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LEHRSTUHL FÜR BODENÖKOLOGIE

Microbial biocontrol of the pathogen *Phytophthora citricola* in the rhizosphere of European beech (*Fagus sylvatica* L.): Impact of elevated O_3 and CO_2 on the antagonistic community structure and function

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Abstract

The Oomycete *Phytophthora citricola* is a common root pathogen in central Europe. Among its many hosts are important tree species like European beech (*Fagus sylvatica*). In recent years it has been shown that beech trees grown under elevated CO_2 conditions are more susceptible to the pathogen, whereas O_3 treated trees showed reduced disease severity. However, the reasons for these observations are still unknown. Besides physiological responses within the plant, which might alter susceptibility, the possibility of changes in the microbial community of beech rhizospheres might be responsible for this phenomenon through an increase or decrease in the abundance of biocontrol active microbes in the rhizosphere.

This study aimed at investigating the composition of the antagonistic microbial community within the rhizosphere of European beeches. Based on an isolation approach microorganisms belonging to the actinobacterial genera *Kitasatospora* and *Streptomyces*, as well as the fungal genera *Trichoderma*, *Penicillium*, *Cylindrocarpon* and *Geomyces* were shown to have great antagonistic effects against *P. citricola in vitro*, thus having the potential to control the disease *in situ*. The mechanism of antagonism of the most common group of isolates, belonging to the genus *Kitasatospora*, was identified using a 12 Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR/MS). The bioactive substance had a molar mass of 281.17 g/mol and was further characterized and identified as the macrolide polyketide cycloheximide using ^{1}H-NMR.

On the basis of these findings a greenhouse experiment was performed to monitor possible changes in diversity of the actinobacterial community on a structural level and type II polyketide synthases responsible for antibiotics production on a functional level. Young beech trees were grown under elevated CO_2, elevated O_3 or ambient conditions for two years. Half of the pots were inoculated with *P. citricola* at the beginning of the second year. Rhizosphere soil was sampled in spring, summer and autumn of 2006. Terminal restriction fragment length polymorphism (t-RFLP) was used to monitor changes in diversity applying specific primers for actinobacterial 16S rRNA genes and the ketosynthase unit of polyketide synthases.

Interestingly, a clone library revealed a highly unique actinobacterial community with 41.1% of the sequences belonging to the only recently described suborder Catenulisporinae and 37.5% of the sequences being unclassified Actinobacteria. When analyzing the actinobacterial 16S rRNA t-RFLP profiles, no qualitative differences were found for neither season, CO_2 and O_3 treatments nor *P. citricola* inoculation. However, quantitative differences in single t-RFs were

observed for different seasons and the major t-RF responsible for this shift could be assigned to organisms corresponding to the mentioned suborder Catenulisporinae. A transient shift in peak height was observed for one major t-RF in O_3 treated summer samples.

On the functional level of type II polyketide synthases no qualitative or quantitative difference could be observed for neither season, CO_2 and O_3 treatments nor *P. citricola* inoculation as analyzed by t-RFLP analysis. A clone library revealed a high diversity of genes potentially responsible for the production of polyketides in soil.

Zusammenfassung

Der Oomyzet *Phytophthora citricola* ist ein häufiges Wurzelpathogen in Mitteleuropa. Zu den vielen Wirten dieses Krankheitserregers zählt auch eine der wichtigesten europäischen Baumarten, die Rotbuche (*Fagus sylvatica*). In den letzten Jahre konnte gezeigt werden, dass Buchen, die unter erhöhten CO_2-Bedingungen angezogen wurden, anfälliger gegenüber diesem Pathogen waren, während Buchen, die unter erhöhten Ozonbedingungen wuchsen, weniger stark ausgeprägte Krankheitssymptome aufwiesen. Die Gründe für diese Beobachtungen konnten bisher noch nicht vollständig geklärt werden. Neben physiologischen Reaktionen der Pflanze, die die Anfälligkeit direkt beeinflussen können, ist nicht auszuschliessen, dass Veränderungen der Zusammensetzung der mikrobiellen Gemeinschaft der Buchenrhizosphäre für dieses Phänomen mitverantwortlich sind. So können Abundanzen biokontroll aktiver Mikroorganismen durch die verschiedenen Behandlung ab- oder zunehmen.

Ziel dieser Untersuchungen war eine Analyse der Zusammensetzung antagonistischer, mikrobieller Gemeinschaften in der Rhizosphäre von Buchen. Anhand von Isolierungen konnte gezeigt werden, dass Organismen der aktinobakteriellen Gattungen *Kitasatospora* und *Streptomyces*, sowie der pilzlichen Gattungen *Trichoderma*, *Penicillium*, *Cylindrocarpon* und *Geomyces*, *in vitro* stark antagonistisch gegenüber *P. citricola* wirkten. Sie haben somit auch das Potential den Krankheitserreger *in situ* zu kontrollieren. Der Mechanismus des Antagonismus durch Isolate der Gattung *Kitasatospora* konnte mit Hilfe eines 12 Tesla Fourier Transform Ionenzyklotronresonanz Massenspektrometer (FT-ICR/MS) in Kombination mit ^{1}H-NMR aufgeklärt werden. Die biokontrollaktive Substanz hatte eine molare Masse von 281.17 g/mol und konnte mittels der NMR-Spektren als das Polyketid Cycloheximid identifiziert werden.

Basierend auf diesen Ergebnissen wurde ein Gewächshausexperiment durchgeführt, um den Einfluss biotischer und abiotischer Faktoren auf die strukturelle und funktionelle Diversität der antagonistischen, mikrobiellen Rhizosphärengemeinschaft zu untersuchen. Die gepflanzten zwei Jahre alten Buchen wuchsen für zwei Jahre bei entweder erhöhter CO_2- oder Ozonbegasung, sowie unter normalen Umgebungsbedingungen. Die Hälfte der Bäume wurde im zweiten Jahr zusätzlich mit *P. citricola* inokuliert. Der Rhizosphärenboden wurde anschliessend im Frühjahr, Sommer und Herbst des Jahres 2006 beprobt. Mittels Terminalem Restriktions Fragment Längen Polymorphismus (t-RFLP) wurde die Diversität aktinobakterieller 16S rRNA Gene und der Ketosynthaseeinheit von Polyketidsynthasen analysiert.

Interessanterweise konnte mit Hilfe einer Klonbank gezeigt werden, dass die unter-

suchte aktinobakterielle Rhizosphärengemeinschaft eine aussergewöhnliche Zusammensetzung aufwies. 41.1% der analysierten Klone wurden der erst kürzlich beschriebenen Unterordnung Catenulisporinae zugeordnet und 37.5% der Klone konnten nur auf der Ebene des Phylums Actinobacteria klassifiziert werden. Anhand der t-RFLP-Analysen zeigte sich kein qualitativer Einfluss der Jahreszeit, der CO_2- und Ozonbehandlung oder der *P. citricola* Inokulierung. Zum Teil konnten jedoch quantitative Unterschiede einzelner t-RFs für verschieden Jahreszeiten festgestellt werden. Das signalintensivste dieser Fragment konnte mit Hilfe der Klonbank der bereits erwähnten Unterordnung Catenulisporinae zugeordnet werden. Eine vorübergehende Veränderung der Signalintensität wurde ebenfalls für ein bedeutendes t-RF im Sommer bei Ozon behandelten Bäumen festgestellt.

Auf der funktionellen Ebene der Typ II Polyketidsynthasen konnte keine qualitative oder quantitative Veränderung durch die Jahreszeiten, die CO_2- und Ozonbehandlung oder die Inokulierung mit *P. citricola* gefunden werden. Eine Klonbank zeigte jedoch eine generell hohe Diversität dieser Gene in dem untersuchten Boden.

Chapter 1

Introduction

Pathogenic microorganisms affecting plant health are a major threat to many plants of economic importance as well as ecosystem stability worldwide. Due to complex interactions in dynamic environments, particularly the rhizosphere for soil borne diseases, control of these plant pathogens is often a great challenge. An increased use of chemicals in plant health management systems has been accompanied by public concerns about the impact of these substances on human and environmental health. The urge to find alternatives has increased rapidly throughout the last centuries. A promising approach is the application of disease suppressing microorganisms to soil or on seeds and planting materials.

Besides studying the introduction of such biocontrol active bacteria and fungi into soil to suppress deleterious microorganisms, the understanding of the ecology of antagonistic microbial communities has emerged as a field of great interest over the last years. Yet, still little is known about the ecology of microbial communities antagonistic to important root pathogens of forest trees, such as the oomycete *Phytophthora citricola*.

1.1 *Phytophthora citricola* as causal agent of root rot on European beeches (*Fagus sylvatica*)

The genus *Phytophthora* (after the Greek for "plant destroyer") is well known to include many species of aggressive pathogens on different plants including many economically and ecologically important species. It belongs to the Order Pythiales within the Phylum Oomycota, which is assigned to the Kingdom Chromista consisting of a group of heterokont, biflagellate organisms (Cooke *et al.*, 2000). The best known example of a devastating disease originating from the genus *Phytophthora* is probably *P. infestans*, the causal agent of late blight of potato. This

disease is infamous for being the cause of the Great Irish famine during the late 1840s which lead to the deaths of over a million people due to starvation (Erwin & Ribeiro, 1996). Other *Phytophthora* spp. are known to be root pathogens of many herbaceous and woody plants including deciduous trees, such as *Quercus* spp. (Jung *et al.*, 2000), *Alnus* spp. (Jung & Blaschke, 2004) and *Fagus* spp. (Jung *et al.*, 2005).

Symptoms of the diseases include root and colar rot often observed as stained, water-soaked lesions. Above ground symptoms can be seen as well, manifesting as chlorotic and wilting leaves, dieback of branches, and bleeding trunk cankers. These symptoms are preceded by changes in physiology of the infected plants. Most commonly a decrease in CO_2 assimilation, transpiration and stomatal conductance are observed before the manifestation of visible symptoms (Oßwald *et al.*, 2004; Fleischmann *et al.*, 2005).

European beech (*Fagus sylvatica* L.) is a host to many different *Phytophthora* species. *F. sylvatica* is of great economical importance since it is the most frequently planted deciduous tree species in central European forests (Jung *et al.*, 2005). *Phytophthora citricola* Sawada, *P. cambivora* (Petri) Buisman and *P. cactorum* (Lebert and Cohn) Schroeter were the most commonly isolated species of the genus in a survey conducted by Jung *et al.* (2005) in 91 forest stands in Bavaria, Germany. Most isolates belonged to the species *P. citricola*, which was present in 56 of the 91 stands. This pathogen has been proven to be a very aggressive pathogen of *F. sylvatica* in many studies (Werres, 1995; Fleischmann *et al.*, 2002b, 2004). It has a broad host range and is also known to cause root rot and trunk cankers on species of the genera *Pinus*, *Acer*, *Aesculus* and *Quercus* (Erwin & Ribeiro, 1996). Of particular concern is the fact that the pathogen has not only been isolated from declining mature trees in the field but also from nurseries, giving it the possibility of quickly spreading over vast areas (Jung *et al.*, 2005). *P. citricola*, like most *Phytophthora* species, is a typical primary invader with limited saprophytic ability (Cooke *et al.*, 2000). It leads to a succession of secondary invasions by organisms including opportunistic fungal pathogens like *Armillaria* spp., *Fomes* spp., *Inonotus* spp., *Hypoxylon* spp., and *Nectria* spp., as well as bark and wood boring insects (Jung *et al.*, 2005).

Management strategies to prevent further spread of *Phytophthora* diseases on *F. sylvatica* have not been put into practice so far. Most means of control, like application of fungicides or organic mulch, are not likely to be feasible in forest stands due to economical and ecological reasons (Jung *et al.*, 2005).

1.2 Biological control of microbial plant pathogens

Controlling soil-borne plant disease has always been a major challenge in agriculture and forestry. Soil itself has long been viewed simply as a substrate for plant growth, providing support for plant roots and a reservoir of essential nutrients necessary for plant growth. Ecological interactions within soils were largely neglected and plant diseases were controlled by the application of chemicals. The most widely used method in the control of soil borne diseases has been the fumigation with methyl bromide, a highly toxic broad spectrum chemical. This and other often used products are not specific and therefore destroy the whole microflora in soil, whether pathogenic or not (Janvier *et al.*, 2007). Methyl bromide has been banned since 2005 (Montreal protocol) and its use is currently phasing out. On the other hand, when applying more specific chemical compounds to control a certain pathogen in order to minimize the effect on non-target organisms, the probability of genetic shifts increases in the population of the pathogen, leading to resistance against the substance. The need to replace or at least supplement chemicals with other methods in integrated management systems is therefore widely recognized and many different approaches are tested.

1.2.1 Biocontrol active organisms

Besides cultural practices to improve soil health, like crop rotation and limiting tillage in agricultural systems, the application of antagonistic microorganisms as biological control agents (BCA) has become of major interest (Whipps, 2001; Haas & Defago, 2005). Antagonistic microorganisms belong to a broad variety of different bacterial and fungal phylogenetic groups.

Among the most commonly isolated and best characterized bacterial antagonists are strains belonging to the fluorescent pseudomonads, a group of rod-shaped Gram-negative bacteria within the γ-subclass of Proteobacteria (Haas & Defago, 2005; Weller *et al.*, 2007). Due to their short generation time, pseudomonads are well adapted to competition in habitats with large amounts of easily degradable compounds such as low molecular carbohydrates and amino acids, as found in the proximity of plant roots in soil. In many cases, researchers focus on the genus *Pseudomonas* as potential antagonists against root pathogens in the rhizosphere due to their fast growth on selective media and their known ecological significance, but other groups are equally important if not as easily isolated. Actinobacteria belong to the Gram-positive bacteria with a high G + C content in their DNA, which are usually able to form branching hyphae at some

stage of their development (Goodfellow & Williams, 1983; Lee & Hwang, 2002; Kämpfer, 2006). Besides their role as bacterial antagonists against several plant diseases, they are known to be soil engineers, who can degrade complex recalcitrant material, often polymeric residues, and are therefore believed to play an important role in decomposition processes in soil (Kämpfer, 2006). Even though their dependency on plant root exudates is likely to be less pronounced than that of pseudomonads, their involvement in root turnover especially for perennial plants has been emphasized. In many studies, their importance in terms of abundance and activity in soil has been demonstrated (Smalla *et al.*, 2001; Gremion *et al.*, 2003; Billings & Ziegler, 2005). Other known bacterial BCAs include strains of *Bacillus* (Milner *et al.*, 1996), *Burkholderia* (Heungens & Parke, 2001), *Serratia* (Okamoto *et al.*, 1998) and *Stenotrophomonas* (Dunne *et al.*, 2000).

The most commonly isolated fungal antagonists belong to the genus *Trichoderma* of the imperfect fungi. In the few cases were sexual stages were identified they were placed within the Ascomycetes in the genus *Hypocrea* (Monte, 2001). In 2004, about 90% of all antagonists used for biocontrol in practice belonged to *Trichoderma* spp. (Benitez *et al.*, 2004), making this group a very interesting target for further investigation. Like the Actinobacteria, they are important for the turnover of organic materials in soils, due to their vast arsenal of lytic enzymes (Viterbo *et al.*, 2002; Harman *et al.*, 2004). Other eukaryotic antagonists include members of the genera *Penicillium* (Berg *et al.*, 2005), *Aspergillus* (Kang & Kim, 2004), non-pathogenic members of known phytopathogenic genera like *Fusarium* (Olivain *et al.*, 2003), *Rhizoctonia* (Cartwright & Spurr jr., 1998) or the oomycetous genus *Pythium* (Picard *et al.*, 2000).

1.2.2 Mechanisms of biological control

Antibiosis by antibiotics production

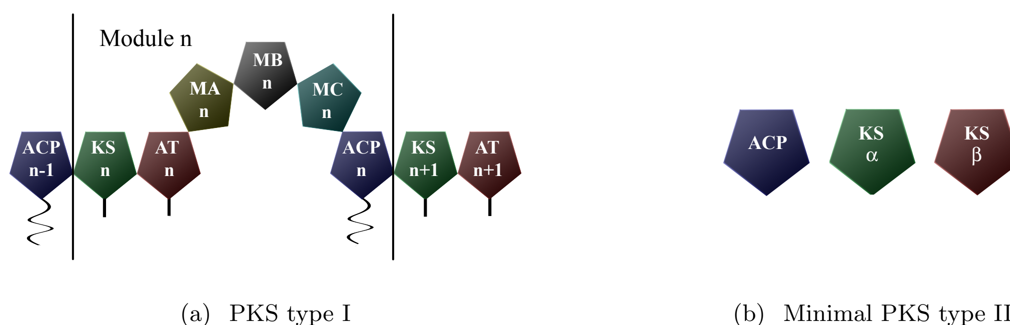
BCAs are able to control diseases by different antagonistic traits. A very common mechanism is antibiosis via the production of antibiotics (Raaijmakers *et al.*, 2002). These substances are a chemically heterogenous group of organic, low-molecular compounds produced by many different microorganisms. They are deleterious to the metabolism and growth of other microorganisms at very low concentrations and are therefore believed to provide the producer with an advantage over its competitors (Fravel, 1988). A variety of antibiotics have been identified, including compounds like 2,4-diacetylphloroglucinol (DAPG), phenazines and cyclic lipopeptides, produced by pseudomonads (Haas & Defago, 2005; Weller *et al.*, 2007), zwittermicin A, kanosamine, munumbicin and platensimycin produced by *Bacillus* spp. and *Streptomyces* spp.

(Silo-Suh *et al.*, 1994; Milner *et al.*, 1996; Castillo *et al.*, 2002; Wang *et al.*, 2006) and gliotoxin and gliovirin produced by *Trichoderma* spp. (Howell, 2003).

Polyketide synthases: a major family of antibiotic producing enzyme complexes Antimicrobials can be produced via many different pathways of the secondary metabolism. As demonstrated later on in this thesis, macrolide antibiotics have the potential to be effective in inhibiting the growth of plant pathogenic microorganisms. This group of antibiotics is produced by polyketide synthases (PKS). Thus, a brief introduction into the organization of the two major PKS classes will be given.

The general biosynthetic pathway for all polyketides is similar. Monomers (e. g. malonyl-CoA and methyl malonyl-CoA) are sequentially condensed by keto-synthases (KS) within the PKS complex to form acyl chains. The process is closely related to fatty acid synthesis, yet a high structural diversity is produced because of different starter unit incorporation, oxidations, cyclisations, and many further modifications (Staunton & Weissman, 2001).

Polyketide synthases exist as large multifunctional proteins (type I) or as dissociable enzymes (type II). In type I PKS systems (modular PKS) multiple domains which carry a series of functional sites produce polyketides in a stepwise fashion. For each module, all functional domains necessary for an elongation of the molecule have to be present and the product is modified and elongated as it moves along this "assembly line" (Fig. 1.1a). These type I PKS systems are very complex and variable. They are commonly found in Actinobacteria, Myxobacteria, Pseudomon-



(a) PKS type I

(b) Minimal PKS type II

Figure 1.1: Schematic illustration of the two major polyketide synthase groups. (a) Modular PKS type I. Detail of one module (n) consisting of all necessary protein domains required for the elongation of the polyketide by one monomer. Polyketides are manufactured in an assembly line fashion by the ketosynthase (KS), acyltransferase (AT) and acyl-carrier-protein (ACP) domains, and further modified by specific enzyme domains (MA-C). (b) Iterative PKS type II. Minimal configuration consists of only three diffusible enzymes: ketosynthase (KS_α), chain length factor (KS_β) and acyl-carrier-protein (ACP), which are reused for each elongation step. Additional enzymes are usually present to specifically modify the polyketide.

ads and Cyanobacteria and are known to produce compounds belonging to the macrolide or polyene antibiotic classes (Jenke-Kodama *et al.*, 2005). Examples belonging to these groups are the antibiotics erythromycin, cycloheximide and monensin A (O'Hagan, 1995).

Type II PKS are organized in a much simpler way, usually consisting of monofunctional enzymes which act together in a multienzyme complex. All type II PKS share a so called minimal PKS which consists of the keto-synthase (KS_α), a chain length factor (KS_β) and an acyl-carrier protein (ACP, Fig. 1.1b). Although this minimal PKS is sufficient to produce a basic carbon skeleton of the polyketide chain, additional enzymes, such as cyclases, aromatases or ketoreductases are required to bring the polyketide into its final folded polycyclic, aromatic structure (Seow *et al.*, 1997). Among the produced antibiotics and anti cancer drugs are tetracyclines, anthracyclines, aureolic acids and many more. These PKS systems have been mainly described for Actinobacteria (Hertweck *et al.*, 2007), but very recently first examples for Gram-negative bacteria were published as well (Brachmann *et al.*, 2007).

Antibiosis by lytic enzyme production and hyperparasitism

The production of lytic enzymes is a second important characteristic of BCAs and their effectiveness has been demonstrated in many studies. The produced enzymes include chitinases, glucanases and proteases, all involved in the cell wall degradation of various organisms (e. g. as reviewed for *Trichoderma* spp. by Viterbo *et al.*, 2002). In the case of oomycetous pathogens, glucanases often seem to be involved in the antagonism since their cell walls contain β-(1,3)-(1,6)-glucans and cellulose rather than chitin as major structural components (Erwin & Ribeiro, 1996; Viterbo *et al.*, 2002). De la Cruz *et al.* (1995) demonstrated the activity of a β-1,3-glucanase produced by a biocontrol active *T. harzianum* strain on cell walls of *Phytophthora syringae* and different true fungi. Also, serine proteases produced by *Stenotrophomonas maltophilia* have been identified to improve biocontrol against *Pythium ultimum* when overexpressed (Dunne *et al.*, 2000). Extracellular enzyme production can be connected either to general antibiosis or hyperparasitism, the direct attack of an organism on another as often observed for many *Trichoderma* species and certain genera of Actinobacteria (El-Tarabily *et al.*, 1997; Benitez *et al.*, 2004).

Siderophore production and degradation of virulence factors

Most organisms require iron as an essential element in different metabolic pathways (Miethke & Marahiel, 2007). Thus efficient acquisition of iron from the habitat can be an important compe-

tition advantage for microorganisms. Due to the scarcity of bioavailable iron in most soil environments, many organisms produce low molecular weight molecules, known as siderophores, to improve their competitiveness for ferric iron (Whipps, 2001). The production of these molecules strongly dependents on environmental factors including pH, the amount of iron and the form of iron ions present, and adequate supply of carbon, nitrogen and phosphorous (Duffy & Defago, 1999).

Detoxification and degradation of virulence factors of a pathogen is another antagonistic trait by which diseases might be suppressed in soil. For example, the phytotoxin fusaric acid produced by various *Fusarium* species can be detoxified by different bacterial strains, e. g. avirulent *Ralstonia solanacearum* (Toyoda *et al.*, 1988). On the other hand, it should be remembered that pathogens are often capable of defending themselves against microbial antagonism via similar mechanisms (Duffy *et al.*, 2003).

Plant growth promotion and induced systemic resistence

Influences of biocontrol active microorganisms on the development of a disease can also be obtained through plant mediated effects caused by the BCAs. Plant growth has been shown to be stimulated by many BCAs including pseudomonads and *Trichoderma* spp. (Whipps, 2001; Harman *et al.*, 2004). Wulff *et al.* (1998) found that isolates of *Pythium oligandrum* can provide improved plant growth to cucumber. Segarra *et al.* (2007) demonstrated that *Trichoderma asperellum* strain T34 increases growth of the same plant. Besides this plant growth promotion effect, Segarra *et al.* (2007) also observed an enhanced resistance to biotic and abiotic stress, which correlated with a change in the plants proteome. This phenomenon, known as induced systemic resistence (ISR), is closely related to systemic aquired resistence (SAR). SAR is the activation of defence mechanisms developed by plants in response to a pathogen attack (Van Loon *et al.*, 1998) via salicylic acid mediated signaling pathways. In contrast, ISR is often associated with jasmonate and ethylene signals (Pieterse & van Loon, 1999). It has first been described for plant growth promoting pseudomonads by Van Peer *et al.* (1991).

Synergism

In many cases antagonism is not based on one of the above mentioned mechanisms alone but a combination of several. Synergisms have been described among lytic enzymes and between enzymes and antibiotics (Howell, 2003; Benitez *et al.*, 2004). Harman *et al.* (2004) highlighted

the role of ISR induced by *Trichoderma* spp. in several plant species in combination with other biocontrol traits. These interactions of antagonistic characteristics of one or several organisms increase the efficiency of the BCAs and reduces the risk of possible resistances of the target pathogen to one mechanism.

However, one trait is of great importance to all biocontrol strain: the ability to be competitive in colonizing the root surface and its close vicinity. Only organisms possessing rhizosphere competence will be effective in controlling deleterious microorganism (Whipps, 2001; Compant *et al.*, 2005).

1.3 Elucidating mechanisms of microbial antagonism

When working with antagonistic microorganisms the identification of mechanisms is often a major challenge. In fact, most studies conducted to investigate the ecology of antagonistic populations ignore the mechanisms involved in biocontrol (e. g. Sid Ahmed *et al.*, 1999; Lee & Hwang, 2002; Wiggins & Kinkel, 2005). Hence, the informations obtained from those studies are often based on *in vitro* or *in vivo* inhibition tests against the target pathogen and underlying principles remain obscure. By doing so, researchers are ignoring a major advantage of culture dependent investigations over culture independent approaches: the possibility to identify possible functions of organisms in a habitat. Nichols (2007) recently stated that only cultivation of environmental isolates gives microbial ecologists a context in which to investigate theoretical molecular findings.

The identification of lytic enzyme production can often be conducted in straight forward approaches by applying enzyme activity assays. These assays have been regularly applied to identify enzymes like chitinases (Lorito *et al.*, 1993; Schirmböck *et al.*, 1994), glucanases (De la Cruz *et al.*, 1995; Kubicek *et al.*, 1996; Sanz *et al.*, 2005), and proteases (Dunne *et al.*, 2000; Liu & Yang, 2007) in relation to biocontrol activities of different microbial antagonists. After elucidating the function of the enzyme, further analyses including purification and identification of the protein can be performed. Subsequent analyses like northern or western blotting are of interest for studying the regulation of the protein production *in vitro* (De la Cruz *et al.*, 1995; Sanz *et al.*, 2005; Liu & Yang, 2007).

While enzyme tests are clear indications that the substance of interest is of protein origin, biochemical characterization of antibiotics is generally more complex. In order to identify these metabolites from biological samples, separation procedures usually need to be applied. These

separation steps are necessary when low-resolving mass analyzers are used (Dettmer *et al.*, 2007) and include high performance or ultra performance liquid chromatography (HPLC and UPLC), gas chromatography (GC) for thermally stable analytes, and capillary electrophoresis (CE) for the separation of charged metabolites. These separations result in a better detection limit and increased mass spectrometry (MS) data quality due to reduced background noise (Oldiges *et al.*, 2007).

An alternative approach is the direct injection of a samples into the ionization source of the mass spectrometer without prior chromatographic separation. In this case, high resolution mass spectrometers are required in order to distinguish between isobars (compounds with the same nominal mass, Dettmer *et al.*, 2007). This technique has been applied using time of flight (TOF) MS and Fourier transform ion cyclotron (FT-ICR) MS. Of the two, FT-ICR/MS offers the highest resolution (>1 500 000 at mass 600) and also the highest currently available mass accuracy (<100 p.p.b., Brown *et al.*, 2005; Rosselló-Mora *et al.*, 2008). The accurate mass determination provided by FT-ICR/MS makes it possible in many cases to identify molecules on the basis of their masses alone even against a background of other ions, without resorting to chromatography (Brown *et al.*, 2005). In cases where the analyte is unknown, accurate mass measurements allow the unambiguous assignment of a molecular formula, at least for metabolites up to a molecular weight of ∼500 Da (Brown *et al.*, 2005). Simple fractionation procedures can be applied to reduce the number of possible candidates for biocontrol activity. This helps to assign the biocontrol activity of the organism to a certain substance produced. To confirm structural characteristics, nuclear magnetic resonance should be utilized, when sample amounts are sufficient.

1.4 Analyzing disease suppression in soil

The suppressiveness of a soil has been defined as its ability to keep disease severity or incidence at a low level, in spite of the presence of a pathogen, a susceptible host and climate conditions favorable for disease development (Baker & Cook, 1974). There are two main conditions recognized which are thought to be responsible for controlling diseases separately or in combination. General suppression is directly linked to the total amount of microbiological activity at a time critical to the pathogen. Not a single microorganism or specific group of microorganisms is responsible by itself for general suppression (Janvier *et al.*, 2007). Specific suppression acts against a background of general suppression but is more qualitative, owing to more specific effects of

individual or selected groups of organisms antagonistic to the pathogen (Janvier *et al.*, 2007). Different methods have been applied to measure possible indicators of these two mechanisms.

1.4.1 Quantitative methods to analyze microbial communities

CFU counts are probably among the most classical approaches to estimate soil microbial populations. A drawback of these methods is the well known bias that the majority of organisms is not readily cultivated by any single isolation approach (Torsvik *et al.*, 1990). Nevertheless, CFU counts are still widely applied and produce very useful information. Increases in bacterial densities for example were associated with increased suppression against the pathogens *Sclerotium rolfsii* on pressing tomatoes (Bulluck III. & Ristaino, 2002) and Phytophthora root rot on alfalfa as well as potato scab (Wiggins & Kinkel, 2005). Yet adverse effects were also observed (Oyarzun, 1998; Benizri *et al.*, 2005).

The possibility to use specific media to monitor microbial groups of interest is also commonly used. Among the most commonly enumerated groups in relation to disease suppression in soils are fluorescent pseudomonads and Actinobacteria (Janvier *et al.*, 2007). Yet, for both groups densities were not consistently associated with disease incidences. The observed variation in microbial densities is likely to depend on the pathosystem and the soil in which the disease occurs.

Soil microbial biomass and activity has been used as bioindicators for suppressive soils. These parameter can be assessed by different methods, among the most commonly used for microbial biomass is the chloroform fumigation-extraction method (Vance *et al.*, 1987). Others like the microwave-extraction method (Islam & Weil, 1998) are also applied. Respiration is a commonly used methods to measure overall microbial activity and it can be estimated either with or without substrate induction (Anderson & Domsch, 1978). As these approaches do not distinguish among microbial groups, they reflect the sum of the responses of different microorganisms present in a soil sample and are therefore closely related to general suppressiveness against soil-borne diseases. A large microbial biomass is expected to create a competitive environment deleterious for the pathogen. Leon *et al.* (2006) found a significant negative correlation between microbial biomass and disease severity of the oomycete *Aphanomyces euteiches* on snap bean. Even though similar effects are often observed, these relationships are not consistent for all pathogens. When studying the effect of cover-crop incorporation in both organic and conventional farming systems on soil suppressiveness to *Pythium aphanidermatum*, Grünwald *et al.* (2000) found no relation

between soil microbial biomass and disease incidence.

1.4.2 Investigating structural diversity of microbial communities

The application of specific isolation protocols for microorganisms has long been the only means to analyze species diversity in soil. Diversity should be a more sensitive indicator for the suppressiveness of a soil than general parameters like microbial biomass and respiration, since it is closely connected to specific disease suppression. In recent years, great advances were made in developing culture independent methods in order to assess a larger and more diverse part of the soil microflora. These techniques are based on several direct extraction procedures for different marker molecules, e. g. nucleic acids or lipids. Morgan & Winstanley (1997) defined biomarkers as any biological component used to indicate a useful feature of a certain organism.

Phospholipid fatty acid (PLFA) analysis is commonly used to overcome the problems of culture based approaches for assessing the total microbial diversity. Many fatty acids have been described to be biomarkers representative for specific microbial groups. Thus, the analyses of PLFAs by gas-liquid chromatography coupled to mass spectrometry provide qualitative and quantitative data about different microbial groups within the communities (Zelles, 1999; Esperschütz *et al.*, 2007). An additional advantage of PLFA analyses is that fatty acids are quickly degraded in soil and therefore the total amount of PLFAs can be used as an indicator of the viable biomass in the habitat (White *et al.*, 1979).

Nucleic acid based methods have proven to be powerful tools in assessing the composition of microbial communities. While analyses relying on DNA extractions of communities should be interpreted as the potential of a community, rRNA or mRNA based approaches should reflect the active part of this community at a certain time point. Genes coding for rRNA are still the primary target for investigations. They are assumed to be good biomarkers for studies of microbial structural diversity, since they are found in all living organisms due to their vital role in protein biosynthesis. Additionally, changes in rRNA gene sequences can be correlated to the evolutionary relationship between organisms (Woese, 1987) thus making it possible to design specific primers or probes for different taxonomic levels (Kämpfer *et al.*, 1996).

Up to today, large collections of rRNA gene sequences have been deposited to publicly accessible databases (Cole *et al.*, 2005). For estimating the rRNA gene diversity of a sample, different methods have been developed throughout the last years. The most straight forward approach is the sequencing of clone libraries obtained from polymerase chain reaction (PCR)

amplifications of environmental DNA. This method is very laborious and time consuming and it can therefore not be performed for many samples in parallel. Recent advances in sequencing technology however have drastically increased the number of sequences that can be analyzed. Roesch *et al.* (2007) were able to amplify 149 000 rRNA gene sequences from three agricultural and one forest soil using a 454 pyrosequencer. This number is more than an order of magnitude higher than numbers obtained in comparable previous studies, giving detailed informations on the composition of the microbial communities in these soils. At the moment this technique is still rarely used due to the high costs of the required machines, but it is to be expected that its use will increase in the future.

Meanwhile, fingerprinting techniques remain the methods of choice for characterizing soil microbial communities. Approaches like terminal restriction length polymorphism (t-RFLP; Liu *et al.*, 1997), denaturing gradient gel electrophoresis (DGGE; Muyzer *et al.*, 1993) or single strand conformation polymorphism (SSCP; Schwieger & Tebbe, 1998) give complex molecular fingerprints of the microbial communities based on PCR amplification. In the case of t-RFLP, fingerprinting is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products. The digested products are mixed with a DNA size standard and fragments are separated by capillary electrophoresis. The end-labeled fragments can then be detected using laser detection analyzers. Due to sequence variations between different taxa end-labeled fragments of different sizes are produced, each size representing an operational taxonomic unit (OTU) (Liu *et al.*, 1997). While each of the mentioned fingerprinting techniques has advantages and drawbacks, Smalla *et al.* (2007) demonstrated that all analyses lead to similar results when directly compared to each other. Additionally, concerning the t-RFLP technique, several studies could show that both sizes and relative signal intensities of individual t-RFs of a samples are reproducible and thus t-RFLP is an excellent method to rapidly compare microbial communities (Osborn *et al.*, 2000; Smalla *et al.*, 2007).

Fingerprinting techniques can be used to analyze a high amount of different samples in relatively little time. The differences in the obtained profiles can than be visualized by multivariate statistical analyses like principal component analysis (PCA) or the distribution independent non-metric multidimensional scaling (NMS). Subsequently OTUs responsible for the separation can be identified (Ramette, 2007).

1.4.3 Assessing functional diversity of microbial communities

In addition to analyzing structural diversity based on rRNA gene sequence variation, functional genes can also be used for monitoring microbial soil communities. While phylogeny and expression of phenotypic traits are often closely connected (Berg, 2000; Oda *et al.*, 2003), a lack of correspondence has also been demonstrated for a variety of soil organisms including antibiotics producing streptomycetes and pseudomonads (Lottmann & Berg, 2001; Metsä-Ketelä *et al.*, 2002; Davelos *et al.*, 2004a; Davelos Baines *et al.*, 2007). Local selection pressure can lead to a diversification within a taxon, while horizontal gene transfer may result in the distribution of specific functional genes between distantly related taxa. Horizontal gene transfer is a well documented phenomenon for antibiotic resistance and biosynthesis genes amongst streptomycetes (Wiener *et al.*, 1998; Egan *et al.*, 2001). Therefore, a targeted analysis of responsible genes in soil is likely to give a more accurate picture of the potential disease suppressiveness by this mechanism.

Fingerprinting techniques like DGGE and t-RFLP have been applied to functional genes related to antibiotics production and disease suppression. Bergsma-Vlami *et al.* (2005) could differentiate seven *phlD* genes responsible for the production of DAPG in the rhizosphere of four different plant species using DGGE, with some genotypes being exclusive to the rhizosphere of specific plants. Costa *et al.* (2007) developed a DGGE based system to analyze the diversity of the global response regulator gene *gacA* required for the production of many secondary metabolites and exoenzymes in plant-associated *Pseudomonas* species. Furthermore, to assess the antibiotics production potential of Actinobacteria in soil, Wawrik *et al.* (2005) designed primers specific for the keto-synthase unit (KS_α) of type II polyketide synthases and utilized t-RFLP analysis to obtain fingerprints of PKS type II diversity in different soil habitats.

1.5 Effects of climate relevant trace gases on plant-soil systems

In 1904, the term rhizosphere was coined by the German scientist Lorenz Hiltner, who defined it as the “soil compartment influenced by the root” (Hiltner, 1904). The rhizosphere is a densely populated area in which soil-borne microorganisms feed on the organic material released from the roots (Ryan *et al.*, 2001). It is estimated that between 5 and 50% of the carbon fixed by plants can be allocated through the roots into the rhizoshpere (Lynch & Whipps, 1991; Marschner, 1995; Kuzyakov & Domanski, 2000). Living roots deposite carbon as exudates, low molecular compounds like amino acids, sugars, organic acids, phenolics and various other secondary metabolites or as high molecular compounds like mucilage or proteins (Neumann & Röhmheld, 2001; Walker *et al.*, 2003). Additional input into the rhizosphere comes from sloughed of root cells and root turn-over (Darrah, 1996).

The quantity and quality of substances released into the rhizosphere by the plant are known to influence the structure and function of soil-borne microbial communities (Darrah, 1996; Hodge *et al.*, 1998). Major factors affecting the composition of these communities are plant species (Smalla *et al.*, 2001; Garbeva *et al.*, 2004; Dohrmann & Tebbe, 2005; Drigo *et al.*, 2007), growth stages of the plants (Baudoin *et al.*, 2002), seasonal shifts (Smalla *et al.*, 2001) and plant nutrition (Yang & Crowley, 2000).

