned. In soil sample C4, only the allele at locus Bb8D6, which was common to all three genotypes, was detected, while for locus Bb4H9 an allele with the size of 215 bp, which was different from the corresponding alleles of any of the three genotypes isolated, was observed. None of the loci were detected in samples C1 and C5.

III.5. Discussion

The goal of this study was to assess the feasibility of cultivation-independent multilocus SSR genotyping of fungal strains in bulk soil DNA extracts. The ascomycete fungus *B. brongniartii*, used in biological control of the European cockchafer, *M. melolontha*, served as a model system to develop a generally applicable strategy. A BCA strain applied in a field experiment was monitored by this strategy, and the results were validated by comparison with those of the established cultivation-dependent approach.

Specificity of SSR detection for reliable cultivation-independent identification of specific fungal isolates from complex samples was achieved by combined use of two criteria: species specificity of SSR PCR primers and multilocus SSR fingerprinting. Species specificity of the six *B. brongniartii* SSR primer pairs was demonstrated by the presence of characteristic SSR amplification products from the target species and their absence from nontargets, respectively (Table 3.1). Species specificity for SSR primers has also been reported for other fungi (Barres *et al.*, 2006; Prospero *et al.*, 2004; Zhou *et al.*, 2001). However, there are also examples of either unspecific SSR

primers, which detect various species (Hayden *et al.*, 2004; Hosid *et al.*, 2005), or highly specific primers, limited to certain strains only (Dalleau-Clouet *et al.*, 2005). Therefore, detection specificity of SSR primers needs to be validated prior to cultivation-independent application to complex DNA samples.

Multilocus SSR fingerprinting of *B. brongniartii* has been reported to discriminate individuals in natural populations with high probabilities of 92 to 99% (Enkerli *et al.*, 2004). Five of the six markers used have been reported to be highly polymorphic, while one marker (Bb8D6) was not (Enkerli *et al.*, 2001). In the present study, amplification from locus Bb8D6 yielded a nontarget amplification product from genomic DNA of *B. bassiana*, which was in accordance with findings by Enkerli (Enkerli *et al.*, 2001). However, with a length of 163 bp, this allele was shorter than any allele recorded for this locus from *B. brongniartii* (172 to 190 bp) by others (Enkerli *et al.*, 2001, Enkerli, 2004 #172) or in the present study. The absence of any unexpected allele or unspecific amplification products in the spiking experiments (Tables 3 and 4) further confirmed specificity of the six SSR primers if applied for detection in bulk soil DNA extracts. In addition, data from the experimental field plot indicated specificity, as they revealed the same genotypes for both analyses (Table 3.5). The allele of 215 bp derived from locus Bb4H9 in soil sample C4 was the only one among 60 analyses that could not be attributed to the three genotypes detected (Table 3.5) and may indicate the presence of a fourth genotype at a very low abundance. All together, these data demonstrated that specificity of *B. brongniartii* SSR PCR primers is also retained in highly complex bulk soil DNA. Specificities of fungal SSR primers within plant DNA extracts have been reported previously (Gobbin *et al.*, 2003; Naef *et al.*, 2006; Zhou *et al.*, 2001), but to our knowledge the present study is the first report on SSR species specificity in bulk soil DNA.

Adjustment of detection sensitivities for multiple SSR loci needs to account both for template quantities in bulk soil DNA extracts and for locus-specific amplification efficiencies. Due to high sequence diversity related to the large numbers of different present in a soil sample, typically, template DNA of individual genotypes is relatively nonabundant in bulk soil DNA (Kirk *et al.*, 2004). Sample-specific template quantities were accounted for by using C_a numbers, which also adjusted for primer-specific detection sensitivities. C_a numbers were set as low as possible in order to minimize the risk of PCR biases and artifact generation. For the 10 soil samples, four classes

of C_a were sufficient to cover the range of all 60 C_T values. This allowed for efficient detection of all markers in all samples. Resulting quantities of all PCR products were similar; thus, no individual dilutions were necessary prior to fragment sizing. The observed reproducibility of $C_{T\text{-rel}}$ values both in genomic DNA and in the tested bulk soil DNA extracts indicated a general stability of SSR marker detection sensitivities for the *B. brongniartii* system.

Detection limits for reproducible cultivation-independent genotyping of *B. brongniartii* were estimated by analyzing either spiked-soil DNA or field soil samples. Unambiguous genotype identification from spiked-soil DNA was possible when 50 ng of bulk soil DNA was spiked with 2 pg genomic DNA of *B. brongniartii* but failed for assays with soil spiked with 0.2 pg genomic DNA (Table 3.4). Because in some reactions alleles were also amplified when present at quantities of 0.6 pg (locus Bb8D6 in mixture e) or 0.2 pg (loci Bb1F4 and Bb5F4 in mixture b and locus Bb4H9 in mixture e), the actual detection limit for *B. brongniartii* may be between 0.2 and 2 pg genomic DNA (Table 3.4). These quantities equal 4.5 or 45 genome copies, respectively, if assuming a genome size of 40 Mbp, as determined for the closest relative, *B. bassiana* (Pfeifer and Khachatourians, 1993). A similar detection limit of 26 copies per PCR has been reported for *Trichoderma atroviride* (Cordier *et al.*, 2007). For field soil samples, the detection limit of the cultivation-independent analysis of *B. brongniartii* genotypes depended on unambiguous identification of the least efficiently amplifying locus, Bb2A3, processed at a maximum of 40 PCR cycles (Table 3.5). The resulting detection limit corresponded to approximately 104 CFU g^{-1} (dry weight) soil (Table 3.5, sample T3). Similar cultivation-independent detection limits were reported for *Metarhizium anisopliae*, 4 × 104 CFU g^{-1} (dry weight) soil (Entz *et al.*, 2005), *Fusarium solani*, 1 × 104 CFU g^{-1} (dry weight) soil (Filion *et al.*, 2003), and *Paecilomyces lilacinus*, 3 × 103 CFU g^{-1} (dry weight) soil (Atkins *et al.*, 2005). With a mean of 275 µg DNA g^{-1} (dry weight) soil, the 50 ng DNA used for PCR corresponded to 0.18 mg soil. Thus, the 104 CFU g^{-1} (dry weight) would represent approximately 2 CFU per PCR. With such low template quantities, stochastic PCR amplification will provide unreliable results (Widmer *et al.*, 1996). However, 1 CFU may represent conidia, as well as mycelium carrying more than one nucleus (Castrillo *et al.*, 2003). In addition, cultivation detects only viable material, whereas cultivation-independent analysis also detects target sequences from unculturable and possibly dead cells (Kirk *et al.*, 2004). For *B. brongniartii*, the relation between the detected

CFU and the number of conidia added to soil was previously estimated to be 1:20; thus, 2 CFU may represent approximately 40 conidia (Keller *et al.*, 2002). This almost perfectly supports the observed detection limit of 104 CFU g^{-1} (dry weight), corresponding to about 40 genome copies per PCR. These considerations are in agreement with the 2 pg detection limit for genomic DNA (Table 3.4). The use of a 100-times-higher quantity of soil, i.e., 20 mg per analysis, for plate counting resulted in a 100-times-higher sensitivity for *B. brongniartii*. This allowed detection of *B. brongniartii* at densities of 261 and 816 CFU g^{-1} (dry weight) in soil samples C3 and C5, respectively, which were not detectable with the cultivation-independent analysis (Table 3.5).

Cultivation-independent multilocus genotyping is an attractive alternative for *B. brongniartii* monitoring. The method has a detection limit of about 104 CFU g^{-1} (dry weight) of *B. brongniartii* as required to induce epizootics in *M. melolontha* infested fields (Keller *et al.*, 2002). In addition, it allows for substantial reduction of the time and cost for BCA monitoring. Analyses can be performed within 1 week, whereas the cultivation-dependent approach may require up to 2 weeks for density analysis and approximately 6 weeks for genotyping due to cultivation and subsequent DNA extraction (Enkerli *et al.*, 2004). Furthermore, the method allowed monitoring of multiple cooccurring strains spiked into bulk soil DNA (Table 3.4). This can be important for fields where genotypic diversity may be high, e.g., 22 different *B. brongniartii* genotypes in a plot of 400 m^2 (J. Enkerli, unpublished data). As only one of the three occurring genotypes was detected per field soil sample (Table 3.5), spiking bulk soil DNA with three different genotypes per sample may cover the expected range of genotype diversity and may represent a realistic model (Table 3.4).

The three genotypes detected in the field experiment were unevenly distributed between the treated and the control plot (Table 3.5), which was in accordance with other studies revealing generally patchy distributions of soil fungi on small scales (Cairney, 2005; Meyling and Eilenberg, 2006; Oros-Sichler *et al.*, 2006). The high plate counts caused by indigenous strains (Table 3.4, soil samples T5 and C2) emphasized the need for genotypic analyses when monitoring applied BCA strains in order to avoid false conclusions.

With a growing need to assess potential risks associated with the release of BCAs into the environment (Hokkanen *et al.*, 2003), effective and efficient monitoring becomes increasingly important (Bidochka, 2001). The cultivation-independent monitoring approach based on multiple SSRs combines species-specific detection of polymorphic markers with strain-level resolution. This allows detection of both BCA and indigenous strains of a target species. Furthermore, this approach will allow for genetic analyses of other organisms (Atkins *et al.*, 2005; Castrillo *et al.*, 2007; Cordier *et al.*, 2007) as well as profiling of microbial community structures within the same DNA extract (Hartmann *et al.*, 2005; Schwarzenbach *et al.*, 2007a). Cultivation-independent fingerprinting using SSR thus could ideally be used to study interactions between released fungal strains and indigenous soil microbial communities.

III.6. Acknowledgements

CHAPTER IV

Effects of biological and chemical insect control agents on fungal community structures in soil microcosms

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Chapter IV. Effects of biological and chemical insect control agents on fungal community structures in soil microcosms

IV.1. Abstract

The entomopathogenic fungus *Beauveria brongniartii* is used in several European countries for specifically controlling the European cockchafer, *Melolontha melolontha*, a pest in grassland and orchards. This biological control agent (BCA) is applied to soil, where it specifically infects the soil dwelling larvae of *M. melolontha*. Application of a fungal BCA to soil may potentially affect indigenous soil fungal populations. Therefore, it was the objective of this study to assess and compare potential effects of the BCA to those of a carbofuran-based chemical control agent (CCA). The experimental system consisted of six soil microcosm treatments with and without larvae of *M. melolontha* and included BCA- and CCA-treatments. Microcosms were maintained under controlled conditions for 91 days and were sampled three times. Untreated larvae survived until the end of the experiment, whereas all treated larvae died within 28 days. Cultivation-independent quantification of *B. brongniartii* in soil was based on quantitative real-time PCR analysis of a specific simple-sequence-repeat marker and significantly correlated with cultivation-dependent quantification. Fungal ribosomal intergenic spacer analysis (RISA) revealed strongest and most significant changes in soil fungal communities for treatments that contained larvae that had died from either control agent. The BCA alone revealed much smaller and transient effects, while effects of the CCA were also small but significantly increased at the end of the experiment. The results revealed that either control strategy induced relatively small effects on soil fungal communities and that molecular genetic tools may be efficiently applied for monitoring and effect assessment of fungal BCAs.