In recent years it has also been demonstrated that elevation of CO_2 and O_3 can alter carbon allocation within plants (Andersen, 2003; Matyssek *et al.*, 2005) leading to changes in rhizodeposition (Andersen, 2003; De Graaff *et al.*, 2006). This is of particular interest since concentrations of CO_2 and O_3 in the Earth’s troposphere have been steadily increasing since the beginning of the industrial revolution. The global atmospheric concentration of carbon dioxide has risen from a pre-industrial value of about 280 ppm to 379 ppm in 2005 and increases are expected to remain at high levels (IPCC, 2007). Studies on background ozone concentrations in the mid-latitude northern hemisphere have suggested an increase of 0.5-2% per year (Vingarzan, 2004).

Elevated CO_2 directly affects ecosystems by increasing carbon fixation through enhanced photosynthesis and thus stimulating plant growth (Ainsworth & Long, 2005). This in turn leads to an increased allocation of resources to the roots and higher amounts of C input into the soil through rhizodeposition (De Graaff *et al.*, 2006). Additionally, Phillips *et al.* (2006) observed that elevated CO_2 had the potential to qualitatively alter root exudation. They demonstrated that efflux rates of amino acids significantly increased for maize (*Zea mays* L.). The effect was shown for six out of 16 specific amino acids and was species specific, since it could not be

demonstrated for rye gras (*Lolium multiflorum* L.) and medic (*Medicago truncatula* Gaertn.).

Ozone, on the other hand, is a major secondary air pollutant, produced by a complex series of photochemical reactions from primary precursor emissions of nitrogen oxides (NO_x) and volatile organic compounds (Ashmore, 2005). Deleterious effects on trees include foliar injuries, premature leaf loss and reduced plant growth (Matyssek & Innes, 1999; Matyssek & Sandermann, 2003), as well as changes in quality and quantity of below ground carbon allocation (Andersen, 2003). O_3 is known to elicit plant responses typically associated with pathogen defence, such as biosynthesis of lignin, increased phenylalanine ammonia-lyase (PAL) activity, and accumulation of phenolic compounds and pathogen-related proteins (Heagle, 1973; Sandermann *et al.*, 1998; Matyssek & Sandermann, 2003).

In comparison, plants fertilized by elevation of CO_2 appear to invest more resources into their growth, while plants under ozone stress seem to activate defence mechanism. Thus, it is likely that environmental condition changing the resource balance between growth and pathogen defence are capable of altering host-pathogen interactions (Matyssek *et al.*, 2005). Changes in susceptibility due to elevation of trace gases have been reported many times, yet the outcomes are highly depend on the pathosystems studied (Chakraborty *et al.*, 2000; Garrett *et al.*, 2006). Therefore, only distinct host-pathogen interactions can be analyzed and no general conclusion can be drawn about the defence status of plants under elevated CO_2 and O_3 (Matyssek *et al.*, 2005).

In the case of *P. citricola* on European beech, Fleischmann *et al.* (2002a) could demonstrate a higher susceptibility of the plants grown under elevated CO_2 for three years, as compared to plants in ambient conditions. When analyzing the same interaction subjected to elevated O_3, Luedemann *et al.* (2005) observed a reduction in biomass caused by the elevation of O_3 in the atmosphere, yet inoculation with *P. citricola* did not further reduce the growth. They interpreted this as an indication of a reduced susceptibility to *P. citricola* through acclimation to increased O_3 levels.

While these effects may be due to direct physiological changes within the plant, it can also be hypothesized that the microbial rhizosphere community is affected by changes in exudation. Thus, beech plants might indirectly influence their defence status by either favoring or disfavoring antagonistic microbial populations in their rhizosphere.

1.6 Objectives

The presented thesis has been part of the Sonderforschungsbereich (SFB) 607 "Growth and Parasite Defence – Competition for Resources in Economic Plants from Agronomy and Forestry". The main hypothesis of this SFB states that "*independent of the factorial scenario, plants do regulate their resource allocation in a way that any increase in growth and competitiveness inherently leads to constraints on parasite defence*" (Matyssek *et al.*, 2005). Within this context it can be expected that factors affecting the plants C allocation have a potential to quantitatively and qualitatively alter the plants rhizodeposition and thereby change the composition of the microbial rhizosphere community. This in turn will have an effect on the suppression of plant pathogens in close vicinity to the fine roots of the plant, if antagonistic microbial populations are affected. In order to identify these populations, antagonists against *P. citricola* were isolated from beech rhizospheres and rhizoplanes. An experiment was designed to evaluate the impact of the factors O_3 and CO_2 on the antagonistic microbial community.

The following hypotheses are stated:

H1 Elevation of O_3 and CO_2 can lead to quantitative changes of the microbial community in the rhizosphere of European beeches

H2 The structure of antagonistic microbial communities in the rhizosphere is affected by elevation of O_3 and CO_2

H3 Functional aspects of the antagonistic population in the rhizosphere are affected by the elevation of O_3 and CO_2

Approaches chosen to confirm these hypotheses were:

H1 Total microbial carbon was measured as indicator for the total microbial biomass and CFU plate counts were performed for an antagonistic microbial population

H2 Culture independent fingerprinting methods were applied to monitor structural changes of an antagonistic microbial population

H3 A mechanism of antibiosis *in vitro* against *P. citricola* was identified and diversity of genes connected to this mechanism were monitored using culture independent methods

Chapter 2

Materials and methods

2.1 Experimental designs

Throughout this study, samples taken from two independent experiments were used. For the isolation of fungal and actinobacterial antagonists against the phytopathogen *Phytophthora citricola* samples from an experiment conducted by Dr. F. Fleischmann (Technical University Munich, Department of Ecology, Chair of Phytopathology of Woody Plants) was used. The focus of the partners in this experiment was to analyze the effect of elevated CO_2 and *P. citricola* inoculation on carbon allocation within the plant.

The main experiment was conducted from November 2004 to September 2006. The objective of this experiment was to investigate the effects of CO_2 and O_3 elevation, *P. citricola* inoculation and season on the antagonistic microbial rhizosphere populations based on the results of the first experiment. In this main experiment however, no isolations were performed but culture independent analyses were carried out (Tab. 2.1).

2.1.1 Soil characteristics

Soil for both experiments was obtained from the mixed beech/spruce forest stand "Eurasburger Forst" (near Augsburg, Germany). The soil is characterized as a podsolic para-brown soil (orthic luvisol, Ah-B horizont, 540 m a.s.l.; (similar to Kreutzer *et al.*, 1991). The soil was sieved (< 2 cm) and homogenized prior to filling into containers.

Table 2.1: Overview of the two experiments used in this study. Analyses performed with the samples are given.

	sampling date	analyses carried out
experiment 1	March 2005	isolation of potential microbial antagonists confrontation tests of isolates vs. *P. citricola*
experiment 2	May, July, September 2006	determination of above and below ground plant biomass quantification of soil microbial carbon structural diversity analysis of the actinobacterial community functional diversity analysis of the actinobacterial community

2.1.2 Greenhouse experiment for the isolation of antagonists

In spring 2003, 32 containers (size of 0.7 x 0.4 m, soil depth of 0.3 m) were planted with six two-year-old saplings of European beech (*Fagus sylvatica* L.). To ensure good drainage a layer of expanded clay Hydroton 8 mm (fleur ami, Tönisvorst, Germany) was placed at the bottom of each container. The expanded clay layer was separated from soil and container with a water permeable Covertan interlayer (Fiberweb, Berlin, Germany). The plants were grown in two climate controlled greenhouse chambers at the Helmholtz Zentrum München, Neuherberg. 16 containers were set under ambient and 16 under twice ambient CO_2 regimes (ambient + 300 ppm). All other factors were according to environmental conditions outside the greenhouse. During winter months plants were kept outside.

In March 2005, eight containers from each CO_2 treatment were inoculated with the root pathogen *P. citricola* (isolate BoGa) as described by Fleischmann *et al.* (2002b) using 120 mL of inoculum (Jung & Blaschke, 1996) in six inoculation holes (each 20 mL) per container. Negative controls were inoculated with the same amount of the sterile culture substrate (Fleischmann *et al.*, 2002b).

Plant material and rhizosphere soil was obtained from six containers (3x ambient, 3x elev. CO_2) at harvest time points 6 - 8 (6 = 6 days, 7 = 11 days and 8 = 16 days after inoculation with *P. citricola*). Only containers that were inoculated with *P. citricola* were used for isolation of antagonists (Figure 2.1). From each container samples from two trees were pooled resulting in three subsamples for each container. For harvesting the rhizosphere soil, the root system of all trees was freed from soil by gentle manual agitation. Rhizosphere soil of each individual plant

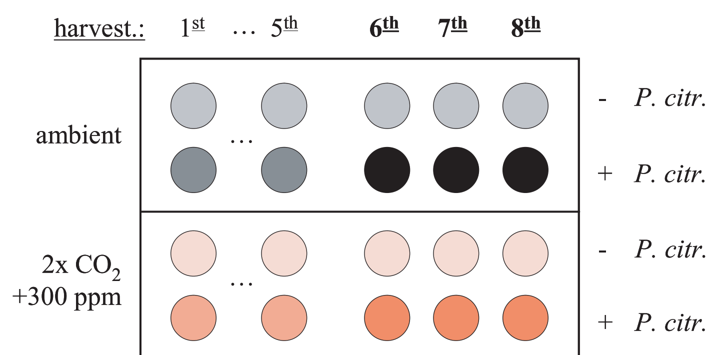


Figure 2.1: Experimental design of the greenhouse experiment for the isolation of antagonists. Harvests at time points 6 - 8 inoculated with *P. citricola* were used for the isolations of antagonists (highlighted in black and red).

was collected in plastic bags by removing soil adhering closely to fine roots (∅ < 5 mm distance to root). These soil samples were used for the isolation of actinobacterial antagonists. Roots were washed with demineralized water and fine root samples were taken from each tree of the mentioned containers for isolation of plant root associated fungi. All samples (roots and soil) were kept at 4°C until further processing.

2.1.3 Greenhouse experiment for culture independent analyses of the microbial rhizosphere community

The experiment was carried out in three climate controlled greenhouse chambers at the Helmholtz Zentrum München, Neuherberg. In November 2004, 54 pots (Teku BC 33, 14 L, Pöppelmann, Lohne, Germany) were planted with three two-year old European beech (*F. sylvatica* L.) saplings. Drainage was ensured as described in section 2.1.2 and plants were kept outside during winters. In May 2005 plants were moved to the greenhouse with treatments being as follows: one chamber (18 pots) was run at twice ambient CO_2 conditions as described in section 2.1.2. Another chamber (18 pots) simulated twice ambient O_3 conditions, yet always remaining below the critical level of 150 ppb. The last chamber (18 pots) was run at ambient conditions. All other parameters were kept at ambient levels for all three chambers and only natural light was used. Irrigation was carried out automatically, starting out with 200 mL every 56 h. Throughout the year, plants were checked regularly and irrigation was adjusting to changing water demands due to increased temperatures (for details refer to Appendix Tab. A.1).

At the end of March 2006 the pots were moved into the greenhouse to initiate bud break. In the first week of April temperature was raised to 18°C during day-time (5 $\underline{00}$ - 19 $\underline{00}$ h) and 12°C

during night. Relative humidity was kept at 70 - 80%. Irrigation started in the second week of April as described above. In the third week of April pots were moved to different chambers according to their treatments. Other climate factors were adjusted to outside conditions.

Figure 2.2: Flooding of the plants for inoculation with *P. citricola* in spring 2006.

In the first week of May inoculation with *P. citricola* (isolate Bu137/7N) was carried out following the protocols referred to in section 2.1.2. For each treatment nine pots received the inoculum according to Jung & Blaschke (1996) and the remaining nine pots per treatment were inoculated with sterile culture substrate. For each pot three inoculation holes (20 mL) were used and 60 mL of inoculum or sterile culture substrate were applied. Subsequently, the pots were flooded for 48 h (Fig. 2.2) to initiate sporulation of the pathogen. Isolate Bu137/7N was preferred over isolate BoGa (used in experiment 1, section 2.1.2) since it was less sensitive to low pH conditions concerning its sporulation in laboratory tests. While isolate BoGa had its pH optimum at 5.0, Bu137/7N sporulated best at 4.5 and was therefore closer to the measured soil pH of 4.1 (F. Fleischmann, pers. comm.). Both strains were cultivated on V8 agar.

Harvests were done at three different time points throughout the year 2006 and three replicates per treatment combination were sampled (Fig. 2.3). Prior to each harvest the pots were flooded again to propagate the infection with the pathogen. The first harvest in May was carried out after the initial inoculation with *P. citricola*. The second harvest followed in July and the last harvest was done in September of 2006. Different time points were chosen in order to evaluate the natural variation caused by seasonal changes on the antagonistic microbial community and to relate those to differences found for the different treatments. Each harvest took three days with one replicate per treatment being harvested each day (= 6 pots a day).

Throughout the experiment modified Hoagland solution (section 2.2.1) was applied as fertilizer. In 2005, 200 mL fertilizer were used once in August (34 mg N per tree) since it was expected that the fresh soil would still be sufficiently rich in nutrients to support tree growth. In 2006, fertilizer was added three times in April, June and August (each time 25.5 mg N per tree).

To monitor changes in soil moisture two pots per chamber were equipped with tensiometers

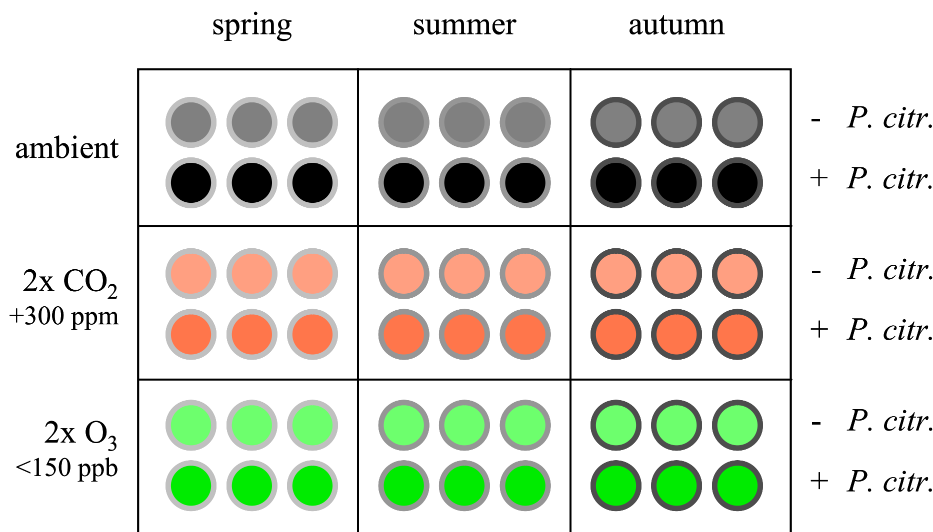


Figure 2.3: Multifactorial experimental design of the greenhouse experiment for culture independent analyses. For each sampling timepoint (spring, summer, autumn) six pots were harvested for each gas treatment (ambient, elevated CO_2, elevated O_3). Of these six pots three were inoculated with *P. citricola* and three with sterile culture substrate as controls.

(Model T5, UMS, Munich, Germany). These pots were not used for harvesting plant or soil material. Harvests began one day after the first reference pot reached a soil water tension of 200 hPa (pF = 2.3). Average soil water contents of the samples are given in Tab. A.2 expressed as percentage of the maximum water holding capacity (MWHC) of the soil. MWHC was determined to be 0.36 g water per g soil (dry weight).

Rhizosphere soil was obtained as mentioned in section 2.1.2. Samples of one pot were pooled to minimize the effect of genetic variation between different beech trees. Soil samples were sieved ($<$ 2 mm) immediately after harvest and aliquoted into two groups. One set of samples was shock frozen in liquid nitrogen and stored at -80°C, the other set was stored at 4°C until further processed. Roots were washed with de-ionized water and fine root samples were taken from each pot. Fine roots were either used for reisolation of the pathogen immediately or shock frozen and stored at -80°C. For reisolation of the pathogen small fine root pieces from the last harvesting time point were incubated on PARPNH agar and growing organisms transfered to V8 agar. Above and below ground biomass was separated (cutting right below leaf scars of cotyledons) and dried at 30°C for $\sim$3 months.

2.2 Materials and recipes

All chemicals used throughout this study were obtained from Sigma (Taufkirchen, Germany) if not indicated otherwise.

2.2.1 Buffers and solutions

Buffer	Component	Amount
TAE buffer	Tris base	242 g
	EDTA	18.6 g
	Acetic acid	57.1 g
	volume was adjusted to 1 L with dH_2O	
	pH was adjusted to 8.0	
TE buffer	Tris-HCl	1.576 g
	EDTA	0.372 g
	volume was adjusted to 1 L with dH_2O	
	pH was adjusted to 7.5	
Washing buffer	Tris-HCl	15.76 g
	EDTA	7.44 g
	NaCl	81.82 g
	volume was adjusted to 1 L with dH_2O	
	pH was adjusted to 8.0	
CTAB buffer	Tris-HCl	15.76 g
	EDTA	7.44 g
	NaCl	81.82 g
	CTAB	10 g
	volume was adjusted to 1 L with dH_2O	
	pH was adjusted to 8.0	

SDS buffer	Tris-HCl	15.76 g
	EDTA	7.44 g
	NaCl	81.82 g
	SDS	20 g

volume was adjusted to 1 L with dH_2O
pH was adjusted to 8.0

modified Hoagland solution (Hoagland & Arnon, 1950)	KNO_3	240 g
	$Ca(NO_3)_2$ x $4H_2O$	120 g
	$NH_4H_2PO_4$	60 g
	$MgSO_4$ x 7 H_2O	120 g
	Trace elements Hoagland	50 mL

volume was adjusted to 100 L with dH_2O

Trace elements Hoagland (Hoagland & Arnon, 1950)	$ZnSO_4$ x 7 H_2O	0.48 g
	$CuSO_4$ x 5 H_2O	0.24 g
	$FeSO_4$ x 7 H_2O	7.2 g
	$MnCl_2$ x 4 H_2O	4.8 g
	H_3BO_3	7.2 g
	Na_2MoO_4 x 2 H_2O	0.36 g

volume was adjusted to 500 mL with dH_2O

Trace element solution 1	$MnCl_2$ x 4 H_2O	0.03 g
	H_3BO_3	0.3 g
	$CoCl_2$ x 6 H_2O	0.2 g
	$CuCl_2$ x 2 H_2O	0.2 g
	$NiCl_2$ x 6 H_2O	0.02 g
	Na_2MoO_4 x 2 H_2O	0.03 g

volume was adjusted to 1 L with dH_2O

Trace element solution 2	EDTA	0.05 g
	$FeSO_4$ x 7 H_2O	0.02 g
	Trace element solution 1	10 mL

volume was adjusted to 100 mL with dH_2O

2.2.2 Media

All media were autoclaved for 20 min at 121°C.

Medium	Component	Amount
Starch Casein Agar (Küster & Williams, 1964)	Starch (watersoluble)	10 g
	Casein (vitamine-free)	0.3 g
	KNO_3	2 g
	K_2HPO_4	2 g
	$MgSO_4$ x 7 H_2O	0.05 g
	$CaCO_3$	0.02 g
	$FeSO_4$ x 7 H_2O	0.01 g
	Cycloheximide	50 mg
	Nystatin	50 mg
	Naldixic acid	25 mg
	Agar	20 g

volume was adjusted to 1 L with dH_2O
pH was adjusted to 7.0 using HCl

Medium	Component	Amount
Oatmeal Agar (Williams & Wellington, 1982)	Oatmeal Agar (Sigma, Seelze, Germany)	72.5 g
	Yeast extract	1 g

volume was adjusted to 1 L with dH_2O

Medium	Component	Amount
Fish-meal extract Agar (Kurtböke *et al.*, 2003)	Fish-meal extract*	20 mL
	NaCl	0.5 g
	Soil extract**	1 mL
	Glucose	20 g
	Peptone	5 g
	$CaCO_3$	3 g
	Agar	20 g

volume was adjusted to 1 L with dH_2O
pH was adjusted to 7.0 using HCl

PARPNH agar (Tsao & Guy, 1977)		
	Vegetable juice	100 mL
	$CaCO_3$	3 g
	Agar	16 g
	Pentachlornitrobenzen	0.01 g
	Ampilicin	0.05 g
	Rifampicin	0.1 g
	Pimaricin (Sigma P-0440)	0.5 mL
	Nystatin	0.06 g
	Hymexazol (Tachigaren) (Sankyo Company Ltd., Tokyo, Japan)	0.05 g

volume was adjusted to 900 mL with dH_2O

Czapek agar (ATCC medium 312)		
	$NaNO_3$	3 g
	K_2HPO_4	1 g
	KCl	0.5 g
	$MgSO_4$ x 7 H_2O	0.5 g
	$FeSO_4$ x 7 H_2O	0.01 g
	Saccharose	30 g
	Agar	15 g

volume was adjusted to 1 L with dH_2O
pH was adjusted to 6.0 - 6.5

ATCC-2 (liquid)		
	Yeast extract	5 g
	Bovine meat extract	3 g
	Peptone	5 g
	Glucose	1 g
	Starch from potato	2 g
	$CaCO_3$	1 g
	NZamine E	5 g

volume was adjusted to 1 L with dH_2O

LB medium (liquid) (Bertani, 1951)		
	Tryptone	10 g
	NaCl	5 g
	Yeast extract	5 g

volume was adjusted to 1 L with dH_2O
pH was adjusted to 7.5

Medium	Component	Amount
V8 agar (Erwin & Ribeiro, 1996)	Vegetable juice	200 mL
	$CaCO_3$	3 g
	Agar	16 g
	volume was adjusted to 1 L with dH_2O	

Medium	Component	Amount
Analysis medium (AM)	$MgSO_4$ x 7 H_2O	0.2 g
	K_2HPO_4	2 g
	NaCl	2 g
	NH_4NO_3	1 g
	Glucose	3 g
	Casein (vitamine-free)	0.3 g
	Thiamine hydrochloride	1 mg
	$CaCl_2$ x 2 H_2O	10 mg
	$ZnSO_4$ x 7 H_2O	1 mg
	trace element solution 2	1 mL
	volume was adjusted to 1 L with dH_2O	
	pH was adjusted to 7.0	

Medium	Component	Amount
***Trichoderma* selective agar (modified)** (Elad & Chet, 1983)	$MgSO_4$ x 7 H_2O	0.2 g
	K_2HPO_4	0.9 g
	KCl	0.15 g
	NH_4NO_3	1 g
	Glucose	3 g
	Chloramphenicol	0.25 g
	Rose Bengal	0.15 g
	Agar	20 g
	volume was adjusted to 1 L with dH_2O	

Medium	Component	Amount
Malt extract agar (MEA)	Malt extract	30 g
	Peptone	5 g
	Agar	16 g
	volume was adjusted to 1 L with dH_2O	

***Fish-meal extract** 100 g of fish-meal (97%) was boiled in 200 mL dH_2O for 3 h in a water bath. Subsequently the extract was produced by filtrating the pulp through a nylon gauze type 1152 (Bückmann GmbH, Mönchengladbach, Germany).

****Soil extract** 400 g of air dry "Eurasburger Forst" soil was autoclaved with 800 mL dH_2O, incubated at RT for 24 h and autoclaved again. The supernatant was filtered through a nylon gauze type 1152 (Bückmann GmbH, Mönchengladbach, Germany).

2.2.3 Reference strains

Table 2.2: Reference strains specifications

classification	species	source
Actinobacteria	*Arthrobacter citreus* BI90	AG Schloter*
	Arthrobacter globiformis	DSM 20124
	Bifidobacterium animalis subsp. *lactis*	DSM 10140
	Cellulomonas biazotea	DSM 20112
	Cellulomonas flavigena	DSM 20109
	Corynebacterium efficiens	DSM 44549
	Corynebacterium glutamicum	DSM 20300
	Curtobacterium citreum	DSM 20528
	Curtobacterium luteum	DSM 20542
	Frigoribacterium faeni	DSM 10309
	Nocardia carnea	DSM 43397
	Nocardioides simplex	DSM 20130
	Pseudoclavibacter helvolus	DSM 20419
	Rathayibacter rathayi	DSM 7485
	Rathayibacter tritici	DSM 7486
	Rhodococcus fascians	DSM 20669
	Streptomyces anulatus	DSM 40361
	Streptomyces griseus subsp. *griseus*	DSM 40236
Firmicutes	*Bacillus azotoformans*	DSM 1046
	Bacillus cereus UW85	DSM 6791
	Bacillus subtilis subsp. *subtilis*	DSM 10
	Lactobacillus sp.	AG Hartmann*
α-Proteobacteria	*Azospirillum brasilense*	DSM 1690
	Bradyrhizobium japonicum 110 spc.4	AG Schloter*
	Ensifer meliloti	DSM 5171
	Hyphomicrobium denitrificans	DSM 1869
	Ochrobactrum anthropi	DSM 20150
	Paracoccus denitrificans	DSM 50405
β-Proteobacteria	*Achromobacter xylosoxidans* subsp. *xylosoxidans*	DSM 2402
	Alcaligenes faecalis subsp. *faecalis*	DSM 30030
	Burkholderia cepacia	DSM 50181
	Chromobacterium violaceum	DSM 30191
	Cupriavidus necator H16	DSM 428
	Variovorax paradoxus	DSM 30034
γ-Proteobacteria	*Acinetobacter sp.*	DSM 586
	Idiomarina loihiensis	DSM 15497

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classification	species	source
γ-Proteobacteria	*Pseudomonas aeruginosa*	DSM 50071
	Pseudomonas alcaligenes	DSM 50342
	Pseudomonas fluorescens	DSM 8567
	Xanthomonas campestris pvar. *campestris*	DSM 3586
Archaea	*Haloarcula marismortui*	DSM 3752
	Haloferax denitrificans	DSM 4425

* Helmholtz Zentrum München

2.2.4 Oligonucleotides

Table 2.3: Oligonucleotide specifications

target	primer	sequence (5'-...-3')	reference
actinobacterial 16S	**20f**	tca cgg aga gtt tga tcc tg	Kataoka *et al.* (1997)
	500r	gcg gct gct ggc acg tag tt	Kataoka *et al.* (1997)
	124seq	agt aac acg tgg gca atc tg	Kataoka *et al.* (1997)
	243f	gga tga gcc cgc ggc cta	Heuer *et al.* (1997)
	AB1165r	acc ttc ctc cga gtt rac	Lüdemann & Conrad (2000)
	Act-283f	ggg tag ccg gcc tga gag gg	McVeigh *et al.* (1996)
	Act-1360r	ctg atc tgc gat tac tag cga ctc c	McVeigh *et al.* (1996)
	S-C-Act-235-a-S-20*	cgc ggc cta tca gct tgt tg	Stach *et al.* (2003)
	S-C-Act-878-a-A-19	ccg tac tcc cca ggc ggg g	Stach *et al.* (2003)
universal bacterial 16S	**B27f**	aga gtt tga tcm tgg ctc ag	Orphan *et al.* (2001)
	1401r	cgg tgt gta caa gac cc	Orphan *et al.* (2001)
	1492r	tac ggy tac ctt gtt acg act t	
P. citricola ITS	**CITR1**	tct tgc ttt ttt tgc gag cc	Schubert *et al.* (1999)
	CITR2	cgc acc gag gtg cac aca aa	Schubert *et al.* (1999)
fungal ITS	**ITS1**	tcc gta ggt gaa cct gcg g	White *et al.* (1990)
	ITS4	tcc tcc gct tat tga tat gc	White *et al.* (1990)
type I PKS	**KS-BEf**	ccg cgc gag gcg ctg gcc gtc gac	Ayuso *et al.* (2005)
	KS-BEr	ccg cgc cgg gcg ggg gtc tcg tcg ttc ggc atc agc ggc acc aac gcg	Ayuso *et al.* (2005)
	K1f	tsa agt csa aca tcg gbc	Ayuso-Sacido & Genilloud (2005)
	K2r	cvt tcg gvv tca gcg gsa cba a	Ayuso *et al.* (2005)
	M6r	cgc agg tts csg tac cag ta	Ayuso-Sacido & Genilloud (2005)
	ksf	gax ccs mts gcs rtc atc gsc atg	Chuck *et al.* (2006)
	ksar	ags gcs acs ags sws sws swg ca	Chuck *et al.* (2006)

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target	primer	sequence (5'-. . . -3')	reference
type I PKS	**atr**	xcc ctg xcc sgg gaa sas sma	Chuck *et al.* (2006)
type II PKS	**540f-PKS***	ggx tgc acs tcx ggx mts gac	Wawrik *et al.* (2005)
	1100r-PKS	ccg ats gcx ccs agx gag tg	Wawrik *et al.* (2005)
rep	**GR-Inter-LINE**	gag ttt ggc aaa gac cc	Smida *et al.* (1996)
	BOXA1r	cta cgg caa ggc gac gct gac	Rademaker *et al.* (1998)
plasmid sequencing	**T7**	taa tac gac tca cta tag gg	
	M13r	gga aac agc tat gac cat g	

* primers were used with fluorescent dye Cy5 for t-RFLP

r = reverse primer, f = forward primer

Degenerate alphabet, m = c or a, r = a or g, y = c or t, s = c or g, k = g or t, d = a or g or t, b = c or g or t, n = a or t or g or c, x = inosine.

2.3 Soil microbial biomass

To estimate total microbial biomass in the rhizosphere soil microbial carbon was quantified applying the chloroform fumigation extraction method (Vance *et al.*, 1987) as defined by DIN (ISO 14240-2:1999-10). Samples were processed within three days after harvest and stored at 4°C for the time in between. All extractions were performed in duplicates for each sample. Soil samples were divided into two subsamples equivalent to 5 g oven-dried soil. For chloroform fumigation 25 mL of ethanol free chloroform was placed in a desiccator together with one subset of the samples. The desiccator was then evacuated until the chloroform had boiled for 2 min. It was subsequently sealed and samples were incubated in chloroform saturated atmosphere for 24 h. Before the extraction, the desiccator was aerated and remaining chloroform removed from samples by evacuating 4 to 6 times. Fumigated and non fumigated subsamples were then suspended in 20 mL of 0.5 M K_2SO_4 (extraction ratio 1:4 w/v) and filtered through a pre-washed paper filter Schleicher & Schuell 595$\frac{1}{2}$ (Whatman, Dassel, Germany). Extracts were stored at -20°C until measured. Total organic carbon concentrations were measured as CO_2 (non-dispersive infrared gas analyzer) in a Total Carbon Analyzer (TOC 5050, Shimadzu Corporation, Tokyo, Japan). Microbial biomass C (C_{mic}) was calculated as $C_{mic} = E_C / k_{EC}$, where E_C = (organic C extracted from fumigated soil) - (organic C extracted from non-fumigated soil) and conversion factor $k_{EC} = 0.45$ (Wu *et al.*, 1990).

2.4 Isolation of microbial antagonists and confrontation tests

2.4.1 Bacterial antagonists (Actinobacteria)

Isolations from rhizosphere soil

For isolation of Actinobacteria, rhizosphere soils were air dried for nine days at RT to reduce the numbers of viable vegetative bacterial cells (Williams *et al.*, 1972) and subsequently sieved (< 2 mm). Prior to dilution the air dried soil was incubated for an additional 2 h at 45°C to further reduce the number of viable vegetative bacterial cells. Actinobacteria were then isolated and enumerated using the soil dilution plate method (Johnson & Curl, 1972) on Actinobacteria favoring starch casein agar (SCA). Briefly, plating was done as follows: 2 g of dry soil were added to a 50 mL BD Falcon™ Tube (BD Biosciences, Erembodegem, Belgium) containing 20 mL of a 0.1% agar solution and 4 g of steril glass beads (∅ 5 mm). The soil suspension was shaken for 30 min at 120 rpm using a Reax 2 overhead shaker (Heidolph, Schwabach, Germany). 1 mL of the suspension was used for a tenfold serial dilution and 100 μL of dilutions 10^{-2}-10^{-5} were plated in three replicates for each subsample (= nine plates per true replicate). Plates were incubated at 27°C in the dark for seven days. Dilutions 10^{-3} were used for counts of colony forming units (cfu) and isolations. All colonies were transferred onto modified oatmeal agar plates and stored at 4°C. Additionally, spore suspensions were stored in 10% glycerol at -20°C (Wellington & Williams, 1978). When isolates were cultivated in liquid medium, ATCC-2 was used.

Confrontation tests

Actinobacteria isolates were examined for their ability to inhibit *P. citricola*. Four isolates were tested simultaneously on one plate of fish-meal extract agar. Actinobacteria were placed 1 cm from the rim of the plate two days prior to inoculation with *P. citricola* to allow production and diffusion of metabolites into the agar. A plug (∅ 5 mm) from the edge of an actively growing *P. citricola* colony was transferred to the center of a confrontation plate. The Actinobacteria isolates were streak inoculated in a 90° angle to the central Oomycete. *P. citricola* mycelial plugs were also placed on uninoculated fish-meal extract agar plates separately as controls. Cultures were incubated in the dark at 25°C (± 2°C) for five days and then examined for inhibition.

Inhibition was calculated as percentage of growth distance of an oomycetous colony toward an actinomycete in relation to growth on control plates. The isolates were then grouped into four

Table 2.4: Inhibition classes of actinobacterial isolates.

Class	Specification	Inhibition range *
0	no inhibition	less than 10%
1	weak inhibition	between 10 - 30%
2	moderate inhibition	between 30 - 50%
3	strong inhibition	more than 50%

* Given as percent of growth inhibition in comparison to control plates without actinobacterial isolates.

classes according their inhibitory potential (Tab. 2.4). Inhibition of less then 10% was classified as no inhibiton (class 0) accounting for the fact that the Actinobacteria cultures themselves obtained a considerable size and thereby were able to reduce the growth of *P. citricola* simply via a deadlock system (two organisms that can not overgrow each other). Deadlock systems were not considered as inhibition. If strong inhibitors were present growth of *P. citricola* was often impaired not only for this organism but also for its neighbours. In those cases the isolates in question were repeated on new plates.

2.4.2 Fungal antagonists

Isolation from fine roots

Active hyphae were isolated from fine roots applying a washing method (Frankland *et al.*, 1990). Pre-washed fine roots were cut into 5 mm long pieces put on a sterile 200 μm sieve and washed with 1 L of sterile dH_2O to remove spores. Washed roots were than put onto a modified *Trichoderma* favouring medium and incubated at 25°C (± 2°C) in the dark. Plates were checked regularly and growing fungal colonies were transferred to Malt extract agar. All fungi were stored on plate at 4°C.

Confrontation tests

Confrontation tests of fungi vs *P. citricola* were done on V8 agar. 5 mm mycelial plugs from actively growing colonies of each organism were placed 5 cm apart from each other on V8 plates. Plates were incubated at 25°C (± 2°C) in the dark and checked on days 3-5 after inoculation. If the isolate overgrew *P. citricola* a plug from the interaction zone was taken after seven days and transfered to *Phytophthora*/*Phytium* selective PARPNH agar to check for viability of *P. citricola*. In those cases confrontation tests were repeated on Czapek agar to see if similar effects could

be obtained from a less complex synthetic medium.

2.5 Metabolite analysis

In order to identify a mechanism of actinobacterial antagonism, isolate 116A+4 was chosen as a representative. The antagonist was grown in a fully synthetic analysis medium (AM). First, a starting culture was prepared by inoculating 25 mL AM with $3*10^8$ spores of strain 116A+4 in a 100 mL Erlenmeyer flask. 10 sterile glass beads (∅ ∼5 mm) were added and the culture was incubated at 25°C at 160 rpm on a horizontal shaker for three days. 0.5 mL of the starting culture were then added to a 100 mL Erlenmeyer flask containing 30 mL AM and incubated for seven days as described above. The culture was centrifuged at 6000 g for 20 min and subsequently the supernatant was filtrated using 0.2 μm filters (Millipore, Eschborn, Germany). Samples were stored at -20°C until further processed.

Desalting of the samples was performed as follows: 1.8 mL of a sample was acidified with 18 μL of formic acid for positive electrospray ionisation (ESI) and loaded onto preconditioned (1 mL dH_2O) Supelco solid phase extraction column Discovery DSC-18 (Sigma, Taufkirchen, Germany). Samples were washed on the column with 1 mL 0.1% formic acid in dH_20 and eluated in 500 μL methanol.

To check for biocontrol activity in the supernatant, a *P. citricola* plug (∅ 5 mm) was placed in the center of a V8 agar plate and incubated for four days at 25°C (± 2°C) in the dark. Methanol was removed from the samples by evaporation using a Univapo 100 ECH vacuum concentrator (UniEquip, Martinsried, Germany). The dried sample was then dissolved in 1 mL dH_2O. The solution was again sterilized by filtration and 200 μL were pipetted into holes (∅ ∼5 mm) at the edge of the V8 agar plate. Growth inhibition of *P. citricola* was checked after two days.

2.5.1 Fourier transform ion-cyclotron (FT-ICR) mass spectrometry

FT-ICR mass spectra were acquired with a Bruker Daltonik (Bremen, Germany) Apex Qe 12 T system equipped with a microelectrospray source operated at ± 3000 V on the spray shield and ± 3500 V on the capillary with a sample flow rate of 250 μL/h, a drying gas flow rate of 4 L/s, a nebulizer gas pressure of 1 bar and a source temperature of 200°C. Ions were stored for 0.5 s in the source hexapole. Typically, 256 scans were accumulated for one spectrum. FTMS parameters were tuned within typical ranges for optimum sensitivity and resolution in a mass range of ca. 200-400 Da using 50 μg/mL arginine in methanol as test solution.

Spectra were acquired in broadband mode and with a time domain size of 1 MWord with a mass range of ca. 150-2000 m/z. A single sine apodization was performed before Fourier transformation of the time domain transient with a processing size of 2 MWord. Spectra were calibrated externally on clusters of arginine using the above mentioned test solution within the required mass range (m/z 175.11895, m/z 349.23062, m/z 523.34230 and m/z 697.453979). In order to increase mass accuracy, spectra were internally recalibrated using peaks of ubiquitary solvent impurities (protonated and sodium-cationized phthalate diesters, m/z 207.15908, m/z 229.14103, m/z 251.12778, m/z 273.10973, m/z 279.15908, m/z 301.14103, m/z 315.25298, m/z 337.23493, m/z 391.28428, m/z 413.26623, m/z 447.34688 and m/z 469.32883). A maximum deviation of 0.1 ppm from those reference masses was allowed.

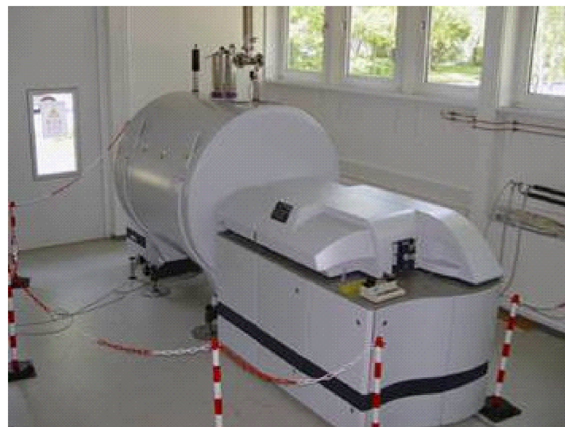


Figure 2.4: 12 Tesla fourier transform ion-cyclotron mass spectrometry (FT-ICR/MS) located at the Institute of Ecological Chemistry, GSF.

Signals were exported to peak lists. The resulting text files with mass/intensity pairs were mass-aligned using a window-based algorithm (Frommberger, unpublished). Elemental formulae were calculated utilizing the Bruker DataAnalysis tool using a maximum error of 0.5 ppm. In order to identify the substance of interest, elemental formulae were submitted to the ChemIDplus Advanced database (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp). Most likely hits were obtained as standards for NMR analysis.

2.5.2 Nuclear magnetic resonance (NMR)

To confirm the identity of the bioactive compound inhibiting *P. citricola* ^{1}H NMR was utilized. The sample exhibiting biocontrol activity was dried using a Univapo 100 ECH vacuum concentrator (UniEquip, Martinsried, Germany). ^{1}H NMR spectra of methanolic microbial extracts (methanol-d_4; 99.95% ^{2}H, Merck, Darmstadt) were acquired at 303 K with a 5 mm z-gradient ^{13}C/^{1}H dual cryogenic probe using 90° excitation pulses (90°(^{1}H) = 9.5 μs, acquisition time: 5 s) on a Bruker DMX 500 spectrometer; HD_2COD was used as internal reference: 3.30 ppm.

2.6 Characterization of pure microbial cultures

All polymerase chain reaction (PCR) amplifications were carried out with a T3 Thermocycler (Biometra, Göttingen, Germany). For all oligonucleotide sequences refer to table 2.3.

2.6.1 Nucleic acid extraction from microorganisms

Nucleic acid extraction from bacteria

DNA from all bacterial isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturers instructions. Liquid cultures were grown in either LB or ATCC-2 medium. For Gram-positive bacteria a lysozyme digestion at 37°C for 60 min was performed prior to extraction. Quality of DNA was evaluated with 1% agarose gels stained with ethidium bromide (Sambrook *et al.*, 1989). As molecular standard a GeneRuler™ 1 kb ladder (MBI Fermentas, St. Leon-Rot, Germany) was used. DNA solutions were quantified using the Nanodrop ND-100 spectrophotometer (Peqlab, Erlangen, Germany).

Nucleic acid extraction from fungi

DNA extraction from fungal isolates was performed according to Kuhad *et al.* (2004) with modifications. Briefly, fungi were grown in liquid malt extract medium (section 2.2.2). Mycelium was washed with sterile dH_2O and washing buffer. 1 g of mycelium was incubated at 50°C for 10-12 min in 6 mL CTAB-buffer and subsequently centrifuged at 25.000 g for 10 min using a Sorvall® $Evolution_{RC}$ centrifuge (Thermo Fisher Scientific Inc., Waltham, USA). Mycelial mass was transferred to 14 mL BD Falcon™ Tubes containing 6 mL SDS buffer supplemented with 120 μL mercaptoethanol. The suspension was incubated for 3 h at 68°C and homogenized 3-4 times by pipetting. 6 mL of equilibrated and stabilized phenol was added to the hot suspension and centrifuged for 1 h at maximum speed using a Heraeus® Megafuge® 1.0R (Thermo Fisher Scientific Inc., Waltham, USA). Supernatant was cleaned and DNA percepitated following the protocol. RNase treatment was performed as described by Sambrook *et al.* (1989). Quality and quantity of the DNA solutions were evaluated as described above.

2.6.2 Genomic fingerprinting of isolates

Molecular fingerprinting using interspersed repetitive DNA elements (rep-PCR technique) was applied to dereplicate isolate strains. For Actinobacteria the BOX primer described by Rade-

maker *et al.* (1998) was used. Fungal isolates were dereplicated using the rep-PCR primer GR-Inter-LINE (Smida *et al.*, 1996). 10 μL of the PCR products were separated on 2% QA-Agarose™ (Qbiogene, Heidelberg, Germany) gels run for 4 h at 80 volts. As molecular standard a GeneRuler™ 1 kb DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) was loaded onto the gel.

BOX-PCR

Reaction mixtures for BOX-PCR contained 1x buffer (Gibco BRL, Germany), 0.3 μM of BOX A1R primer (Thermo Hybaid, Ulm, Germany), 2.5 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.25 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 100 ng DNA template and 1.5 U Taq polymerase (Gibco BRL, Karlsruhe, Germany) in a final volume of 50 μL. The PCR cycle program consisted of an initial denaturation at 95°C for 3 min and 30 sec followed by 35 cycles of 94°C for 1 min and 10 sec, annealing at 56°C for 40 sec and elongation at 72°C for 2 min and 10 sec. A final elongation at 72°C for 6 min concluded the program.

Inter-LINE PCR

Reaction mixtures for Inter-LINE PCR contained 1x buffer (Gibco BRL, Karlsruhe, Germany), 3.2 μM of GR-Inter-LINE primer (Thermo Hybaid, Ulm, Germany), 5 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.1 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 50 ng DNA template and 1.25 U Taq polymerase (Gibco BRL, Karlsruhe, Germany) in a final volume of 25 μL. The PCR cycle program was preceded by a hot start at 95°C for 5 min followed by 4 cycles of 37°C for 1 min and 30 sec, 72°C for 2 min and 93°C for 1 min and 30 sec. Subsequently, 24 cycles of 52°C for 1 min, 72°C for 1 min and 30 sec and 93°C for 1 min were performed. The program ended with a final elongation at 72°C for 10 min.

2.6.3 Sequencing of PCR products

Sequencing of partial 16S rRNA genes for Actinobacteria and internal transcribed spacer (ITS) rRNA regions for fungal isolates was performed for further characterization and identification of dereplicated isolates. Sequencing was carried out using the BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, USA) with different sequencing primers and annealing temperatures as indicated below. Sequence reactions were done according to manufacturers instructions on an ABI 3730 sequencer (Applied Biosystems, Foster City, USA) and were purified either by ethanol

precipitation (as suggested by the manufacturer) or using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany).

Partial 16S rRNA gene sequencing

For Actinobacteria, partial 16S rRNA gene sequencing was applied following the protocol of Kataoka *et al.* (1997). The resulting 120 bp long sequences included the highly variable *c* region of the 16S rRNA gene (Kataoka *et al.*, 1997; Kämpfer, 2006). First, a 500 bp fragment was amplified. For this, a total of 50 μL of reaction mix contained 1x Stoffel buffer (Applied Biosystems, Foster City, USA), 0.2 μM of primers 20f and 500r (Thermo Hybaid, Ulm, Germany), 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, USA), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 50-100 ng of template DNA and 1.25 U of AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, USA). The PCR program was preceded by a hot start at 95°C for 5 min followed by 32 cycles of 97°C for 30 sec, 50°C for 1 min and 72°C for 1 min. A final elongation at 72°C for 10 min was done at the end of the program. Amplicons were purified using a PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the sequencing primer 124f (Thermo Hybaid, Ulm, Germany) at 50°C. Sequences were distinguished by NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) and by calculating a phylogenetical tree using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/) applying a PHYLIP algorithm.

Internal transcribed spacer (ITS) rRNA region sequencing

For the identification of fungal isolates the ITS regions (ITS1, 5.8S, ITS2) were sequenced as recommended by Druzhinina *et al.* (2005). Amplification of the ITS regions was performed as follows: each 50 μL reaction contained 1x buffer (Gibco BRL, Karlsruhe, Germany), 0.4 μM of primers ITS1 and ITS4 (Thermo Hybaid, Ulm, Germany), 5 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 10% betaine, 50-100 ng of template DNA and 2.5 U Taq polymerase (Gibco BRL, Karlsruhe, Germany). The cycler program was as follows: 95°C for 3 min, 30 cycles of 95°C for 45 sec, 55°C for 30 sec, 72°C for 30 sec and a final elongation at 72°C for 10 min. Amplicons were purified using the PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the ITS1 primer at a annealing temperature of 47°C. Non-*Trichoderma* fungi sequences were distinguished by NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) whereas *Trichoderma* sequences were differentiated using the *Trich*OKEY identification tool version 2.0 (Druzhinina *et al.*, 2005).

2.6.4 Cloning and sequencing of plasmids

Amplification of the 16S rRNA gene

For the actinobacterial isolate 116A+4 a longer fragment of the 16S rRNA gene was amplified using the universal eubacterial primer set B27f/1401r with an initial denaturation step at 95°C for 10 min, followed by 30 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 30 sec and a final extension at 72°C for 10 min. The reaction mixture contained 1 x buffer, 10% betaine, 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 0.2 μM of each primer, 2.5 U Taq polymerase (Gibco BRL, Karlsruhe, Germany) and 50 ng of DNA template in a final volume of 50 μL. Amplicons were purified using the PCR Purification Kit (Qiagen, Hilden, Germany).

Cloning of the PCR product

The PCR product was cloned into a pCR® 2.1-TOPO® vector with the TA Cloning® Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For ligation the amount of the PCR product was increased to 50 ng in a 10 μL reaction mixture of 1x ligation buffer, 50 ng pCR® 2.1-TOPO® vector and 4.0 U of T4 DNA Ligase. The ligation was incubated at 14°C over night. Selection of positive clones was done by standard blue-white screening (Sambrook *et al.*, 1989). Colonies were grown over night in 2-5 mL LB broth containing 50 μg/mL kanamycin and used for plasmid extraction according to Bimboim & Doly (1979).

Plasmids containing inserts of the correct size were selected after digestion with *Eco*RI (MBI Fermentas, St. Leon-Rot, Germany). Reaction mixtures containing 2 μL plasmid extraction, 5 U *Eco*RI and 2 μL *Eco*RI buffer in a 20 μL total volume were incubated for 1 h at 37°C, followed by 20 min at 65°C to inactivate the enzyme.

Sequencing was carried out according to section 2.6.3 using primers T7 and M13r at 45°C and 47°C respectively.

2.6.5 Species specific PCR for the detection and identification of *P. citricola*

To confirm the identity of the reisolated *P. citricola* from inoculated fine roots, a species specific PCR was conducted following the protocol of Schubert *et al.* (1999). The isolates were grown for 5 d at RT in liquid MEA (section 2.2.2) and DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The PCR reaction mixture contained 1x buffer (Gibco BRL,

Karlsruhe, Germany), 0.25 μM of primers CITR1 and CITR2 (Thermo Hybaid, Ulm, Germany), 5 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 15-40 ng of template DNA and 1.25 U Taq polymerase (Gibco BRL, Karlsruhe, Germany). The cycler program consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min. A final elongation at 72°C for 10 min was carried out. Amplicons were checked for the correct size of 701 bp on 1% agarose gels with a 100 bp ladder as molecular standard after staining with ethidium bromide.

2.7 PCR based analyses of environmental samples

2.7.1 DNA extraction from environmental samples

Environmental DNA (root and soil) was extracted using the Fast Spin DNA Extraction Kit for Soil (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions with modifications. For rhizosphere soil samples 0.5 g of homogenized and sieved soil was used, while 0.1 g of fine root material was used for extraction after grinding in liquid nitrogen. The original protocol was modified by adding two washing steps of the silica binding matrix using 5.5 M guanidine thiocyanate (GTC) solution to remove inhibitory substances. For this, DNA was bound to the silica matrix and allowed to settle for 3-4 min. The supernatant was removed and the silica matrix was washed with 1 mL of 5.5 M GTC solution. Again, the matrix was allowed to settle for 3-4 min and the supernatant was removed. This procedure was repeated twice, then the matrix was resuspended in 1 mL GTC solution and transfered to the SPIN™ Filter. To remove remaining guanidine thiocyanate washing the silica with 500 μL of SEWS-M solution was repeated twice as well.

2.7.2 PCR amplification of structural and functional genes

Actinobacterial 16S rRNA gene amplification

To evaluate the diversity of Actinobacteria in beech rhizospheres the specific primers S-C-Act-235f/Act-1360r were used. Amplification was performed as follows: each 50 μL reaction contained 1x buffer (Gibco BRL, Karlsruhe, Germany), 0.2 μM of each primer (Thermo Hybaid, Ulm, Germany), 2 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 5% Dimethyl Sulfoxide (DMSO), 0.3% bovine serum albumin (BSA), 20 ng of template DNA and 2.5 U Taq polymerase (Gibco BRL, Karlsruhe,

Germany). A hot start was applied with a denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 45 sec, 72°C for 1 min and 45 sec and a final elongation at 72°C for 5 min.

In order to confirm specificity of the primers used, amplification was checked for all reference strains listed in section 2.2.3. In addition, a clone library was established from rhizosphere soil DNA (ambient, summer, not inoculated with *P. citricola*) according to the procedure described in section 2.6.4 using sequencing primer T7 primer only. Sequences were classified using the higher-order bacterial taxonomy implemented in the Ribosomal Database Project II Release 9.54 (Cole *et al.*, 2005) naïve Bayesian rRNA classifier (http://rdp.cme.msu.edu/) (Wang *et al.*, 2007).

Type II polyketide synthase (PKS) amplification

For a culture independent functional analysis of microbial communities the type II polyketide synthase specific primer pair PKS-540f/PKS-1100r (Wawrik *et al.*, 2005) was used. The expected amplicon is a 550-560 bp long fragment of the ketoacyl-synthase domain (KS_α) of the minimal polyketide synthase. For amplification, a 50 μL PCR reaction consisted of 1x buffer (Gibco BRL, Karlsruhe, Germany), 0.4 μM of each primer (Thermo Hybaid, Ulm, Germany), 2 mM $MgCl_2$ (Gibco BRL, Karlsruhe, Germany), 0.2 mM of each dNTP (MBI Fermentas, St. Leon-Rot, Germany), 5% Dimethyl Sulfoxide (DMSO), 0.3% bovine serum albumin (BSA), 20 ng of template DNA and 5 U Taq polymerase (Gibco BRL, Karlsruhe, Germany). The cycle program was initiated with a hot start for 5 min at 95°C followed by 40 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 45 sec. A final elongation at 72°C for 10 min ended the program.

The specificity of the primers was checked by creating a clone library from rhizosphere soil DNA (ambient, summer, not inoculated with *P. citricola*) according to the procedure described in section 2.6.4 using T7 primer.

2.7.3 Terminal restriction fragment length polymorphism analysis (t-RFLP)

To analyze the diversity of structural and functional genes in microbial rhizosphere communities terminal restriction fragment length polymorphism analysis (t-RFLP) was applied.

Actinobacterial 16S rRNA genes

PCR amplification for actinobacterial 16S rRNA genes was done as described in section 2.7.2 applying primer S-C-Act-235f fluorescently labeled with Cy5 at the 5'-terminal end. For each

soil sample DNA was extracted in duplicates and PCR was repeated for each DNA extract in triplicates. PCR reactions were then pooled for each DNA extract and purified using the PCR Purification Kit (Qiagen, Hilden, Germany). Double digestions were carried out using the enzymes *Mbo*I (New England Biolabs, Frankfurt am Main, Germany) and *Fau*I (SibEnzyme, Zweibrücken, Germany). For the first digestion reaction, a total volume of 10 μL contained 1x NEBuffer 1 (New England Biolabs, Frankfurt am Main, Germany), 2.5 U of *Mbo*I and 100-150 ng of the PCR product. The digestion mixture was incubated for 16 h at 37°C. Then, 10 μL of *Fau*I solution containing 1 U of enzyme in 1x NEBuffer 1 was added. This reaction mixture was incubated at 55°C for another 16 h, subsequently heated to 65°C for 20 min to inactivate the enzymes and cleaned using the Minelute PCR Purification Kit (Qiagen, Hilden, Germany).

Type II PKS genes

PCR amplification for type II PKS genes was performed as described in section 2.7.2 reducing the number of cycles to 35 and using primer PKS-540f fluorescently labeled with Cy5 at the 5'-terminal end. DNA for each sample was extracted in duplicates and PCR was repeated for each DNA extract in triplicates. PCR reactions were pooled and purified using the PCR Purification Kit (Qiagen, Hilden, Germany). 100-150 ng of the PCR product was digested with 20 U *Hha*I (New England Biolabs, Frankfurt am Main, Germany) in a total of 20 μL of 1x NEBuffer 4 (New England Biolabs, Frankfurt am Main, Germany) supplemented with 2 μg BSA. DNA was digested for 18 h at 37°C followed by an enzyme inactivation at 65°C for 20 min. The reaction mix was purified using the Minelute PCR Purification Kit (Qiagen, Hilden, Germany).

Detection and analysis of fragments

For detection of labeled fragments 2.5 μL (= 1-5 ng DNA) of the purified digestion reaction was mixed with 0.25 μL GenomeLab™ DNA Size Standard 600 (Beckman Coulter GmbH, Krefeld, Germany) and 27.25 μL SLS buffer (Beckman Coulter GmbH, Krefeld, Germany) and was covered with a drop of mineral oil to prevent evaporation. Separation of the fragments was conducted using a CEQ™ 2000 XL sequencer (Beckman Coulter GmbH, Krefeld, Germany) with program parameters indicated in table 2.5. Each reaction was run three times on different capillaries to minimize capillary effects. One representative profile was taken for each sample for further analysis. To analyze peak profiles the CEQ™ 8000 Genetic Analysis System software version 8.0.52 (Beckman Coulter GmbH, Unterschleißheim, Germany) was used. Peak recogni-

tion was checked and edited manually to include all peaks within a profile. Matrices were then exported to Microsoft® Excel (Microsoft Corporation, Redmond, USA) and peak heights were expressed relative to total peak height within a sample. Subsequently, all peaks below 0.5% of the total peak height within a sample were excluded from the analysis and 0.5% was set to zero for the rest of the peaks. Rare peaks with only one occurrence throughout all samples were excluded. Mean values were calculated for each peak from the duplicate DNA extractions for each soil sample.

Table 2.5: Program definition for t-RFLP runs using the CEQ™ 2000 XL sequencer.

step	action	parameter
1	heating capillary	raise temperature to 50°C
2	denaturation of DNA	90°C for 2 min
3	injection	at 2.0 kV for 30 sec
4	separating sample	at 4.8 kV for 60 min

2.7.4 Quantitative real-time PCR

For the quantification of genes in beech fine roots or rhizosphere soil, SYBR® green quantitative real-time PCR assays were used. Amplifications were carried out using a 7300 Real Time PCR System (Applied Biosystems, Foster City, USA). A plasmid standard was produced as described below and quantified using the Nanodrop ND-100 spectrophotometer (Peqlab, Erlangen, Germany). Tenfold dilutions of the standards were prepared and standard curves were generated utilizing the ABI 7300 SDS Software System (Applied Biosystems, Foster City, USA) by plotting the threshold cycle number for the standard DNA against the $\log^{10}$ of the copy number as described by Henry *et al.* (2006) with PCR efficiency E being calculated by $E = (10^{-1/\text{slope}}) - 1$.

Detection of *P. citricola* in fine roots

Plasmid standards were prepared cloning the ITS fragment of *P. citricola* (isolate Bu137/7N) using the pSTBlue-1 AccepTor Vector kit (Novagen, Madison, USA). Ligation reaction containing 1 μL pSTBlue-1 AccepTor vector, 2 μL purified PCR product and 5 μL clonables 2x ligation in a total of 10 μL was carried out at RT overnight. The transformation of the competent cells and screening of the colonies were performed according to manufacturer's instructions. The identity of the insert was confirmed by *Eco*RI digestion and sequencing as described in section 2.6.4. For

the external standard tenfold dilutions of the plasmid solution were done (10^5-10^1).

PCR amplification from the extracted fine root DNA and standards was performed as follows: each 25 μL reaction contained 5 μL qPCR ROX & Go Green Mastermix (QBiogene, Grünberg, Germany), 0.25 μM of each primer CITR1 and CITR2 (Thermo Hybaid, Ulm, Germany), 0.06% BSA and 2 μL of 1:2 diluted DNA extract. The PCR cycle program consisted of an initial denaturation of the templates and activation of the *Taq* DNA polymerase at 95°C for 15 min followed by 40 cycles of 95°C for 30 sec, 62°C for 30 sec and 72°C for 1 min and a detection of the fluorescence at 80°C. Each sample and the standards were repeated in triplicates.

To calculate the recovery rate of DNA from beech fine roots, DNA extractions as described in section 2.7.1 were performed spiking the sample with $2.28 * 10^7$ copies of the standard per 100 mg fine roots. Dilutions of these spiked samples (1:2, 1:4, 1:8) were used for quantitative PCR to detect possible inhibitory substances in the DNA extracts.

2.8 Statistical analyses

Univariate statistics

All univariate statistics were carried out using the S-Plus® package Version 6.2 (Insightful Corp., Seattle, USA). To check for normal distribution of the data Kolmogorov-Smirnov Goodness-of-Fit test was applied. Histograms and qqnorm plots of data distribution were used for visual confirmation of normality. If normal distribution of data was not given, transformations were performed as indicated in the relevant passages or non-parametric tests were applied.

Multivariate statistics

Non-metric multidimensional scaling (NMS) on the basis of Euclidean distance measure was used as an unconstrained ordination method to visualize patterns for multivariate data sets utilizing PCR-ORD version 5.0 (MjM Software, Gleneden Beach, USA). To verify linear relationships among variables the length of gradient as calculated for the first axis of a detrended correspondence analysis (DCA) was used. For all data sets the length of gradient was below 1 indicating linear relationships. The best dimensionality for the data sets was assessed by comparing stress values of 250 runs performed for 1-D to 6-D solutions. Additional dimensions were considered useful if they reduced the final stress by five or more (based on the Kruskal's stress formula 1 multiplied by 100). For all data sets 2-D solutions fitted this criterion. To evaluate whether

NMS extracted stronger axes than expected by chance this procedure was repeated with randomized versions of the data sets and compared with the real data (Monte Carlo test). For all solutions the P-value was $p < 0.01$. For final solutions a maximum of 500 iterations was set using a stability criterion of < 0.0000001 for the last 10 iterations (McCune & Grace, 2002).

To test for differences in composition and relative abundance of the multivariate data between samples from different treatments or groups non-parametric multivariate analysis of variance (PerMANOVA) was used (Anderson, 2001). This test can be based on any distance measure (in this case Euclidean distance) and can partition variation directly among individual terms in a multifactorial ANOVA (analysis of variance) model. It is also independent of the assumption that the data conform to a multivariate normal distribution, yet it is sensitive to dispersion and therefore results have to be treated with caution and should be interpreted with the help of ordination methods. For each term in the analysis, 4999 permutaions of raw data units were done to obtain P-values. Individual pair-wise multiple comparisons by permutation were performed for factors showing significant differences (again performing 4999 permutations). In some cases, there were not enough permutations possible to get a reasonable test. For these situations, 4999 Monte Carlo samples were drawn from the theoretical asymptotic permutation distribution (Anderson & Robinson, 2003; Anderson & Millar, 2004). Analyses were carried out using the FORTRAN program PerMANOVA by M. J. Anderson (http://www.stat.auckland.ac.nz/~mja/Programs.htm).

To contrast the abundance of t-RFs across different groups of samples, first, indicator species analysis was performed according to Dufrêne & Legendre (1997) as implemented in PC-ORD version 5.0 (MjM Software, Gleneden Beach, USA). For all peaks giving significant results for the indicator species analysis, univariate ANOVA was performed on the relative abundance of those peaks. Due to the large variability and highly skewed nature of the data, it was not reasonable to assume normality for any of the variables. Thus, all tests were done using a permutation procedure (with 4999 permutations) as described for PerMANOVA above. Significant results were also tested with the above mentioned "a posteriori" pair-wise comparison, with again 4999 random permutations to obtain a P-value or Monte Carlo samples drawn from the theoretical asymptotic permutation distribution.

For all tests untransformed data was used, due to the lower reproducibility of peaks with low intensities (Osborn *et al.*, 2000). Standard procedures like square root or logarithmic transformations would have only increased the importance of smaller peaks in following analyses.

Phylogenetic trees

Phylogenetic trees were either calculated from nucleic acid sequences using the neighbour joining method implemented in the ClustalW WWW Service from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw/) and visualized using the PHYLIP output format with TreeView version 1.6.6 (http://taxanomy.zoology.gla.ac.uk/rod/rod.html) or from protein sequences (for PKS clone library) using the maximum-likelihood algorithm implemented in ARB (http://www.arb-home.de) after aligning the sequences with the ARB Fast Aligner tool.

Gel images were analyzed with the program GelCompar II (Applied Maths, Belgium), using the Dice coefficient and the unweighted pair group clustering method with arithmetic averages (UPGMA).

Rarefaction analysis were performed to estimate the completeness of sampling of communities by clone libraries. Rarefaction curves were generated using the software Analytic Rarefaction (http:/www.uga.edu/strata/software/Software.html) by Steven Holland.

Chapter 3

Results

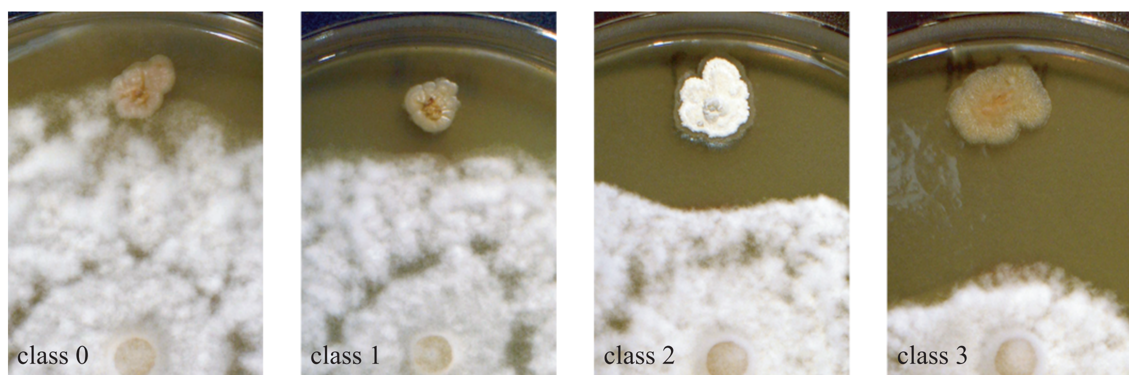
3.1 Fungal and actinobacterial antagonists against *Phytophthora citricola*

3.1.1 Actinobacteria isolated from beech rhizosphere soil and confrontation tests with *P. citricola*

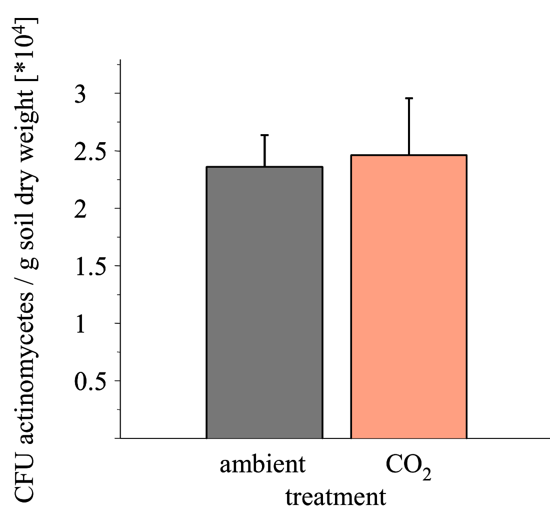
Potential actinobacterial antagonists against *P. citricola* were isolated and cultured from rhizosphere soil of European beeches. All bacterial colonies showing mycelial growth patterns characteristic for Actinobacteria were isolated and colony-forming units (CFU) were counted on casein-starch agar. The CFUs represent the amount of actinobacterial spores in soil, since pretreatments were performed to minimize vegetatively growing bacteria (as described in section 2.4.1). As expected, Actinobacteria could be isolated readily from beech rhizosphere soil, showing a wide variety of morphologically diverse groups. Isolation frequencies ranged from 1.8 to $3.0*10^4$ CFU per gram air dried rhizosphere soil. With the given amount of three replicates for each treatment it was not possible to detect a significant difference between the treatments using Student's t-test. Although, figure 3.1b strongly suggested similar CFUs for rhizosphere communities of CO_2 and ambient treated plants.

A total of 243 Actinobacteria were isolated and subsequently tested for antagonism against *P. citricola*. 117 isolates (48%) inhibited the growth of the Oomycete. Antagonistic isolates were then subdivided into three inhibition classes: weak (class 1) = 10 - 30%, moderate (class 2) = 30 - 50% and strong inhibition (class 3) $\geq$ 50% reduction in growth of *P. citricola* (Fig. 3.1a).

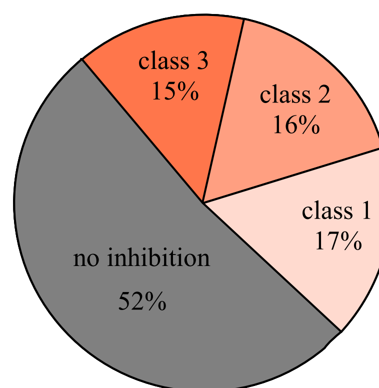
41 isolates (17%) showed weak inhibition, 40 isolates (16%) moderate and 36 isolates (15%) strong inhibition (Fig. 3.1c). There was no difference in isolation frequencies of the different antagonistic classes between the treatments. From the 36 strong and 40 moderate antagonistic



(a) Inhibition classes



(b) Isolation frequencies



(c) Inhibition class distribution

Figure 3.1: Summary of Actinobacteria isolations (a) Inhibition classes: no inhibition (class 0) = less then 10% , weak inhibition (class 1) = 10 - 30%, moderate inhibition (class 2) = 30 - 50% and strong inhibition (class 3) $\geq$ 50% reduction of growth of *P. citricola* compared to control plates, (b) Isolation frequencies of Actinobacteria compared between ambient and elevated CO_2 treated plants expressed as colony forming units (CFU) per gram air dried soil (n=3, error bars represent standard deviations) and (c) Inhibition class distribution amongst total actinobacterial isolates (n = 243).

isolates half were isolated from each treatment, for the 41 weak antagonists 20 were isolated from elevated CO_2 treated plants and 21 from ambient conditions.

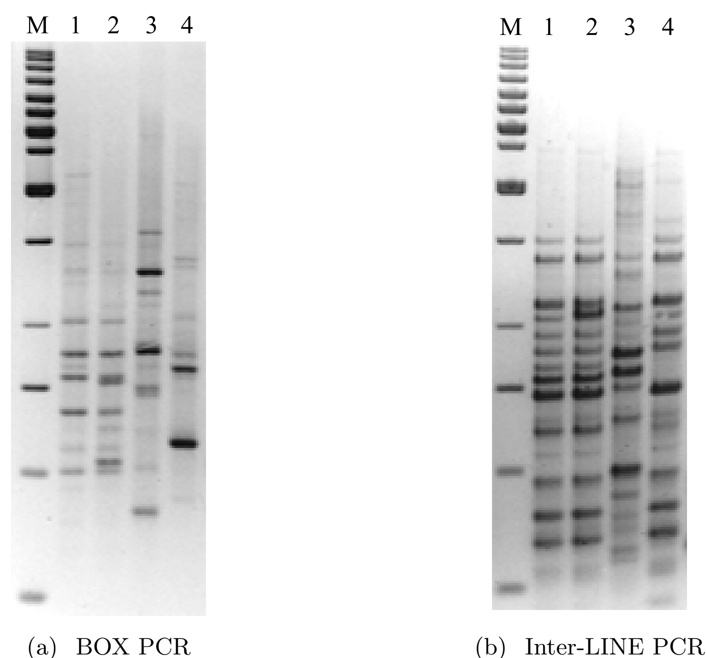
3.1.2 Characterization of the actinobacterial isolates

All isolates belonging to the strong and moderate antagonists (classes 2 and 3) were characterized using a combination of repetitive DNA elements fingerprints (rep-PCR, Fig. 3.2a) and partial sequencing of a highly variable region of the 16S rRNA gene. In accordance with Davelos *et al.*

(2004b), dereplication of the isolates was based on a threshold of 90% similarity for the rep-PCR profiles, using the Dice coefficient. 47 unique isolates were identified. These were then submitted to partial sequencing of the 16S rRNA gene resulting in nine different phylotypes (NCBI accession numbers: EU139022 - EU139029 and EU139032). This classification allowed a grouping of the isolates with a resolution close to species level (Kataoka *et al.*, 1997; Kämpfer, 2006). According to this method eight of the nine phylotypes belonged to different groups of the genus *Streptomyces*. The most common strong antagonistic group (phylotype 1) was classified as belonging to the genus *Kitasatospora* (Fig. 3.3). To verify the identity of this phylotype a longer fragment (~1400 bp) of the 16S rRNA gene was sequenced for one of the isolates (116A+4, accession number EU139032). Using the Ribosomal Database Classifier the isolate was confirmed to be a member of the genus *Kitasatospora*.

When investigating the BOX fingerprint UPGMA dendrograms for all isolates belonging to phylotype 1 a very high diversity could be seen. From a total of 24 isolates 18 strains were differentiated after dereplication (Fig. 3.4). All of those isolates were categorized as strong antagonists.

Phylotype 102 (Fig. 3.3) encompassed another large group of antagonists with a total of 25



(a) BOX PCR (b) Inter-LINE PCR

Figure 3.2: Exemplary gel pictures of rep-PCR fingerprinting for actinobacterial and fungal isolates shown on high resolution agarose gels (a) BOX PCR for actinobacterial isolates. Lanes: 1 - 3 = phylotype 102, 4 = phylotype 104, M = 1 kb molecular weight marker (b) Inter-LINE PCR for fungal isolates. Lanes: 1, 2, 4 = *Trichoderma asperellum*, 3 = *Cylindrocarpon destructans*, M = 1 kb molecular weight marker.

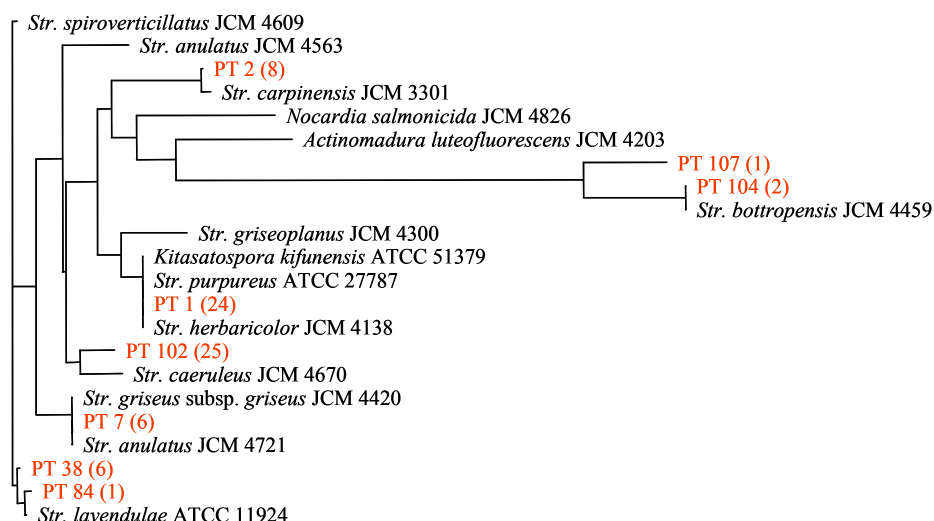


Figure 3.3: Neighbour joining tree of partial 16S rRNA gene PCR following the protocol of Kataoka *et al.* (1997). Nine phylotypes were differentiated and are shown in red, while reference strains are black. Numbers in brackets indicate isolation frequencies of the phylotypes isolated from rhizosphere soil. *Str* = *Streptomyces*, PT = phylotype, JCM = Japan Collection of Microorganisms, ATCC = American Type Culture Collection.

isolates, 18 of which showed a unique rep-PCR profile. Out of those 18 isolates 13 belonged to the group of moderate antagonists, while 5 were categorized as strong antagonists. No relationship between the fingerprint patterns and inhibition classes could be seen (Appendix Fig. C.4).

Phylotypes 2, 7 and 38 (Fig. 3.3) included a total of 20 isolates, showing six unique rep-PCR patterns. In detail, phylotype 2 consisted of one unique individual, phylotype 7 of two unique individuals and phylotype 38 of three unique individuals (Fig. C.1 - C.3). While phylotypes 2 and 38 exhibited only moderate inhibition of *P. citricola*, all isolates belonging to phylotype 7

Table 3.1: Distribution of different actinobacterial phylotypes between treatments.

phylotypes	total	CO_2	ambient
PT 1	24	13	11
PT 2	8	0	8
PT 7	6	0	6
PT 38	6	5	1
PT 84	1	1	0
PT 95	1	1	0
PT 102	25	15	10
PT 104	2	0	2
PT 107	1	1	0
not identified	2	2	0
total	76	38	38

PT = phylotype

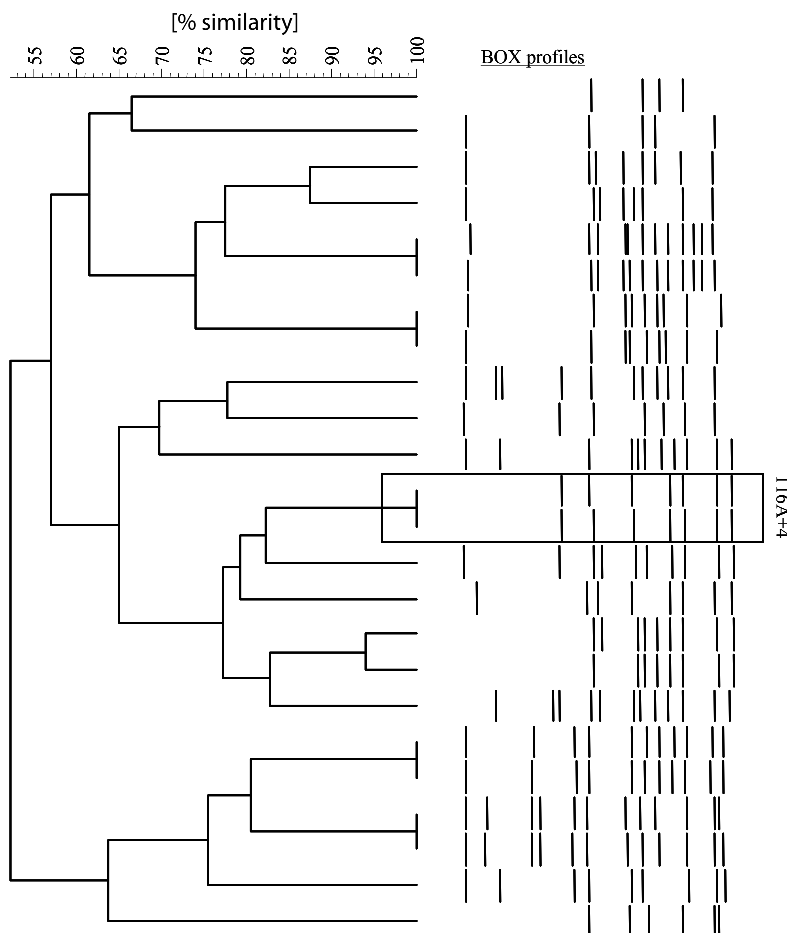


Figure 3.4: UPGMA dendrogram for BOX fingerprints of *Kitasatospora* isolates. All isolates were strong antagonists. Isolate 116A+4 was chosen for metabolite analysis.

were considered strong antagonists.