IV.2. Introduction

Melolontha melolontha L. (Coleoptera: Scarabaeidae), the European cockchafer or May beetle, represents a widespread pest throughout Central Europe (Keller and Zimmermann, 2005). Adult beetles emerge from soil in April or May and swarm along forest borders, where they preferentially feed on leaves of oak and beech. After some weeks, female beetles migrate to breeding sites and deposit their eggs into the soil of perennial ley. Larvae hatch after about 6 weeks and develop in soil where they feed predominantly on roots of grasses and fruit trees and thereby can cause severe economic damage (Keller and Zimmermann, 2005; Strasser, 2004). After the larvae have completed three instars and a pupal stage of about three weeks adult beetles emerge and swarm in spring, which closes the three to four year cycle.

For chemical control of *M. melolontha,* carbofuran-containing chemical control agents (CCA), such as Curaterr®, can be used. Curaterr® granulate contains 5% carbofuran and the recommended dosage for control of *M. melolontha* is 50 kg ha^{-1} in grasslands, while control of other insects in various crops, such as maize requires 10 kg ha^{-1} (Swiss Federal Office for Agriculture, 2007). Because carbofuran is toxic also for beneficial insects, specific approval is required for use against *M. melolontha* in Switzerland (Swiss Federal Office for Agriculture, 2007).

The ascomycete fungus *Beauveria brongniartii* (Saccardo) Petch has long been known as the most important and specific natural pathogen of *M. melolontha* (Dufour, 1894). A *B. brongniartii*-based biological control agent (BCA) for *M. melolontha* has been developed in form of fungus colonized barley kernels (FCBK; Keller, 2000). This BCA is commercially available since 1991 and can be applied in the field by use of standard seeding machinery (Keller, 2000). Its application has become a widely used measure for integrated pest management in several European countries, such as Germany, Austria, Italy, and Switzerland. In Switzerland, for instance, about 1200 ha grassland have been treated with this BCA between 1992 and 2007 (S. Keller, pers. comm.). The application of 40 kg FCBK ha^{-1} has been demonstrated to result in spore concentrations of up to 10^5 g^{-1} soil three to ten months after application and to effectively control *M. melolontha* populations (Keller *et al.,* 2002). It has also been shown that applied *B. brongniartii* strains can persist in soil if the host is present and

can mediate long-term control for more than ten years by keeping host densities below the damage threshold (Enkerli *et al.*, 2004; Kessler *et al.*, 2004).

Insect control in soil, either based on CCA or BCA, may affect soil quality and thereby may pose a potential risk for the environment (Watrud and Seidler, 1998). Ecological risks of a specific agent depend on its presence in the environment as well as on the effects it may have on the environment. Therefore, ecological risk has been defined as the product of the two factors 'exposure to an agent' and the 'negative effects' it may have (EPA, 1998). Assessment of risks associated with the application of a BCA therefore requires quantitative monitoring of its presence as well as detailed analyses of potentially negative effects it may cause. These effects may be described by physical, chemical, as well as biological soil characteristics (Karlen *et al.*, 1997). Many aspects of these characteristics are mediated by soil fungi, which play an important role in soil ecosystem functioning (Sinsabaugh, 2005). If soil indigenous fungi are affected, e.g. by mass introduction of a fungal species (Cooke and Rayner, 1984b), these functions may be altered or lost, thus representing potential damages to soil ecosystems (Griffiths *et al.*, 2001; Wardle *et al.*, 2004).

Analyses of soil biological and in particular soil microbiological characteristics have gained much attention in the recent past and a variety of methods has been developed, which allow to gain a more profound understanding of the complex interactions of soil microbial ecology and soil quality (Bloem *et al.*, 2006). Monitoring of the presence of specific fungal species or strains advanced with the use of molecular genetic markers, which allow for genotype-specific identification of fungi (Bidochka, 2001). However, monitoring has mostly depended on cultivation and subsequent enumeration of colonies (Hagn *et al.*, 2003b; Strasser *et al.*, 1996) as well as identification of isolates using genetic markers such as simple sequence repeats (SSR, so called microsatellites; Enkerli *et al.*, 2001), randomly amplified polymorphic DNA (RAPD; Hagn *et al.*, 2003b), or restriction fragment length polymorphisms (RFLP; Viaud *et al.*, 2000). Cultivation-dependent analyses are often time consuming and limited to culturable fungal species (Bridge and Spooner, 2001). Cultivation-independent analyses enable to analyze non-culturable fungal species and provide information on the distribution of fungal populations and communities in soil (Anderson and Cairney, 2004; Cairney, 2005). Fungal BCAs, for example have been identified in soil DNA extracts by use of SSR markers (Schwarzenbach *et al.*, 2007b) or sequence characterized amplified region (SCAR) markers (Cordier *et al.*,

2007; Dauch *et al.*, 2003). In addition, SSR markers have allowed to distinguish co-occurring indigenous genotypes of the same species (Schwarzenbach *et al.*, 2007b). For cultivation-independent analyses of changes in fungal community structures, other molecular genetic markers have been used (Gomes *et al.*, 2003; Prosser, 2002). Such a marker is the internal transcribed spacer (ITS) of the ribosomal RNA (rRNA) operon (Anderson and Cairney, 2004) that has been used for ribosomal intergenic spacer analysis (RISA; Fisher *et al.*, 2000; Ranjard *et al.*, 2001) and can yield high-resolution profiles of bacterial or fungal communities (Anderson and Cairney, 2004).

A variety of statistical tools has become available for detailed analyses of genetic community profiles. Multivariate statistical analyses of RISA profiles allowed for sensitive detection of changes in soil microbial communities, for instance in response to exposure to heavy metals (Hartmann *et al.*, 2005), soil types (Ranjard *et al.*, 2001), grassland types (Kennedy *et al.*, 2006), or agricultural management (Hartmann *et al.*, 2006). Explorative statistics such as cluster analysis (Hartmann *et al.*, 2006) or principle component analysis (Ranjard *et al.*, 2001) were used to assess changes in RISA profiles of microbial communities. Moreover, constrained ordination methods allowed to test for significant effects of environmental variables using Monte Carlo permutation testing based on redundancy analysis (RDA; Hartmann *et al.*, 2006) or canonical correspondence analysis (Kennedy *et al.*, 2006). Specific components of the community profiles, also referred to as operational taxonomic units (OTU), may be affected and can be determined by discriminant statistics (Hartmann *et al.*, 2005; Kennedy *et al.*, 2006; Schwarzenbach *et al.*, 2007a; Supaphol *et al.*, 2006). Variation partitioning (Borcard *et al.*, 1992) has been applied to microbial profiling data, for example to determine effects of farming systems and crop rotations (Hartmann *et al.*, 2006). The indicator value (IndVal) method has been developed for the identification of indicator plant species (Dufrene and Legendre, 1997) and holds potential for use in identification of microbial indicators. These tools, either used individually or in combination, currently allow for detailed analyses of potential effects of various factors, including BCAs or CCAs, on soil microbial communities.

The objective of this study was to assess and compare potential effects of the *B. brongniartii*-based BCA and the carbofuran-based CCA Curaterr® on soil fungal community structures. For this purpose a controlled microcosm system was used.

The specific goals were first, to establish an SSR marker-based cultivation-independent quantification of the BCA in soil and to apply it to assess exposure of soil organisms to the BCA. Second, effects of the control agents on soil fungal community structures were assessed based on RISA profiling followed by multivariate statistical analyses.

IV.3. Material and methods

IV.3.1. *M. melolontha* larvae *and B. brongniartii* BCA strain

Second and third instar larvae of *M. melolontha* were collected from an infested hay-meadow in Wiesenberg (8° 22' 3'' east, 46° 55' 42'' north), Central Switzerland, in June 2005. Each Larva was placed in a peat containing cylindrical plastic container (4.5 cm diameter and 6 cm high), maintained at room temperature in the dark, and fed with carrot slices every ten days (Kessler, 2004). They were kept in quarantine for 11 weeks in order to select for healthy individuals that had not previously been infected by entomopathogens. During this period all larvae developed to third instar.

The BCA used in this study was *B. brongniartii* strain 996 (in the following referred to as BCA strain), a re-isolate of strain DSM 15205 previously applied to a *M. melolontha* infested test field in Lungern, Central Switzerland (Schwarzenbach *et al.*, 2007b). Genotyping based on the SSR markers developed by Enkerli *et al.* (2004), was used to verify genotype identity of strains 996 and DSM 15205. Conidiospores were produced according to Kessler (2004) and DNA was extracted from pure cultures as described by Schwarzenbach *et al.* (2007b).

IV.3.2. Soil used for the microcosm study

In August 2005 soil (Cambisol, pH 7.5, 27.9% clay, 30.6% sand, 5.9% humus, 3.4% organic material) was collected from a permanent pasture in northern Zurich (Holderbach, 8° 29' 57'' east, 47° 25' 6'' north), next to a deciduous forest. Over the last 30 years, annual mean temperature was 8.5°C, and annual mean precipitation was 1042 mm (MeteoSwiss, 2007). The top 5 cm, containing grass and plant roots, were removed with a spade. Approximately 20 kg soil were collected from the 5 to 20

cm depth fraction and transported in plastic bags to the laboratory. Sieved soil (according to Keller *et al.*, 2002) was spread in a plastic box, covered and equilibrated in the dark at 20°C and 85% relative humidity for two weeks. Soil water content was 38% (w/w), corresponding to 51% (w/w) of its water holding capacity and was monitored by weighing the entire box. Cultivation-dependent (Kessler *et al.*, 2004) as well as cultivation-independent analyses (Schwarzenbach *et al.*, 2007b) of *B.* brongniartii (see also below) revealed its absence from this soil.