Phylotype 104 (Fig. 3.3) consisted of two isolates both of which had a unique rep-PCR pattern. While one isolate was identified as a strong antagonist the other one showed moderate inhibition. Finally, there were three phylotypes (84, 95, 107) which were only present once (Fig. C.5). All three showed moderate inhibition of *P. citricola*.

When looking at the distribution of the different phylotypes between the two treatments, the picture was not as homogeneous as compared to the distribution of inhibition classes. While the common phylotypes 1 and 102 were distributed equally between the treatments, all rare phylotypes were more or less isolated from only one treatment (Tab. 3.1). Due to the low isolation frequency for single phylotypes statistical analysis could not be performed.

3.1.3 Isolation of fungi from beech fine roots and confrontation tests

Fungi were isolated from fine roots of European beeches using a medium favoring the genus *Trichoderma*. In total 220 fungi were isolated 25 of which exhibited characteristic growth morphology of the genus *Trichoderma*. All fungi belonging to this group had the capability of overgrowing *P. citricola* and reducing or stopping the growth of the pathogen. Yet, the type of interaction between those fungal antagonists and *P. citricola* varied. Isolates that quickly overgrew *P. citricola* and completely inhibited further growth, also led to a collapse of the oomycetous aerial mycelium (*T. asperellum* and *viridescens*).

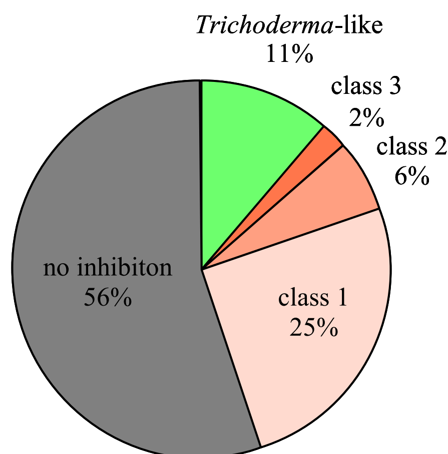


Figure 3.5: Isolation frequencies of the different groups of isolated fungi. Class 1 = weak inhibition, class 2 = moderate inhibition and class 3 = strong inhibition.

Isolates that overgrew slowly and did not completely inhibit the pathogen, did not cause the aerial mycelium to collapse (*T. harzianum*). *T. citrinoviride* resembled an intermediate type, which slowly overgrew *P. citricola* and caused a collapse of the mycelium after longer incubation time. (Fig. 3.6a). Growth of *P. citricola* was not inhibited by the antagonists before colonies were overgrown.

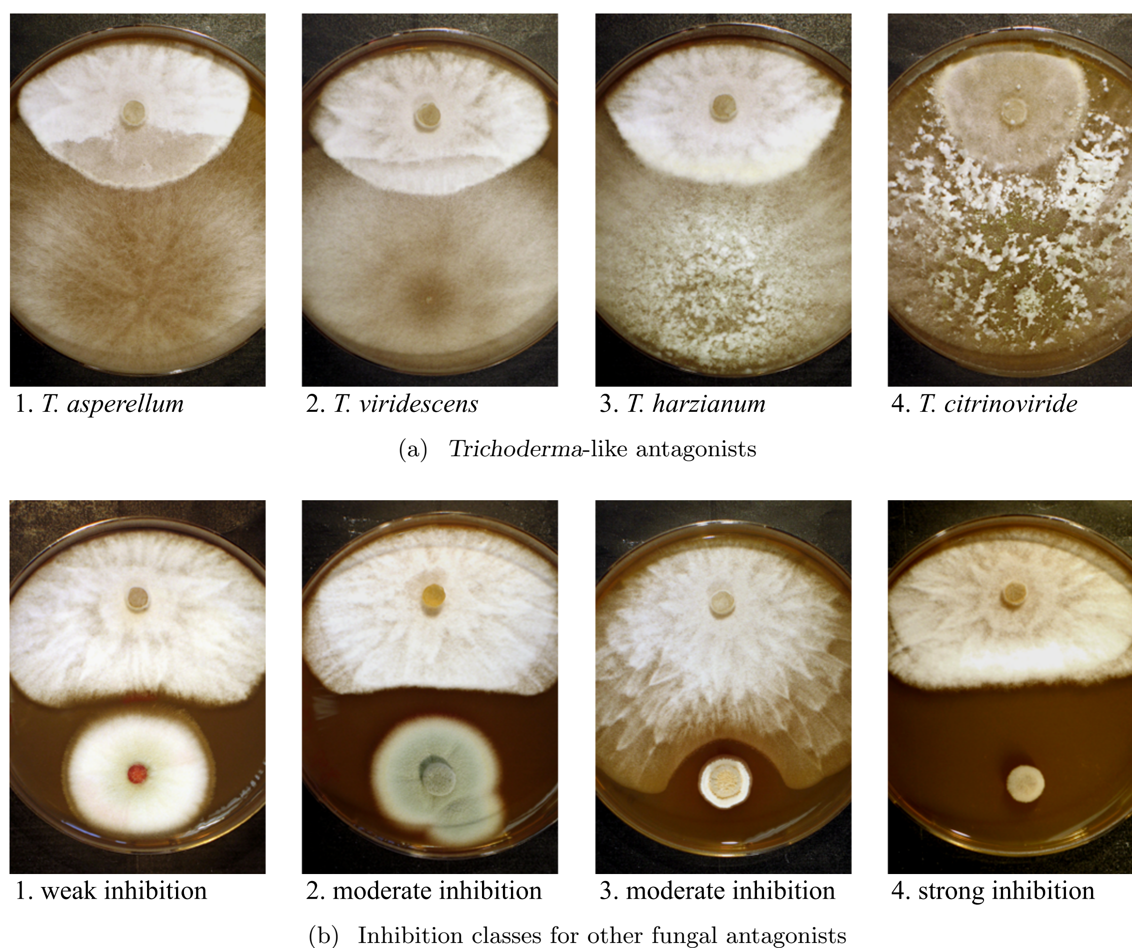
Of the remaining isolates 121 showed no inhibition of *P. citricola*. 55 isolates exhibited weak inhibition, 13 moderate and 5 isolates showed strong inhibition of the pathogen (Fig. 3.6b). Isolation frequencies for all isolates are shown in Figure 3.5. All confrontation tests with non *Trichoderma*-like fungi that showed no inhibition, resulted in deadlock systems. This type of interaction was characterized as two organisms that did not overgrow each other *in vitro*.

3.1.4 Characterization of fungal isolates

All inhibiting fungal isolates, excluding weak antagonists, were dereplicated utilizing rep-PCR (Inter-LINE, see Fig. 3.2b) and subsequently identified by sequencing of the ITS region. Based on a threshold of 90% similarity 20 unique isolates were identified and the corresponding ITS regions sequenced (NCBI accession numbers: EU139033-EU139057). Taxon identification was carried out using the oligonucleotide BarCode *Trich*OKEY online tool version 2.0 for *Trichoderma* species and NCBI BLAST for all other fungi. In total nine rep-PCR phylotypes were

found for *Trichoderma*-like fungi. Three phylotypes belonged to the taxon *T. asperellum* (16 isolates), four to the taxon *T. harzianum* (7 isolates) and one to each taxon *T. citrinoviride* and *T. viridescens* (Fig. 3.7). Each of the latter were isolated only once.

The remaining 18 fungal isolates were grouped into 11 rep-PCR phylotypes. Seven of those phylotypes (12 isolates) could be classified as belonging to the genus *Penicillium*. Two had the closest hit *P. janthinellum* ATCC 4845, three *P. ochrochloron* NRRL 926 and the remaining two phylotypes had close hits that were only identified to the genus level *Penicillium* (Fig. C.6). Five of the remaining isolates had three unique rep-PCR profiles and were classified as *Cylindrocarpon*



(a) *Trichoderma*-like antagonists

(b) Inhibition classes for other fungal antagonists

Figure 3.6: Growth in confrontation tests with fungal isolates (a) Examples of all *Trichoderma* species isolated from beech fine roots. For pictures 1 and 2 (*T. asperellum* and *T. viridescens*) collapse of the oomycetous mycelium was observed. *P. citricola* was overgrown rapidly. For *T. harzianum* (picture 3) no collapse of *P. citricola* was observed. *T. citrinoviride* (picture 4) overgrew slowly yet after longer incubation (7 d) a collapse of the oomycetous mycelium could be seen. All pictures were taken after 4 d. (b) Inhibition classes for non *Trichoderma*-like fungi. 1 = weak inhibition (isolate not identified), 2 + 3 = moderate inhibition (2 = *Penicillium sp.*, 3 = *Geomyces pannorum*) and 4 = strong inhibition (*Cylindrocarpon destructans*). Pictures 1, 2 and 4 taken after 5 d, picture 3 taken after 7 d.

destructans (strain IFO31882 as closest hit) and the last isolate was classified as *Geomyces pannorum*. For the latter, no culture collection reference was given, therefore the identity of this isolate has to be taken with caution (accession number of the closest hit: AJ509872). For all BLAST results E-values were 0.0.

Concerning inhibition of *P. citricola in vitro*, all *C. destructans* isolates were considered strong as antagonists, while the remaining 13 isolates exhibited moderate inhibition (Fig. 3.6b). The distribution of isolation frequencies between different treatments is given in table 3.2. No effect of elevated CO_2 was detected.

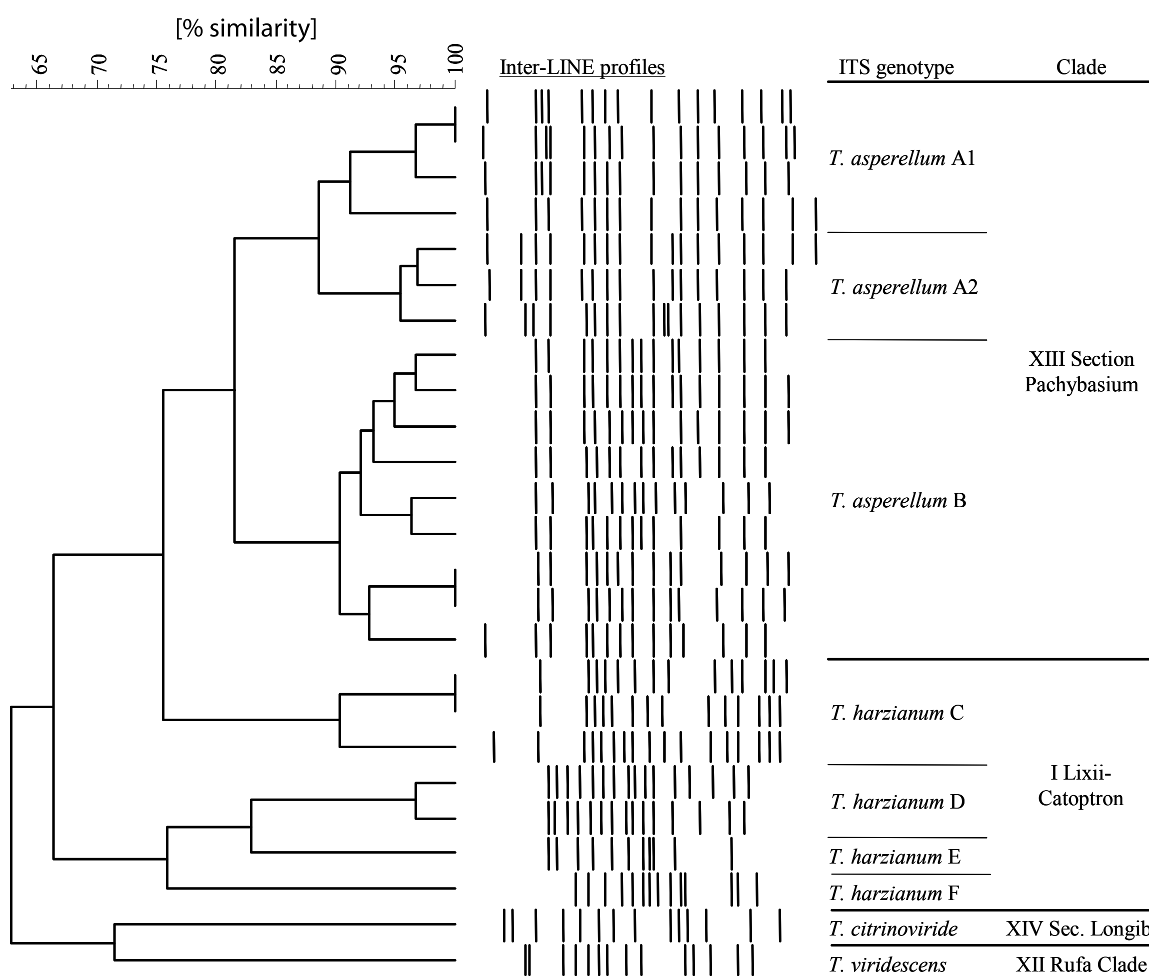


Figure 3.7: UPGMA dendrogram of Inter-LINE fingerprints for all *Trichoderma* isolates. Short vertical bars indicate 90% similarity between isolates. Long vertical bars delineate taxa (with corresponding *Trichoderma* clades) as identified by ITS sequencing and *Trich*OKEY version 2.0. Sec. Longibr. = Section *Longibrachiatum*.

Table 3.2: Distribution of isolated antagonistic fungi between treatments based on identification with *Trich*OKEY or NCBI BLAST.

Taxon	total	CO_2	ambient
Trichoderma spec.	25	12	13
T. asperellum	16	8	8
T. harzianum	7	2	5
T. citrinoviride	1	1	0
T. viridescens	1	1	0
Penicillium sp.	12	4	8
Cylindrocarpon destructans	5	2	3
Geomyces pannorum	1	0	1
total	43	18	25

3.1.5 Viability of *P. citricola* in interaction zones with *Trichoderma* spec.

To check survival of *P. citricola* in the interaction zones with *Trichoderma* isolates, plugs from these zones were incubated on *Phytophthora/Phytium* selective medium. All fast overgrowing isolates which inhibited growth of the pathogen upon contact (*T. asperellum* and *T. viridescens*), killed *P. citricola* on both tested media (V8 agar and Czapek agar). Only one phylotype (D) of *T. harzianum* was able to retard the growth of *P. citricola* from plugs on V8 agar and in one case stopped the pathogen from growing. *T. citrinoviride* killed *P. citricola* on V8 agar, but had no effect on the pathogen after seven days on Czapek agar (Tab. 3.3).

Table 3.3: Viability test of *P. citricola* in interaction zones with *Trichoderma* species.

Taxon	Inter-LINE	interaction		viability in interaction zone	
	phylotype	type I	type II	Czapek agar	V8 agar
T. asperellum	A1	+	-	-	-
T. asperellum	A2	+	-	-	-
T. asperellum	B	+	-	-	-
T. harzianum	C	-	+	+	+
T. harzianum	D	-	+	+	+/-
T. harzianum	E	-	+	+	+
T. harzianum	F	-	+	+	+
T. citrinoviride		-	+	+	-
T. viridescens		+	-	-	-

Interaction types: I = fast overgrowing and completely inhibiting *P. citricola* (Fig. 3.6a pictures 1 and 2), II = slowly overgrowing and inhibition of *P. citricola* was not always complete (Fig. 3.6a pictures 3 and 4). Two different media were compared.

3.2 Metabolite analysis

3.2.1 Detection and characterization of a bioactive compound (FT-ICR/MS)

To elucidate a mechanism of antagonism, actinobacterial isolate 116A+4 was chosen for metabolite analysis as a representative of phylotype 1. First, the presence and activity of the inhibiting substance was confirmed for the desalted, dried and in dH_2O redissolved culture supernatant. Biocontrol activity was still detectable in the prepared solution (Fig. 3.8) and therefore further analyses were performed.

When analyzing the desalted supernatant using the FT-ICR/MS in positive electrospray ionization (ESI) mode clear spectra with a high resolution of different peaks were obtained without further separation of the compounds, e.g. with HPLC (Fig. 3.9a). If the substances of interest are known and exact masses can be predicted, this method offers the possibility for a quick and accurate detection. In the present case, the substance in question was not known, therefore a fractionation of the sample had to be performed in order to narrow down the group of possible biocontrol active molecules.

As a quick and easy method for fractionation of the sample the elution procedure as described in section 2.5.1 was modified. Instead of eluting the complete sample at once with 100% methanol, increasing methanol concentrations in dH_2O were used. This rough fractionation was performed with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% methanol, in order to separate molecules on the basis of their polarity. Upon testing the dried and redissolved fractions on V8 plates as described in section 2.5.1, biocontrol activity could only be detected for the fraction eluted with 40% methanol. When analyzing fractions 30% - 50% with the FTMS three major peaks could be seen in fraction 40%. These peaks, m/z 282.1699, m/z 344.0918 and m/z 304.1519, were not present or only present in much lower intensities in the other fractions. The signal at m/z 282.1699 showed the highest intensity (Fig. 3.9b). Peak m/z 304.1519 fitted the m/z

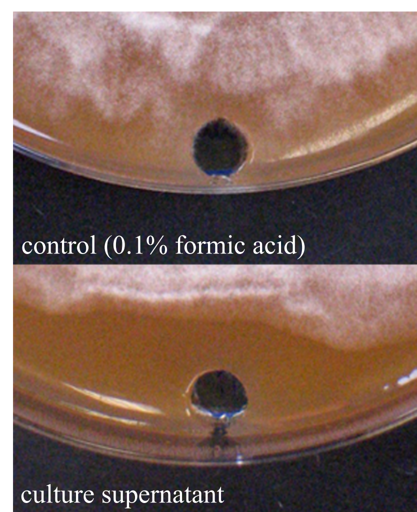
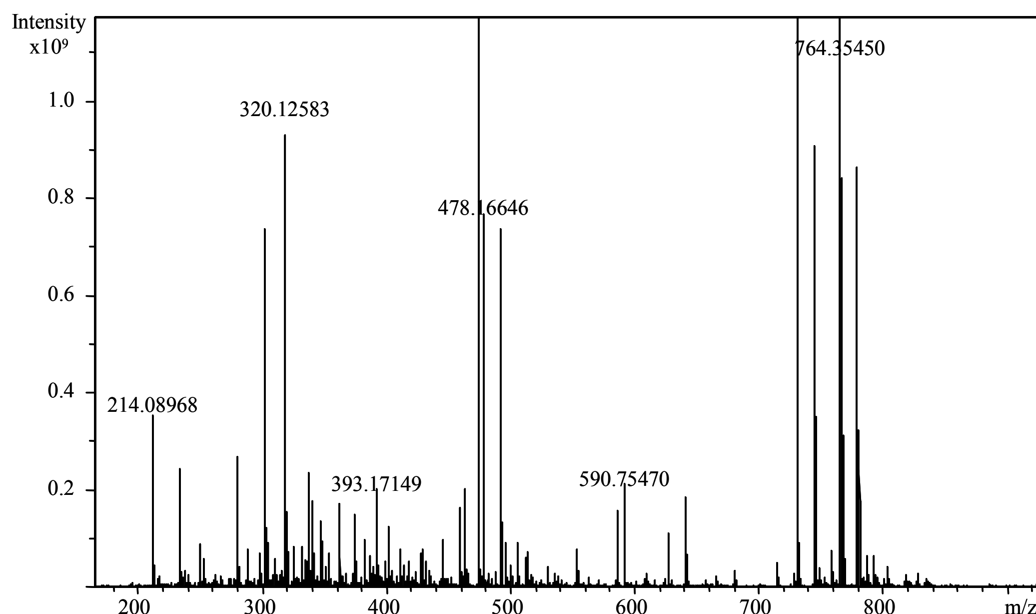
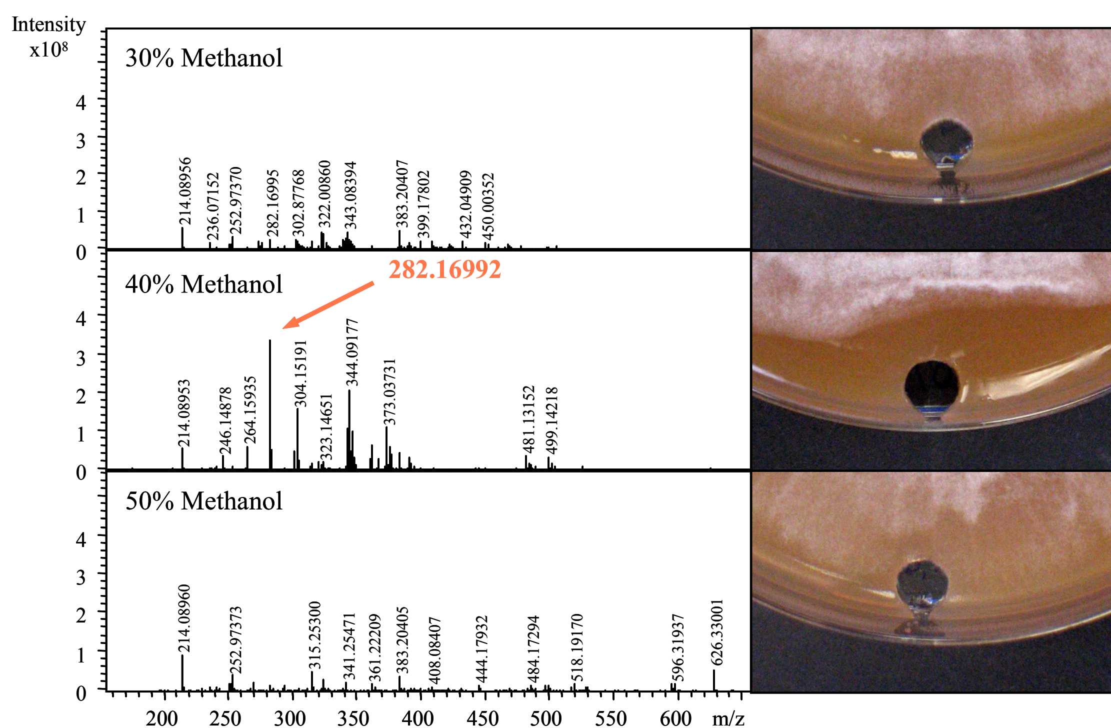


Figure 3.8: Occurance of biocontrol activity in isolate 116A+4 culture supernatant after desalting, drying and redissolving in dH_2O.



(a) Complete FT-ICR/MS spectrum of isolate 116A+4 culture supernatant



(b) FT-ICR/MS spectra of fractions eluted with 30%, 40% and 50% methanol

Figure 3.9: FT-ICR/MS spectra of isolate 116A+4 culture supernatant measured in positive electrospray ionization mode (a) Analysis of unfractionated culture supernatant. (b) Analysis of fractions eluted with 30%, 40% and 50% methanol. Dominant peak m/z 282.1699 is indicated in red. Pictures to the right show the inhibition of growth caused by fraction 40%, while no inhibition was seen for any other fraction.

expectations for the Na^+-adduct of m/z 282.1699:

$$304.1524 \text{ Da} = 282.1699 \text{ Da} + 22.9898 \text{ Da} - 1.0073 \text{ Da}$$

where 304.1524 Da is the expected molecular mass of the Na^+-adduct and 22.9898 Da and 1.0073 Da are the molecular masses of Na^+ and H^+ respectively. Taking this into account, only two major peaks were left as candidates for the biocontrol active substance.

When submitting the expected elemental formula for peak m/z 282.1699, $C_{15}H_{23}NO_4 + H^+$, to the ChemIDplus Advanced database the best hit was the macrolide polyketide cycloheximide. This substance is a known antibiotic against a wide variety of eukaryotic organisms produced by *Streptomyces* species and therefore a reasonable molecule being responsible for the inhibition of *P. citricola*. Interestingly, it has not been described to be produced by strains belonging to the genus *Kitasatospora* so far.

Different expected elemental formulae for m/z 344.0918 were also submitted to the ChemIDplus Advanced database, but no reasonable hit was identified. No substance with known biological activity could be found.

Inhibition of *P. citricola* by a pure cycloheximide solution was tested on agar plates as described for testing culture supernatant (section 2.5). Inhibition of the growth of *P. citricola* could be shown when 2 μg of cycloheximide in 200 μL dH_20 were applied to plates (recommended working concentration by the manufacturer = 10 μg per mL).

3.2.2 Identification of the bioactive compound (NMR)

Analysis with FT-ICR/MS and an inhibition test with pure cycloheximide indicated this polyketide to be the biocontrol active substance, therefore ^{1}H NMR was applied to verify this hypothesis. As analytical standard cycloheximide (PESTANAL®, Riedl-de Haën, Seelze) was used. Comparison of ^{1}H NMR spectra of microbial extracts with methanol-d4 solution of cycloheximide acquired under identical conditions confirmed the identity of the complex ^{1}H NMR spectral patterns (Fig. 3.10). Considering the fair visibility of several relevant, non-superimposed NMR resonances derived from cycloheximide, further NMR characterization (e. g. by two-dimensional NMR spectroscopy) was considered not necessary.

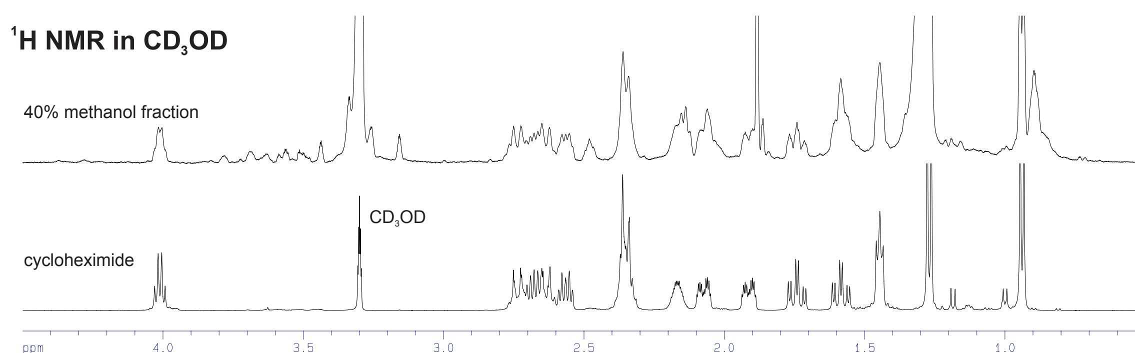


Figure 3.10: Comparison of ^{1}H NMR spectra of cycloheximide standard and the bioactive fraction obtained from eluting with 40% methanol.

3.3 Validation of specific primer sets for structural and functional analyses

3.3.1 Actinobacteria 16S rDNA primers

Validation of different Actinobacteria-specific 16S rDNA primers

In order to analyze the actinobacterial community in beech rhizospheres with PCR based methods, a suitable pair of primers had to be found. First, an *in silico* comparison of published Actinobacteria-specific primers was performed using the PROBE_MATCH function of the Ribosomal Database Project (RDP) online tool (http://rdp.cme.msu.edu/) version 9.54. Zero missmatches were allowed and default parameters were used (Tab. 3.4).

From the table shown, large differences concerning the specificity of the different primers can be seen. The most narrow potential amplification range was given for primer F243 designed by Heuer *et al.* (1997). This primer matched only 5862 actinobacterial sequences, most of which belonged to the genus *Streptomyces*. On the other hand, primers like Act283f which potentially amplify the most actinobacterial templates submitted to the databases, are also likely to bind to a large variety of different organisms outside the Actinobacteria. Thus, using primers like this would only be feasible in combination with another group specific primer.

The most commonly used Actinobacteria-specific primer is forward primer F243, regardless of the limitations mentioned above. Additionally, the primer is also known to produce unspecific bands depending on the universal reverse primer used (Wawrik *et al.*, 2005), making it unsuitable for t-RFLP analysis in many cases. In the present study, F243 was tested with reverse primers 1401r and 1492r. In both cases, a band of the expected size could be seen after gel electrophoresis, as well multiple unspecific bands. Changing PCR conditions did not eliminate the problem, therefore a different pair of primers had to be used.

Table 3.4: Comparison of Actinobacteria-specific primers.

Taxon	Act1360r	Act283f	S-C-Act -878-a-A-19	S-C-Act -235-a-S-20	F243	AB1165r
Aquificae	0	227	235	0	0	0
Thermotogae	0	32	1	0	0	0
Thermodesulfobacteria	0	90	94	0	0	0
Deinococcus-Thermus	0	28	3	0	0	0
Chloroflexi	0	4	1	1	0	1
Thermomicrobia	0	0	1	1	0	0
Nitrospira	0	1	512	2	1	0
Deferribacteres	0	159	17	0	0	0
Cyanobacteria	0	0	38	1	0	2
Proteobacteria	98	100	2406	59	16	117
Firmicutes	19	1503	1537	11	0	153
Actinobacteria	10325	22076	16994	19788	5862	14079
Planctomycetes	1	6	14	3	1	2
Spirochaetes	0	1227	0	0	0	0
Fibrobacteres	0	0	0	0	0	0
Acidobacteria	10	132	428	3	1	2
Bacteroidetes	6	3	11	3	0	5
Fusobacteria	0	444	0	0	1	0
Verrucomicrobia	1	2	1	563	19	2
Dictyoglomi	0	6	6	0	0	0
Gemmatimonadetes	1	1	389	35	0	1
G. i. s. BRC1	0	0	1	0	0	0
G. i. s. OP10	0	0	0	4	0	0
G. i. s. TM7	2	0	0	0	0	1
G. i. s. WS3	0	30	5	0	0	0
putative Chimera	0	0	0	2	0	0
unclassified Bacteria	53	1286	1529	260	28	90
total	10516	27357	24223	20736	5929	14455
% Actinobacteria	98.7	84.7	74.9	96.6	99.3	98.0

Information obtained using the PROBE_MATCH function of the Ribosomal Database Project.

Date: 17.08.2007

Zero missmatches were allowed.

G. i. s. = Genera incertae sedis.

For all other primers tested here, it has been described that non-actinobacterial 16S rDNA was also amplified from environmental samples in the original studies (Lüdemann & Conrad, 2000; McVeigh *et al.*, 1996; Stach *et al.*, 2003). Primers S-C-Act-235-a-S-20 and Act1360r were chosen, since both are highly specific for the class Actinobacteria (1.3% and 3.4% of non-actinobacterial hits, Tab. 3.4) and should complement each other concerning the exclusion of non-actinobacterial groups. A minor drawback of primer Act1360r was that it gave 1 missmatch for 55% of the sequences from the family Streptomycetaceae, while all other primers had zero missmatches for more than 98% of all sequences submitted from this familiy when checked with the PROBE_MATCH function. But when 1 missmatch was allowed all primers had more than 98% hits within the family.

PCR amplification with this primer pair from rhizosphere DNA showed a clear band with

the expected size of ∼1100 bp. No unspecific bands were amplified. When testing the primers on single cultures, for all 27 actinobacterial strains tested, equal amplificiation of the target could be shown. These strains represented a wide variety of phylogenetically diverse Actinobacteria strains, including all nine phylotypes of *Streptomyces* and *Kitasatospora* isolated during the course of this study (Tab. 3.5). 24 non-actinobacterial reference strains were chosen to represent the most important bacterial phylogenetic lineages in soil, including α-, β-, γ-Proteobacteria and Firmicutes, as well as two Archeae (Tab. 2.2).

Table 3.5: Validation of specificity of Actinobacteria 16S rDNA primers.

Species	Strain code / phylotype	Actinobacteria 16S PCR	PKS Type II
Arthrobacter citreus BI90		+	+
Arthrobacter globiformis	DSM 20124	+	-
Bifidobacterium animalis subsp. *lactis*	DSM 10140	+	-
Cellulomonas biazotea	DSM 20112	+	-
Cellulomonas flavigena	DSM 20109	+	+
Corynebacterium efficiens	DSM 44549	+	-
Corynebacterium glutamicum	DSM 20300	+	-
Curtobacterium citreum	DSM 20528	+	-
Curtobacterium luteum	DSM 20542	+	-
Frigoribacterium faeni	DSM 10309	+	-
Nocardia carnea	DSM 43397	+	-
Nocardioides simplex	DSM 20130	+	-
Pseudoclavibacter helvolus	DSM 20419	+	-
Rathayibacter rathayi	DSM 7485	+	-
Rathayibacter tritici	DSM 7486	+	-
Rhodococcus fascians	DSM 20669	+	-
Streptomyces anulatus	DSM 40361	+	+
Streptomyces griseus subsp. *griseus*	DSM 40236	+	-
Kitasatospora sp. 1164A+4	PT 1	+	+
Streptomyces sp. 116A-7	PT 2	+	+
Streptomyces sp. B118C-6	PT 7	+	+
Streptomyces sp. 217B-2	PT 38	+	-
Streptomyces sp. 216A-7	PT 84	+	+
Streptomyces sp. 217B-4	PT 95	+	+
Streptomyces sp. A218B-4	PT 102	+	-
Streptomyces sp. B118B-4	PT 104	+	+
Streptomyces sp. A218A-1	PT 107	+	-
non-actinobacterial references* (24 strains)		-	n. t.

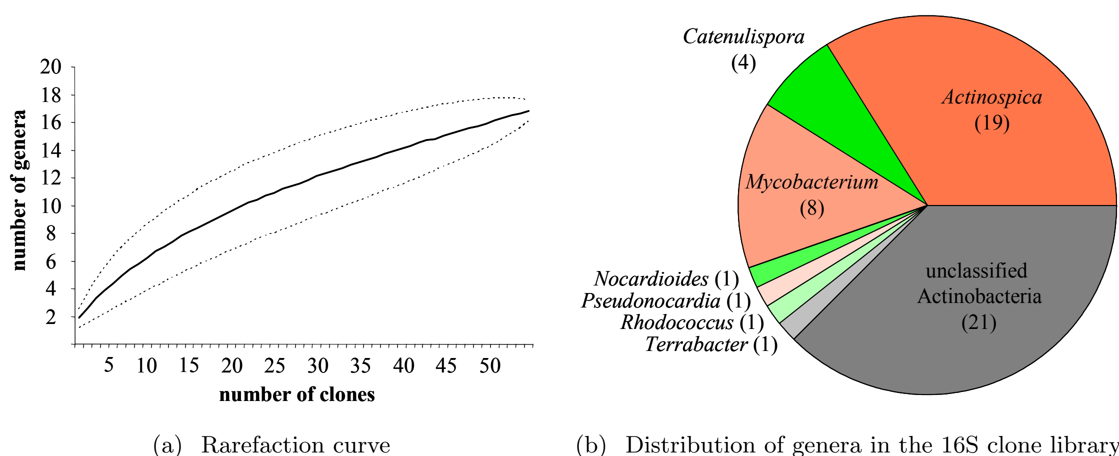
* for detailed information on non-actinobacterial reference strains refer to Tab. 2.2.
n. t. = not tested, PT = 16S rDNA phylotype.

Validation of 16S rDNA primer specificity by clone library

The specificity of the chosen primer pair S-C-Act-235-a-S-20/Act1360r was demonstrated by establishing a clone library from rhizosphere DNA from the main experiment. DNA was taken from an ambient sample, summer harvest, which was not inoculated with *P. citricola*.

In total, 58 clones were picked and analyzed. One of the clones did not contain a fragment of the correct size and another clone produced a mixed sequence. Therefore, 56 sequences were compared. 16S rRNA gene sequences were classified using the Ribosomal Database Project (RDP) classifier. 21 sequences could only be assigned to a genus with less than 95% probability (mostly below 60%) and were considered as unclassified. Nevertheless, these sequences were assigned to the phylum Actinobacteria by the RDP classifier, confirming the specificity of the chosen primer set. The remaining clones belonged to the genus *Actinospica* (19 clones), *Mycobacterium* (8 clones), *Catenulispora* (4 clones) and one clone of each genus *Nocardioides*, *Pseudonocardia*, *Rhodococcus* and *Terrabacter* (Fig. 3.11b). Clones belonging to the genera *Streptomyces* and *Kitasatospora* were not found in the library.

A rarefaction analysis was performed in order to estimate if the number of analyzed clones was sufficient to represent the diversity in the environmental sample. All clones were grouped according to the genus assigned to them by the RDP classifier. For unclassified clones, groups corresponding to the genus with the nearest hit were used. For the 56 clones the rarefaction curve did not reach a plateau (Fig. 3.11a), indicating that an increase in clones to analyze is



(a) Rarefaction curve

(b) Distribution of genera in the 16S clone library

Figure 3.11: 16S clone library (a) Analytic rarefaction analysis showing the number of different bacterial genera plotted as a function of number of clones. Dotted lines indicate 95% confidence intervals. (b) Distribution of genera in the 16S clone library. Genera were defined as unclassified, if prediction probability by RDP classifier was below 95%. Numbers of clones in the library are written in brackets.

necessary to reflect the full diversity of the community. All sequences were submitted to the NCBI database (accession numbers: EU138966-EU139021).

3.3.2 Polyketide synthase (PKS) specific primers

In this study, the macrolide polyketide cycloheximide has been found to be responsible for the inhibition of the growth of *P. citricola* by isolates belonging to the genus *Kitasatospora* (section 3.2). This genus was the most commonly isolated strong actinobacterial antagonist. Therefore, it was hypothesized that the diversity of genes responsible for the production of polyketides in soil could act as an indicator for the antagonistic potential of the community.

Validation of different PKS specific primer sets

During the last years a number of primer pairs have been described, which amplify different parts of either type I or type II PKS systems. In most cases these primers have been tested on pure cultures and not environmental DNA.

An exception is the primer set 540f-PKS/1100r-PKS for PKS type II systems by Wawrik *et al.* (2005), which has been designed for amplification from soil DNA. The suitability of these primers for rhizosphere DNA used in this study was tested and a band of the expected size of ~550 bp was amplified with no unspecific bands (Fig. 3.12). When applying this system to actinobacterial pure cultures, amplicons of the correct size were amplified for *Arthrobacter citreus* BI90, *Cellulomonas flavigena* DSM 20109 and *Streptomyces anulatus* DSM 40361. From the nine phylotypes of *Streptomyces* and *Kitasatospora* isolated during the course of this study, six produced an amplicon of the expected size (Tab. 3.5).

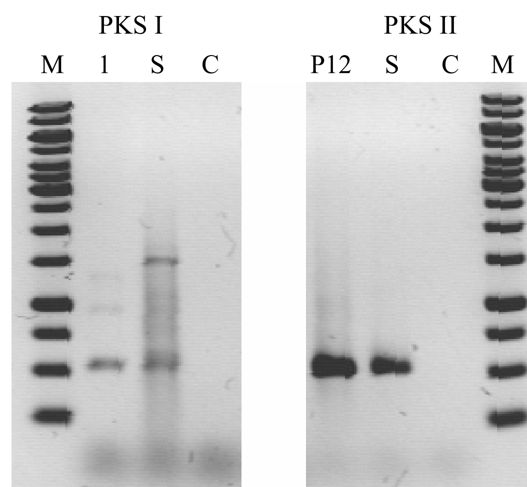


Figure 3.12: 1.5% agarose electrophoresis for generated PKS type I (primers ksf/ksar) and PKS type II (primers 540f-PKS/1100r-PKS) products. Lanes: 1 = isolate 116A+4, P12 = plasmid standard, S = rhizosphere DNA, C = no template control, M = 1 kb molecular weight marker.

Due to the very complex structures of Type I PKS systems, primer design is much more challenging than for type II systems. All primer pairs tested so far had only been used on pure culture DNA and in most cases produced bands of the expected sizes as well as unspecific bands,

making them unsuitable for ecological analyses like t-RFLP or qPCR. To find a suitable primer pair, first, amplifications were performed for pure culture DNA of *Kitasatospora sp.* 116A+4 and *Streptomyces anulatus* DSM 40361. Primer sets tested were KS-BEf/KS-BEr and K1f/K2r (Ayuso *et al.*, 2005), ksf/atr and ksf/ksar (Chuck *et al.*, 2006) as well as the primer pair K1f/M6r (Ayuso-Sacido & Genilloud, 2005, Tab. 2.3).

The combination K1f/M6r gave clear bands of the expected size of ∼1200 - 1400 bp for both organisms. Yet, the reverse primer M6r was designed for methyl-malonyl-CoA specific acyltransferase (AT) domains, excluding all systems utilizing malonyl-CoA as a starter unit, like cycloheximide (O'Hagan, 1995). A primer system having both binding sites within the keto synthase (KS) domain of the enzym complex should therefore be preferred for molecular ecological studies.

When combining primer K1f with the KS domain specific primer K2r a band of the expected size of ∼250 bp was amplified for *Kitasatospora sp.* 116A+4 but not for *Streptomyces anulatus* DSM 40361. Yet, for both organisms multiple bands of other sizes were amplified as well. An additional problem with this combination was, that the amplicon is likely to be too small for t-RFLP based diversity analysis.

Similar negative results were obtained for the primer combinations KS-BEf/KS-BEr and ksf/atr. Either multiple bands were present or no amplification occurred.

The primer combination ksf/ksar met most of the preconditions expected of a suitable primer pair. Its predicted PCR product had the size of ∼500 bp, being big enough for t-RFLP diversity analysis and still small enough to establish a qPCR SYBR green assay. Both binding sites are within the KS domain and a dominant band of the correct size was obtained from the tested isolates. When applying this PCR protocol to soil DNA a dominant band of the expected size was also obtained. Yet, the quality of the PCR product was not satisfactory. From the agarose electrophoresis a very strong smear could be seen and an additional band at ∼1500 bp was visible (Fig. 3.12). Changing the PCR conditions did not optimize the result to meet the quality requirements necessary for either t-RFLP analysis or qPCR assays. Nevertheless, it is to be expected, that by optimizing the primers ksf/ksar, it will be possible to perform culture independent analysis of PKS type I diversity and quantity in soil in the future. This, however, was beyond the scope of this study.

Due to the lack of a suitable primer pair for PKS of type I, further analyses were performed using only the PKS type II system 540f-PKS/1100r-PKS. A wide variety of aromatic polyketide

antibiotics are produced via the type II systems (e.g. actinorhodin, oxytetracycline) making them an interesting target as indicators for the biological control potential in soil.

Validation of PKS type II primer specificity by clone library

Analogous to the approach used for 16S rRNA gene analysis, a clone library was established to verify the specificity of the chosen primer pair 540f-PKS/1100r-PKS. In total 54 clones were sequenced, of which 51 sequences had polyketide type II KS-domains as closest hits when submitted to the NCBI database (blastn). The remaining three did not show any homology to known proteins when submitted to the database.

The nucleic acid codes were then translated into protein sequences and aligned as described in section 2.8. Upon translation it was discovered that two of the sequences included internal stop codons, hence they were very likely to be pseudogenes and were therefore excluded from the phylogenetic analysis (NCBI accession numbers: EU138920 and EU138948). For the remaining 49 sequences, two were only partially sequenced and could therefore not be included in the phylogenetic tree.

A maximum-likelihood tree (Fig. 3.14) was calculated for the 47 clone and 32 reference protein sequences. The references were obtained from the NCBI database and included sequences of known actinobacterial PKS type II biosynthetic clusters. Additionally, two outgroups were included, *fabB* (beta-ketoacyl-ACP synthase I) from *Escherichia coli* involved in fatty acid synthesis and a PKS type II from *Photorhabdus luminescens* TTO1 (γ-Proteobacteria), one of only two known PKS type II systems outside the Actinobacteria, responsible for the production of an anthraquinone pigment.

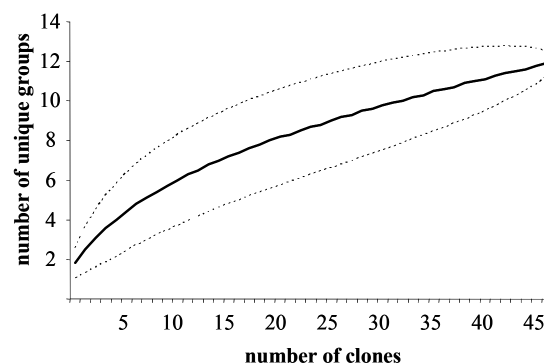


Figure 3.13: Analytic rarefaction analysis for the PKS type II clone library. Dotted lines indicate 95% confidence intervals.

When looking at the maximum-likelihood tree (Fig. 3.14) eleven groups could be differentiated on the basis of 95% similarity of the protein sequences. Of the two clones only partially sequenced, one showed a unique sequence and was therefore considered as own group for the following rarefaction analysis. Rarefaction analysis revealed a curve that did not reach a plateau (Fig. 3.13), implying that an increase in clone numbers would be necessary for an estimation

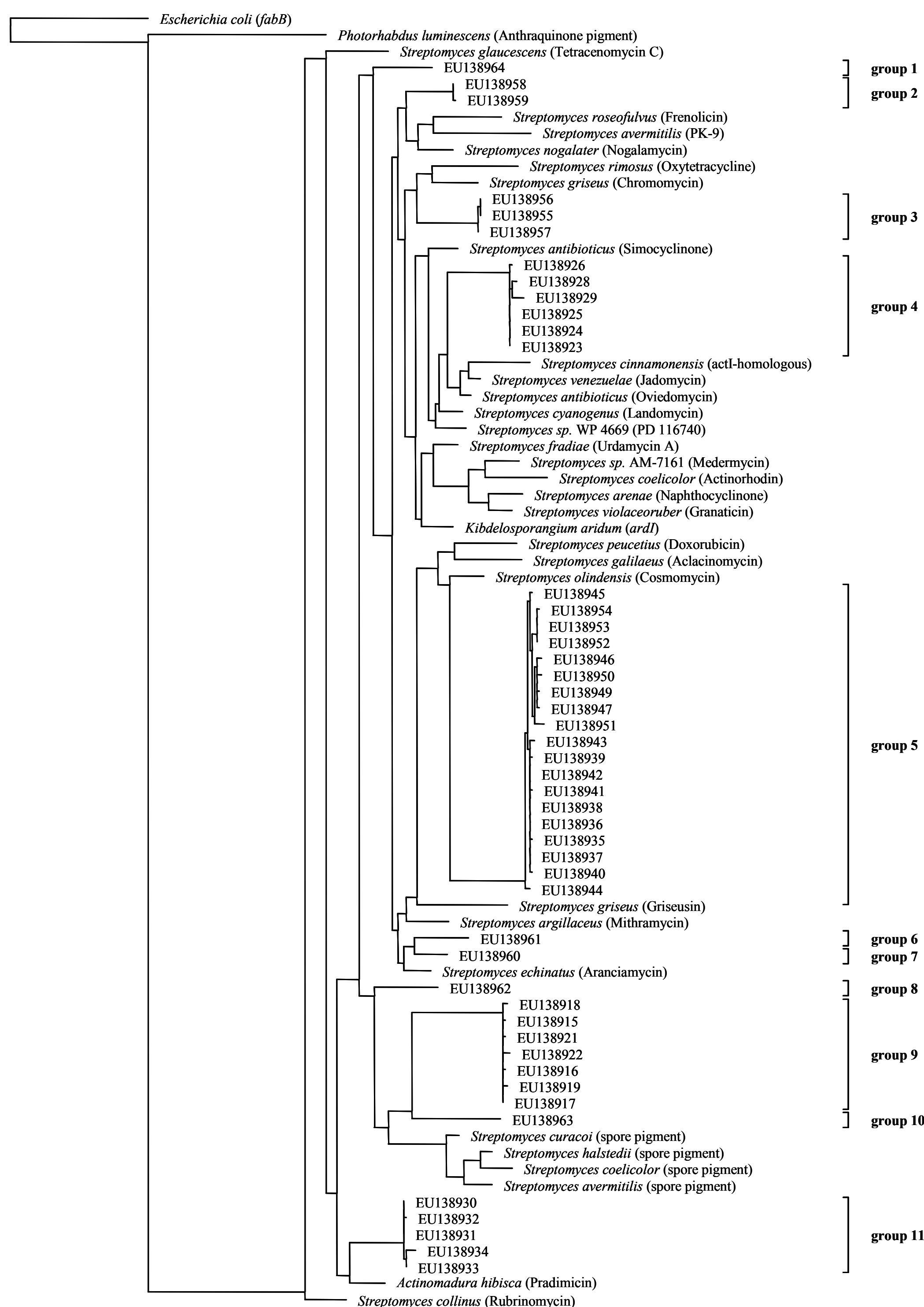


Figure 3.14: Maximum-likelihood tree based on partial PKS type II protein sequences (185 amino acid positions) from cultured polyketide producers with known PKS Type II sequences (NCBI database) and sequences from beech rhizosphere clone library (accession numbers indicated). Tree topology is supported by parsimony and neighbor-joining methods. Products of the reference PKS systems are given in parentheses. Groups are assigned on the basis of 95% similarity of the protein sequences. Bar indicates 10% dissimilarity in protein sequences.

of the total number of unique sequences present in soil. Sequences obtained from the clone library were very diverse as seen by the wide distribution of the different groups throughout the tree. Nine of the eleven groups clustered alone or close to known antibiotics producing PKS type II keto synthase domains (groups 1-8 and 11), while two groups clustered in close vicinity to known spore pigment producing keto synthase domains (groups 9 and 10). All sequences were submitted to the NCBI database (accession numbers: EU138915-EU138965).

3.4 Effects of elevated carbon dioxide, elevated ozone and inoculation with *P. citricola* on a plant-soil system

3.4.1 Plant growth

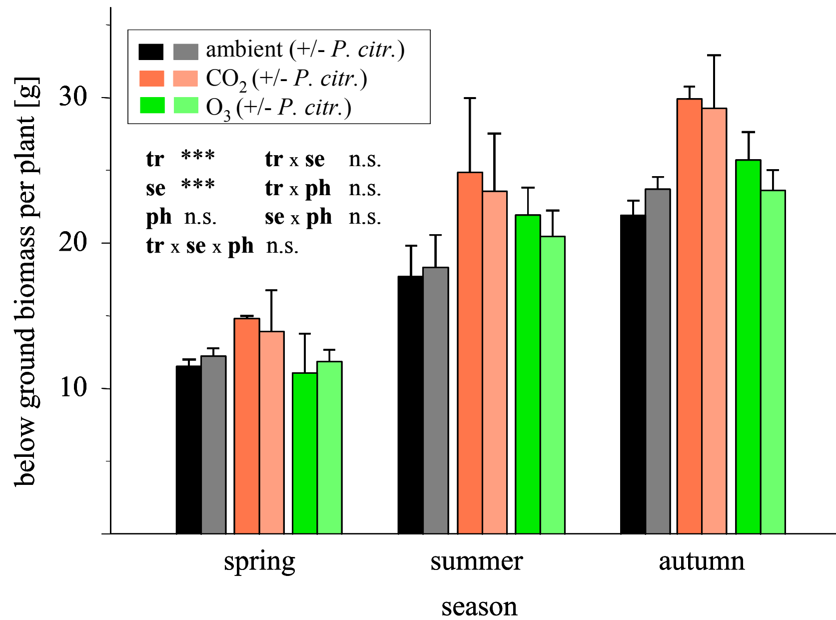
In order to evaluate effects of elevated O_3 and CO_2 treatments as well as seasonal effects on the plants, biomass data was obtained from the main experiment. As one sample the mean of three trees planted in each pot was considered a replicate. The most pronounced effect of the season and treatments was observed for below ground plant biomass. In spring it ranged for ambient treated plants from 11.06 - 12.71 g, for O_3 treated plants between 7.96 - 13.18 g and for CO_2 treated plants from 10.69 - 15.95 g per plant. In autumn below ground biomass had more than doubled in all treatments ranging for ambient conditions between 20.70 - 24.62 g, O_3 21.96 - 27.77 g and CO_2 conditions from 25.47 - 32.76 g per plant (see Fig. 3.15a). For statistical analysis data was log(10) transformed and multifactorial analysis of variance (ANOVA) and "a posteriori" multiple pair-wise comparisons (Tukey's HSD method) were applied.

Both, treatments ($p < 0.001$) and seasonal effects ($p < 0.001$) showed significant influence on the below ground biomass (Appendix Tab. B.1), whereas inoculation with *P. citricola* had no effect. When analyzing with pairwise comparisons according to Tukey's HSD method there was a significant difference between the pairs ambient - CO_2 and O_3 - CO_2, whereas differences between ambient and O_3 treated plants could not be shown. All harvesting time points varied significantly from each other.

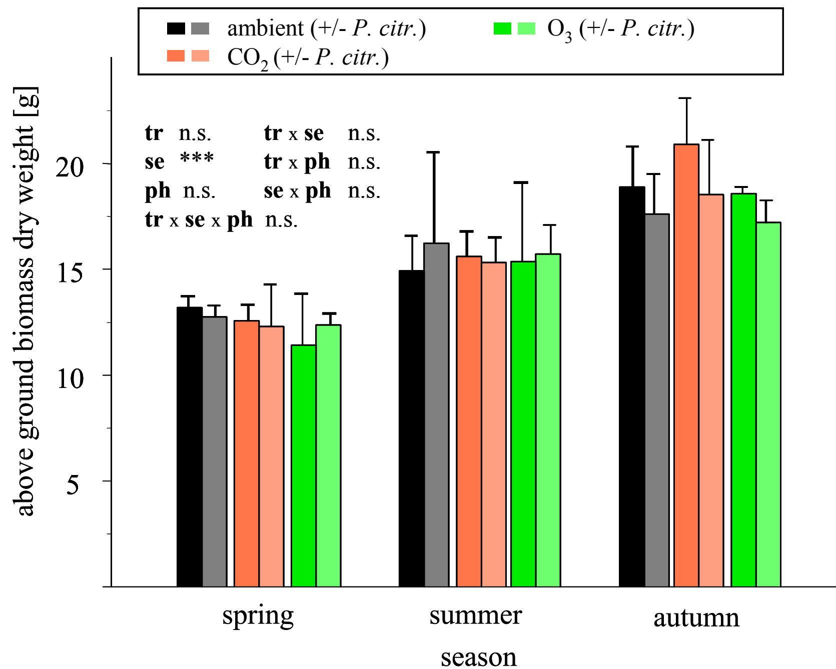
For total above ground biomass only seasonal variation showed a significant effect ($p < 0.001$) when analyzed by multifactorial ANOVA (Tab. B.2), with all seasons being significantly different from each other as detected by Tukey's HSD method. The data was log(10) transformed. In spring total above ground biomass for all samples ranged between 8.71 - 14.12 g, in summer from 12.18 - 21.13 g and in autumn from 15.87 - 22.41 g per plant (Fig. 3.15b). No additional information was obtained when leaf and woody biomasses were analyzed separately.

3.4.2 Soil microbial biomass

Microbial biomass carbon (C_{mic}) was measured as indicator for microbial biomass. Measurements were carried out for summer and autumn harvests of 2006 for all treatments. In summer C_{mic} values ranged from 664 mg per kg soil dry weight to 856 mg kg^{-1} soil. While both, ambient and O_3 treatments, showed values of around 700 mg kg^{-1} soil, measurements for CO_2 treat-



(a) Below ground biomass of beeches from main experiment



(b) Above ground biomass of beeches from the main experiment

Figure 3.15: Plant biomass of beeches from the main experiment (a) Dry weight of the below ground biomass per tree for harvests throughout the year 2006 with respect to the different treatments. (b) Dry weight of the above ground biomass per tree for harvests throughout the year 2006 with respect to the different treatments. Data represent means ± standard deviation, n=3 (nine plants in three pots) for each treatment combination. Factors: se = season, tr = treatment (O_3, CO_2, ambient), ph = *Phytophthora citricola* inoculation. The levels of significance for multifactorial ANOVA is given in each graph, n.s. = not significant ($p > 0.05$), *** $p < 0.001$.

ments were at an average C_{mic} value of 822 mg kg^{-1} soil. In autumn, all treatments were at the same C_{mic} levels as the CO_2 treated samples. C_{mic} values ranged from 730 - 951 mg kg^{-1} soil (Fig. 3.16). Multifactorial ANOVA (Tab. B.3) showed that the factor season had a significant influence ($p < 0.001$) on microbial biomass carbon, while different treatments did not ($p = 0.080$). Interaction between the factors season and treatment was significant ($p = 0.018$), highlighting the difference of the CO_2 treatment in summer compared to ambient and O_3 treatments, while no difference was seen in autumn (Fig. 3.16).

Inoculation with *Phytophthora citricola* showed no statistically significant effect.

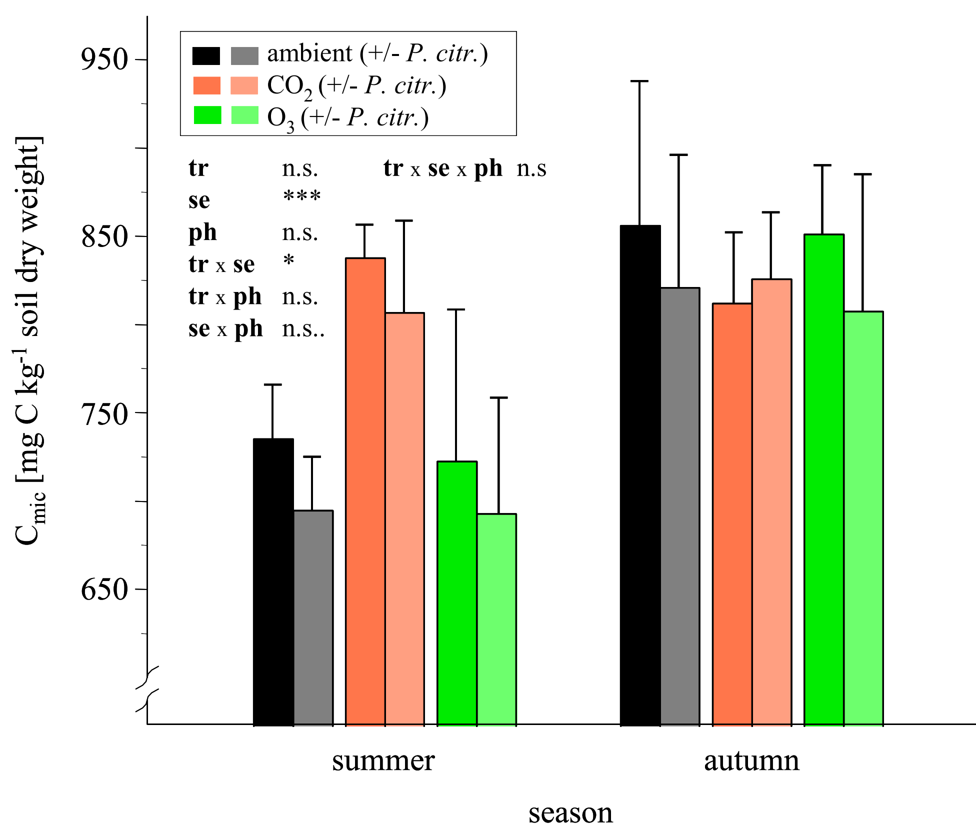


Figure 3.16: Microbial biomass C (C_{mic}) for harvesting time points summer and autumn from greenhouse experiment 2006 expressed in mg microbial C per kg dry weight rhizosphere soil. Data represent means ± standard deviation, n=3 (rhizosphere soil of three plants was pooled for one measurement per pot) for each treatment combination. Factors: se = season, tr = treatment (O_3, CO_2, ambient), ph = *Phytophthora citricola* inoculation. The levels of significance for multifactorial ANOVA is given, n.s. = not significant ($p > 0.05$), * $p < 0.05$, *** $p < 0.001$.

3.4.3 *Phytophthora citricola*

Two different methods were applied to provide evidence for the establishment of a *P. citricola* infection in beech fine roots.

First, a qualitative detection was performed. The pathogen was isolated from fine roots in autumn using *Phytophthora*/*Phytium* specific isolation agar PARPNH (section 2.1.3). Isolates were identified by growth morphology and utilizing a *P. citricola* specific PCR reaction (section 2.7.4). The isolations were successful for two of the inoculated O_3 treated plants. Identity of the isolates was confirmed (specific PCR yielded a fragment of the expected size of ~711 bp). From control plants and inoculated ambient and CO_2 treated plants no *P. citricola* was isolated.

The second method to analyze the infection level was to apply SYBR green quantitative real-time PCR. To check the purity of the DNA obtained by the applied extraction procedure, twofold dilutions of spiked fine root DNA was carried out (1:2, 1:4, 1:8) and quantified. All dilutions resulted in an expected ΔCt of ~1 cycle, therefore no inhibitory substances were present in the extracts. The efficiency of the PCR ranged between $E = 0.85$ and $E = 0.94$ (slope of the

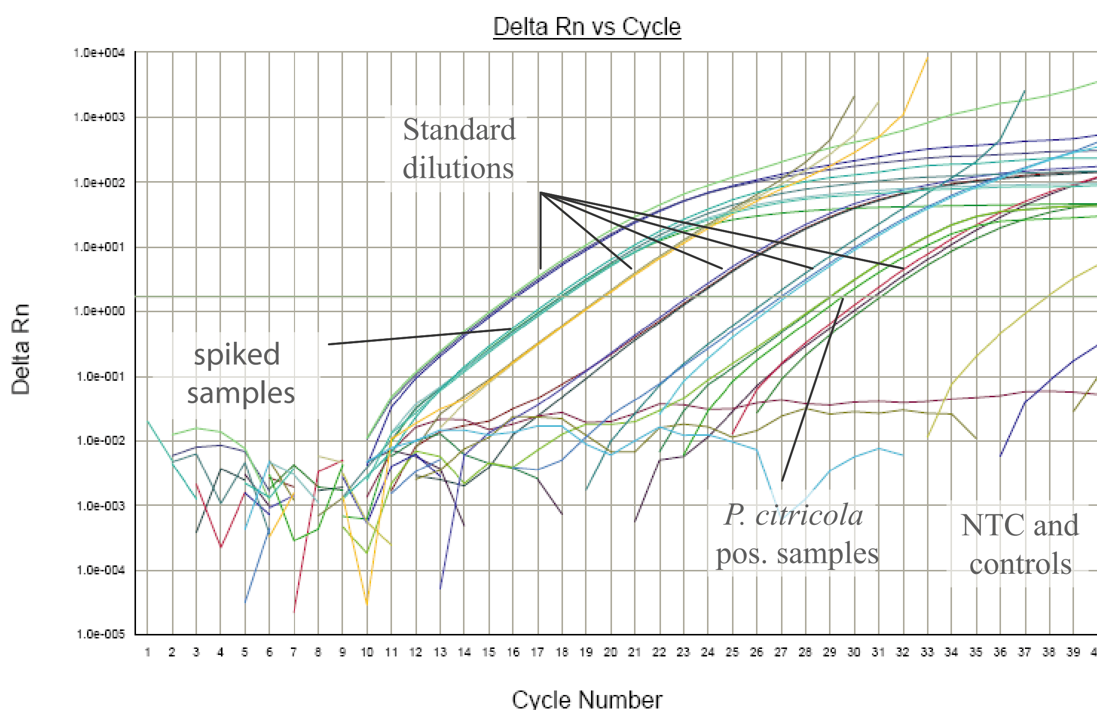


Figure 3.17: qPCR amplification plot for the ITS PCR product obtained with primers CITR1/CITR2 for the detection of *P. citricola* in beech fine roots. Plasmid standards were used in tenfold serial dilutions (final quantities: $2*10^5$ - 10^1). Amplifications from spiked root sample is shown as well as a sample with normal infection levels. Horizontal line indicates fluorescence threshold. NTC = no template control.

standard curve being between -3.47 and -3.73). The coefficient of determination was always $R^2 > 0.99$.

The results of the qPCR were highly variable, ranging from no detectable amplification for some of the inoculated samples to up to $5.9*10^6$ copies of ITS template per gram fine root in others (Fig. 3.18). Due to this variation no tendency for neither season nor trace gas treatments was observed for inoculated plants. Additionally, it was clear that there was a low level of natural *P. citricola* infection in the control plants at the beginning of the experiment (Fig. 3.18). The level of infection within these control pots increased in autumn. The statistical significance of the different factors on the infection rate were tested utilizing the Kruskal-Wallis rank sum test. Neither season nor treatment showed statistically significant effects, while inoculation with *P. citricola* was statistically significant ($p < 0.001$) (Fig. 3.18).

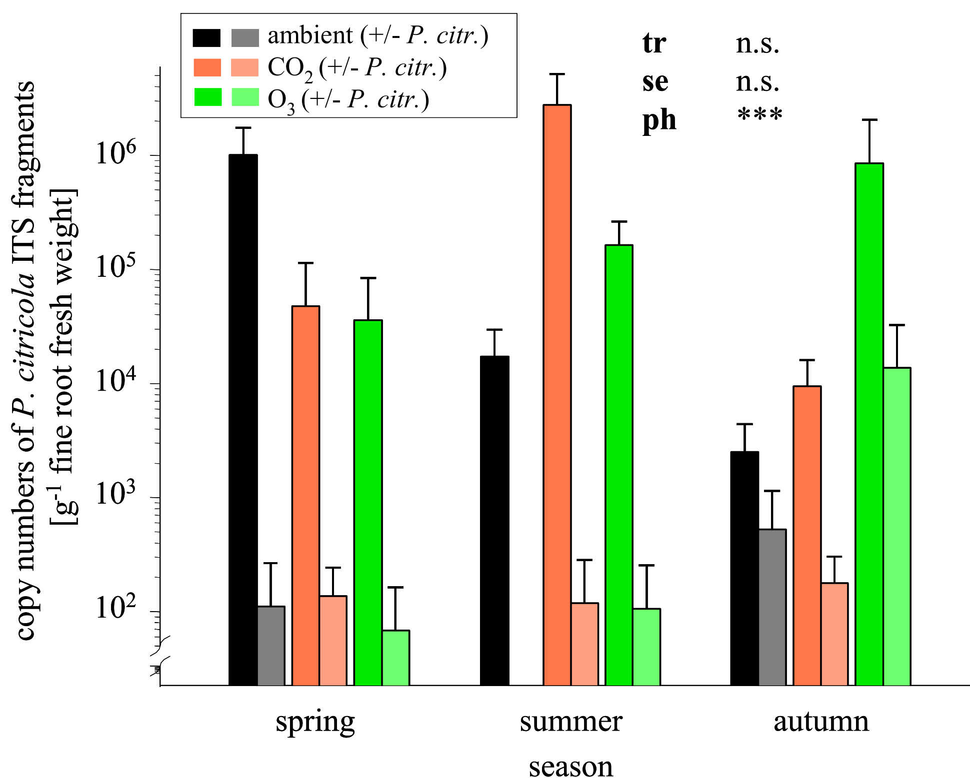


Figure 3.18: *P. citricola* distribution in beech fine roots using the primer pair CITR1/CITR2. Copy number quantity of the ITS template in the samples is expressed as copies per g fine root (fresh weight) and shown on a logarithmic scale. Error bars represent standard deviations. The levels of significance for Kruskal-Wallis rank sum test is given, n.s. = not significant ($p > 0.05$), *** $p < 0.001$.

3.5 Structural and functional diversity of actinobacterial rhizosphere communities

3.5.1 Actinobacterial structural diversity

From the 54 samples included in the analysis, 39 different t-RFs were identified after relativization and removal of background noise. Six of these t-RFs (15.4%) had an average peak height between 5-16.6%, nine (23.1%) between 0.5-5% and the majority of 24 (61.5%) were below 0.5%.

The over all frequency of the major t-RFs was very high. All 15 t-RFs between 0.5 and 16.6% relative peak height had a frequency of >95% throughout all samples. This indicated a rather homogenous composition of all samples concerning the major components of the t-RFLP profiles.

Non-metric multidimensional scaling

To visualize changes in actinobacterial 16S rDNA t-RFLP patterns, non-metric multidimensional scaling (NMS) was used as a method of ordinating the data. A two dimensional plot captured most of the variance in the t-RFLP profiles, with the first two dimensions containing 84.2% and 13.8% of the information in the analytical data set respectively (cumulative = 98.0%). To assess the quality of the ordination a "stress" value was calculated (Kruskal, 1964; McCune & Grace, 2002). The obtained value of 6.11 indicated that the ordination could be considered good with no real risk of drawing false inferences.

The most dominant effect seen in the NMS plot was the separation of samples collected in spring (upright triangles, Fig. 3.19). This effect was independent of the treatments (ambient, CO_2, O_3). The distribution of summer and autumn samples (circles and squares, Fig. 3.19) was more heterogeneous than that of spring samples. While no clear separation of summer and autumn harvests was visible for ambient and CO_2 treated plant rhizospheres, summer samples of the O_3 treatments appeared to be distinct. Inoculation with *P. citricola* did not result in any separation of samples in the NMS plot.

These observations were in agreement with the results of a non-parametric multivariate analysis of variance (PerMANOVA) and "a posterior" performed multiple pair-wise comparisons (Tab. B.4 and B.5). For the PerMANOVA, the effects of factors season and treatment were statistically significant (each $P = 0.0002$), while inoculation with *P. citricola* had no effect (Tab. B.4). When performing a multiple pair-wise comparison between the three levels of the

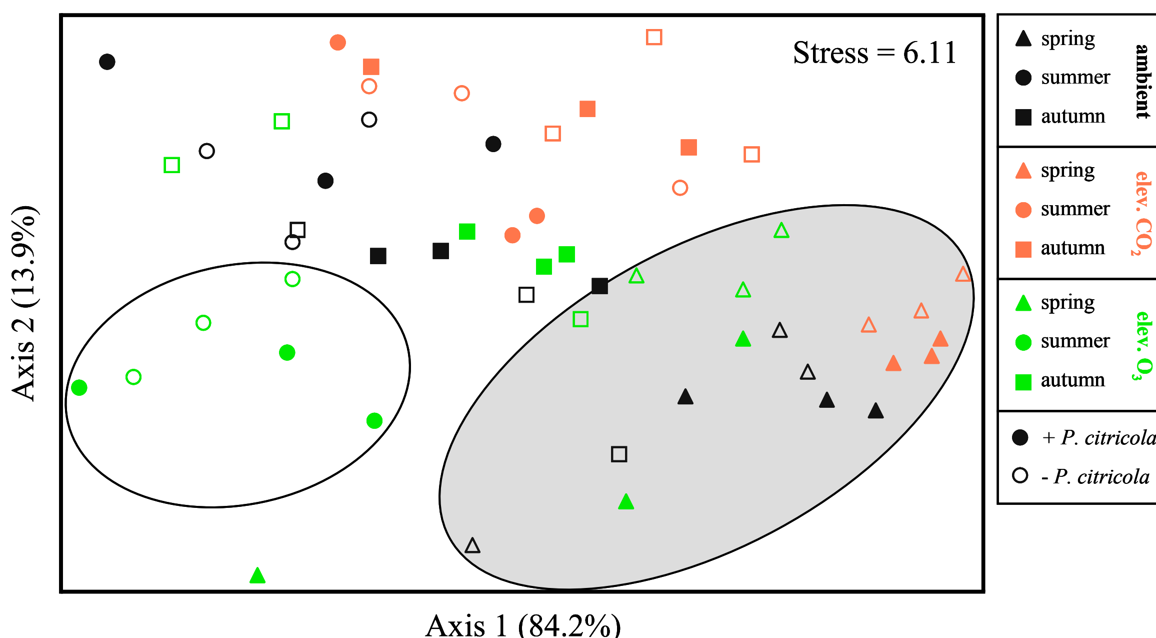


Figure 3.19: Non-metric Multidimensional Scaling (NMS) plot of 16S rDNA t-RFLP profiles for rhizosphere DNA of the greenhouse experiment 2006. Filled symbols indicate samples inoculated with *P. citricola*. Circles highlight clearly separating groups (gray = spring samples, transparent = ozone summer samples). Stress value is given according to Kruskal's stress formula 1 multiplied by 100.

factor season, it was apparent that all seasons were statistically significantly different from each other. For the three levels of the factor treatment, significant differences could be shown for ambient vs. CO_2 and CO_2 vs. O_3 samples, while ambient vs. O_3 samples exhibited no statistically significant separation (Tab. B.5). The separation of rhizosphere samples from ozone treated plants was statistically significant in summer ($P < 0.01$, Tab. B.6).

Indicator species analysis

In order to find t-RFs responsible for the separation of different groups, an indicator species analysis was performed (Dufrêne & Legendre, 1997; McCune & Grace, 2002). Groups chosen for a detailed analysis were, first, spring samples vs. autumn/summer samples and, secondly, a contrasting of the treatments (ambient, O_3, CO_2) for samples harvested in summer.

Since only very small peaks showed changes of their frequency throughout the samples, the more dominant t-RFs were expected to be characterized primarily by changes in abundance. T-RFs with a significant result in the indicator species analysis were further analyzed by means of a permutation based univariate ANOVA with "a posterior" multiple pair-wise comparison. The results for selected peaks are summarized in table 3.6.

Of the t-RFs tested positive for an indication of spring samples, t-RF 102 stands out as the most dominant peak (Tab. 3.6a). It can be considered as negative indicator for spring since the average relative peak height of this t-RF doubled from 10.7% in spring to 21.4% in summer, followed by a small reduction toward autumn to 17.6% (Fig. 3.20a). These differences were statistically significant between all groups ($P < 0.001$).

Other major t-RFs like peaks 69, 162, 226 and 380 were positive indicators of spring (Tab. 3.6a). Yet, the observed differences in relative abundance for those peaks were generally rather small and should therefore not be overinterpreted. Still it was obvious that a change in the overall composition of the actinobacterial rhizosphere community took place throughout the year.

When comparing the fragment sizes of the indicator t-RFs with peaks obtained from the clone library, it was possible to identify actinobacterial genera which were likely to be represented by those t-RFs. Organisms belonging to the family Catenulisporaceae (genera *Actinospica* and *Catenulispora*) were very likely to be responsible for the t-RF with the fragment size of 102 bp. While all clones belonging to the genus *Actinospica* had the same size, the four

Table 3.6: Results of indicator species analysis in combination with univariate ANOVAs on the selected t-RFs.

(a) Indicator species analysis contrasting spring vs. summer and autumn samples.

t-RF	IG	mean relative peak height within groups [%]			ANOVA	pair-wise comparisons		
		sp (SD)	su (SD)	au (SD)	P-value	sp vs. su	sp vs. au	su vs. au
69	sp	**10.418 (1.556)**	**7.987 (0.819)**	**7.629 (1.598)**	0.0002	0.0002	0.0002	0.4094
102	su	**10.716 (3.609)**	**21.427 (3.398)**	**17.640 (3.694)**	0.0002	0.0002	0.0002	0.0052
162	sp	**14.974 (1.485)**	**12.404 (1.651)**	**12.888 (1.470)**	0.0002	0.0002	0.0002	0.3644
226	sp	3.233 (0.363)	2.268 (0.543)	2.078 (0.715)	0.0002	0.0002	0.0002	0.3896
362	su/au	0.505 (0.176)	1.491 (0.954)	1.518 (0.805)	0.0002	0.0002	0.0002	0.9316
367	sp	2.668 (0.348)	1.824 (0.592)	1.974 (0.353)	0.0002	0.0002	0.0004	0.3702
380	sp	6.964 (0.907)	4.685 (0.942)	5.769 (1.259)	0.0002	0.0002	0.0032	0.0074

(b) Indicator species analysis contrasting ambient vs. CO_2 vs. O_3 samples in summer.