IV.3.3. Microcosm experiment

Microcosms were set up in transparent plastic pots of 9.5 cm diameter and 13 cm height, filled with 200 g equilibrated soil (see above) and screw-lids loosely attached to allow air flow. A total of 36 microcosms was established, representing six treatments with six replicates each (Table 4.1). All microcosms were maintained in the dark at 20°C and 85% relative humidity for the entire duration of the experiment, i.e. 91 days. Soil water content was graphymetrically determined and maintained at the initial level of 38% (w/w). At day 0 the six different treatments were established (Table 4.1). For the treatments "BCA" and "L+BCA" the commercially available *B. brongniartii* BCA product 'Beauveria-Schweizer' (E. Schweizer Seeds Ltd., Thun, Switzerland) was used. This product consisted of fungus colonized barley kernels (FCBK) overgrown with *B. brongniartii* strain 996. Eight FCBKs were added to each corresponding microcosm. For the treatments "CCA" and "L+CCA" (Table 4.1) 350 mg of the insecticide Curaterr[®] (containing 5% carbofuran; Bayer, Leverkusen, Germany) were added to each corresponding microcosm. According to guidelines for the assessment of side-effects of control agents (Gerber *et al.*, 1991), BCA and CCA quantities applied were ten times higher than the recommended 40 kg ha^{-1} for *B. brongniartii* FCBKs (Keller *et al.*, 2002) and 50 kg ha^{-1} for Curaterr (Swiss Federal Office for Agriculture, 2007). Therefore, potential effects of the two control agents in the here presented microcosm experiment may be stronger than expected from an actual field application. For the treatments "Larva", "L+BCA", and "L+CCA", one *M. melolontha* larva was added to each microcosm corresponding to approximately 150 larvae m^{-2}, i.e. 10 times the density reported as damage threshold in grassland. Throughout the experiment, living larvae were fed every ten days with one carrot slice per microcosm and leftovers were removed three days after feeding. Larvae

were analyzed daily for treatment-characteristic symptoms, i.e. desiccation and dark brown coloration of the insect body for the CCA treatment or red coloration of the insect body followed by emergence of white mycelium for the BCA treatment. Larvae were considered dead if they revealed these symptoms and showed no reaction upon touching with forceps.

Table 4.1: Treatments used to analyze effects of the fungal biocontrol agent *B. brongniartii* and the insecticide Curaterr[®] on the community composition of non-target fungi in presence or absence of targeted *M. melolontha* larvae.

Label[a]	Treatment
Ctrl	200 g soil
BCA	200 g soil and 8 FCBK[b]
CCA	200 g soil and 350 mg Curaterr®[c]
Larva	200g soil and 1 *M. melolontha* larva
L+BCA	200g soil and 1 *M. melolontha* larva and 8 FCBK
L+CCA	200g soil and 1 *M. melolontha* larva and 350 mg Curaterr®

[a] Control (Ctrl), biological control agent (BCA), chemical control agent (CCA), *M. melolontha* larva (Larva or L)

[b] fungus colonized barley kernels (FCBK)

[c] insecticide, containing 5% carbofuran

IV.3.4. Soil-sampling and DNA sample preparation

After an initial equilibration phase of seven days, each microcosm (treatments and controls) was sampled three times, i.e. at days 7, 42, and 91 of the experiment. Dry weights were determined from 10 g fresh soil dried at 105°C for 24 h. Nucleic acids were extracted three consecutive times from approximately 600 mg fresh soil using a bead beating procedure according to Hartmann *et al.* (2005). The three extracts from each soil were combined, precipitated, and dissolved in 1 ml TE buffer per gram extracted dry soil. DNA content was quantified fluorometrically with PicoGreen (Molecular Probes, Eugene, OR), and adjusted to 20 ng DNA μl^{-1} for use in PCR (Hartmann *et al.*, 2005). Pooled soil DNA extracts, in the following referred to as "pools", were prepared by mixing equal volumes of DNA extract from each of the six replicates and by adjustment of DNA concentration to 20 ng μl^{-1} as described above.

IV.3.5. Quantification of the *B. brongniartii* BCA strain in soil

For cultivation-dependent quantification of *B. brongniartii*, twenty grams fresh soil were added to 100 ml 4 mM tetra-sodiumpyrophosphate and suspended at room temperature for 2 h at 110 rpm on a rotary shaker (Kessler *et al.*, 2004). After sedimentation for 15 s, 100 µl of the supernatant were plated in three analytical replicates on solid selective medium (SM; Strasser *et al.*, 1996). Supernatants of the two BCA-containing treatments were diluted by a factor 10^{-2} and 10^{-3}. After incubation for 14 days at 22°C in the dark, densities of *B. brongniartii* were determined as colony forming units per gram soil dry weight (cfu g^{-1} dw).

For cultivation-independent quantification of *B. brongniartii* in soil DNA, copy numbers of the *B. brongniartii*-specific SSR marker Bb5F4 were determined using real-time PCR (Enkerli *et al.*, 2001; Schwarzenbach *et al.*, 2007b). In order to scavenge PCR-inhibitory substances in soil DNA extracts, 50 ng soil DNA from each sample were incubated with 30 µg bovine serum albumin (BSA) for 20 min at 65°C in a total volume of 10.25 µl water (Kreader, 1996; Schwarzenbach *et al.*, 2007a). To each BSA-treated DNA sample, 14.75 µl PCR mixture were added, containing 12.5 µl iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.75 µl 10 µM forward primer Bb5F4 (5'-CTCGATCACTTTTCCATCAA-3'; HEX-labeled) and 1.5 µl 10 µM reverse primer Bb5F4 (5'-TGGTTGGGTCATGTTAGTCA-3'; Enkerli *et al.*, 2001). Primers were purchased from Microsynth (Balgach, Switzerland). PCR conditions consisted of 3 min initial denaturation at 95°C, followed by 45 PCR cycles of 40 s at 94°C, 40 s at 58°C and 30 s at 72°C. Real-time PCR was performed in two analytical replicates using an iCycler iQ Real Time PCR Detection System with Software version 3.1 (Bio-Rad). Resulting C_t values were used to calculate SSR marker numbers in soil DNA extracts. Serial ten-fold dilutions with 10^1 to 10^7 copies of a plasmid containing the SSR marker Bb5F4 were used as quantification standards. Independent triplicate quantifications revealed a logarithmic standard curve with a linear regression of $r^2 = 0.997 \pm 0.002$ and a PCR efficiency of 85.2% $\pm$ 2.4 %.

IV.3.6. RISA profiling

Fungal ITS fragments were specifically amplified with forward primer fRISAfor (5'-GTTTCCGTAGGTGAACCTGC-3'; HEX-labeled; position 1769 – 1788,

Saccharomyces cerevisiae SSU rRNA, GenBank accession DQ888227) and reverse primer fRISArev (5'-ATATGCTTAAGTTCAGCGGGT-3'; position 124 – 145; *S. cerevisiae* LSU rRNA; GenBank accession DQ888227) (Sequerra *et al.*, 1997) both purchased from Microsynth. Prior to PCR, 50 ng soil DNA extract was incubated with 30 µg BSA in a volume of 15 µl as described above. PCR was performed in a final volume of 50 µl containing 2 U of HotStar Taq DNA polymerase (Qiagen, Hilden, Germany), 1x PCR buffer, 0.4 mM dNTP (Invitrogen, Carlsbad, CA), 0.4 µM of each primer and 2.5 mM $MgCl_2$ using an iCycler (Bio-Rad). Cycling conditions were 15 min initial activation of the polymerase and DNA denaturation at 95°C, followed by 35 PCR cycles of 30 s at 94°C, 40 s at 63°C and 40 s at 72°C, followed by a final elongation for 7 min at 72°C. By using these stringent conditions, the selected primer combination did not amplify the *B. brongniartii* ITS fragment of 554 bp and therefore analyses were not biased by different quantities of *B. brongniartii* present in the different samples. Quality of amplification products was confirmed by gel electrophoresis in 1% agarose gels and ethidium bromide staining. Twenty µl of each PCR product were precipitated with 55 µl ethanol (70% vol/vol), and 2 µl 7.5 M ammoniumacetate followed by centrifugation at room temperature for 30 min at 4°C (Amersham-Biosciences, 1999) and subsequent re-dissolving in 40 µl ddH_2O. For RISA 2 µl purified amplification product were mixed with 12 µl formamide and 0.2 µl X-Rhodamine (ROX) labeled internal electrophoretic size standard MapMarker1000 (BioVentures, Murfreesboro, TN). ITS fragment sizes were analyzed on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA) equipped with sixteen 36 cm capillaries filled with POP-7 polymer (Applied Biosystems). Run conditions were 7 kV for 75 min at 60°C. GeneMarker version 1.5 (SoftGenetics LLC, State College, PA) was used to identify unambiguously scorable ITS fragments (referred to as OTUs) in the size-range of 50 to 1000 relative migration units (rmu; relative to the internal electrophoretic size standard) and minimal signal intensities of 20 relative fluorescent units (rfu) (2007a). Average profiles were determined as arithmetic means of the six replicate profiles from each treatment and in the following will be referred to as "means".

IV.3.7. Statistical analyses

Data from cultivation-dependent and -independent quantification of *B. brongniartii* were compared by using Pearson product-moment correlation. Analytical precision of the two methods was compared based on variations of analytical replications as well as replicated microcosms. Variation among analytical as well as experimental replicates was determined as mean variation coefficients. Student's t-test was used to determine statistical significance of Pearson's correlations and other numerical comparisons.

Cluster analyses (CA) were used for comparison of RISA profiles and were performed with default settings in Statistica version 6.1 (StatSoft, Tulsa, OK). Hierarchical CA was performed with the Ward method based on pairwise squared Euclidean distances of normalized profile data (Schwarzenbach *et al.*, 2007a). Non-hierarchical k-means CA was used to test, whether separation of the six treatments was hierarchic (Dufrene and Legendre, 1997). Statistical significance of differences among fungal RISA profiles were determined with Monte Carlo permutation testing using Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY) as described by Schwarzenbach *et al.* (2007a). To minimize type I errors in multiple comparisons of RISA data, Bonferroni-Holm correction was applied (Hartmann *et al.*, 2006; Wright, 1992). Significant differences among treatments were indicated at cluster nodes in Ward dendrograms.

Variation partitioning (Borcard *et al.*, 1992; Hartmann *et al.*, 2005) was used to compare effect magnitudes of treatments and was determined with Canoco for Windows 4.5 (Microcomputer Power). These analyses were based on two RDA models: i) the BCA-model, which was based on RISA profiles from the treatments "BCA", "Larva", and "L+BCA" and ii) the CCA-model, which was based on RISA profiles from the treatments "CCA", "Larva", and "L+CCA".

The indicator value (IndVal) method (Dufrene and Legendre, 1997) was applied for identifying OTUs in the RISA profiles that were positively correlated with specific treatments or treatment groups, and in the following will be referred to as "indicative OTUs". The indicator value is given in percent and is a measure for the specificity and the fidelity of an OTU. The indicator value of an OTU is maximal, i.e. 100%, if it is only detected in one treatment and occurs in all six replicates of this treatment. OTUs

with indicator values exceeding the threshold of 30% were tested for significant (p < 0.05) indication of a treatment or a treatment group using a randomized permutation approach (Dufrene and Legendre, 1997).