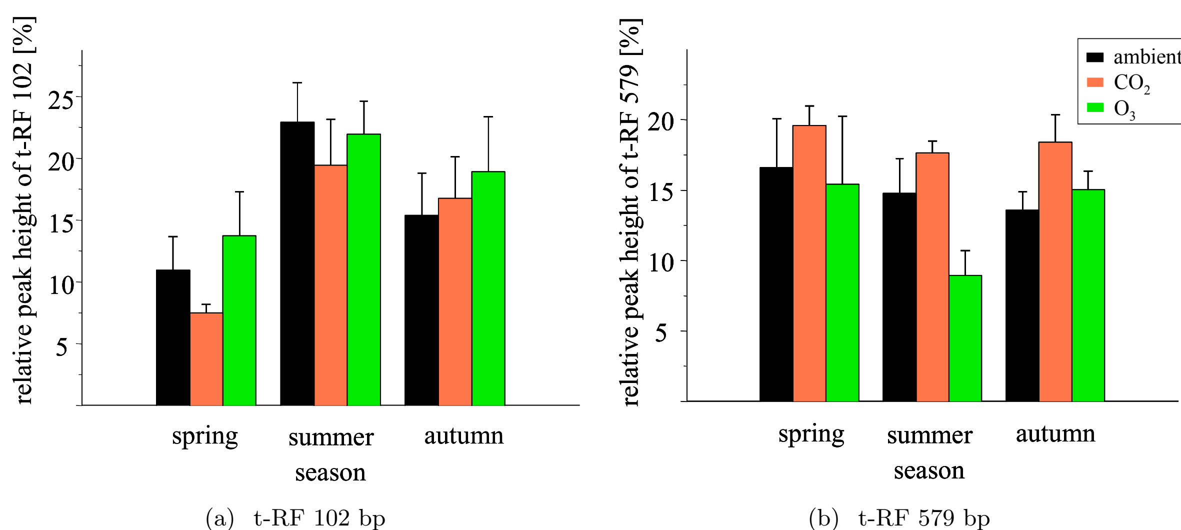
t-RF	IG	mean relative peak height within groups [%]			ANOVA	pair-wise comparisons		
		am (SD)	CO_2 (SD)	O_3 (SD)	P-value	am vs. CO_2	am vs. O_3	CO_2 vs. O_3
411	am	1.258 (0.339)	0.865 (0.194)	0.512 (0.151)	0.0002	*0.0256*	*0.0006*	*0.0056*
579	CO_2	**14.776 (2.473)**	**17.648 (0.849)**	**8.915 (1.801)**	0.0002	*0.0182*	*0.0008*	*0.0002*

Selected indicator peaks are shown. Values in bold highlight major peaks that were most likely to be responsible for the separation of the groups.
P-values for univariate ANOVA were obtained by 4999 permutations for each peak. P-values in italics were obtained using 4999 Monte Carlo samples from the asymptotic permutation distribution.
IG = contrast group indicating the group with the highest abundance of the peak, sp = spring, su = summer, au = autumn, am = ambient, SD = standard deviation.

Catenulispora clones produced three different t-RF fragment sizes (Tab. 3.7), with one corresponding t-RF (362 bp) following approximately the same seasonal shift that was observed for t-RF 102 (Tab. 3.6a).

In order to find t-RFs responsible for the separation of t-RFLP profiles from the ozone treated rhizosphere samples in summer, a similar analysis as for seasonal indicators was performed (Tab. 3.6b). In general, most of the t-RFs identified were only very minor peaks, with the exception of t-RF 579. This peak was less abundant in the rhizosphere of ozone treated plants. The ozone effect was statistically significant, but could only be seen at the summer harvest (Fig. 3.20b). Therefore the separation of ozone treated samples during summer was very likely due to a change in the abundance of this peak. t-RF 411 was a second good, yet only minor, indicator showing statistical significance for different treatments (again especially O_3 as a negative indicator).

None of the classified clones from the 16S rRNA gene library corresponded to neither t-RF 579 nor 411. Yet, a group of unclassified clones were shown to have a t-RF of 578 bp (data not shown). Also, isolates belonging to the genera *Kitasatospora* and *Streptomyces* gave signals at 411 and 409 bp respectively (Tab. 3.7) and were therefore probably represented by this peak in the profiles. Considering this, t-RF 411 was surprisingly small compared to the high isolation frequency of the genera based on the culture dependent approach.



(a) t-RF 102 bp

(b) t-RF 579 bp

Figure 3.20: Relative heights of t-RFs 102 and 579. Average relative peak heights of (a) t-RF 102 bp and (b) t-RF 579 bp for each season and treatment [%]. Data represent mean relative peak heights within a t-RFLP profile ± standard deviation, n=6.

Table 3.7: T-RF sizes of the 16S rRNA genes from clones or pure cultures (double digest: *Mbo*I/*Fau*I).

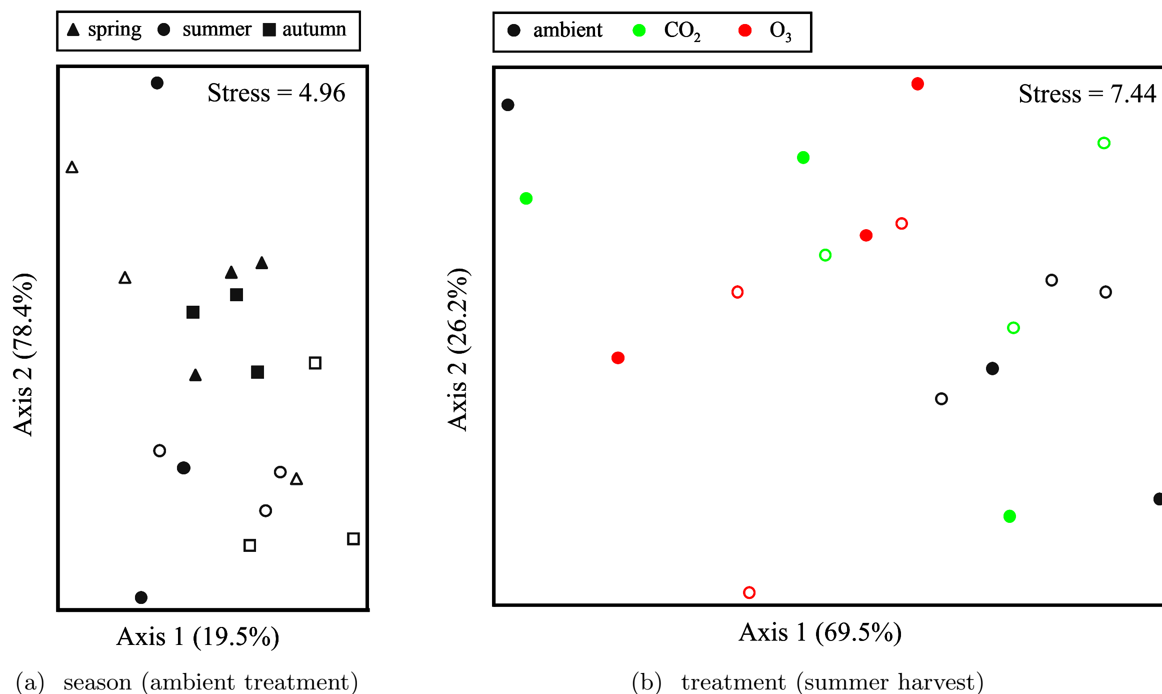
clone no./ strain*	genus	family	enzyme	expected fragment size [bp]	actual fragment size [bp]
A15	*Actinospica*	Catenulisporaceae	*Fau*I	106	102
A7	*Catenulispora*	Catenulisporaceae	*Fau*I	106	102
A19	*Catenulispora*	Catenulisporaceae	*Fau*I	366	362
A37	*Catenulispora*	Catenulisporaceae	*Fau*I	388	381
A2	*Mycobacterium*	Mycobacteriaceae	*Fau*I	230	226
A6	*Mycobacterium*	Mycobacteriaceae	*Fau*I	378	371
A27	*Mycobacterium*	Mycobacteriaceae	*Fau*I	375	367
A49	*Nocardioides*	Nocardioidaceae	*Mbo*I	154	149
A13	*Pseudonocardia*	Pseudonocardineae	*Fau*I	476	471
A17	*Rhodococcus*	Nocardiaceae	*Fau*I	473	464
A39	*Terrabacter*	Intrasporangiaceae	*Mbo*I	596	596
PT-1	*Kitasatospora*	Streptomycetaceae	*Mbo*I	415	411
PT-7	*Streptomyces*	Streptomycetaceae	-	-	409

*A = clone from actinobacterial 16S rDNA clone library, PT = 16S rDNA phylotype of isolate

3.5.2 Actinobacterial PKS type II diversity

To study changes in PKS type II diversity two separate analyses were performed. In order to analyze possible seasonal shifts, one analysis was done from all ambient treated samples for each season (a total of 18 samples). The other analysis was performed with all 18 summer samples to analyze the effects of the different treatments (ambient, CO_2 and O_3). The summer harvest was chosen based on the observations that the clearest separation of actinobacterial 16S rDNA t-RFLP profiles was seen for this season, as described in section 3.5.1. After relativization and removal of background noise 26 (for seasonal analyses) and 28 (for treatment analyses) t-RFs were included in the matrices respectively. Average relative peak heights of the included t-RFs varied between 29.7% and <0.01%. Of these t-RFs, five (17.9%) had an average peak height of >5%, seven (25.0%) had an average peak height bewteen 0.5-5% and 16 (57.1%) were below 0.5%.

The frequency of the t-RFs throughout the samples was very high for major peaks. The five highest peaks (>5% average relative peak height) were all present in every sample. From the seven t-RFs between 0.5-5%, four were present in every sample, while the remaining three could only be found in half of the samples. For t-RFs below 0.5% the average frequency was around 32.5%. In comparison to the 16S rDNA t-RFLP profiles, PKS type II profiles were still very homogenous for the major peaks, yet variablity increased for peaks below 5% average peak height.



(a) season (ambient treatment) (b) treatment (summer harvest)

Figure 3.21: Non-metric Multidimensional Scaling (NMS) plots of PKS Type II t-RFLP profiles for rhizosphere DNA of the greenhouse experiment 2006. (a) Ordination of ambient treated rhizosphere samples for all seasons and (b) samples from each treatment (ambient, CO_2, O_3) at the summer harvesting time point. Filled symbols indicate samples inoculated with *P. citricola*. Stress values are given according to Kruskal's stress formula 1 multiplied by 100.

Non-metric multidimensional scaling

For the two different matrices, independent NMS plots were created. In both cases a two dimensional plot covered most of the variance within the t-RFLP profiles.

For the analysis of seasonal changes, the first two dimensions covered 19.5% and 78.4% of the information in the analytical data set respectively (cumulative = 97.9%). A stress value of 4.96 was calculated for the NMS, indicating a very good ordination with no risk of drawing false conclusions. By visually analyzing the resulting ordination plot, no separation of any season could be observed (Fig. 3.21a).

When creating a NMS plot for the analysis of different treatments, the first two dimensions covered 69.5% and 26.2% of the information in the analytical data set (cumulative = 95.7%). Again the stress value was low (7.44) indicating a good and solid ordination. Yet, as seen for the ordination of the seasonal matrix, no effects could be observed for the different treatments (Fig. 3.21b).

To verify these visual observations non-parametric multivariate analysis of variance (Per-

MANOVA) was performed. For all factors, season (Tab. B.7), treatment (Tab. B.8) and inoculation with *P. citricola*, no statistically significant differences were calculated.

Chapter 4

Discussion

The present study aimed to investigate the potential biocontrol activity of rhizosphere microorganisms against *P. citricola*. Bacterial and fungal groups known to be antagonistic against other oomycetous pathogens were targeted and isolated from rhizosphere soil and fine roots of European beeches (*Fagus sylvatica*) and tested for antagonism against *P. citricola in vitro*. Furthermore, a possible mechanism of the biocontrol activity of the isolated antagonists was elucidated and key genes were identified. Thus, making it possible to culture independently monitor changes within the antagonistic community on a functional and structural level. Recent studies indicated that elevation of O_3 and CO_2 could have significant influences on the susceptibility of *F. sylvatica* to *P. citricola*. Therefore, the effect of an elevation of these trace gases on the composition of the antagonistic rhizosphere community was investigated. It was hypothesized that changes in the antagonistic microbial community might be responsible for the observed changes in susceptibility. This study could be of interest not only to microbial ecologists, but also to forestry and nurseries, where the pathogen might pose an increasing threat under changing climate conditions in the future.

4.1 Occurance of microbial antagonism against *P. citricola*

4.1.1 Actinobacteria

Actinobacteria isolated from the experimental setup showed a very high frequency of antagonism against *P. citricola in vitro* (48% of all tested isolates). Organisms belonging to the moderate and strong antagonistic classes, were classified by sequencing the variable *c*-region of the 16S rRNA gene after dereplication by BOX-PCR. All isolates were assigned to the genera *Strepto-*

myces and *Kitasatospora* respectively. Both genera are closely related and belong to the family Streptomycetaceae (Kämpfer, 2006), which includes three genera of Actinobacteria forming an extensively branched substrate and spore forming aerial mycelium (Kämpfer, 2006). Members of all three genera are primarily isolated from soil habitats. They are responsible for the degradation of complex recalcitrant plant and animal material, e.g. polymeres such as polysaccharides, proteins, lignocellulose, and aromatic compounds, due to their ability to produce a variety of extracellular enzymes (Kämpfer, 2006). In terrestrial habitats, streptomycetes constitute the most abundant group of Actinobacteria (Schrempf, 2006). The ability of these Actinobacteria to inhibit diverse groups of phytopathogens *in vitro* and *vivo* is well known (Raaijmakers *et al.*, 2002; Paulitz & Belanger, 2001; Kämpfer, 2006) and some formulations, like Mycostop®, are already available as licensed products (Paulitz & Belanger, 2001). In accordance with the isolations performed in this study, Lee & Hwang (2002) observed that 80% of all Actinobacteria isolated from different soils belonged to the genus *Streptomyces* and the frequency among isolates with antifungal activity was between 40% and 87%. Their ability to control oomycetous plant pathogens *in vitro* and *in vivo* has been demonstrated in serveral studies (Rothrock & Gottlieb, 1981; Yuan & Crawford, 1995; You *et al.*, 1996; Crawford *et al.*, 1993; El-Tarabily *et al.*, 1997; Lee & Hwang, 2002; Xiao *et al.*, 2002).

Actinobacterial CFU counts in soil typically range from 10^4 to 10^7 per gram dry weight of soil (Lee & Hwang, 2002; Takahashi & Omura, 2003; Kämpfer, 2006), yet they vary strongly between different soils and the isolation procedures applied. The observed CFU counts for the studied soil of 10^4-10^5 can therefore be considered normal, yet below the average. Comparing these results to other studies can not easily be done, due to the mention differences in isolation procedures and soil characteristics. While, for example, Lee & Hwang (2002) determined rather constant CFU counts between 1 to $4*10^6$ per gram dried soil for different soils in Korea including mountain forest and grassland sites, Garbeva *et al.* (2006) obtained count numbers between $3*10^4$ to a maximum of $6*10^5$, when comparing grasslands and arable land under different agricultural regimes. Similar high variability between different soils were also reported by Martinez *et al.* (2002) who studied 45 soils in Canada. In this case CFU counts varied from not detectable to $2.5*10^6$, with a mean value of $2*10^5$.

Besides environmental factors like the type of soil studied, the season and year of sampling, a major influence on CFU counts has to be attributed to the isolation procedure itself. Members of the Streptomycetaceae can readily be isolated using general selective isolation methods like

heat pretreatments and subsequent serial dilution plating on starch-casein or humic acid agar (Williams *et al.*, 1972; Goodfellow & Williams, 1983; Hayakawa & Nonomura, 1987; Kämpfer, 2006). These methods are generally accepted and yield reproducible results, yet some groups of Actinobacteria might not be obtained as easily. Since most streptomycetes have a growth optimum at neutral pH values, most media are adjusted to pH 7.0 - 7.5 (Kämpfer, 2006). While acidophilic Actinobacteria are often known to be neutrotolerant (Khan & Williams, 1975; Kim *et al.*, 2004; Xu *et al.*, 2006), it has been demonstrated that some acidophilic groups can only be cultivated on specialized media (Khan & Williams, 1975; Hagedorn, 1976; Kim *et al.*, 2003; Busti *et al.*, 2006a; Cavaletti *et al.*, 2006). Considering the low pH of the studied soil (∼4.1), an increase in CFUs would be expected for pH adjusted media (Hagedorn, 1976; Goodfellow & Williams, 1983; Cavaletti *et al.*, 2006).

Another important factor is the sources of carbon and nitrogen in the media. Joseph *et al.* (2003), for example, could demonstrate that regardless of a high reisolation frequency of known Actinobacteria, novel isolates and lineages could still be obtained simply by changing media compositions in terms of C-sources. Therefore, counts of CFUs must always be compared with regards to the limitations of the isolation procedures. In the case of the present study, it can be assumed that a fraction of the actinobacterial population could not be isolated by means of the applied isolation technique and the results need to be compared to culture independent analysis, as is discussed later on.

When analyzing the diversity of the isolates within the identified phylotypes by utilizing repetitive element-sequence-based BOX-PCR, the highest profile diversity was seen for the most common phylotypes PT 1 and PT 102. Less abundant phylotypes (PT 2, 7 and 38) were represented by only one or two distinct clones. This is interesting to note, since for streptomycetes, a poor correspondence between 16S rDNA genotypes and phenotypical traits like production of and resistence to antibiotics have been reported (Davelos *et al.*, 2004a; Davelos Baines *et al.*, 2007), while significant correlations between antibiotic phenotypes and BOX-PCR could be shown (Davelos Baines *et al.*, 2007). Therefore it has to be considered that the true diversity of an actinobacterial population can not be seen on species level as determined by the conserved 16S rRNA gene sequences but rather by identifying genotypical diversity (Cohan, 2002). This heterogeneity also indicates more functionally diverse populations within the phylotypes PT 1 and PT 102 potentially facing higher selective pressure in the soil environment (Nwosu, 2001; Davelos Baines *et al.*, 2007). The structural diversity, as observed on 16S rRNA gene level,

is thus very likely not to be directly correlated to the functionality of the population, which has to be kept in mind when applying culture independent approaches to study the microbial community. Interestingly, differences in strength of the inhibition of *P. citricola* were observed for phylotype 102, yet no correlation could be shown to characteristic BOX-PCR profiles of the isolates.

4.1.2 Fungal isolates

In the present study, *Trichoderma* spp. have proven to be very effective antagonists against *P. citricola in vitro*. All *Trichoderma* strains isolated showed antagonism against *P. citricola*, with isolates belonging to *T. asperellum* and *T. viridescens* completely inhibiting the growth of the pathogen. This is in accordance with numerous studies which have shown *Trichoderma* spp. to be very potent antagonists against oomycetous (Lumsden & Locke, 1989; Smith *et al.*, 1990; Lederer *et al.*, 1992; Chambers & Scott, 1995; Sid Ahmed *et al.*, 1999; Limon *et al.*, 2004) and fungal plant pathogens (Lorito *et al.*, 1993; Schirmböck *et al.*, 1994; Hermosa *et al.*, 2000; Paulitz & Belanger, 2001; Sanz *et al.*, 2005). Additionally, the majority of all commercially available biocontrol agents belong to this genus (Paulitz & Belanger, 2001; Benitez *et al.*, 2004). Chambers & Scott (1995) reported that isolates of *Trichoderma* exhibited strong antagonism against *P. citricola* in the only study on biocontrol conducted with this pathogen to date.

A collapse of hyphae and killing of *P. citricola*, as observed for *T. asperellum* and *T. viridescens* after seven days of *in vitro* dual cultures, indicates a complete disintegration of the pathogen, while it was still viable after the interaction with *T. harzianum*. For the isolate of *T. citrinoviride* a media specific interaction was observed. While *P. citricola* was killed in the interaction zone on V8 agar, the oomycete could survive on the synthetic Czapek agar. Similar effects were observed by Sid Ahmed *et al.* (1999) who demonstrated a higher inhibition of *Phytophthora capsici* by *T. harzianum* on V8 agar when compared to Czapek agar. Besides changes in lytic enzyme or antibiotic production of *T. citrinoviride* as a direct effect of the medium, another explanation for this effect may be a slower growth of both organisms on the synthetic medium. Due to this retardation the actual time of interaction is reduced and the chances of survival for *P. citricola* increases. Regardless of the reasons for this phenomenon, it demonstrates that results obtained from *in vitro* tests are always biased by the composition of the medium and culture conditions used, as remarked by Fravel (1988). Interactions should therefore be checked on more then one medium if this is feasible.

It is interesting that none of the isolated *T. harzianum* strains were able to completely inhibit *P. citricola*, considering that this species comprises the highest number of the biocontrol agents reported (Hermosa *et al.*, 2000; Paulitz & Belanger, 2001; Benitez *et al.*, 2004). Yet, this might not be surprising due to the fact that *T. harzianum* is a species aggregation which includes a large and heterogeneous group of strains with a high degree of intraspecific variability (Hermosa *et al.*, 2000; Druzhinina *et al.*, 2005). In addition, biocontrol activity of a strain always needs to be analyzed in respect to the pathogen involved. Interactions are often very specific and a certain strain might not suppress one pathogen but can be effective against a broad range of other organisms (Whipps, 2001). Also, several traits of a strain can not be sufficiently tested *in vitro*, such as competition for nutrients, growth promotion effects on the plant and the induction of resistance in the plant (Whipps, 2001; Paulitz & Belanger, 2001; Howell, 2003; Harman *et al.*, 2004).

Of the remaining antagonistic fungal isolates all genera, *Penicillium*, *Cylindrocarpon* and *Geomyces*, are well known to be abundant in soil habitats (Hamelin *et al.*, 1996; Götz *et al.*, 2006; De Bellis *et al.*, 2007; Cosgrove *et al.*, 2007). Particularly interesting is the presence of isolates belonging to the genus *Cylindrocarpon*. Many isolates of this genus have been reported to produce not only antifungal and antibacterial compounds (Quaghebeur *et al.*, 1994) but are also known to be opportunistic plant pathogens in nurseries (Hamelin *et al.*, 1996). *Cylindrocarpon* spp. have a broad host range, infecting deciduous trees as well as other annual and perennial plants (Hamelin *et al.*, 1996). Members of this genus have also lead to considerable damage on young beech trees in a phytotron experiment carried out within the SFB 607 (Ritter, pers. comm.).

Many species belonging to the genus *Penicillium* are well known for their biocontrol activity (Berg *et al.*, 2005). Yet this genus also includes plant pathogens which are often associated with fruit and post harvesting diseases (Zamani *et al.*, 2006; Neri *et al.*, 2006; Deng *et al.*, 2007). For *Geomyces* spp. no deleterious interaction with plants have been published so far, but members of this keratinophilic genus are frequently reported to be opportunistic pathogens to humans (Gianni *et al.*, 2003).

Trichoderma spp., even though they are good colonizers of plant roots, are usually not pathogenic to plants. Pathogenicity has only been demonstrated in few cases. In many instances, inoculation with *Trichoderma* spp. even lead to a growth promoting effect (Harman *et al.*, 2004), making this genus an even more interesting group for an application in biocontrol. Pathogenicity

towards immunosuppressed humans however has also been demonstrated for several species of this genus, including isolates belonging to *T. citrinoviride* (Kuhls *et al.*, 1999).

Based on these reports, the benefits for the plants from these interactions should be checked for every microbial partner involved. The possibilities of negative effects of potential biocontrol active organisms on the target plants or human health have to be kept in mind when dealing with these complex interactions.

4.2 Mechanisms of antagonism

In order to identify antagonism related genes, an important goal of this study was to elucidate mechanisms with which antagonistic microorganisms are potentially able to suppress *P. citricola* in soil.

4.2.1 The actinobacterial antibiotic cycloheximide and its relevance in soils

By means of a high resolution FT-ICR/MS analysis in combination with ^{1}H NMR it was possible to prove that the actinobacterial isolate 116A+4, belonging to the genus *Kitasatospora* (PT 1), was able to produce the glutarimide antibiotic cycloheximide. This antibiotic is a macrolide polyketide, which inhibits protein biosynthesis in eukaryotic organisms (Obrig *et al.*, 1971) and is very likely to be produced via a type I polyketide synthase (O'Hagan, 1995). Yet, even though the biosynthesis pathway is well characterized (Kominek, 1975; Jeffs & McWilliams, 1981; Shimada *et al.*, 1981), the enzymatic complexes involved have not been described to date. Cycloheximide inhibits the binding of the aminoacyl-tRNA to the ribosome, the transfer of the amino acids from the aminoacyl-tRNA to the elongating peptide and the release of the deacylated tRNA from the ribosome. Additionally, the translocation of the aminoacyl-tRNA from the acceptor to the donor position of the ribosome is impaired (Obrig *et al.*, 1971).

Cycloheximide has been well know to be produced by a broad range of streptomycetes including *S. griseus*, *S. albulus*, *S. noursei*, *S. naraensis* and many more (Ford *et al.*, 1958; Vanek *et al.*, 1967, 1969; Jeffs & McWilliams, 1981). No reports have been published so far on any cycloheximide producing strains of the genus *Kitasatospora*. This is probably due to the fact that there have been several changes in the phylogenetic position of this genus over the last decades (Kämpfer, 2006). The taxon was proposed by Omura *et al.* (1982), subsequently subsumed within the genus *Streptomyces* (Wellington *et al.*, 1992) and re-established by Zhang *et al.* (1997). It is therefore likely that in some cases cycloheximide production was attributed

to *Streptomyces* spp. instead of *Kitasatospora* spp..

The role of antibiosis in biological control has been under debate for decades (Gottlieb, 1976; Rothrock & Gottlieb, 1981; Fravel, 1988; Handelsman & Stabb, 1996; Raaijmakers *et al.*, 2002) and proving the effectiveness of antibiosis in biocontrol has been challenging. Up to today, no generalized answer can be given for all antibiotics or enzymes produced. Inactivation of antibiotic production by mutagenesis has, in many cases, resulted in a reduced ability of the antagonistic bacteria to control pathogens (Raaijmakers *et al.*, 2002). While most of these studies have focused on antagonism by *Pseudomonas* spp. (Raaijmakers *et al.*, 2002; Weller *et al.*, 2007), some reports on successful mutagenesis of antibiotics producing genes have also been published on *Burkholderia cepacia* (Heungens & Parke, 2001), *Serratia marcescens* (Okamoto *et al.*, 1998) and *Bacillus cereus* (Silo-Suh *et al.*, 1994). A second convincing line of evidence for the role of antibiotics in biocontrol has been the introduction of antibiotic biosynthetic genes in heterologous, non-producing strains. Timms-Wilson *et al.* (2000) demonstrated that the introduction of genes for phenazine-1-carboxylic acid (PCA) production into the chromosome of a PCA-nonproducing *Pseudomonas fluorescens* strain significantly increased its protection against *Pythium ultimum* on pea seedlings when compared to the parental strain. Similarly did the introduction of a gene responsible for the production of the polyketide 2,4-diacetylphloroglucinol (DAPG) into a DAPG-nonproducing strain of *P. fluorescens* significantly increase its effectiveness against *P. ultimum* on sugar beet (Fenton *et al.*, 1992).

For actinobacterial antagonists, similar investigations are still lacking. So far only indirect proof for the relevancy of *in vitro* antibiosis for biocontrol has been provided for this group and the data is controversial. These studies are also biased by culture conditions. In some cases isolates selected based on *in vitro* antibiotic activity could control the pathogens *in vivo* while others could not (Rothrock & Gottlieb, 1981; O'Brien *et al.*, 1984; El-Tarabily *et al.*, 1997; You *et al.*, 1996; Schottel *et al.*, 2001; Xiao *et al.*, 2002). These contradicting results are very likely caused by a number of factors, including the abiotic conditions in soil and the chemical properties of the substance responsible for the inhibition (Raaijmakers *et al.*, 2002). Some of the physical factors which have been reported to affect antibiotic production and activity are temperature, soil moisture and pH (Raaijmakers *et al.*, 2002). It has been addressed in most reviews that a major drawback of any biological control agent is the necessity of introducing it into a new habitat where it faces environmental conditions which might not be favorable to its biocontrol traits (Handelsman & Stabb, 1996; Whipps, 2001). Additionally as stated above, it

is believed that most antagonists suppress pathogens by a variety of different mechanisms and therefore antibiosis *in vitro* is only one possible criterion for effective antagonism (Trejo-Estrada *et al.*, 1998; Howell, 2003; Compant *et al.*, 2005).

In the presented study, the antagonistic potential of a natural population is investigated and no introduction into different habitats is intended. Therefore, it should be considered, whether or not the observed mechanism is likely to be effective in the soil studied. The antibiotic cycloheximide was produced by one of the most common phylotypes of the isolated actinobacterial population and could thus potentially be produced in the soil. The polyketide also has some properties that make it very suitable for this particular acidic soil. It is well known that cycloheximide is rather stable under acidic conditions, while it is quickly inactivated at a basic pH (O'Neil *et al.*, 2001). For example, boiling of the substance at pH 2 for 1 h does not lead to an inactivation (O'Neil *et al.*, 2001), suggesting a high stability of the compound in the studied soil. Additionally, due to the neutral character of the molecule, it is less likely to be permanently bound to clay particles, which is a well documented mechanism of antibiotic inactivation in soils (Gottlieb, 1976; Fravel, 1988).

From the 1950s to the late 1970s cycloheximide was tested and used for many agricultural applications against oomycetous and true fungal pathogens, showing its efficiency in soils (Ford *et al.*, 1958; Brown, 1978). But, due to its high toxicity to many eukaryotic organisms, including humans, it is not utilized anymore. While broad application of the substance in the environment is not desirable, the production of cycloheximide by native and local populations of antagonistic microorganism in their microhabitat is likely to have much less drastic effects and might be very valuable to complement the "tool box" of a successful antagonist. In conclusion, it can be stated that cycloheximide is a very likely candidate to enhance the success of antagonists in the rhizosphere of European beeches in the studied soil.

4.2.2 Possible mechanisms of fungal antagonism

Since no detailed analysis of the mechanisms involved in fungal antagonism were conducted, only a brief discussion of the possibilities for *Trichoderma* spp. will be given. As mentioned above, the most deleterious interaction with *Trichoderma* spp., by *T. asperellum* and *T. viridescens*, lead to disintegration and death of the oomycetous mycelium. No antibiosis was observed before hyphal contact indicating a direct interaction between the two mycelia.

Trichoderma species are well known for their ability to produce a large arsenal of differ-

ent lytic enzymes involved in general antibioses or specific mycoparasitism (Lorito *et al.*, 1994; Viterbo *et al.*, 2002; Sanz *et al.*, 2004; Seidl *et al.*, 2005; Liu & Yang, 2007). Additionally, *Trichoderma* strains produce antibiotics which inhibit the growth of the antagonized microorganisms. Among these metabolites, the production of tricholin, peptaibols, viridin, gliovirin and many others has been described (Benitez *et al.*, 2004). In many cases synergisms between the production of hydrolytic enzymes and antibiotics has been observed (Howell, 2003; Benitez *et al.*, 2004).

Concerning the mechanisms of the isolates, the following analyses could be performed to elucidate the modes of action involved: first, enzyme tests of the interaction zones in comparison to pure cultures (for glucanases and proteases) as well as secondly, FT-ICR/MS metabolic profiling of the interaction zone targeting known antibiotics produced by *Trichoderma* species.

4.3 Influence of abiotic and biotic factors on a forest plant-soil system

4.3.1 Effects on the growth of European beeches

Among the applied treatments only the elevation of CO_2 resulted in a significant effect on plant growth of European beech trees in the experiment performed in 2006. Below ground biomass increased by around 30% for this treatment in comparison to ambient controls. For above ground biomass no significant effect could be shown. The below ground effect is in accordance with most studies conducted on the influence of CO_2 elevation on plant growth. In a meta-analysis carried out by De Graaff *et al.* (2006), the authors found out that the average belowground biomass increase in previous studies was 28.3% compared to untreated controls. They also demonstrated an average increase in above ground biomass for woody plants of around 30.5%. De Graaff *et al.* (2006) also noted that other meta-analysis had obtained different results (Curtis & Wand, 1998; Poorter & Perez-Soba, 2001), most likely due to differences between the studies analyzed (e. g. field vs. pot studies). Therefore, non-optimal growth conditions due to constrains of pots used in the greenhouse experiment could be responsible for the lack of a significant effect on the above ground biomass. No significant changes in above and below ground biomass were observed by Liu *et al.* (2004) for European beeches grown for three years under elevated CO_2, confirming the above ground biomass results in the present study. Additionally, they observed the tendency of an increased below ground biomass, however in their case the effect was not significant.

In contrast to the results presented here, a decrease in the below ground biomass under

elevated O_3 has been demonstrated in many studies for a variety of plant species (King *et al.*, 2001; Andersen, 2003). In comparison to the responses to elevated CO_2, these effects were much more variable and dependent on other factors as well. When investigating the impact of elevated O_3 on European beeches, Liu *et al.* (2004) found no significant effect when the trees were planted in monoculture. There was even a tendency towards an increase in below ground biomass, as was observed in the present study. When beeches were planted in competition with spruce however, significant reductions in the below ground biomass were observed under elevated O_3 (Liu *et al.*, 2004; Luedemann *et al.*, 2005). Therefore, it has been shown that O_3 has an impact on the below ground competitiveness of beeches, yet these effects were not likely to be demonstrated under the factorial set up of the study presented here.

Any effect on the plants caused by the inoculation with *P. citricola*, such as a reduction in root biomass, is likely to be affected by the natural infestation of the planted beeches by the pathogen. As mentioned above, *Phytophthora* infections are increasingly common in Bavarian plant nurseries and very difficult to control (Jung *et al.*, 2005). Furthermore, the plant material is not checked for infections in nurseries, thereby increasing the risk of spreading the disease. While clearly the infection was more pronounced for inoculated plants, no effect on total below ground biomass was measured. In most cases, the infection leads to a decrease in small or fine root biomass as well as a decrease in the number of root tips, while coarse roots are not affected (Nechwatal & Oßwald, 2001; Fleischmann *et al.*, 2002b; Wang, 2003; Fleischmann *et al.*, 2004). Hence, it is likely that possible effects were not observed in this study, since these parameters could not be measured.

4.3.2 Effects on total microbial biomass

For the main experiment, total microbial biomass (C_{mic}) was measured as an indicator for the microbial community size influenced by the factors season, elevation of trace gases and inoculation with *P. citricola*. When looking at the summer harvesting time points, C_{mic} values for ambient and O_3 treatments were significantly lower than the corresponding value for the CO_2 treatment. This is most likely due to an indirect effect of the treatment. In table A.2 the soil water content is given as percentage of the maximal water holding capacity (MWHC) at the different time points. It can be seen that for both, ambient and O_3 treatments, the rhizosphere soils dried much faster than for the corresponding CO_2 treatment in summer. Rhizosphere soil of ambient and O_3 treatments had low soil water contents of around 50% of the MWHC due

to the extremly high temperatures during this summer harvest, while CO_2 treatments remained at a relativly high level of 62% (Tab. A.2). This can be caused by an increased water use efficiency often reported for plants under elevated CO_2 (Garrett *et al.*, 2006). Due to the higher CO_2 concentrations, plants can keep the internal CO_2 levels in the substomatal cavity at a high level while conserving water by partial closure of the stomata (Garrett *et al.*, 2006). Thus, it is likely that the very dry conditions in summer are an explanation for the decrease of the total microbial C. This is in aggreement with findings of Islam *et al.* (2000), who demonstrated that soil under well-watered conditions had 20% higher amounts of C_{mic} than restricted soil water treatments.

When comparing the C_{mic} values at the autumn harvest, no significant differences can be seen for any treatment. This corresponds to the relatively homogeneous soil water contents of the samples. In a similar study investigating the effect of CO_2 elevation on a beech-spruce ecosystem, Wiemken *et al.* (2001) did not find significant differences for the treatment as well. Zak *et al.* (2000b) reported in his review on soil microbial responses to elevated CO_2 that changes in the microbial biomass are characterized by large increases and declines, contributing to a high degree of variability within and between plant species. Of the studies they analyzed, 62% showed an increase, 18% a decrease and 20% exhibited no change in microbial biomass, while 95% of the studies showed a significant increase in microbial respiration due to elevated CO_2 (Zak *et al.*, 2000b). Similar results were obtained in a more recent meta-analysis by De Graaff *et al.* (2006), in which the authors observed an overall increase in C_{mic} by 7.7% and 17.1% for microbial respiration under elevated CO_2. This data suggest that while microbial respiration seems to be a more sensitive indicator for changes in the microbial community, both parameters have similar tendencies. It can therefore be assumed from the presented data, that under optimal water supply there is no significant influence of any of the applied treatments on the microbial community size and thus on the potential general suppressiveness of the habitat against deleterious microorganisms.

4.4 Structural and functional diversity of the actinobacterial rhizosphere community

Due to the fact that total community analysis often fails to indicate changes induced by elevated CO_2, Jossi *et al.* (2006) stated the necessity to identify putative responsive groups which perform important functions in the soil-plant system in order to analyze effects with more focus. In

this study, the main focus lay on actinobacterial antagonists due to their vital role in forest ecosystems. They have been identified as major degraders of residue plant materials, often associated with perennial plants (Kämpfer, 2006), and are considered strong antagonists against diverse root rot pathogens as mentioned above. Their active role in soils has recently been demonstrated in a free air carbon enrichment forest site under elevated CO_2 by Billings & Ziegler (2005). The authors observed an incorporation of a ^{13}C-label into the fatty acid 10Met18:0, a known marker for Actinobacteria. Hence, they concluded that this group is an active part of this plant-soil forest system.

4.4.1 Diversity assessment by means of clone libraries

Structural analysis of the community

In order to assess the accuracy and to identify constrains of the applied primer pair a clone library from environmental DNA was established. 100% of the sequenced clones contained a fragment originating from the phylum Actinobacteria (section 3.3.1). Similar specificities have not been achieved in previous studies with any of the tested primer pairs. Lüdemann & Conrad (2000) reported for the primer AB1165r in combination with the universal forward primer 27f, that 33% of the PCR products belonged to bacterial lines other than Actinobacteria, mainly to the group of Gram-positve bacteria with low G+C content. McVeigh *et al.* (1996) showed that 13% of the PCR products, obtained with primers Act283f/Act1360r, belonged to organisms outside the Actinobacteria. In the case of Stach *et al.* (2003), the designed primers S-C-Act-235-a-S-20/S-C-Act-878-a-A-19 resulted in 25% of the sequenced clones belonging to the classes Gemmatimonadetes and Planctomycetes respectively. Even the most conservative primer, F243 by Heuer *et al.* (1997), amplified sequences outside the Actinobacteria in some studies. Dohrmann & Tebbe (2005) observed that 33% of the clones produced with F243 and the reverse primer R1387 contained fragments of the phylum Verrucomicrobia.

Interestingly, none of the clones obtained in this study belonged to the genera *Streptomyces*/*Kitasatospora* which were the most frequently isolated antagonistic groups. Even though there was one missmatch for primer Act-1360r for 55% of the Streptomycetaceae, it is very unlikely that this had a major influence on the outcome of the analysis. The observed missmatch was not located within the terminal three bases of the 3' end of the primer which are known to be crucial for the selectivity (Sommer & Tautz, 1989). Additionally, the ability of the primer pair S-C-Act-235-a-S-20 and Act-1360r to amplify sequences originating from members of the

genus *Streptomyces* from environmental DNA has been shown by Stach *et al.* (2003). They used a semi-nested PCR with Act-1360r in the first round to produce a clone library from environmental DNA and achieved a specificity of 99% for the phylum Actinobacteria, including members of *Streptomyces*.

Thus, it can be assumed that Actinobacteria belonging to the Streptomycetaceae might not be the dominant part of the actinobacterial population despite their dominance among the isolates. Considering that 37.5% of all clones from the clone library could not be classified on genus level and another 41.1% belonged to genera only very recently described (*Actinospica* and *Catenulispora*, Busti *et al.*, 2006a; Cavaletti *et al.*, 2006) it is likely that the actinobacterial community in the studied soil is very unique. This would explain the relatively low CFU count numbers obtained from the investigated soil. Both genera, *Actinospica* and *Catenulispora*, were isolated on a rather specific gellan gum based medium and cultures appeared after a long incubation period of eight weeks (Busti *et al.*, 2006a). These findings emphasize the necessity to compare and complement results from culture dependent and independent studies (Nichols, 2007). With this in mind it would be very interesting to apply the isolation technique described by Busti *et al.* (2006a) to the studied soil, compare the results to the standard procedure applied in this thesis and find possible antagonists in these newly described genera.

Analyzing functional diversity related to polyketide synthase enzyme complexes

When testing different PKS primer systems, it became obvious that only the application of the PKS type II primer set by Wawrik *et al.* (2005) was suitable for the amplification of fragments from soil environmental DNA. The lack of a good amplification by PKS type I targeting systems can be explained when looking at the complex structure of these proteins.

PKS type I genes possess a modular organization, with a repetition of similar gene segments within a single gene cluster (as seen from the protein arrangement in Fig. 1.1a). This implies that the actual diversity of PKS type I sequences is not only determined by the number of PKS type I gene clusters in the genome of an organism, but also by the number of modules within each of these clusters (Weissman, 2004). Designing primers suitable for the amplification from soil for complex enzyme machineries like that is very challenging and clear amplifications have not even been possible from pure culture DNA in most cases (Ayuso *et al.*, 2005; Ayuso-Sacido & Genilloud, 2005; Chuck *et al.*, 2006). The results obtained by the primer combination ksf/ksar

are however very promising. First culture independent studies could be performed with this system on the basis of clone libraries to evaluate the diversity of the amplified sequences from soil. Yet, since it was necessary to analyze large amounts of samples in the present study, this system was not applicable.

PKS type II systems are of great interest when investigating the antibiotics production potential of actinobacterial soil populations. This especially holds true since a large portion of the actinobacterial community in the studied soil belongs to the newly described suborder Catenulisporinae. For this new lineage of Actinobacteria, Busti *et al.* (2006b) described a high potential to produce secondary metabolites with a polyketide scaffold. This is a feature they share with members of *Streptomyces* and related genera. All strains analyzed by Busti *et al.* (2006b) belonging to this group yielded distinct bands when checked with specific primers for PKS type I and II. For one of the strains, the production of a bioactive molecule similar to the well studied antibiotic actinorhodin could be demonstrated. This antibiotic is synthesized by a PKS type II system (Hopwood, 1997).

Concerning the accuracy of the PKS type II PCR from environmental DNA, three sequences (∼6%) showed no homology to known proteins and two (∼4%) revealed internal stop codons upon translation into protein sequence. These latter two have to be considered pseudogenes, nonfunctional homologes to known genes (Vanin, 1985), due to their lack of protein-coding ability. Therefore, almost 10% of all clones have to be considered junk DNA and will only lead to enhanced background noise of the analysis. Their distribution is likely to be random since no selective pressure takes effect on them.

Additional nine sequences (16.7%) were closely related to spore pigment producing genes. The necessity to differentiate between antibiotics and spore pigments producing PKS systems has been stressed by Metsä-Ketelä *et al.* (1999) due to different ecological functions of the resulting molecules. In the present study this homology could only be demonstrated on protein level, showing the need to analyze functional gene sequences by comparing amino acid sequences. While the property of a molecule to act as a pigment does not yield any information about its chemical properties concerning bioactivity, a major difference is that spore pigments are presumably covalently attached to macromolecular components of spores (Lee *et al.*, 2005). Therefore they might act as protectives against grazing by the microfauna of the soil, but are very likely not involved in antibiosis against competing microorganisms.

From analyzing the clone library of the chosen PKS primer pair, it can already be concluded

that the background noise will be enhanced for t-RFLP analysis in comparison to the 16S rRNA gene. Additionally, any change observed needs to be interpreted with caution, since the functionality of the genes have not been demonstrated. None of the PKS genes amplified from the studied soil had a sequence identical with a known PKS type II gene. On the other hand, this indicates that the PKS type II primers by Wawrik *et al.* (2005) have a very broad amplification range. They were designed on the bases of only 69 actinobacterial KS_α gene sequences obtained from GenBank and thus it had to be expected that they were biased by the limited availability of sequences. These doubts are however not supported by the results obtained from the clone library in this study.

4.4.2 Monitoring structural changes in the actinobacterial rhizosphere community of European beeches

Using t-RFLP as a culture independent method to monitor changes in the actinobacterial beech rhizosphere community, the overall variability observed between different samples was rather low. For all major peaks detected, the differences observed were merely on the level of peak intensities and no differences could be seen based on the presence or absence of these peaks. It can therefore be concluded that none of the applied factors (CO_2, O_3, season or *P. citricola* inoculation) had the capability of qualitatively changing the actinobacterial community concerning its major components and that the general influence of the factors applied could thus be considered small. Nevertheless quantitative differences between samples for some t-RFs could be observed and clearly assigned to the influence of certain factors.

The clearest separation of samples was detected to be caused by seasonal shifts. Unique profiles were observed for spring and the major t-RF 102 bp responsible for this separation could be assigned to represent genera from the order Catenulisporinae based on comparisons with the clone library. Yet, it is very likely that more taxa which were not present in the library did contribute to this peak. The phenomenon that several species or even genera are represented by the same peak in t-RFLP and other fingerprinting techniques has been reported in many studies (e. g. Osborn *et al.*, 2000; Lord *et al.*, 2002; Smalla *et al.*, 2007), thus interpretations of the results have to be done cautiously.

The drastic increase of t-RF 102 bp from spring to summer was statistically significant and similar for all trace gas treatments. Seasonal shifts of microbial rhizosphere communities have been demonstrated in several studies (Smalla *et al.*, 2001; Thirup *et al.*, 2001; Baudoin

et al., 2002; Jossi *et al.*, 2006) and in some cases these responses were associated specifically with Actinobacteria. Smalla *et al.* (2001) recorded a strong seasonal shift at the beginning of the vegetation period for rhizosphere communities of strawberries, oil seed rape and potato plants. Based on DGGE analysis of the rhizosphere communities they found indications that the abundances of bacterial high G + C populations were different during the developmental stages of all plants studied. Successional changes have also been described for plant associated Actinobacteria based on CFU counts and quantitative PCR methods (Thirup *et al.*, 2001). The authors could show that at later time points in the season the abundance of Actinobacteria in the vicinity of barley roots increased significantly. They concluded that Actinobacteria are persistent during microbial succession beyond the early stages of root growth in annual plants due to their capability to penetrate and solubilize dead root litter (Thirup *et al.*, 2001). Additionally, the active role of Actinobacteria in rhizospheres of diverse plants has been demonstrated in numerous recent studies (Gremion *et al.*, 2003; Billings & Ziegler, 2005; Hjort *et al.*, 2007). In the case of European beech the process of decomposition of old roots is likely to be more constant throughout the growing season compared to annual plants. The seasonal effect observed can thus probably not be assigned to increasing decomposition processes throughout the year. Additionally, the t-RF in question (t-RF 102 bp) peaked in summer and exhibited a slight decrease toward the end of the growing season. If this part of the bacterial population was mostly dependent on dead root litter as opposed to root exudates, it would rather be expected to peak in autumn, when the balance between new fine root production and older dead roots is largely in favor of the latter (Hertel & Leuschner, 2002).

A second statistically significant separation was observed in summer, where ozone samples showed unique profiles due to the relative reduction of one major t-RF (579 bp). Yet, as mentioned above, the effect was very subtle and in this case transient, since autumn harvest of ambient and O_3 treatments could not be differentiated any more. Clones from the library possibly representing this peak could not be identified by the RDP classifier. The identity and ecology of this putative O_3 responsive group would be of great interest and efforts should be made to isolate corresponding organisms from the studied soil. Only then, their functionality within the soil and possible implications on suppression of soil borne diseases can be investigated.

Responses of the microbial community to elevation of tropospheric ozone on a structural level have been shown in recent studies, but as observed in this thesis, the effects were relatively small in most cases. Dohrmann & Tebbe (2005) reported that from neither general Bacteria nor group

specific SSCP profiles an ozone effect could be seen for the rhizosphere communities of different ozone-sensitive and insensitive plants. The only exception in this study was the ozone sensitive composite *Sonchus asper* L., where changes were observed exclusively for the Actinobacteria-specific profiles under elevated O_3. In other studies effects on the fungal rhizosphere community were shown. Chung *et al.* (2006) demonstrated that elevation of O_3 significantly altered the fungal community composition in a free-air enrichment experiment under three deciduous tree species utilizing DGGE. They also observed an increase in fungal relative abundance by PLFA analysis. In contrast to these result, Phillips *et al.* (2002) observed a decrease in fungal PLFAs while the relative proportion of Gram-positive and Gram-negative indicator PLFAs were not affected. To verify the results obtained from this study it would be interesting to analyze the effects of a prolonged fumigation with elevated ozone on beeches in the same soil and thereby confirm the results obtained for t-RF 579 bp.

A tendency towards a separation of CO_2 samples was also observed, yet the effect was too small to be meaningfully interpreted. Of the studies that have investigated the influence of changing C allocations to the rhizosphere under elevated CO_2 mixed results were produced. Some works found similar undetectable or only subtle changes as the present study (Zak *et al.*, 2000a; Bruce *et al.*, 2000; Klamer *et al.*, 2002; Lipson *et al.*, 2005; Grüter *et al.*, 2006), while others observed more pronounced effects (Montealegre *et al.*, 2000; Janus *et al.*, 2005; Lipson *et al.*, 2005; Jossi *et al.*, 2006). While the majority of these studies worked on the level of DNA, Jossi *et al.* (2006) compared results obtained from DNA analysis with the active fraction of the microbial community as assessed by RNA based analysis. They reported a strong effect on the microbial rhizosphere community of the grass *Lolium perenne* L. after nine years of elevated CO_2 treatment mainly on the active fraction of the community. Interestingly, a high proportion of DGGE bands associated with elevation of CO_2 corresponded to sequences affiliated to Actinobacteria. Those sequences were generally retrieved from the active fraction of the community. The authors conclude that Actinobacteria might function as key organisms in the response to elevated CO_2. In the present study, a comparison between DNA and RNA based analysis could not be performed because of difficulties in the extraction procedure of nucleic acids from the studied soil. RNA amounts obtained were very low and the quality of the nucleic acids was extremely poor, due to shredding of the molecules and contamination with PCR inhibitory substances. This was expected to have major influences on the outcome of the analysis and therefore it was preferred to work with a high quality yielding DNA extraction protocol.

4.4.3 Monitoring PKS type II diversity in the rhizosphere of European beeches

This is the first study performed to investigate the effects of elevation of trace gases on genes potentially responsible for antibiotics production in the rhizosphere. When analyzing the diversity of PKS type II genes, no effect was discovered for neither seasonal shifts, nor an influence of the trace gas treatments.

Since PKS type II genes do not follow the trend observed for 16S rRNA genes, it can be concluded that phylotypes responsible for the observed changes do either not contain similar PKS genes or they do not possess PKS type II genes at all. Either way it can be stated that in this case no correlation could be seen between phylogenetic trends and the genotypical trait PKS type II. This is in line with the findings of Metsä-Ketelä *et al.* (2002) who observed that the phylogenies of 16S rRNA genes and PKS genes in Actinobacteria soil isolates were not congruent. They concluded therefore that the phylogenetic grouping of Actinobacteria is an inadequate predictor for the type of secondary metabolites they produce.

Only few analyses have been performed to investigate the impact of elevation of trace gases on functions in soil. The most commonly applied methods are enzyme assays. Changes observed by these approaches can be directly linked to differences in substrate availability and quality and are thus very informative. They are therefore also presumed to be affected more directly by changes in the physiology of the plants than traits like antibiotic production. The effects of CO_2 or O_3 elevations on enzyme activities in the rhizosphere have been detected in different studies (Phillips *et al.*, 2002; Pritsch *et al.*, 2005; Chung *et al.*, 2006, 2007) and were generally discussed as results of an increase in the corresponding substrates availability in the habitat. The only analysis on the effects on antagonistic traits besides emzyme activities under elevated CO_2 has been performed by Tarnawski *et al.* (2006). Using a combination of isolations of *Pseudomonas* spp. and phenotypical characterization of the isolates they observed a shift in the *Pseudomonas* rhizosphere community of *Lolium perenne*. In their study, the percentage of siderophore and hydrogen cyanide producers increased under elevated CO_2 indicating a potential of this factor to change the suppressiveness of soils.

From the PKS t-RFLP analysis performed in this study it is possible to state two conclusions. First, the diversity of Actinobacteria possessing these genes is not affected by seasonal changes and secondly, the applied trace gas treatments did not have any effects on the distribution of these genes in the rhizosphere of beeches after two seasons of treatment. Therefore, the proposed changes in susceptibility of beech trees toward *P. citricola* can not be attributed to the potential

production of metabolites belonging to the aromatic polyketides. Yet, it has to be kept in mind, that changes were only analyzed on DNA and not mRNA levels of these genes. Additionally, the limitations of the primer system discussed in section 4.4.1 are also likely to influence the analysis and obscure possible effects of the studied factors.

4.5 Conclusions and perspectives

Microbial populations in the rhizosphere of plants fulfill many functions in this habitat and factors that influence these populations might induce positive or negative feedback responses on the plants. Microorganisms antagonistic against root pathogens can protect the plant from deleterious effects of these pathogens and thereby increase the health status of the plant. Factors changing the carbon allocation of plants, like the elevation of O_3 or CO_2, can influence the microbial rhizosphere community and therefore have the potential to induce feedback responses on the plant. This work aimed to clarify important aspects of microbial antagonism against the root rot pathogen *Phytophthora citricola* on European beeches and the influence of abiotic factors, such as elevation of O_3 or CO_2, on the structure and function of these antagonistic communities in the rhizosphere.

The hypotheses (**H**) stated at the beginning of this thesis have been addressed by a range of techniques and different conclusions can be drawn. First, it can be stated that the possibility to antagonize the oomycetous pathogen was widely distributed throughout the isolated microorganisms indicating a large potential for biological control against this pathogen in beech rhizospheres. By comparing culture dependent and culture independent techniques it became obvious that no single approach can answer all questions, and that it is necessary to apply these approaches complementing each other to get a more complete idea of the antagonistic community and its functions. By utilizing total microbial biomass carbon as an indicator for the overall microbial community size no direct effect of O_3 or CO_2 treatments were observed, suggesting that there was no change of the general suppressiveness of the habitat towards the pathogen induced by the treatments (**H1**).

Following isolations and the classification of antagonists, it was possible to identify the antibiotic cycloheximide produced by *Kitasatospora* spp. to be responsible for the inhibition of *P. citricola in vitro*. This antibiotic exhibits chemical properties suitable for the application in the analyzed soil. Additionally, the potential to produce substances belonging to the same class of antibiotics has been previously reported for the dominant fraction of the uncultured

actinobacterial population (belonging to the order Catenulisporinae). Polyketide synthase genes were therefore hypothesized to act as marker genes for the potential to produce antibiotics in soil. Their distribution as well as actinobacterial 16S rRNA gene distribution was monitored in beech rhizospheres under the influence of elevated trace gases. Elevation of O_3 and CO_2 are known to change the susceptibility of beech plants to *P. citricola* and the possible involvement of the actinobacterial community in this phenomenon was investigated. While a minor and transient effect due to the elevation of ozone could be seen in summer in the conducted experiment, this observation was dominated by a larger seasonal effect. Additionally, since the ecological function of the possibly O_3 sensitive group is not known, no conclusive answer can be given to hypothesis 2. For all samples the overall composition of the actinobacterial population was very homogeneous, indicating no drastic effect on the community by the treatment (**H2**). The diversity of PKS genes in the rhizosphere was neither affected by the season nor by treatments, therefore the described changes in susceptibility of beeches towards *P. citricola* are not likely to be attributed to an altered composition of aromatic polyketide producing microbial rhizosphere populations, yet changes on the transcript levels of these genes in the rhizosphere can not be excluded (**H3**).

While most hypotheses remain to be confirmed based on the results presented here, this study provides valuable information about the ecology and application of antagonistic microorganisms in the rhizosphere of European beeches. Of particular interest for further studies would be the validation of the obtained results for experiments with longer exposure times to O_3 and CO_2, with special regard to the putative O_3 responsive actinobacterial group (t-RF 579 bp). Due to focusing on the actinobacterial antagonists, other important groups like the genus *Trichoderma* could not be analyzed in depth in this study and would be of interest for further studies. Recently, Hagn *et al.* (2007) published specific primers targeting the ITS region of *Trichoderma* species. The designed primers were also tested on the forest soil investigated in this study and good amplifications of the target sequences were obtain. Hence, the utilization of a *Trichoderma* specific t-RFLP system opens up the possibility of monitoring this important genus in soil. Additionally, the surprisingly unique actinobacterial community in the studied soil should be analyzed in greater detail including the application of specialized isolation procedures. The isolation of new taxonomic groups from this soil is very likely and might yield organisms capable of producing novel secondary metabolites. This would not only be of great interest to microbial ecologists, but might reveal new antibiotics or anticancer drugs for medical purposes.

References

Ainsworth, E.A. & Long, S.P. (2005). What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytol*, 165, 351–71.

Andersen, C.P. (2003). Source-sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytol*, 157, 213–28.

Anderson, J. & Domsch, K. (1978). A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biol Biochem*, 10, 215–21.

Anderson, M. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol*, 26, 32–46.

Anderson, M. & Millar, R. (2004). Spatial variation and effects of habitat on temperate reef fish assemblages in northeastern New Zealand. *J Exp Mar Biol Ecol*, 305, 191–21.

Anderson, M. & Robinson, J. (2003). Generalised discriminant analysis based on distances. *Aust NZ J Stat*, 45, 301–18.

Ashmore, M. (2005). Assessing the future global impacts of ozone on vegetation. *Plant Cell Environ*, 28, 949–64.

Ayuso, A., Clark, D., Gonzalez, I., Salazar, O., Anderson, A. & Genilloud, O. (2005). A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Appl Microbiol Biotechnol*, 67, 795–806.

Ayuso-Sacido, A. & Genilloud, O. (2005). New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microb Ecol*, 49, 10–24.

Baker, K. & Cook, R. (1974). *Biological control of plant pathogens*. W.H. Freman and Co., San Francisco, U.S.A.

Baudoin, E., Benizri, E. & Guckert, A. (2002). Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. *Appl Soil Ecol*, 19, 135–45.

Benitez, T., Rincon, A.M., Limon, M.C. & Codon, A.C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *Int Microbiol*, 7, 249–60.

Benizri, E., Piutti, S., Verger, S., Pages, L., Vercambre, G., Poessel, J. & Michelot, P. (2005). Replant diseases: bacterial community structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. *Soil Biol Biochem*, 37, 1738–46.

Berg, G. (2000). Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J Appl Microbiol*, 88, 952–60.

Berg, G., Zachow, C., Lottmann, J., Götz, M., Costa, R. & Smalla, K. (2005). Impact of plant species and site on rhizosphere-associated fungi antagonistic to *Verticillium dahliae* kleb. *Appl Environ Microbiol*, 71, 4203–13.

Bergsma-Vlami, M., Prins, M.E. & Raaijmakers, J.M. (2005). Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* spp. *FEMS Microbiol Ecol*, 52, 59–69.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol*, 62, 293–300.

Billings, S. & Ziegler, S. (2005). Linking microbial activity and soil organic matter transformations in forest soils under elevated CO_2. *Global Change Biol*, 11, 203–12.

Bimboim, H.C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl Acids Res*, 7, 1513–23.

Brachmann, A.O., Joyce, S.A., Jenke-Kodama, H., Schwar, G., Clarke, D.J. & Bode, H.B. (2007). A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*. *Chembiochem*, 8, 1721–28.

Brown, L.W. (1978). High-pressure liquid chromatographic determination of cycloheximide in ointment and suspension formulations. *J Pharm Sci*, 67, 669–72.

Brown, S.C., Kruppa, G. & Dasseux, J.L. (2005). Metabolomics applications of FT-ICR mass spectrometry. *Mass Spectrom Rev*, 24, 223–31.

Bruce, K., Jones, T., Bezemer, T., Thompson, L. & Ritchie, D. (2000). The effect of elevated atmospheric carbon dioxide levels on soil bacterial communities. *Global Change Biol*, 6, 427–34.

Bulluck III., L. & Ristaino, J. (2002). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology*, 92, 181–9.

Busti, E., Cavaletti, L., Monciardini, P., Schumann, P., Rohde, M., Sosio, M. & Donadio, S. (2006a). *Catenulispora acidiphila* gen. nov., sp. nov., a novel, mycelium-forming actinomycete, and proposal of Catenulisporaceae fam. nov. *Int J Syst Evol Microbiol*, 56, 1741–6.

Busti, E., Monciardini, P., Cavaletti, L., Bamonte, R., Lazzarini, A., Sosio, M. & Donadio, S. (2006b). Antibiotic-producing ability by representatives of a newly discovered lineage of actinomycetes. *Microbiology*, 152, 675–83.

Cartwright, D.K. & Spurr jr., H.W. (1998). Biological control of *Phytophthora parasitica* var. *nicotianae* on tobacco seedlings with non-pathogenic binucleate *Rhizoctonia* fungi. *Soil Biol Biochem*, 30, 1879–84.

Castillo, U.F., Strobel, G.A., Ford, E.J., Hess, W.M., Porter, H., Jensen, J.B., Albert, H., Robison, R., Condron, M.A., Teplow, D.B., Stevens, D. & Yaver, D. (2002). Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL 30562, endophytic on *Kennedia nigriscans*. *Microbiology*, 148, 2675–85.

Cavaletti, L., Monciardini, P., Schumann, P., Rohde, M., Bamonte, R., Busti, E., Sosio, M. & Donadio, S. (2006). *Actinospica robiniae* gen. nov., sp. nov. and *Actinospica acidiphila* sp. nov.: proposal for Actinospicaceae fam. nov. and Catenulisporinae subord. nov. in the order Actinomycetales. *Int J Syst Evol Microbiol*, 56, 1747–53.

Chakraborty, S., Tiedemann, A.V. & Teng, P.S. (2000). Climate change: potential impact on plant diseases. *Environ Pollut*, 108, 317–26.

Chambers, S. & Scott, E. (1995). *In vitro* antagonism of *Phytophthora cinnamomi* and *P. citricola* by isolates of *Trichoderma* spp. and *Gliocladium virens*. *J Phytopathol*, 132, 471–7.

Chuck, J.A., Dunn, C., Facultad, F.E., Nakazono, C., Nikodinovic, J. & Barrow, K.D. (2006). Amplification of DNA encoding entire type I polyketide synthase domains and linkers from *Streptomyces* species. *Curr Microbiol*, 53, 89–94.

Chung, H., Zak, D., Reich, P. & Ellsworth, D. (2007). Plant species richness, elevated CO_2, and atmospheric nitrogen deposition alter soil microbial community composition and function. *Global Change Biol*, 13, 980–9.

Chung, H., Zak, D.R. & Lilleskov, E.A. (2006). Fungal community composition and metabolism under elevated CO_2 and O_3. *Oecologia*, 147, 143–54.

Cohan, F.M. (2002). What are bacterial species? *Annu Rev Microbiol*, 56, 457–87.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M. & Tiedje, J.M. (2005). The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res*, 33, 294–6.

Compant, S., Duffy, B., Nowak, J., Clement, C. & Barka, E.A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol*, 71, 4951–9.

Cooke, D.E.L., Drenth, A., Duncan, J.M., Wagels, G. & Brasier, C.M. (2000). A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genet Biol*, 30, 17–32.

Cosgrove, L., McGeechan, P.L., Robson, G.D. & Handley, P.S. (2007). Fungal communities associated with degradation of polyester polyurethane in soil. *Appl Environ Microbiol*, 73, 5817–24.

Costa, R., Gomes, N.C., Krogerrecklenfort, E., Opelt, K., Berg, G. & Smalla, K. (2007). *Pseudomonas* community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. *Environ Microbiol*, 9, 2260–73.

Crawford, D.L., Lynch, J.M., Whipps, J.M. & Ousley, M.A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl Environ Microbiol*, 59, 3899–905.

De la Cruz, J., Pintor-Toro, J.A., Benitez, T., Llobell, A. & Romero, L.C. (1995). A novel endo-beta-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. *J Bacteriol*, 177, 6937–45.

Curtis, P. & Wand, X. (1998). A meta-analysis of elevated CO_2 effects on woody plant mass, form and physiology. *Oecologia*, 113, 299–313.

Darrah, P. (1996). Rhizodeposition under ambient and elevated CO_2 levels. *Plant Soil*, 187, 265–75.

Davelos, A.L., Xiao, K., Flor, J.M. & Kinkel, L.L. (2004a). Genetic and phenotypic traits of streptomycetes used to characterize antibiotic activities of field-collected microbes. *Can J Microbiol*, 50, 79–89.

Davelos, A.L., Xiao, K., Samac, D.A., Martin, A.P. & Kinkel, L.L. (2004b). Spatial variation in *Streptomyces* genetic composition and diversity in a prairie soil. *Microb Ecol*, 48, 601–12.

Davelos Baines, A., Xiao, K. & Kinkel, L.L. (2007). Lack of correspondence between genetic and phenotypic groups amongst soil-borne streptomycetes. *FEMS Microbiol Ecol*, 59, 564–75.

De Bellis, T., Kernaghan, G. & Widden, P. (2007). Plant community influences on soil microfungal assemblages in boreal mixed-wood forests. *Mycologia*, 99, 356–67.

De Graaff, M.A., Van Groenigen, K.J., Six, J., Hungate, B. & Van Kessel, C. (2006). Interactions between plant growth and soil nutrient cycling under elevated CO_2: a meta-analysis. *Global Change Biol*, 12, 2077–91.

Deng, S., Lorito, M., Penttila, M. & Harman, G.E. (2007). Overexpression of an endochitinase gene (ThEn-42) in *Trichoderma atroviride* for increased production of antifungal enzymes and enhanced antagonist action against pathogenic fungi. *Appl Biochem Biotechnol*, 142, 81–94.

Dettmer, K., Aronov, P.A. & Hammock, B.D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrom Rev*, 26, 51–78.

DIN (ISO 14240-2:1999-10). Bodenbeschaffenheit - Bestimmung der mikrobiellen Biomasse von Böden - Teil 2: Fumigations-Extraktionsverfahren (ISO 14240-2:1999-10).

Dohrmann, A.B. & Tebbe, C.C. (2005). Effect of elevated tropospheric ozone on the structure of bacterial communities inhabiting the rhizosphere of herbaceous plants native to Germany. *Appl Environ Microbiol*, 71, 7750–8.

Drigo, B., Kowalchuk, G., Yergeau, E., Bezemer, T., Boschker, H. & Van Veen, J.A. (2007). Impact of elevated carbon dioxide on the rhizosphere communities of *Carex arenaria* and *Festuca rubra*. *Global Change Biol*, 13, 2396–410.

Druzhinina, I.S., Kopchinskiy, A.G., Komon, M., Bissett, J., Szakacs, G. & Kubicek, C.P. (2005). An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet Biol*, 42, 813–28.

Duffy, B.K. & Defago, G. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol*, 65, 2429–38.

Duffy, B.K., Schouten, A. & Raaijmakers, J.M. (2003). Pathogen self-defense: mechanisms to counteract microbial antagonism. *Annu Rev Phytopathol*, 41, 501–38.

Dufrêne, M. & Legendre, P. (1997). Species assemblages and indicator species: the need for a flexible asymmetrical approach. *Ecol Monogr*, 67, 345–66.

Dunne, C., Moenne-Loccoz, Y., de Bruijn, F.J. & O'Gara, F. (2000). Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81. *Microbiology*, 146, 2069–78.

Egan, S., Wiener, P., Kallifidas, D. & Wellington, E.M. (2001). Phylogeny of *Streptomyces* species and evidence for horizontal transfer of entire and partial antibiotic gene clusters. *Ant v Leeuwenhoek*, 79, 127–33.

El-Tarabily, K., Hardy, G.E.S.J., Sivasithamparam, K., Hussein, A.M. & Kurtböke, D.I. (1997). The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. *New Phytol*, 137, 495–507.

Elad, Y. & Chet, I. (1983). Improved selective media for isolation of *Trichoderma* spp. or *Fusarium* spp. *Phytoparasitica*, 11, 55–8.

Erwin, D.C. & Ribeiro, O.K. (1996). *Phytophthora Diseases Worldwide*. 1st edn. APS Press, St. Paul.

Esperschütz, J., Gattinger, A., Mader, P., Schloter, M. & Fliessbach, A. (2007). Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. *FEMS Microbiol Ecol*, 61, 26–37.

Fenton, A.M., Stephens, P.M., Crowley, J., O'Callaghan, M. & O'Gara, F. (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl Environ Microbiol*, 58, 3873–8.

Fleischmann, F., Göttlein, A., Rodenkirchen, H., Lütz, C. & Oßwald, W. (2004). Biomass, nutrient and pigment content of beech (*Fagus sylvatica*) saplings infected with *Phytophthora citricola*, *P. cambivora*, *P. pseudosyringae* and *P. undulata*. *For Path*, 34, 79–92.

Fleischmann, F., Koehl, J., Portz, R., Beltrame, A. & Oßwald, W. (2005). Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin "citricolin" to pathogenesis. *Plant Biol*, 7, 650–8.

Fleischmann, F., Matyssek, R. & Oßwald, W. (2002a). Impact of CO_2 and nitrogen fertilization on the infection of beech with *Phytophthora citricola*. In: *Botanikertagung*. Freiburg, Germany, p. 313.

Fleischmann, F., Schneider, D., Matyssek, R. & Oßwald, W. (2002b). Investigations on net CO_2 assimilation, transpiration and root growth of *Fagus sylvatica* infested with four different *Phytophthora* species. *Plant Biol*, 4, 144–52.

Ford, J.H., Klomparens, W. & Hamner, C.L. (1958). Cycloheximide (acti-dione) and its agricultural uses. *Plant Disease Reporter*, 42, 680–95.

Frankland, J., Dighton, J. & Boddy, L. (1990). Methods for studying fungi in soil and forest litter. *Methods Microbiol*, 22, 343–404.

Fravel, R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Ann Rev Phytopathol*, 26, 75–91.

Garbeva, P., Postma, J., van Veen, J.A. & van Elsas, J.D. (2006). Effect of above-ground plant species on soil microbial community structure and its impact on suppression of *Rhizoctonia solani* AG3. *Environ Microbiol*, 8, 233–46.

Garbeva, P., van Veen, J.A. & van Elsas, J.D. (2004). Microbial diversity in soil: selection microbial populations by plant soil type and implications for disease suppressiveness. *Annu Rev Phytopathol*, 42, 243–70.

Garrett, K.A., Dendy, S.P., Frank, E.E., Rouse, M.N. & Travers, S.E. (2006). Climate change effects on plant disease: genomes to ecosystems. *Annu Rev Phytopathol*, 44, 489–509.

Gianni, C., Caretta, G. & Romano, C. (2003). Skin infection due to *Geomyces pannorum* var. *pannorum*. *Mycoses*, 46, 430–2.

Goodfellow, M. & Williams, S.T. (1983). Ecology of actinomycetes. *Ann Rev Microbiol*, 37, 189–216.

Gottlieb, D. (1976). The production and role of antibiotics in soil. *J Antibiot (Tokyo)*, 29, 987–1000.

Gremion, F., Chatzinotas, A. & Harms, H. (2003). Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. *Environ Microbiol*, 5, 896–907.

Grünwald, N., Hu, S. & Van Bruggen, A. (2000). Short-term cover-crop decomposition in organic and conventional soils: soil microbial and nutrient cycling indicator variables associated with different levels of soil suppressiveness to *Pythium aphanidermatum*. *Eur J Plant Pathol*, 106, 51–65.

Grüter, D., Schmid, B. & Brandl, H. (2006). Influence of plant diversity and elevated atmospheric carbon dioxide levels on belowground bacterial diversity. *BMC Microbiol*, 6, 68–75.

Götz, M., Nirenberg, H., Krause, S., Wolters, H., Draeger, S., Buchner, A., Lottmann, J., Berg, G. & Smalla, K. (2006). Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods. *FEMS Microbiol Ecol*, 58, 404–13.

Haas, D. & Defago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol*, 3, 307–19.

Hagedorn, C. (1976). Influences of soil acidity on *Streptomyces* populations inhabiting forest soils. *Appl Environ Microbiol*, 32, 368–75.

Hagn, A., Wallisch, S., Radl, V., Charles Munch, J. & Schloter, M. (2007). A new cultivation independent approach to detect and monitor common *Trichoderma* species in soils. *J Microbiol Meth*, 69, 86–92.

Hamelin, R.C., Berube, P., Gignac, M. & Bourassa, M. (1996). Identification of root rot fungi in nursery seedlings by nested multiplex PCR. *Appl Environ Microbiol*, 62, 4026–31.

Handelsman, J. & Stabb, E.V. (1996). Biocontrol of soilborne plant pathogens. *Plant Cell*, 8, 1855–69.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. & Lorito, M. (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nat Rev Microbiol*, 2, 43–56.

Hayakawa, M. & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J Ferment Technol*, 65, 501–9.

Heagle, A. (1973). Interactions between air pollutants and plant parasites. *Ann Rev Phytopathol*, 11, 365–88.

Henry, S., Bru, D., Stres, B., Hallet, S. & Philippot, L. (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Appl Environ Microbiol*, 72, 5181–9.

Hermosa, M.R., Grondona, I., Iturriaga, E.A., Diaz-Minguez, J.M., Castro, C., Monte, E. & Garcia-Acha, I. (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl Environ Microbiol*, 66, 1890–8.

Hertel, D. & Leuschner, C. (2002). A comparison of four different fine root production estimates with ecosystem carbon balance data in a *Fagus-Quercus* mixed forest. *Plant Soil*, 239, 237–51.

Hertweck, C., Luzhetskyy, A., Rebets, Y. & Bechthold, A. (2007). Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. *Nat Prod Rep*, 24, 162–90.

Heuer, H., Krsek, M., Baker, P., Smalla, K. & Wellington, E.M. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol*, 63, 3233–41.

Heungens, K. & Parke, J. (2001). Postinfection biological control of oomycete pathogens of pea by *Burkholderia cepacia* AMMDR1. *Phytopathology*, 91, 383–91.

Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. *Arbeiten der Deitschen Landwirtschaftlichen Gesellschaft*, 98, 59–78.

Hjort, K., Lembke, A., Speksnijder, A., Smalla, K. & Jansson, J.K. (2007). Community structure of actively growing bacterial populations in plant pathogen suppressive soil. *Microb Ecol*, 53, 399–413.

Hoagland, D. & Arnon, D. (1950). The water-culture method for growing plants without soil. The College of Agriculture, University of California, Berkeley, vol. 347.

Hodge, A., Paterson, E., Grayston, S., Campell, C., Ord, B. & Killham, K. (1998). Characterization and microbial utilization of exudate material from rhizosphere of *Lolium perenne* grown under CO_2 enrichment. *Soil Biol Biochem*, 30, 1033–43.

Hopwood, D.A. (1997). Genetic contributions to understanding polyketide synthases. *Chem Rev*, 97, 2465–98.

Howell, C.R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Plant Dis*, 87, 4–10.

IPCC (2007). Summary for policymakers. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, UK and New York, NY, USA.

Islam, K., Mulchi, C. & Ali, A. (2000). Interactions of tropospheric CO_2 and O_3 enrichments and moisture variations on microbial biomass and respiration in soil. *Global Change Biol*, 6, 255–65.

Islam, K. & Weil, R. (1998). Microwave irradiation of soil for routine measurement of microbial biomass C. *Biol Fert Soils*, 27, 408–16.

Janus, L.R., Angeloni, N.L., McCormack, J., Rier, S.T., Tuchman, N.C. & Kelly, J.J. (2005). Elevated atmospheric CO_2 alters soil microbial communities associated with trembling aspen (*Populus tremuloides*) roots. *Microb Ecol*, 50, 102–9.

Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T. & Steinberg, C. (2007). Soil health through soil disease suppression: Which strategy from descriptors to indicators? *Soil Biol Biochem*, 39, 1–23.

Jeffs, P. & McWilliams, D. (1981). Carbon-13 nuclear magnetic resonance study of the biosynthesis of cycloheximide. Stereospecific incorporation of malonate into glutarimide ring. *J Am Chem Soc*, 103, 6185–92.

Jenke-Kodama, H., Sandmann, A., Müller, R. & Dittmann, E. (2005). Evolutionary implications of bacterial polyketide synthases. *Mol Biol Evol*, 22, 2027–39.

Johnson, L. & Curl, E. (1972). *Methods for research on the Ecology of soil-borne plant pathogens.* Burgess Publishing Company, Minneapolis, MN, USA.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A. & Janssen, P.H. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl Environ Microbiol*, 69, 7210–5.

Jossi, M., Fromin, N., Tarnawski, S., Kohler, F., Gillet, F., Aragno, M. & Hamelin, J. (2006). How elevated pCO_2 modifies total and metabolically active bacterial communities in the rhizosphere of two perennial grasses grown under field conditions. *FEMS Microbiol Ecol*, 55, 339–50.

Jung, T. & Blaschke, H. (1996). Phytophthora root-rot in declining forest trees. *Phyton (Austria)*, 36, 95–102.

Jung, T. & Blaschke, H. (2004). *Phytophthora* root and collar rot of alders in Bavaria: distribution, modes of spread, and possible management strategies. *Plant Pathol*, 53, 197–208.