IV.4. Results

IV.4.1. Efficacy of insect control

None of the *M. melolontha* larvae had died in any treatment up to day seven (Fig. 4.1). The insecticide-treated larvae ("L+CCA"; Table 4.1) died between days 8 and 14, while larvae exposed to the BCA strain ("L+BCA"; Table 4.1) died between days 18 and 28. All dead larvae from treatments "L+BCA" and "L+CCA" showed the treatment-characteristic symptoms. The larvae from the control ("Larva"; Table 4.1) survived the experiment, indicating 100% efficiency of the treatments "L+BCA", and "L+CCA" (Fig. 4.1).
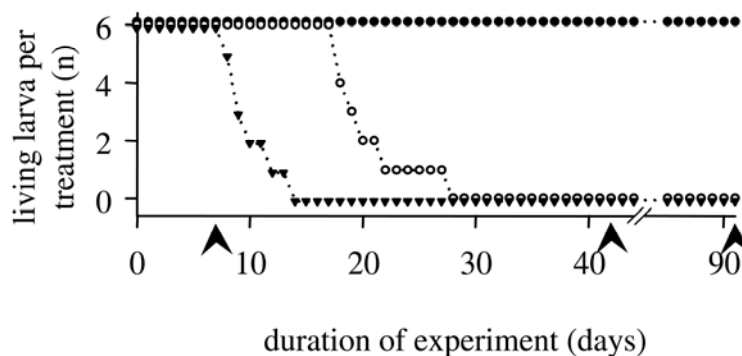
Fig. 4.1 Number of replicates with living *M. melolontha* larvae among a total of six replicated microcosms with the treatments containing larvae only (closed circles, Larva), larvae and the *B. brongniartii* BCA strain (open circles, L+BCA), or larvae and the chemical control agent Curaterr® (triangle, L+CCA). Survival of the larvae was monitored during the entire experiment, i.e. for 91 days. Arrowheads indicate time points of soil sampling, at day 7, 42, and 91 of the experiment.

IV.4.2. DNA quantities in soil samples

Mean DNA quantities extracted from the microcosm soils were 202 ± 22 µg g^{-1} dw at day 7, 183 ± 10 µg g^{-1} dw at day 42 and 165 ± 30 µg g^{-1} dw at day 91 (Table 4.2). This general decrease of extracted DNA quantities from day 7 to day 91 was significant (p < 0.05) for the treatments "Ctrl", "BCA", "CCA", and "L+BCA". After 91

days, treatment "CCA" revealed the significantly lowest average DNA quantity of 107 μg DNA g^{-1} soil dw. With 160 μg DNA g^{-1} soil dw treatment "L+CCA" followed the same trend, but was not significantly different from the control due to a large standard deviation.

Table 4.2: Soil biomass contents in the microcosms expressed as soil DNA quantities extracted from the six replicates of each treatments after 7, 42, and 91 days continuous incubation. Data are presented as means and standard deviations of the six replications.

Treatment[a]	DNA quantities [μg DNA g^{-1} soil dw]		
	7 days	42 days	91 days
Ctrl	$231 \pm 61^{(a)\,[xy]}$	$179 \pm 14^{(ab)\,[x]}$	$171 \pm 11^{(b)\,[x]}$
BCA	$191 \pm 15^{(ab)\,[x]}$	$200 \pm 15^{(a)\,[y]}$	$183 \pm 9^{(b)\,[xz]}$
CCA	$182 \pm 21^{(a)\,[x]}$	$171 \pm 15^{(a)\,[x]}$	$107 \pm 7^{(b)\,[y]}$
Larva	$189 \pm 11^{(a)\,[x]}$	$188 \pm 13^{(a)\,[xy]}$	$185 \pm 12^{(a)\,[xz]}$
L+BCA	$230 \pm 27^{(a)\,[y]}$	$183 \pm 30^{(b)\,[xy]}$	$185 \pm 6^{(b)\,[z]}$
L+CCA	$189 \pm 26^{(a)\,[x]}$	$178 \pm 17^{(a)\,[x]}$	$160 \pm 35^{(a)\,[xz]}$

[a] according to Table 4.1

(a, b,c) significance groups within treatments across time points (p<0.05)

[x, y, z] significance groups within time points across treatments (p<0.05)

IV.4.3. Quantification of the BCA strain

Cultivation-dependent quantification of *B. brongniartii* revealed mean densities between 1.3 x 104 and 2.4 x 107 cfu g-1 dw soil for the two treatments "BCA" and "L+BCA" at the three time points (Fig. 4.2 a and b, left panels). In the treatments where no *B. brongniartii* BCA was applied, no colonies of this fungus were detected during the course of this experiment (data not shown). The mean of variation coefficients was 0.74 for replicated microcosms and 0.37 for analytical replicates. In treatment "BCA" mean plate counts significantly (p < 0.001) increased from 1.6 x 104 cfu g-1 dw at day 7, to 2.5 x 106 cfu g-1 dw at day 42, which was followed by a slight (p > 0.05) decrease to 1.5 x 106 cfu g-1 dw at day 91 (Fig. 4.2a, left panel). In treatment "L+BCA", plate counts increased (p > 0.05) from 1.3 x 104 cfu g-1 dw at day 7 to 8.4 x 106 cfu g-1 dw at day 42, followed by a further increase (p > 0.05) to

2.4 x 107 cfu g-1 dw at day 91 (Fig. 4.2b, left panel). Total increases of *B. brongniartii* colony counts from day 7 to day 91 were significant (p<0.001) for treatments "BCA" and "L+BCA". *B. brongniartii* plate counts did not differ for the two treatments at days 7 and 42 (p > 0.05), but were significantly higher in treatment "L+BCA" at day 91 (p < 0.001; Fig. 4.2).
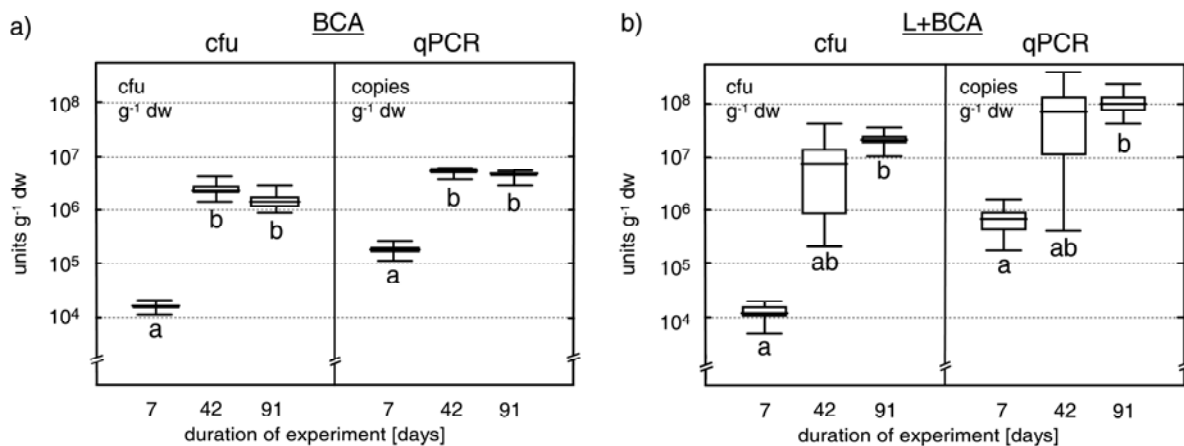

Fig IV.2: Means (middle-bar), standard errors (box) as well as min and max (bottom- and top-bar) of *B. brongniartii* densities from six replicated soil microcosms amended with a) eight fungus colonized barley kernels (FCBK) colonized with the *B. brongniartii* BCA strain ("BCA"), b) eight FCBKs and one *M. melolontha* larva ("L+BCA"). *B. brongniartii* densities were determined on selective medium as colony forming units per gram soil dry weight (cfu g-1 dw) and as copy numbers of SSR-marker Bb5F4 (copies g-1 dw) determined with quantitative real-time PCR. Significance groups (a, b, c) within treatments and type of detection represent the level of p<0.05.

Cultivation-independent quantification of *B. brongniartii* with quantitative PCR of SSR-marker Bb5F4 revealed mean numbers between 1.9 x 105 and 1.2 x 108 for the two treatments "BCA" and "L+BCA" at the three time points (Fig. 4.2 a and b, right panels). Mean of variation coefficients were 0.75 for replicated microcosms and 0.18 for analytical replicates. Quantities of SSR-marker Bb5F4 revealed a significant (p < 0.001) increase in treatment "BCA" from 1.9 x 105 at day 7, to 5.7 x 106 at day 42, followed by a decrease (p > 0.05) to 4.8 x 106 at day 91 (Fig. 4.2a, right panel). In treatment "L+BCA" mean SSR-marker numbers increased (p > 0.05) from 7.2 x 105 at day 7 to 7.9 x 107 at day 42, followed by a further increase (p > 0.05) to 1.2 x 108 at day 91. Total increase of SSR-marker numbers from day 7 to day 91 were significant in treatments "BCA" (p < 0.001) and "L+BCA" (p<0.01). The cultivation-dependent and -independent quantifications significantly correlated (p < 0.05) with correlation coefficients of r = 0.82 for treatment "BCA" and r = 0.89 for treatment "L+BCA".
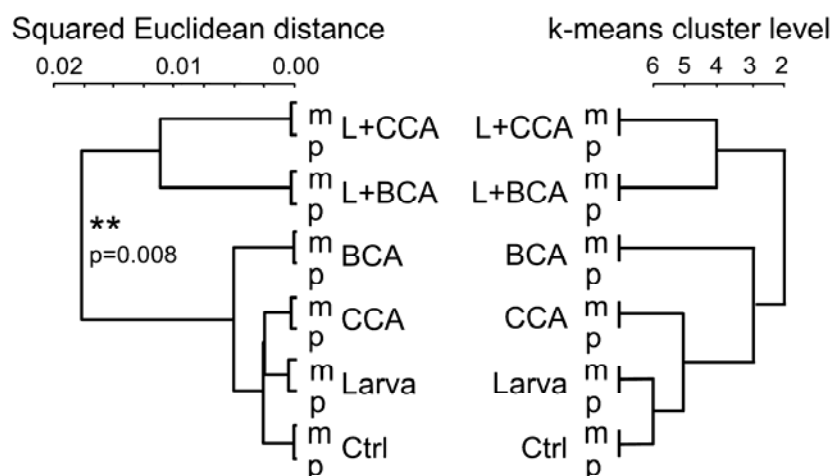
IV.4.4. Soil fungal community structures

A total of 57 fungal RISA OTUs was scorable across all samples, ranging in size from 362 to 884 rmu. Numbers of OTUs ranged from 21 to 54 for individual samples, and standard deviations ranged from 1.6 to 12.2 OTUs. The only significant change in OTU numbers was observed in treatment "BCA" between days 42 (48.5 ± 2.3 OTUs) and 91 (40.5 ± 7.6 OTUs), however, both values were not significantly different from the one at day 7 (46.0 ± 5.2 OTUs). Shannon diversity indices for the RISA profiles ranged from 2.94 to 3.15 and standard deviations from 0.04 to 0.19. The only significant change in diversity was observed in treatment "CCA" between days 42 (3.09 ± 0.09) and 91 (2.94 ± 0.14), but also these values were not significantly different from the one at day 7 (3.05 ± 0.18). RISA profiles from corresponding pooled soil DNA extracts (pools; p in Fig. 4.3) and calculated averages of RISA profiles (means; m in Fig. 4.3) clustered for all six treatments and all three time points in the Ward dendrograms. With k-means CA they mostly clustered, with two exceptions for the treatments "Larva" and "L+CCA" at sampling day 7 (Fig. 4.3a). Dendrogram topologies of Ward and k-means CA were clearly different at day 7 (Fig. 4.3a) and Monte Carlo permutation testing revealed no significant differences among the six treatments or treatment groups. At day 42 the two cluster analyses revealed largely congruent dendrogram topologies except for the two lowest cluster levels involving treatments "CCA", "Larva", and "Ctrl" (Fig. 4.3b). Monte Carlo permutation testing identified a significant (p < 0.001) separation of the treatment group containing dead larvae, i.e. "L+BCA" & "L+CCA". After 91 days, both clustering methods yielded identical dendrogram topologies (Fig. 4.3c). Significance testing with Monte Carlo permutations revealed that the differentiation between the treatment group containing dead larvae and the group containing all other treatments became more significant at day 91. In addition, the difference between the control agents, i.e. CCA versus BCA, also became significant, both if applied in treatments with larvae (p<0.01) or without larvae (p<0.05; Fig. 4.3c). Analyses revealed no significant differences among the treatments "Ctrl", "Larva", and "BCA" (Fig. 4.3).
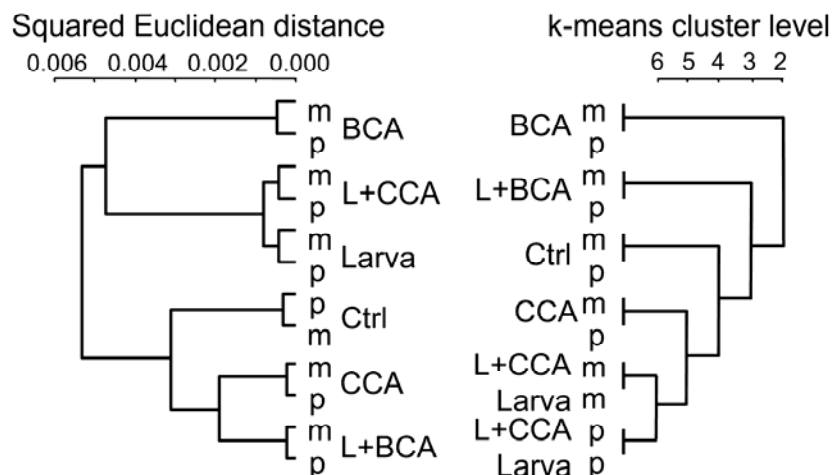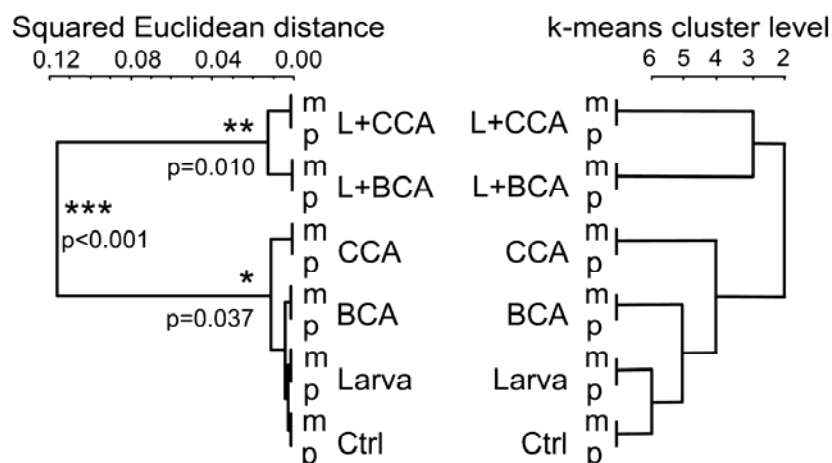
Fig. 4.3: Cluster analyses of fungal community structures based on RISA of pooled replicate DNA (p) and calculated means of replicate RISA profiles (m) for all six replicates of each treatment after 7 days (a), 42 days (b), and 91 days (c). Wards hierarchical clustering (left panels) was based on squared Euclidean distances calculated from normalized data of RISA profiles. The scales for squared Euclidean distances differ among panels a – c. Non-hierarchical k-means clustering (right panel) was calculated for 2 to 6 groups (cluster levels) based on normalized data of RISA profiles. Significances determined with Monte Carlo permutations were based on redundancy analysis of RISA profiles from all six replicates of each treatment. Significance levels are * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Treatments included biological control agent (BCA), chemical control agent (CCA), *M. melolontha* larva (Larva or L), and an untreated control (Ctrl).

Variation partitioning of RISA profile data allowed for determination of effects of specific treatments on soil fungal communities (Table 4.3). In the BCA-model, treatment factors "L+BCA", "BCA" and "Larva" were considered and in the CCA-model the factors "L+CCA", "CCA" and "Larva". Total explained data variation increased from day 7 to day 91 from 8.9% to 47.8% in the BCA-model and from 15.5% to 51.2% in the CCA-model. At day 7 neither of the two models revealed significant specific partitions of explained variation. At day 42 the treatments "L+BCA" and "L+CCA" explained significant partitions of total variations at $p < 0.01$ while treatment "BCA" was significant at $p < 0.05$. At day 91 treatment "CCA" became a significant factor ($p < 0.05$) while treatment "BCA" was no longer significant ($p > 0.05$). The partitions of total variations of the treatments "L+BCA" and "L+CCA" strongly increased and were highly significant ($p < 0.001$). If interactions were excluded, only variations explained by treatments containing dead larvae, i.e. "L+BCA" or "L+CCA", were significant (Table 4.3).

Table 4.3 Variation partitioning of RISA profiles of soil fungal communities in microcosms treated with biological (BCA) or chemical (CCA) control agents and containing larvae of *Melolontha melolontha* (Larva or L). The fungal BCA was a *Beauveria brongniartii* strain and the CCA was Curaterr®. Microcosms were sampled three times, i.e. after 7, 42, and 91 days. Variation partitioning was based on separate models for each control agent, i.e. a BCA-model and a CCA-model. Treatment-specific variation partitioning including and excluding shares with other treatments are shown and are divided by a slash.

day	Variation partition [Percent of total variation]					
	BCA-model			CCA-model		
	BCA[a]	Larva	L+BCA	CCA	Larva	L+CCA
7	4.4 / 3.0	1.8 / 1.6	3.8 / 2.7	12.1 / 5.0	9.7 / 5.9	10.3 / 6.9
42	7.9[q] / 3.5	3.3 / 3.0	12.0[r] / 8.5[q]	4.8 / 2.2	2.7 / 2.5	11.7[r] / 8.2[q]
91	5.3 / 1.6	12.7 / 1.0	44.7[s]/ 31.8[s]	12.3[q]/3.7	5.5 / 1.3	45.0[s]/ 28.6[s]

[a] Treatments according to Table 4.1
q, r, s significance levels q p<0.05, s p<0.01, s p<0.001

Indicative fungal OTUs were determined for sampling days 42 and 91, which revealed significant effects of treatments or groups of treatments (Table 4.3 and Fig. 4.3). The group of treatments including "L+BCA" and "L+CCA" was characterized by two and four indicative OTUs at days 42 and 91 respectively (Table 4.4). The two OTUs at 521 and 525 rmu were indicative at both sampling days and their indicator values increased to more than 80%.

Table 4.4: Indicator Value (IndVal) analysis of fungal RISA profiles. Analyses were performed for sampling days 42 and 91, which revealed significant treatment effects on fungal community structures. Indicative operational taxonomic units (OTU) significantly (p<0.05) indicated treatments or groups of treatments and had indicator values larger than 30%

Treatment[a]	Indicative OTU [rmu]	Indicator Value (k-means level[b])	
		day 42	day 91
L+BCA & L+CCA	394	-	46.0 (2)
L+BCA & L+CCA	521	76.1 (2)	83.4 (2)
L+BCA & L+CCA	525	70.7 (2)	85.5 (2)
L+BCA & L+CCA	603	-	67.9 (2)
L+BCA	521	71.2 (4)	-
L+CCA	621	57.9 (3)	80.3 (3)
L+CCA	684	-	57.6 (3)
L+CCA	706	-	55.1 (3)
L+CCA	711	48.5 (4)	48.5 (3)
BCA	545	54.7 (3)	-
BCA	562	95.7 (3)	47.9 (5)
BCA	570	42.7 (3)	-
BCA	575	45.3 (3)	-
BCA	601	58.2 (3)	-
BCA	642	37.4 (3)	-
BCA	643	36.5 (3)	-
BCA	662	50.7 (3)	-
BCA	687	38.3 (3)	-
BCA	689	37.9 (3)	-
CCA	429	-	42.6 (4)
CCA	529	-	31.2 (4)
CCA	559	49.0 (5)	-
CCA	568	49.2 (5)	40.8 (4)
CCA	655	32.7 (5)	-
Larva	376	-	46.6 (6)

[a]according to Table 4.1

[b] k-mean levels according to Fig. 4.3

At day 42 the OTU at 521 rmu was also indicative for treatment "L+BCA", which represented one component of the treatment group described above (Fig. 4.3 and Table 4.4). The two OTUs at 621 rmu and 711 rmu were at both sampling days indicative for treatment "L+CCA" and the indicator value of the OTU at 621 rmu increased to more than 80%, while the other three OTUs at 684 rmu, 706 rmu, and 711 rmu revealed lower values at that day. At day 42 treatment "BCA" was characterized by ten indicative OTUs. OTU 562 revealed the highest indicator value of 95.7%, and was the only one that remained indicative at day 91. Treatment "CCA" was characterized by three indicative OTUs at both sampling days but only the OTU at 568 rmu was found indicative at both days, although with indicator values below 50%. A single OTU was indicative for treatment "Larva" at day 91 and none for the control treatment.

Indicative fungal OTUs were determined for sampling days 42 and 91, which revealed significant effects of treatments or groups of treatments (Table 4.3 and Fig. 4.3). The group of treatments including "L+BCA" and "L+CCA" was characterized by two and four indicative OTUs at days 42 and 91 respectively (Table 4.4). The two OTUs at 521 and 525 rmu were indicative at both sampling days and their indicator values increased to more than 80%. At day 42 the OTU at 521 rmu was also indicative for treatment "L+BCA", which represented one component of the treatment group described above (Fig. 4.3 and Table 4.4). The two OTUs at 621 rmu and 711 rmu were at both sampling days indicative for treatment "L+CCA" and the indicator value of the OTU at 621 rmu increased to more than 80%, while the other three OTUs at 684 rmu, 706 rmu, and 711 rmu revealed lower values at that day. At day 42 treatment "BCA" was characterized by ten indicative OTUs. OTU 562 revealed the highest indicator value of 95.7%, and was the only one that remained indicative at day 91. Treatment "CCA" was characterized by three indicative OTUs at both sampling days but only the OTU at 568 rmu was found indicative at both days, although with indicator values below 50%. A single OTU was indicative for treatment "Larva" at day 91 and none for the control treatment.

IV.5. Discussion

Soil represents a highly complex ecosystem and analyses of changes in soil quality are often difficult due to variability induced by climatic, edaphic, or management factors. Therefore, microcosm approaches are often used for effect assessment of specific factors and to dissect effects that may co-occur in a field situation. The controlled conditions reduce stochastic variation and allow to detect changes with higher sensitivity than in a field experiment (Birch *et al.*, 2007). However, microcosm studies also have limitations, especially with regard to maintenance of soil structure or exchange with surrounding soil compartments (Seidler, 1992). In the current microcosm study, average soil DNA contents declined over the course of the experiment (Table 4.2), indicating a decrease of soil biomass (Fliessbach and Widmer, 2006). This was observed in most microcosms (Table 4.2) and was in agreement with reports from similar studies (Kessler, 2004; Rooney *et al.*, 2006). This decline may be explained with the limited nutrient pool available in a microcosm and starvation of soil microbial communities. Therefore, microcosm studies should be limited in time (Gerber *et al.*, 1991). No decrease in DNA quantities was observed in the treatment "Larva", which was continuously supplied with carrots to feed the animals. Treatments "L+BCA" and "L+CCA" also received carrots but only until larvae had died, i.e. the longest for 27 and 13 days respectively (Fig. 4.1). Both these treatments tended to lower soil DNA contents at the end of the experiment. The significantly lowest soil DNA content was observed in treatment "CCA" at the end of the experiment (Table 4.2). Curaterr$^®$ has been shown to potentially reduce numbers of soil arthropods as well as of soil fungi (Hassal, 1990b).

Quantities of *B. brongniartii* in all BCA treated microcosms were relatively high (Fig. 4.2) and reached maximum levels of 6.2×10^7 cfu g^{-1} dw. This was about hundred times the densities detected in meadows, where applied BCAs had induced epizootics in *M. melolontha* populations (Keller *et al.*, 2002). In treatment "BCA" maximal average densities of *B. brongniartii* were detected at day 42 (Fig. 4.2a), while in treatment "L+BCA" they increased until day 91 (Fig. 4.2b). Even though the fungus saprophytically grows on barley kernels, presence of a larva may have allowed for improved growth of the fungus, which was in agreement with findings of Kessler *et al.* (2004). Variation in *B. brongniartii* colony counts and corresponding SSR-marker numbers was high but mainly due to differences among experimental

replicates, as revealed by variation coefficients of 0.74 and 0.75 respectively, as well as large standard errors (Fig. 4.2). This variation may be due to variable proliferation of the BCA strain among experimental replicates or due to small differences in spore quantities that were present on individual FCBKs (Keller *et al.*, 2002). Colony counts correlated well with SSR Bb5F4 copy numbers. However, mean SSR marker numbers were consistently higher than cfu values, ranging from 2.3 to 56 SSR markers per cfu detected (Fig. 4.2). This supported a previous estimate that about 1 cfu may be detectable for 20 *B. brongniartii* spores applied to soil (Keller *et al.*, 2002) and may be explained with reduced culturability of spores recovered from soil or with multiple nuclei contained in a single cfu, possibly derived from a fragment of hypha (Castrillo *et al.*, 2003). The new cultivation-independent method was shown to be reliable and of higher analytical precision. In addition, it allowed for reduction of the time required for quantification to about half as compared to the cultivation-dependent approach.

Averaging genetic profiles of microbial communities either by pooling of replicate DNA extracts or by calculation of means of replicate profiles has successfully been used to assess robustness of genetic profiles and differences among treatments (Hartmann *et al.*, 2006; Schwarzenbach *et al.*, 2007a). Only for the treatments "L+CCA" and "Larva" at day 7, did the data from pools and means not cluster in the k-means analysis (Fig. 4.3a). Decreasing differences in dendrogram topologies were found with the two clustering methods between days 7 and 91, when they became identical (Fig. 4.3). Cluster analysis of RISA profiles obtained from pooled DNA of replicate samples and from means of replicate profiles was thus confirmed as a stringent test for analytical quality of microbial community profiles.

Monte Carlo permutation testing revealed no significant difference in fungal RISA profiles among treatment "BCA" and treatments "Larva" and "Ctrl" (Fig. 4.3) even though variation partitioning indicated a significant but transient effect of this treatment (Table 4.3). In agreement with this transient effect, IndVal analysis identified ten indicative OTUs for treatment "BCA" at day 42, i.e. the highest number of indicative OTUs detected among all treatments in this study (Table 4.4). However, only the OTU at 562 rmu remained indicative also at day 91. It is important to note that this was the only significant difference detected for treatment "BCA" at day 91 and that among the treatments "BCA", "Larva", and "Ctrl" no significant differences

were detected (Fig. 4.3). These data clearly suggested that *B. brongniartii* may cause only minor and transient disturbances of soil fungal community structures.

A significant ($p < 0.05$) effect on fungal community structures was detected at day 91 in treatment "CCA". This was supported by cluster analyses (Fig. 4.3c), variation portioning (Table 4.3), and by the significant decline in soil DNA contents (Table 4.2). IndVal analysis revealed three OTUs, which were significantly correlated with the application of the CCA at day 42 as well as at day 91 (Table 4.4). Fungal species represented by these OTUs may have indirectly profited from the CCA-application, i.e. possibly from nutrients released form dead soil arthropods (Das and Mukherjee, 2000). However, OTUs may also be negatively correlated with a specific treatment, but the currently available version of the IndVal program does not allow to identify such possibly important negative correlations (Dufrene, 2004). For instance, Beatty and Sohn (1986) reported growth inhibition for five soil fungal species in the range from 6 to 106 ppm carbofuran, whereas 1 ppm did not significantly affect these fungi. Application of 47 ppm carbofuran in a field experiment, caused a reduction of isolated soil fungal taxa from 102 to 68 (Stanton *et al.*, 1981). In the current experiment 87.5 ppm carbofuran were applied to soil, thus observable effects were in agreement with the earlier reports. If the applied CCA had reduced growth of only certain fungal groups, the numbers of fungal OTUs in treatment "CCA" should be lower at the end of the experiment. However, mean numbers of scorable OTUs increased insignificantly from 43.5 to 47.2. Thus, the significant ($p < 0.05$) effect detected by fungal community profiling may mainly reflect a shift in fungal community structure.

Treatments containing dead larvae, i.e. "L+BCA" and "L+CCA" had significant effects on soil fungal communities at day 42 and 91 (Tables 3 and 4; Fig. 4.3). This was supported by both types of cluster analyses as well as by variation partitioning and two consistently indicative OTUs. Total numbers of scorable OTUs insignificantly increased from day 7 to day 91 by a mean of 5.9 OTUs in treatment "L+BCA" and 2.2 OTUs in treatment "L+CCA". The clear separation of these two treatments from the others is most likely due to the dead larvae present in the microcosms. However, by the end of the experiment, the treatments "L+CCA" and "L+BCA" developed a significant ($p < 0.01$) differentiation, which was supported by cluster analyses (Fig. 4.3) as well as four indicative OTUs for treatment "L+CCA" (Table 4.4). This indicated a stronger effect of treatment "L+CCA", which was in accordance with the stronger effect observed for treatment "CCA" as compared to treatment "BCA".

The aims of the here presented study were to develop and apply genetic diagnostics to quantify abundance of a *M. melolontha*-specific *B. brongniartii* BCA in soil and to assess potential effects it may have on soil fungal communities. Effects were compared to those of the broad spectrum CCA Curaterr. Molecular genetic and statistical tools were successfully used in this microcosm study for ranking effects of various treatments and for quantitatively monitoring the BCA. Although the CCA is less specific for controlling *M. melolontha* as compared to the fungal BCA, their effects on soil fungal communities in this microcosm study were comparable and very small. In natural ecosystems stochastic variation can be high and effects may quickly be compensated (Ritz, 2005). Therefore, it may be difficult to detect the here reported small effects on soil fungal communities of both control agents in a field study. Furthermore, effects on soil fungal communities in the field may be even smaller because maximum densities of the *B. brongniartii* BCA in field studies have been reported to be hundred times lower than detected in this microcosm study (Keller *et al.*, 2002) and because ten times lower quantities of the BCA and the CCA are applied in the field. If, however, specific effects of the *B. brongniartii* BCA on fungal communities were detected under field conditions, indicative OTUs may represent candidate indicators and may be taxonomically identified (2006a). Risk assessment programs for BCAs are currently adapted to better meet requirements for microbial BCAs (Mensink and Scheepmaker, 2007). As demonstrated in this study a combination of cultivation-independent monitoring and effect analyses may offer a feasible approach and offer support for risk assessment of microbial BCAs. The methodology for the *B. brongniartii* BCA may be adapted to other BCAs and may also be combined with complementary approaches to monitor soil quality (Winding *et al.*, 2005).

IV.6. Acknowledgments

Chapter IV

CHAPTER V

General Discussion

Chapter V. General Discussion

V.1. Monitoring and effect studies for a fungal BCA

This thesis was initiated to improve the level of knowledge in the field of environmental biosafety and was financed by the Swiss Federal Office for the Environment (FOEN, 2008). The general goal was to improve risk assessment of existing or new biological control agents (BCA), including both naturally occurring or genetically modified microorganisms. In this context, the objective of this thesis was to test and improve molecular genetic tools for monitoring of a fungal biocontrol agent and for assessment of BCA effects on soil fungal communities.

The *Beauveria brongniartii* biocontrol agent was chosen as a model system because it is a well studied example of an indigenous entomopathogenic fungus used as a BCA. *B. brongniartii* has been commercially applied for over 17 years without any known negative impact. Applied to soil, *B. brongniartii* resides at the area of application for several years (Enkerli *et al.,* 2004), resulting in long-term protection from economic losses through infestations by the European cockchafer, *Melolontha melolontha* (Keller, 2000). The *B. brongniartii* model system allowed to develop fast and reliable tools to monitor the *B. brongniartii* fungal BCA in soil and to analyze potential effects on soil fungal communities. The general conclusions from the conducted studies and their possible contributions to risk assessment of BCAs are discussed in this section.

V.2. Evaluation of methods used in this thesis

V.2.1. Soil DNA extraction used in cultivation-independent analyses

Cultivation-independent molecular genetic analyses are based on environmental DNA extracts. The DNA extraction protocol used (chapters II, III, IV) has been developed by Bürgmann *et al.* (2001) and has allowed for efficient and reproducible recovery of DNA from various soil types. Total quantities of extracted DNA were

shown to correlate with total soil microbial-carbon content (C_{mic}) determined by the chloroform fumigation extraction method (Hartmann *et al.*, 2006; Hartmann *et al.*, 2005). After the protocol has successfully been used for sensitive and reproducible analyses of both bacteria and archaea (Chen *et al.*, 2007; Hartmann *et al.*, 2006; Hartmann *et al.*, 2005; Pesaro *et al.*, 2004; Widmer *et al.*, 2006b) results of the current thesis showed its successful application also for studying fungi (chapters II, III, IV).

V.2.2. Profiling soil fungal communities analyzing ribosomal marker genes

Both molecular genetic profiling methods used in this thesis, namely T-RFLP (chapter II) and RISA (chapter IV), produced highly reproducible fungal community profiles. Dependent on the research question, either individual samples or pooled samples were analyzed. Profiles derived from individual soil samples were suitable to assess heterogeneity of fungal communities within a field (chapter II) or among replicated microcosms (chapter IV), while pooled samples were representative for either the field plot they were derived from (chapter II) or for corresponding treatments (chapter IV). Calculated means of individual profiles were shown suitable references to test representativity of community profiles derived from pooled soil samples for analyses of soil fungal (chapters II and IV) communities. This was well in accordance with findings from pools and means of soil bacterial communities (Hartmann *et al.*, 2006).

V.2.3. Cultivation-independent monitoring using SSR markers

Cultivation-independent analyses of simple sequence repeat (SSR) markers in soil DNA extracts have been shown suitable for genotyping (chapter III) and quantifying (chapter IV) *B. brongniartii*. This approach thus meets the regulatory requirement to identify a microorganism at the strain-level in a BCA product (European Union, 2005) and facilitates the registration of individual BCA-strains, as required by most regulatory boards (EPA, 2006; European Union, 2001; OECD, 2003; Swiss Federal Office for Agriculture, 2005).

Whether SSR-based monitoring can be used with BCAs other than *B. brongniartii* depends first on the availability of suitable SSR markers and second on the

specificity of these primers in soil DNA extracts. For a number of soil fungi, representing putative or registered BCAs, SSR markers are available. These are for example *M. anisopliae* (Enkerli *et al.*, 2005a), *B. bassiana* (Rehner and Buckley, 2003), *Paecilomyces fumosoroseus* (Dalleau-Clouet *et al.*, 2005) or *Ampelomyces quisqualis* (Harvey, 2006). However, except for the six SSR markers of *B. brongniartii* (see chapter III), no other reports on cultivation-independent analysis of SSRs in soil have been published so far.

V.2.4. Approaching the heterogeneity of soil fungal populations and communities

Heterogeneity of soil fungal populations and communities was observed in all three studies of this thesis. Samples derived from different locations within a field showed spatially differing fungal communities (chapter II) and a heterogeneous distribution of *B. brongniartii* populations (chapter III). Despite controlled conditions, soil fungal communities also differed among replicated microcosms (chapter IV). Under natural conditions, fungal heterogeneity can derive from factors such as competition among fungi (Cooke and Rayner, 1984a), patchy distribution of suitable substrates (Standing *et al.*, 2005), differences in the distribution of soil pores (Young and Ritz, 2005), or patches of different soil acidity (Bardgett *et al.*, 1993). At a larger scale, grazing schemes (Ritz *et al.*, 2004; Wardle, 2005) or agricultural management practices (Brodie *et al.*, 2003; Kennedy *et al.*, 2005b) can affect patterns of plant species distribution, which in turn influence fungal community structures (Brodie *et al.*, 2003; Kennedy *et al.*, 2005b; Ritz *et al.*, 2004; Wardle *et al.*, 2004). Fungi have been shown to be less abundant in intensively managed soils, where bacteria dominate and are typically homogeneously distributed (Drijber *et al.*, 2000; Zak, 1992). Under extensive management regimes fungi dominate and show high diversity, with a typical tendency to remain at a locality (Green *et al.*, 2004). This can result in a more heterogeneous distribution of fungal taxa compared to bacteria (Horner-Devine *et al.*, 2004). Differences in abundance and distribution between fungi and bacteria require specific attention when analyzing fungal communities using methods which were previously developed to analyze soil bacterial communities (Anderson and Cairney, 2004). With this regard, averaging samples of different characteristics can be essential to allow for comparisons of fungal community structures of samples derived

either from microcosm experiments (see chapter IV) or field experiments (see chapter II).

The heterogeneous distribution of fungi may also be viewed under the aspect of soil functions, their diversity as well as possible implications of averaging. There may be different reasons for the heterogeneities observed in the studies of this thesis: Spatial variability of fungal populations, e.g. different *B. brongniartii* genotypes found within a field plot (chapter III) may derive from mutual exclusion of different genotypes with identical physiological requirements (Setala *et al.*, 2005). Spatial variability of fungal communities, as observed in chapter II, may derive from an unequal distribution of fungal taxa with differing physiological requirements (Ettema and Wardle, 2002; Morris, 1999) and variability among fungal community structures of microcosm replicates (see chapter IV) may derive from stochastic differences in development of different fungal taxa initially present at the beginning of the incubation period. Different species within soil fungal communities may adapt to various conditions in soil microhabitats due to individual capacities to perform specific functions (Dighton *et al.*, 2001; Ponge, 2005; Sinsabaugh, 2005). Diversity of these functions has been considered as an indicator of soil quality as discussed in chapter IV (Hunt and Wall, 2002; Karlen *et al.*, 2003; O'Donnell *et al.*, 2001; Winding *et al.*, 2005). Reduction of soil microbial diversity could result in losses of functions and thus may represent a negative impact, i.e. a hazard, to soil quality (Griffiths *et al.*, 2004; Kennedy, 1999). Not every reduction of a particular soil microbial population causes a reduction of soil functionality. A possible damage may be compensated by other soil microorganisms performing the lost functions (Bradford *et al.*, 2002; Mikola *et al.*, 2002; Walker, 1992). Such functional redundancy has experimentally been demonstrated for fungal communities if their diversity was high (Setala and McLean, 2004; Tiunov and Scheu, 2005). Differences in fungal community structures as revealed in chapter II or IV in this thesis, may therefore not necessarily cause differences in soil functions. With regard to this aspect, averaging soil fungal communities can be essential for correct interpretations of effects on fungal community structures caused by an agricultural management factor such as the application of a fungal BCA (see chapter IV).

V.3. Contributions to a risk assessment in biocontrol

V.3.1. Analyses of exposure to microbial biocontrol agents

Risk analyses of a microbial BCA requires reliable and quantitative assessment of exposure of other organisms to the agent (EPA, 2007b; European Union, 2005; Swiss Federal Office for Agriculture, 2005). For *B. brongniartii* this requirement is met by a set of cultivation-dependent methods (Enkerli *et al.*, 2001; Strasser *et al.*, 1996).

Cultivation-independent monitoring of *B. brongniartii*, i.e. identification and quantification using specific SSR markers (chapters III and IV) was shown an attractive addition to cultivation-dependent methods previously established. Application of the cultivation-independent method in field soil samples allowed for rapid and reliable screening of the distribution of the introduced BCA strain as well as autochthonous *B. brongniartii* strains. As recently predicted, newly developed genetic tools can indeed facilitate accurate and efficient tracking of biocontrol agents in the field (Fravel, 2005). Cultivation-independent monitoring may later be used in long-term studies in order to assess dissemination of *B. brongniartii*, a problem formulated by Enkerli *et al.* (2005b) or to investigate population dynamics of the *M. melolontha* / *B. brongniartii* host pathogen interaction as proposed by Enkerli *et al.* (2004).

Cultivation-independent monitoring based on the analysis of SSR markers may also be considered in exposure studies with BCAs other than *B. brongniartii*. However, development and specificity testing of respective SSR markers requires considerable effort for each BCA individually.

V.3.2. Analyses of effects on non-target soil microorganisms

Molecular genetic analysis of a microbial community structure can be used to measure the non-target effects of a fungal BCA on soil fungi. The same approach has also been applied to assess possible effects of bacterial BCAs on bacterial communities (Sigler *et al.*, 2001; Thirup *et al.*, 2003; Timms-Wilson *et al.*, 2004). Analysis of microbial community structures based on the rRNA gene cluster is an explorative approach to assess possible changes in the composition of phylogenetic entities in a community. Effects on specific soil ecological functions would, however,

require in-dept studies on individual functions of specific organisms, i.e. indicators, affected by a BCA (Fig. 5.1).

Detectable effects on a fungal community composition may either result from direct interactions between the applied fungal BCA and the soil fungi of the community or from interactions between the BCA and soil organisms others than fungi which indirectly can influence soil fungal community structures. Direct interactions may derive from nutritive competition (Cooke and Rayner, 1984a; Laine and Nuorteva, 1970) or antagonisms (Tuininga, 2005) such as physical exclusion (Boddy, 2000), antibiotics (Ghisalberti and Sivasithamparum, 1991) or diffusible metabolites (Bruce *et al.*, 1984; Strasser *et al.*, 2000). In case of fungus-pathogenic fungi such as *Trichoderma sp.* applied as BCA, pathogenicity to non-target microorganisms may specifically have to be analyzed (Brimner and Boland, 2003). Indirect effects can develop if affected non-fungal organisms interact with the fungal community (Morris and Robertson, 2005). For example, if fungivores such as collembola or nematodes were affected, their grazing pressure on fungi may change (Bardgett *et al.*, 1993; Klironomos and Kendrick, 1995; Mulder *et al.*, 2003) .

In this thesis possible effects were assessed under controlled conditions and at BCA quantities ten times higher than used for field applications (chapter IV). Data obtained revealed suitability of both the microcosms as an experimental system and the analysis methods applied. In absence of the host, *M. melolontha*, the *B. brongniartii* BCA revealed a transient effect on the investigated fungal community structures (chapter IV). Comparable studies on non-target effects of bacterial BCAs using soil microcosms and molecular genetic profiling methods either found transient (Sigler *et al.*, 2001; Timms-Wilson *et al.*, 2004) or no effects (Bjorklof *et al.*, 2003; Thirup *et al.*, 2003) on either bacterial or fungal community structures.

Experiments under controlled conditions are more sensitive for detecting effects than field experiments (Winding *et al.*, 2004). Due to their limited size, microcosms essentially exclude higher trophic levels. Thus, microcosms limit the possibility for indirect effects of an agent, because many organismic interactions were excluded (Hajek and Goettel, 2007). Field experiments may therefore be carried-out with two goals: 1) to validate effects on indicator organisms (chapter IV), 2) assessment of effects not detectable under controlled conditions, 3) to discriminate between effects caused by the BCA and effects caused by natural variability within fungal

communities in the field. For this discrimination, the strategy to derive representative fungal community profiles from a field (chapter II) may be applied. Furthermore, long-term studies in the field were required to assess whether the effects of the BCA persist and whether they vary seasonally.

The results of molecular genetic profiling methods to analyze effects of a BCA are well in accordance with effects caused by other agronomical practices. For example, molecular genetic profiling has successfully been applied to analyze effects of crop rotation and farming systems (Hartmann *et al.*, 2006), transgenic potatoes (Rasche *et al.*, 2006) or transgenic bacteria (Timms-Wilson *et al.*, 2004) on soil bacterial community structures. Profiling was also shown effective to derive differences in soil fungal community structures caused by pasture fertilization (Clegg, 2006; Kennedy *et al.*, 2005a), pesticide application (Girvan *et al.*, 2004) or farming systems (Hagn *et al.*, 2003b). Depending on the kind of soil functions mediated by affected microorganisms, any effect on a microbial community potentially can cause harm to the environment. A generally applicable approach to risk assessment based on analyses of soil microbial communities is therefore of interest for the (re-) evaluation of many agronomical practices.

V.3.3. Risk characterization of microbial biocontrol agents

Risk characterization is a final step in risk assessment and may be used in decisions, for example to define tolerable exposure levels of an agent (EPA, 1998; European Union, 2000). Fundamentally based on exposure and effect analyses (Fig. 1.5), risk characterization requires the identification of those effects that are considered adverse (European Union, 2000), i.e. the assessment of specific traits which cause harm to the environment (EPA, 1998). For microbial BCAs several harmful traits have been formulated, such as their potential to infect bees, or toxicity to earthworms (EPA, 2007b; European Union, 2001; Swiss Federal Office for Agriculture, 2005). Deficits exist for the characterization of risks for microorganisms. It is currently not known, whether or which changes in microbial communities represent adverse, neutral or beneficial effects (Swift, 2005).

Based on exposure and effect analyses, the methods and strategies used in this thesis present a straightforward approach to assess whether more detailed studies were necessary in order to evaluate risks of a BCA to soil fungal communities. If no

significant effects are detected on fungal community structures, no risk can be assumed from the application of a BCA towards fungi. If effect studies revealed significant changes on fungal community structures, analyses of soil functions possibly affected by a BCA may be required. However, because most soil functions depend on the occurrence of specific organisms this requires improved understanding of the functional aspects of soil fungal communities (Robinson *et al.*, 2005; Stromberger, 2005).

The present thesis provides important contributions to the understanding of changes in fungal soil communities due to the application of an entomopathogenic BCA. This provides a framework for the risk assessment of a BCA on non-target microorganisms. Additional research is now required to identify indicator organisms associated with specific soil functions.

V.4. Perspectives

V.4.1. Further uses of cultivation-independent analyses using SSR markers

Cultivation-independent genotypic analyses using SSR markers may also have potential for other groups of fungi. The mycorrhizae, both arbuscular mycorrhizal fungi (AMF) as well as ectomycorrhizal fungi (ECM), are the most thoroughly studied fungal groups. This is possibly because of their important contributions to nutrient uptake of plants or their respective protection from pathogens (Leake *et al.*, 2005). Identification of mycorrhizal species often rely on ITS markers. These mostly allow for species-specific resolutions (Horton, 2002), but possible occurrence of multiple copies of the rRNA gene cluster in a nucleus (Hillis and Dixon, 1991; Tsuchiya and Taga, 2001) can result in individual mutations and overestimations of mycorrhizal diversity (Fatehi and Bridge, 1998). Genetic markers without sequence-specific primers, such as amplified fragment length polymorphism (AFLP) or inter simple sequence repeat (ISSR) profiles have also successfully been used for the analysis of mycorrhizae (Koch *et al.*, 2006; Vandenkoornhuyse *et al.*, 2001). However, all these techniques require cultivation and isolation of genomic DNA. SSR markers may be advantageous in these respects. They are typically found once per genome, i.e. each genotype produces a single fingerprint (Matula and Kypr, 1999; Tautz, 1989) and

may be applied within bulk soil DNA extracts (see chapter III of this thesis). A number of SSR markers have been developed so far for several mycorrhizal species (e.g.Croll *et al.*, 2008; Dunham *et al.*, 2003; Jany *et al.*, 2006; Jany *et al.*, 2003; e.g.Lefrancois *et al.*, 2003) and may potentially be applicable for cultivation-independent analyses. This would facilitate monitoring of mycorrhizal species in natural environments and would help to increase knowledge on the associations mycorrhizae undergo with other organisms in natural ecosystems (Cairney, 2005; Croll *et al.*, 2008).

V.4.2. Further research towards improved risk characterization of BCAs

Based on the methods developed and the findings of this thesis, Figure 5.1 summarizes points of further investigations possibly required to specifically characterize the risks involved with the application of a BCA to soil.

Strain-specific quantification

As registrations for BCAs are issued for specified strains (EPA, 2007b; European Union, 2005; Swiss Federal Office for Agriculture, 2005) strain-specific exposure analysis is required to ascribe possible environmental effects to a BCA. For the *B. brongniartii* BCA, cultivation-independent identification has been established (chapter IV). A species-specific quantification has been developed, based on the analysis of a single SSR marker (chapter IV). A strain-specific quantification is likely possible, when all six SSR markers were quantified in parallel. Whether this approach to exposure analysis may be applicable to other fungal BCAs depends on the availability and specificity of suitable SSR markers.

Effects under field conditions

The methods developed in this thesis will have to be used in field tests in order to assess possible effects of the *B. brongniartii* BCA under natural conditions. If effects were detectable, treatment-specific indicators could be determined as presented for the controlled system in chapter IV.

Indicators and validation

For any treatment-specific indicator a tool may be developed that allows its specific identification. Such identification tools may subsequently be used for the validation of expected effects after the application of a BCA at any new site. Identification of indicators would also allow for dose-response analyses as required by the European risk assessment procedure (European Union, 2000).

Analysis of functions mediated by indicators

If a BCA applied to a field causes significant effects on the soil fungal community structure, functions of affected soil fungi need to be known. Therefore, functions of validated indicators need to be assessed. This would allow to identify possible harm to the soil ecosystem and finally to characterize risks involved with the application of a BCA.
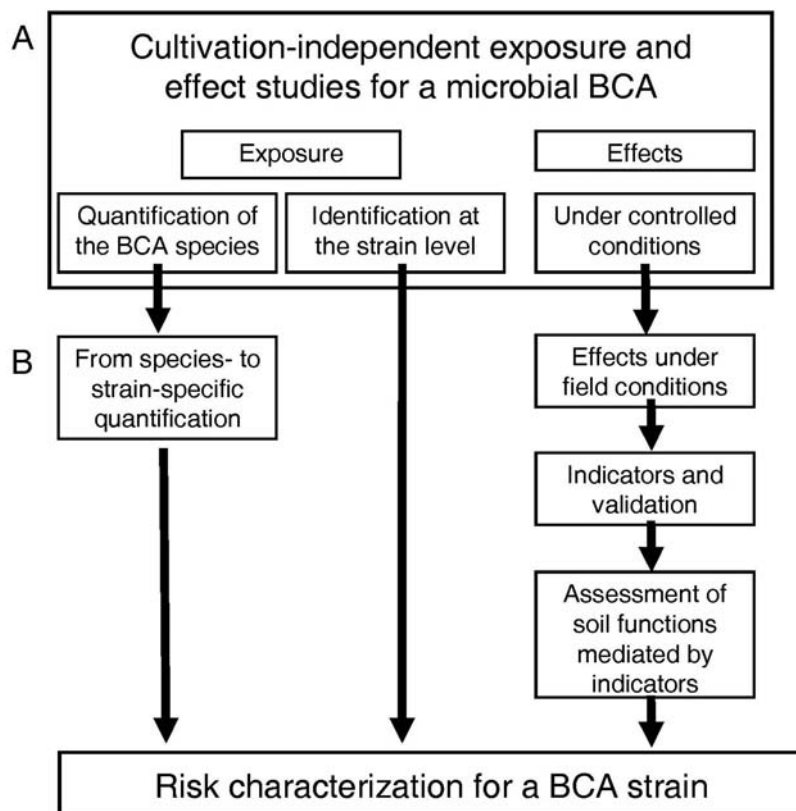

Fig. 5.1 Proposed scheme for the risk characterization of a BCA applied to soil, based on the analysis of soil microbial communities. The top box (A) summarizes the findings of the current thesis; boxes below (B) indicate further research needs.

V.5. Conclusions

The soil dwelling fungus *B. brongniartii* can be used to control the European Cockchafer, *M. melolontha* in grasslands. The results of this thesis show that this fungal biocontrol agent (BCA) has no significant effect on soil fungal community structures. In the contrary, application of a chemical control agent or presence of dead *M. melolontha* larvae can cause significant effects. Molecular genetic profiling methods were successfully used to sensitively and rapidly assess these effects. The same methods are also suitable to assess the spatial distribution of fungal communities within a field and to derive community profiles representative for an entire field. Moreover, molecular genetic identification tools allow for rapid cultivation-independent monitoring of *B. brongniartii* strains in the environment. This can be used for analyses of time and intensity other soil dwelling organisms may be exposed to a BCA strain. Together, effect- as well as exposure-analyses are basic elements of any environmental risk assessment. In this thesis we showed that the application of cultivation-independent exposure- and effect-analyses is suitable to improve risk assessment for BCAs applied to soil.

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Curriculum vitae

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Publications

Schwarzenbach K, Enkerli J, Widmer F (2007a) Objective criteria to assess representativity of soil fungal community profiles. *Journal of Microbiological Methods* **68**, 358-366.

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