Jung, T., Blaschke, H. & Oßwald, W. (2000). Involvement of soilborne *Phytophthora* species in Central European oak decline and the effect of site factors on the disease. *Plant Pathol*, 49, 706–18.

Jung, T., Hudler, G., Jensen-Tracy, S., Griffiths, H., Fleischmann, F. & Oßwald, W. (2005). Involvement of *Phytophthora* species in the decline of European beech in Europe and the USA. *Mycologist*, 19, 159–66.

Kämpfer, P. (2006). The Family Streptomycetaceae, Part I: Taxonomy. In: *The Prokaryotes.* Springer, New York, vol. 3, pp. 538–604.

Kämpfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M. & Amann, R. (1996). Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus *in situ* identification using group- and genus-specific rRNA-targeted oligonucleotide probes. *Microb Ecol*, 32, 101–21.

Kang, S. & Kim, S. (2004). New antifungal activity of penicillic acid against *Phytophthora* species. *Biotechnol Lett*, 26, 695–98.

Kataoka, M., Ueda, K., Kudo, T., Seki, T. & Yoshida, T. (1997). Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus *Streptomyces*. *FEMS Microbiol Lett*, 151, 249–55.

Khan, M. & Williams, S.T. (1975). Studies on the ecology of actinomycetes in soil - VIII. *Soil Biol Biochem*, 7, 345–8.

Kim, S.B., Lonsdale, J., Seong, C.N. & Goodfellow, M. (2003). *Streptacidiphilus* gen. nov., acidophilic actinomycetes with wall chemotype I and emendation of the family Streptomycetaceae (Waksman and Henrici (1943)AL) emend. Rainey et al. 1997. *Ant v Leeuwenhoek*, 83, 107–16.

Kim, S.B., Seong, C.N., Jeon, S.J., Bae, K.S. & Goodfellow, M. (2004). Taxonomic study of neutrotolerant acidophilic actinomycetes isolated from soil and description of *Streptomyces yeochonensis* sp. nov. *Int J Syst Evol Microbiol*, 54, 211–4.

King, J., Pregitzer, K., Zak, D., Sober, J., Isebrands, J., Dickson, R., Hendrey, G. & Karnosky, D.F. (2001). Fine-root biomass and fluxes of soil carbon in young stands of paper birch and trembling aspen as affected by elevated atmospheric CO_2 and tropospheric O_3. *Oecologia*, 128, 237–50.

Klamer, M., Roberts, M.S., Levine, L.H., Drake, B.G. & Garland, J.L. (2002). Influence of elevated CO_2 on the fungal community in a coastal scrub oak forest soil investigated with terminal-restriction fragment length polymorphism analysis. *Appl Environ Microbiol*, 68, 4370–6.

Kominek, L.A. (1975). Cycloheximide production by *Streptomyces griseus*: alleviation of end-product inhibition by dialysis-extraction fermentation. *Antimicrob Agents Chemother*, 7, 861–3.

Kreutzer, K., Göttlein, A., Pröbstle, P. & Zuleger, M. (1991). Höglwaldforschung 1982-1989. Zielsetzung, Versuchskonzept, Basisdaten. In: *Ökosystemforschung Höglwald. Forstwissenschaftliche Forschungen* (eds. Kreutzer, K. & Göttlein, A.). Verlag Paul Parey, Hamburg, Berlin, pp. 11–21.

Kruskal, J. (1964). Nonmetric multidimensional scaling: a numerical method. *Psychometrika*, 29, 115–29.

Kubicek, C.P., Bolzlbauer, U.M., Kovacs, W., Mach, R.L., Kuhls, K., Lieckfeldt, E., Borner, T. & Samuels, G.J. (1996). Cellulase formation by species of *Trichoderma* sect. *Longibrachiatum* and of *Hypocrea* spp. with anamorphs referable to *Trichoderma* sect. *Longibrachiatum*. *Fungal Genet Biol*, 20, 105–14.

Kuhad, R.C., Kapoor, R.K. & Lal, R. (2004). Improving the yield and quality of DNA isolated from white-rot fungi. *Folia Microbiol (Praha)*, 49, 112–6.

Kuhls, K., Lieckfeldt, E., Börner, T. & Guého, E. (1999). Molecular reidentification of human pathogenic *Trichoderma* isolates as *Trichoderma longibrachiatum* and *Trichoderma citrinoviride*. *Med Mycol*, 37, 25–33.

Kurtböke, D.I., Hayakawa, M., Terekhova, L. & Okazaki, T. (2003). *Selective Isolation of Actinomycetes*. Queensland Complete Printing Services, Nambour, Queensland.

Küster, E. & Williams, S.T. (1964). Selection of media for isolation of streptomycetes. *Nature*, 202, 928–9.

Kuzyakov, Y. & Domanski, G. (2000). Carbon inputs by plants into the soil. *J Plant Nutr Soil Sci*, 163, 421–31.

Lederer, W., Lorenz, K.H. & Seemüller, E. (1992). Studies on antagonistic effects of *Trichoderma* isolates against *Phytophthora cactorum*. *J Phytopathol*, 136, 154–64.

Lee, J.Y. & Hwang, B.K. (2002). Diversity of antifungal actinomycetes in various vegetative soils of Korea. *Can J Microbiol*, 48, 407–17.

Lee, T.S., Khosla, C. & Tang, Y. (2005). Orthogonal protein interactions in spore pigment producing and antibiotic producing polyketide synthases. *J Antibiot (Tokyo)*, 58, 663–6.

Leon, M., Stone, A. & Dick, R. (2006). Organic soil amendments: impacts on snap bean common root rot (*Aphanomyces euteiches*) and soil quality. *Appl Soil Ecol*, 31, 199–210.

Limon, M.C., Chacon, M.R., Mejias, R., Delgado-Jarana, J., Rincon, A.M., Codon, A.C. & Benitez, T. (2004). Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. *Appl Microbiol Biotechnol*, 64, 675–85.

Lipson, D.A., Wilson, R.F. & Oechel, W.C. (2005). Effects of elevated atmospheric CO_2 on soil microbial biomass, activity, and diversity in a chaparral ecosystem. *Appl Environ Microbiol*, 71, 8573–80.

Liu, W.T., Marsh, T.L., Cheng, H. & Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol*, 63, 4516–22.

Liu, X., Kozovits, A., Grams, T., Blaschke, H., Rennenberg, H. & Matyssek, R. (2004). Competition modifies effects of enhanced ozone/carbon dioxide concentrations on carbohydrate and biomass accumulation in juvenile Norway spruce and European beech. *Tree Physiol*, 24, 1045–55.

Liu, Y. & Yang, Q. (2007). Cloning and heterologous expression of aspartic protease SA76 related to biocontrol in *Trichoderma harzianum*. *FEMS Microbiol Lett*, 277, 173–81.

Lord, N.S., Kaplan, C.W., Shank, P., Kitts, C.L. & Elrod, S.L. (2002). Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. *FEMS Microbiol Ecol*, 42, 327–37.

Lorito, M., Harman, G., Hayes, C., Broadway, R., Tronsmo, A., Woo, S. & Di Pietro, A. (1993). Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology*, 83, 302–7.

Lorito, M., Hayes, C., Di Pietro, A., Woo, S. & Harman, G.E. (1994). Purification, characterization, and synergistic activity of a glucan 1,3-beta-glucosidase and an N-acetyl-beta-glucosaminidase from *Trichoderma harzianum*. *Phytopathology*, 84, 398–405.

Lottmann, J. & Berg, G. (2001). Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants. *Microbiol Res*, 156, 75–82.

Lüdemann, H. & Conrad, R. (2000). Molecular retrieval of large 16S rRNA gene fragments from an Italian rice paddy soil affiliated with the class Actinobacteria. *Syst Appl Microbiol*, 23, 582–4.

Luedemann, G., Matyssek, R., Fleischmann, F. & Grams, T.E. (2005). Acclimation to ozone affects host/pathogen interaction and competitiveness for nitrogen in juvenile *Fagus sylvatica* and *Picea abies* trees infected with *Phytophthora citricola*. *Plant Biol*, 7, 640–9.

Lumsden, R.D. & Locke, J. (1989). Biological control of damping-off caused by *Phytium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. *Phytopathology*, 79, 361–6.

Lynch, B.A. & Whipps, J.M. (1991). *Substrate flow in the rhizosphere*. Kluwer Academic Publisher, Dodrecht, Netherlands.

Marschner, H. (1995). *Mineral Nutrition of Higher Plants*. 2nd edn. Academic Press, London.

Martinez, C., Michaud, M., Bélanger, R. & Tweddell, R. (2002). Identification of soils suppressive against *Helminthosporium solani*, the causal agent of potato silver scurf. *Soil Biol Biochem*, 34, 1861–8.

Matyssek, R., Agerer, R., Ernst, D., Munch, J.C., Oßwald, W., Pretzsch, H., Priesack, E., Schnyder, H. & Treutter, D. (2005). The plant's capacity in regulating resource demand. *Plant Biol*, 7, 560–80.

Matyssek, R. & Innes, J. (1999). Ozone - a risk factor for trees and forests in Europe? *Water Air Soil Poll*, 116, 199–226.

Matyssek, R. & Sandermann Jr., H. (2003). Impact of ozone on trees: an ecophysiological perspective. In: *Progress in Botany* (eds. Esser, K., Lüttge, U., Beyschlag, W. & Hellwig, F.). Springer Verlag, Berlin, Heidelberg, pp. 349–404.

McCune, B. & Grace, J. (2002). *Analysis of Ecological Communities*. MjM Software Design, Gleneden Beach, USA.

McVeigh, H., Munro, J. & Embley, T. (1996). Molecular evidence for the presence of novel actinomycete lineages in a temperate forest soil. *J Ind Microbiol Biotechnol*, 17, 197–204.

Metsä-Ketelä, M., Halo, L., Munukka, E., Hakala, J., Mäntsälä, P. & Ylihonko, K. (2002). Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various *Streptomyces* species. *Appl Environ Microbiol*, 68, 4472–9.

Metsä-Ketelä, M., Salo, V., Halo, L., Hautala, A., Hakala, J., Mäntsälä, P. & Ylihonko, K. (1999). An efficient approach for screening minimal PKS genes from *Streptomyces*. *FEMS Microbiol Lett*, 180, 1–6.

Miethke, M. & Marahiel, M.A. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol Rev*, 71, 413–51.

Milner, J.L., Silo-Suh, L., Lee, J.C., He, H., Clardy, J. & Handelsman, J. (1996). Production of kanosamine by *Bacillus cereus* UW85. *Appl Environ Microbiol*, 62, 3061–5.

Monte, E. (2001). Understanding *Trichoderma*: between biotechnology and microbial ecology. *Int Microbiol*, 4, 1–4.

Montealegre, C., Van Kessel, C., Blumenthal, J., Hur, H.G., Hartwig, U. & Sadowsky, M. (2000). Elevated atmospheric CO_2 alters microbial population structure in a pasture ecosystem. *Global Change Biol*, 6, 475–82.

Morgan, J. & Winstanley, C. (1997). Microbial biomarkers. In: *Modern soil microbiology* (ed. Trevors, J.). Marcel Dekker, New York, pp. 331–47.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*, 59, 695–700.

Nechwatal, J. & Oßwald, W. (2001). Comparative studies on the fine root status of healthy and declining spruce and beech trees in the Bavarian Alps and occurrence of *Phytophthora* and *Pythium* species. *Forest Pathol*, 31, 257–73.

Neri, F., Mari, M., Menniti, A.M. & Brigati, S. (2006). Activity of trans-2-hexenal against *Penicillium expansum* in 'Conference' pears. *J Appl Microbiol*, 100, 1186–93.

Neumann, G. & Röhmheld, V. (2001). The release of root exudates as affected by plant physiological status. In: *The Rhizosphere* (eds. Pinton, R., Varanini, Z. & Nannipieri, P.). Marcel Dekker, Inc., New York, pp. 41–93.

Nichols, D. (2007). Cultivation gives context to the microbial ecologist. *FEMS Microbiol Ecol*, 60, 351–7.

Nwosu, V.C. (2001). Antibiotic resistance with particular reference to soil microorganisms. *Res Microbiol*, 152, 421–30.

O'Brien, J., Blanchette, R. & Sutherland, J. (1984). Assessment of *Streptomyces* spp. from elms for biological control of Dutch elm disease. *Plant Dis*, 68, 104–6.

Obrig, T.G., Culp, W.J., McKeehan, W.L. & Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J Biol Chem*, 246, 174–81.

Oda, Y., Star, B., Huisman, L.A., Gottschal, J.C. & Forney, L.J. (2003). Biogeography of the purple nonsulfur bacterium *Rhodopseudomonas palustris*. *Appl Environ Microbiol*, 69, 5186–91.

O'Hagan, D. (1995). Biosynthesis of fatty acid and polyketide metabolites. *Nat Prod Rep*, 12, 1–32.

Okamoto, H., Sato, M., Sato, Z. & Isaka, M. (1998). Biocontrol of *Phytophthora capsici* by *Serratia marcescens* F-1-1 and analysis of biocontrol mechanisms using transposon-insertion mutants. *Ann Phytopathol Soc Jpn*, 64, 287–93.

Oldiges, M., Lutz, S., Pflug, S., Schroer, K., Stein, N. & Wiendahl, C. (2007). Metabolomics: current state and evolving methodologies and tools. *Appl Microbiol Biotechnol*, 76, 495–511.

Olivain, C., Trouvelot, S., Binet, M.N., Cordier, C., Pugin, A. & Alabouvette, C. (2003). Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogenic strains of *Fusarium oxysporum*. *Appl Environ Microbiol*, 69, 5453–62.

Omura, S., Takahashi, Y., Iwai, Y. & Tanaka, H. (1982). *Kitasatosporia*, a new genus of the order Actinomycetales. *J Antibiot (Tokyo)*, 35, 1013–9.

O'Neil, M., Smith, A. & Heckelman, P. (2001). *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals*. 13th edn. Merck + Co., Inc., Rahway, N.J., USA.

Orphan, V.J., Hinrichs, K.U., Ussler W., r., Paull, C.K., Taylor, L.T., Sylva, S.P., Hayes, J.M. & Delong, E.F. (2001). Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Appl Environ Microbiol*, 67, 1922–34.

Osborn, A.M., Moore, E.R. & Timmis, K.N. (2000). An evaluation of terminal-restriction fragment length polymorphism (t-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol*, 2, 39–50.

Oßwald, W., Koehl, J., Heiser, I., Nechwatal, J. & Fleischmann, F. (2004). New insights in the genus *Phytophthora* and current diseases these pathogens cause in their ecosystems. In: *Progress in Botany* (eds. Esser, K., Lüttge, U., Beyschlag, W. & Murata, J.). Springer Verlag, Berlin, Heidelberg, pp. 436–466.

Oyarzun, P. (1998). Factors associated with soil receptivity to some fungal root rot pathogens of peas. *Appl Soil Ecol*, 10, 151–69.

Paulitz, T.C. & Belanger, R.R. (2001). Biological control in greenhouse systems. *Annu Rev Phytopathol*, 39, 103–33.

Phillips, D., Fox, T. & Six, J. (2006). Root exudation (net efflux of amino acids) may increase rhizodeposition under elevated CO_2. *Global Change Biol*, 12, 561–7.

Phillips, R., Zak, D., Holmes, W. & White, D. (2002). Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone. *Oecologia*, 131, 236–44.

Picard, K., Tirilly, Y. & Benhamou, N. (2000). Cytological effects of cellulases in the parasitism of *Phytophthora parasitica* by *Pythium oligandrum*. *Appl Environ Microbiol*, 66, 4305–14.

Pieterse, C.M. & van Loon, L.C. (1999). Salicylic acid-independent plant defence pathways. *Trends Plant Sci*, 4, 52–8.

Poorter, H. & Perez-Soba, M. (2001). The growth response of plants to elevated CO_2 under non-optimal environmental conditions. *Oecologia*, 129, 1–20.

Pritsch, K., Luedemann, G., Matyssek, R., Hartmann, A., Schloter, M., Scherb, H. & Grams, T.E. (2005). Mycorrhizosphere responsiveness to atmospheric ozone and inoculation with *Phytophthora citricola* in a phytotron experiment with spruce/beech mixed cultures. *Plant Biol*, 7, 718–27.

Quaghebeur, K., Coosemans, J., Toppet, S. & Compernolle, F. (1994). Cannabiorci- and 8-chlorocannabiorcichromenic acid as fungal antagonists from *Cylindrocarpon olidum*. *Phytochemistry*, 37, 159–61.

Raaijmakers, J.M., Vlami, M. & de Souza, J.T. (2002). Antibiotic production by bacterial biocontrol agents. *Ant v Leeuwenhoek*, 81, 537–47.

Rademaker, J., Louws, F. & de Bruijn, F.J. (1998). Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: *Molecular Microbial Ecology Manual* (ed. Akkermans, A.). Kluwer Academic Publ., Dordrecht, vol. 3.4.3, pp. 1–27.

Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol*, 62, 142–60.

Roesch, L., Fulthorpe, R., Riva, A., Casella, G., Hadwin, A., Kent, A., Daroub, S., Camargo, F., Farmerie, W. & Triplett, E. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal*, 1, 283–90.

Rosselló-Mora, R., Lucio, M., Pena, A., Brito-Echeverría, J., López-López, A., Valens-Vadell, M., Frommberger, M., Antón, J. & Schmitt-Kopplin, P. (2008). Metabolic evidence for biogeographic isolation of the extremophilic bacterium Salinibacter ruber. *The ISME Journal*, in press.

Rothrock, C.S. & Gottlieb, D. (1981). Importance of antibiotic production in antagonism of selected Streptomyces species to two soil-borne plant pathogens. *J Antibiot (Tokyo)*, 34, 830–5.

Ryan, P., Delhaize, E. & Jones, D. (2001). Function and mechanism of organic anion exudation from plant roots. *Annu Rev Plant Physiol Plant Mol Biol*, 52, 527–60.

Sambrook, J., Fritsch, E. & Maniatis, T. (1989). *Molecular cloning. A laborytory manual.* Cold Spring Harbor Laboratory press,, New York.

Sandermann Jr., H., Ernst, D., Heller, W. & Langebartels, C. (1998). Ozone: an abiotic elicitor of plant defence reactions. *Trends Plant Sci*, 3.

Sanz, L., Montero, M., Grondona, I., Vizcaino, J., Llobell, A., Hermosa, M.R. & Monte, E. (2004). Cell wall-degrading isoenzyme profiles of Trichoderma biocontrol strains show correlation with rDNA taxonomic species. *Curr Genet*, 46, 277–86.

Sanz, L., Montero, M., Redondo, J., Llobell, A. & Monte, E. (2005). Expression of an alpha-1,3-glucanase during mycoparasitic interaction of Trichoderma asperellum. *FEBS J*, 272, 493–9.

Schirmböck, M., Lorito, M., Wang, Y., Hayes, C., Arisan-Atac, I., Scala, F., Harman, G. & Kubicek, C. (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of Trichoderma harzianum against phytopathogenic fungi. *Appl Environ Microbiol*, 60, 4364–70.

Schottel, J., Shimizu, K. & Kinkel, L.L. (2001). Relationship of in vitro pathogen inhibition and soil colonization to potato scab biocontrol by antagonistic Streptomyces spp. *Biol Control*, 20, 102–12.

Schrempf, H. (2006). The Family Streptomycetaceae, Part II: Molecular Biology. In: *The Prokaryotes*. Springer, New York, vol. 3, pp. 605–622.

Schubert, R., Bahnweg, G., Nechwatal, J., Jung, T., Cooke, D.E.L., Duncan, J.M., Müller-Starck, G., Langebartels, C., Sandermann Jr., H. & Oßwald, W. (1999). Detection and quantification of *Phytophthora* species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. *Eur J For Pathol*, 27, 1–19.

Schwieger, F. & Tebbe, C.C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microbiol*, 64, 4870–6.

Segarra, G., Casanova, E., Bellido, D., Odena, M.A., Oliveira, E. & Trillas, I. (2007). Proteome, salicylic acid, and jasmonic acid changes in cucumber plants inoculated with *Trichoderma asperellum* strain T34. *Proteomics*, 7, 3943–52.

Seidl, V., Huemer, B., Seiboth, B. & Kubicek, C.P. (2005). A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J*, 272, 5923–39.

Seow, K.T., Meurer, G., Gerlitz, M., Wendt-Pienkowski, E., Hutchinson, C.R. & Davies, J. (1997). A study of iterative type II polyketide synthases, using bacterial genes cloned from soil DNA: a means to access and use genes from uncultured microorganisms. *J Bacteriol*, 179, 7360–8.

Shimada, H., Noguchi, H., Iitaka, Y. & Sankawa, U. (1981). Biosynthesis of cycloheximide stereospecific incorporation of [1,2,3-13c3] malonate. *Heterocycles*, 15, 1141–6.

Sid Ahmed, A., Pérez-Sánchez, C., Egea, C. & Candela, M. (1999). Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathol*, 48, 58–65.

Silo-Suh, L., Lethbridge, B., Raffel, S., He, H., Clardy, J. & Handelsman, J. (1994). Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl Environ Microbiol*, 60, 2023–30.

Smalla, K., Oros-Sichler, M., Milling, A., Heuer, H., Baumgarte, S., Becker, R., Neuber, G., Kropf, S., Ulrich, A. & Tebbe, C.C. (2007). Bacterial diversity of soils assessed by DGGE, t-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results? *J Microbiol Methods*, 69, 470–9.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H. & Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol*, 67, 4742–51.

Smida, J., Leibhard, S., Nickel, A.M., Eckardt-Schupp, F. & Hieber, L. (1996). Application of repetitive sequence-based PCR (inter-LINE PCR) for the analysis of genomic rearrangements and for the genome characterization on different taxonomic levels. *Genet Anal*, 13, 95–8.

Smith, V., Wilcox, W. & Harman, G. (1990). Potential for biological control of phytophthora root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology*, 80, 880–885.

Sommer, R. & Tautz, D. (1989). Minimal homology requirements for PCR primers. *Nucleic Acids Res*, 17, 6749.

Stach, J.E., Maldonado, L.A., Ward, A.C., Goodfellow, M. & Bull, A.T. (2003). New primers for the class Actinobacteria: application to marine and terrestrial environments. *Environ Microbiol*, 5, 828–41.

Staunton, J. & Weissman, K. (2001). Polyketide biosynthesis: a millennium review. *Nat Prod Rep*, 18, 380–416.

Takahashi, Y. & Omura, S. (2003). Isolation of new actinomycete strains for the screening of new bioactive compounds. *J Gen Appl Microbiol*, 49, 141–54.

Tarnawski, S., Hamelin, J., Jossi, M., Aragno, M. & Fromin, N. (2006). Phenotypic structure of *Pseudomonas* populations is altered under elevated pCO_2 in the rhizosphere of perennial grasses. *Soil Biol Biochem*, 38, 1193–201.

Thirup, L., Johnsen, K. & Winding, A. (2001). Succession of indigenous *Pseudomonas* spp. and actinomycetes on barley roots affected by the antagonistic strain *Pseudomonas fluorescens* DR54 and the fungicide imazalil. *Appl Environ Microbiol*, 67, 1147–53.

Timms-Wilson, T.M., Ellis, R.J., Renwick, A., Rhodes, D.J., Mavrodi, D.V., Weller, D.M., Thomashow, L.S. & Bailey, M.J. (2000). Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact*, 13, 1293–300.

Torsvik, V., Salte, K., Sorheim, R. & Goksoyr, J. (1990). Comparison of phenotypic diversity and dna heterogeneity in a population of soil bacteria. *Appl Environ Microbiol*, 56, 776–81.

Toyoda, H., Hashimoto, H., Utsumi, R., Kobayashi, H. & Ouchi, S. (1988). Detoxification of fusaric acid by a fusaric acid-resistent mutant of *Pseudomonas solanacearum* and its application to biological control of *Fusarium* wilt of tomato. *Phytopathology*, 78, 1307–11.

Trejo-Estrada, S., Paszczynski, A. & Crawford, D.L. (1998). Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. *J Ind Microbiol Biot*, 21, 81–90.

Tsao, P. & Guy, S. (1977). Inhibition of *Mortierella* and *Phytium* in a *Phytophthora* isolation medium containing hymexazol. *Phytopathology*, 67, 796–801.

Van Loon, L.C., Bakker, P.A. & Pieterse, C.M. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol*, 36, 453–83.

Van Peer, R., Niemann, G. & Schippers, B. (1991). Induced resistence and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology*, 81, 728–34.

Vance, E.D., Brookes, P.C. & Jenkinson, D.S. (1987). An extraction method for measuring soil microbial biomass C. *Soil Biol Biochem*, 19, 703–7.

Vanek, Z., Cudlin, J. & Vondracek, M. (1967). *Cycloheximide and Other Glutarimide Antibiotics*. vol. II of *Antibiotics*. Springer-Verlag, New York.

Vanek, Z., Puza, M., Cudlin, J., Vondracek, M. & Rickards, R.W. (1969). Metabolites of *Streptomyces noursei*. X. Biogenesis of cycloheximide. *Folia Microbiol (Praha)*, 14, 388–97.

Vanin, E.F. (1985). Processed pseudogenes: characteristics and evolution. *Annu Rev Genet*, 19, 253–72.

Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmos Environ*, 38, 3431–42.

Viterbo, A., Ramot, O., Chemin, L. & Chet, I. (2002). Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Ant v Leeuwenhoek*, 81, 549–56.

Walker, T.S., Bais, H.P., Grotewold, E. & Vivanco, J.M. (2003). Root exudation and rhizosphere biology. *Plant Physiol*, 132, 44–51.

Wang, J., Soisson, S.M., Young, K., Shoop, W., Kodali, S., Galgoci, A., Painter, R., Parthasarathy, G., Tang, Y.S., Cummings, R., Ha, S., Dorso, K., Motyl, M., Jayasuriya, H., Ondeyka, J., Herath, K., Zhang, C., Hernandez, L., Allocco, J., Basilio, A., Tormo, J.R., Genilloud, O., Vicente, F., Pelaez, F., Colwell, L., Lee, S.H., Michael, B., Felcetto, T., Gill, C., Silver, L.L., Hermes, J.D., Bartizal, K., Barrett, J., Schmatz, D., Becker, J.W., Cully, D. & Singh, S.B. (2006). Platensimycin is a selective FabF inhibitor with potent antibiotic properties. *Nature*, 441, 358–61.

Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. (2007). Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*, 73, 5261–7.

Wang, Z. (2003). The influence of *Phytophthora citricola* on rhizosphere soil solution chemistry and the nutritional status of European beech seedlings. *J Phytopathol*, 151, 365–8.

Wawrik, B., Kerkhof, L., Zylstra, G.J. & Kukor, J.J. (2005). Identification of unique type II polyketide synthase genes in soil. *Appl Environ Microbiol*, 71, 2232–8.

Weissman, K.J. (2004). Polyketide biosynthesis: understanding and exploiting modularity. *Philos Transact A Math Phys Eng Sci*, 362, 2671–90.

Weller, D.M., Landa, B.B., Mavrodi, O.V., Schroeder, K.L., De La Fuente, L., Blouin Bankhead, S., Allende Molar, R., Bonsall, R.F., Mavrodi, D.V. & Thomashow, L.S. (2007). Role of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. in the defense of plant roots. *Plant Biol*, 9, 4–20.

Wellington, E.M., Stackebrandt, E., Sanders, D., Wolstrup, J. & Jorgensen, N.O. (1992). Taxonomic status of *Kitasatosporia*, and proposed unification with *Streptomyces* on the basis of phenotypic and 16s rRNA analysis and emendation of *Streptomyces* Waksman and Henrici 1943, 339AL. *Int J Syst Bacteriol*, 42, 156–60.

Wellington, E.M. & Williams, S. (1978). Preservation of actinomycete inoculum in frozen glycerol. *Microbios Lett*, 6, 151–7.

Werres, S. (1995). Influence of the Phytophthora isolate and the seed source on the development of beech (*Fagus sylvatica*) seedling blight. *Eur J For Pathol*, 25, 381–90.

Whipps, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot*, 52, 487–511.

White, D., Davis, W., Nickers, J., King, J. & Bobbie, R. (1979). Determination of sedimentary microbial biomass by extractable lipid phosphate. *Oecologia*, 40, 51–62.

White, T., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A Guide to Methods and Applications* (eds. Innis, M., Gelfand, D., Sninsky, J. & White, T.). Academic Press Inc., New York, pp. 315–321.

Wiemken, V., Laczko, E., Ineichen, K. & Boller, T. (2001). Effects of elevated carbon dioxide and nitrogen fertilization on mycorrhizal fine roots and the soil microbial community in beech-spruce ecosystems on siliceous and calcareous soil. *Microb Ecol*, 42, 126–35.

Wiener, P., Egan, S., Huddleston, A.S. & Wellington, E.M. (1998). Evidence for transfer of antibiotic-resistance genes in soil populations of streptomycetes. *Mol Ecol*, 7, 1205–16.

Wiggins, B. & Kinkel, L.L. (2005). Green manures and crop sequences influence alfalfa root rot and pathogen inhibitory activity among soil-borne streptomycetes. *Plant Soil*, 268, 271–283.

Williams, S., Shameemullah, M., Waltson, E. & Mayfield, C. (1972). Studies on the ecology of actinomycetes in soil. VI. The influence of moisture tension on growth and survival. *Soil Biol Biochem*, 4, 215–25.

Williams, S.T. & Wellington, E.M. (1982). Principles and problems of selective isolation of microbes. In: *Bioactive microbial products: Search and discovery* (eds. Bu´lock, J., Nisbet, L. & Winstanley, D.). Academic Press, London, UK.

Woese, C.R. (1987). Bacterial evolution. *Microbiol Rev*, 51, 221–71.

Wu, J., Joergensen, R., Pommerening, B., Chaussod, R. & Brookes, P. (1990). Measurement of soil microbial biomass C by fumigation-extraction - an automated procedure. *Soil Biol Biochem*, 25, 553–9.

Wulff, E., Pham, A., Chérif, M., Rey, P., Tirilly, Y. & Hockenhull, J. (1998). Inoculation of cucumber roots with zoospores of mycoparasitic and plant pathogenic *Pythium* species: differential zoospore accumulation, colonization ability and plant growth response. *Eur J Plant Pathol*, 104, 69–76.

Xiao, K., Kinkel, L.L. & Samac, D.A. (2002). Biological control of Phytophthora root rots on alfalfa and soybean with *Streptomyces*. *Biol Control*, 23, 285–95.

Xu, C., Wang, L., Cui, Q., Huang, Y., Liu, Z., Zheng, G. & Goodfellow, M. (2006). Neutrotolerant acidophilic *Streptomyces* species isolated from acidic soils in China: *Streptomyces guanduensis* sp. nov., *Streptomyces paucisporeus* sp. nov., *Streptomyces rubidus* sp. nov. and *Streptomyces yanglinensis* sp. nov. *Int J Syst Evol Microbiol*, 56, 1109–15.

Yang, C.H. & Crowley, D.E. (2000). Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol*, 66, 345–51.

You, M., Sivasithamparam, K. & Kurtboke, D.I. (1996). Actinomycetes in organic mulch used in avocado plantations and their ability to suppress *Phytophthora cinnamomi*. *Biol Fertil Soils*, 22, 237–42.

Yuan, W.M. & Crawford, D.L. (1995). Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl Environ Microbiol*, 61, 3119–28.

Zak, D., Pregitzer, K., Curtis, P. & Holmes, W. (2000a). Atmospheric CO_2 and the composition and function of soil microbial communities. *Ecol Appl*, 10, 47–59.

Zak, D., Pregitzer, K., King, J. & Holmes, W. (2000b). Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: a review and hypothesis. *New Phytol*, 147, 201–22.

Zamani, M., Tehrani, A.S., Ahmadzadeh, M. & Abadi, A.A. (2006). Effect of fluorescent pseudomonades and *Trichoderma* spp. and their combination with two chemicals on *Penicillium digitatum* caused agent of citrus green mold. *Commun Agric Appl Biol Sci*, 71, 1301–10.

Zelles, L. (1999). Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol Fert Soils*, 29, 111–29.

Zhang, Z., Wang, Y. & Ruan, J. (1997). A proposal to revive the genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982). *Int J Syst Bacteriol*, 47, 1048–54.

Appendix A

Supplementary informations

Table A.1: Irrigation details for the the main experiment.

2005		2006	
changed on	to	changed on	to
02.06.	200 mL every 56 h	12.04.	150 ml every 48 h
22.07.	150 mL every 24 h	12.06.	100 ml every 24 h
27.07.	150 mL every 12 h	20.06.	100 ml every 12 h
22.08.	150 mL every 24 h		
08.09.	150 mL every 48 h		

Table A.2: Soil water content [% of maximum water holding capacity (MWHC)] at different harvesting time points for the main experiment. MWHC was determined to be at 0.36 g water per g soil (dry weight).

season	date	ambient [% of MWHC]	SD	CO_2 [% of MWHC]	SD	O_3 [% of MWHC]	SD
spring	16.-18.05.	74.3	5.2	74.8	3.4	69.3	6.6
summer	24.-26.07.	49.5	7.3	62.3	6.4	49.9	10.8
autumn	13.-15.09.	83.5	7.4	86.4	3.4	72.1	6.7

SD = standard deviation.

Appendix B

Statistical tables

Table B.1: Multifactorial ANOVA with response variable below ground biomass.

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Treatment	2	0.108	0.054	19.377	< 0.001
Season	2	0.933	0.467	167.125	< 0.001
Phytophthora	1	0.000	0.000	0.020	0.888
Treatment:Season	4	0.013	0.003	1.181	0.336
Treatment:*Phytophthora*	2	0.005	0.003	0.963	0.391
Season:*Phytophthora*	2	0.001	0.001	0.207	0.814
Treatment:Season:*Phytophthora*	4	0.005	0.001	0.424	0.790
Residuals	36	0.101	0.003		

Table B.2: Multifactorial ANOVA with response variable total above ground biomass.

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Treatment	2	0.004	0.002	0.699	0.504
Season	2	0.279	0.140	44.590	< 0.001
Phytophthora	1	0.001	0.001	0.225	0.638
Treatment:Season	4	0.006	0.002	0.506	0.732
Treatment:*Phytophthora*	2	0.002	0.001	0.394	0.677
Season:*Phytophthora*	2	0.007	0.003	1.115	0.339
Treatment:Season:*Phytophthora*	4	0.002	0.001	0.168	0.953
Residuals	36	0.113	0.003		

Table B.3: Multifactorial ANOVA with response variable microbial biomass carbon (C_{mic}).

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Treatment	2	18735.34	9367.67	2.811	0.080
Season	1	58430.46	58430.46	17.533	< 0.001
Phytophthora	1	6884.23	6884.23	2.066	0.164
Treatment:Season	2	31789.35	15894.68	4.769	0.018
Treatment:*Phytophthora*	2	1642.89	821.45	0.246	0.783
Season:*Phytophthora*	1	333.24	333.24	0.100	0.755
Treatment:Season:*Phytophthora*	2	1347.97	673.99	0.202	0.818
Residuals	24	79983.53	3332.65		

Table B.4: Non-parametric multivariate analysis of variance (PerMANOVA) for 16S t-RFLP based on Euclidean distance measure.

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Phytophthora	1	5.9443	5.9443	0.2486	0.8332
Season	2	1414.1309	707.0655	29.5734	0.0002
Treatment	2	512.112	256.056	10.7097	0.0002
Phytophthora:Season	2	42.8689	21.4344	0.8965	0.4584
Phytophthora:Treatment	2	62.779	31.3895	1.3129	0.2632
Season:Treatment	4	188.0658	47.0164	1.9665	0.0682
Phytophthora:Season:Treatment	4	119.2743	29.8186	1.2472	0.2746
Residual	36	860.7172	23.9088		

P-values were obtained using 4999 permutations.

Table B.5: *P*-values for PerMANOVA pair-wise comparisons among factor levels for seasons and treatments.

Factor	Pair-wise comparison	*P*
Season	sp vs. su	0.0002
	sp vs. au	0.0002
	su vs. au	0.0040
Treatment	am vs. CO_2	0.0218
	am vs. O_3	0.1780
	CO_2 vs. O_3	0.0014

Using 4999 permutations. No corrections have been made for multiple tests.
sp=spring, su=summer, au=autumn, am=ambient.

Table B.6: *P*-values for PerMANOVA pair-wise comparisons for each level of factors season and treatment.

Factor	Tested within level (of factor)	Pair-wise comparison	*P*
season	am (treatment)	sp vs. su	0.0004
		sp vs. au	0.0046
		su vs. au	0.0116
	CO_2 (treatment)	sp vs. su	0.0002
		sp vs. au	0.0006
		su vs. au	0.2402
	O_3 (treatment)	sp vs. su	0.0022
		sp vs. au	0.0354
		su vs. au	0.0066
treatment	sp (season)	am vs. CO_2	0.0110
		am vs. O_3	0.2216
		CO_2 vs. O_3	0.0104
	su (season)	am vs. CO_2	0.0406
		am vs. O_3	0.0022
		CO_2 vs. O_3	0.0008
	au (season)	am vs. CO_2	0.0246
		am vs. O_3	0.2050
		CO_2 vs. O_3	0.0528

P-values were obtained using 4999 Monte Carlo samples from the asymptotic permutation distribution, since too few permutations were possible. No corrections have been made for multiple tests. sp=spring, su=summer, au=autumn, am=ambient.

Table B.7: Non-parametric multivariate analysis of variance (PerMANOVA) for PKS type II t-RFLP profiles of the ambient treatment for all seasons based on Euclidean distance measure.

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Season	2	332.29	166.15	1.5771	0.1978
Phytophthora	1	200.39	200.39	1.9022	0.1502
Season:*Phytophthora*	2	195.45	97.726	0.92766	0.4402
Residual	12	1264.2	105.35		

P-values were obtained using 4999 permutations.

Table B.8: Non-parametric multivariate analysis of variance (PerMANOVA) for PKS type II t-RFLP of the summer harvest for all treatments based on Euclidean distance measure.

Source	*df*	Sum of Sq	Mean Sq	*F*	*P*
Season	2	222.21	111.10	0.81053	0.5524
Phytophthora	1	130.67	130.67	0.95323	0.3954
Season:*Phytophthora*	2	224.50	112.25	0.81888	0.5470
Residual	12	1644.9	137.08		

P-values were obtained using 4999 permutations.

Appendix C

rep-PCR dendrograms

Supplementary Actinobacteria BOX-PCR dendrograms

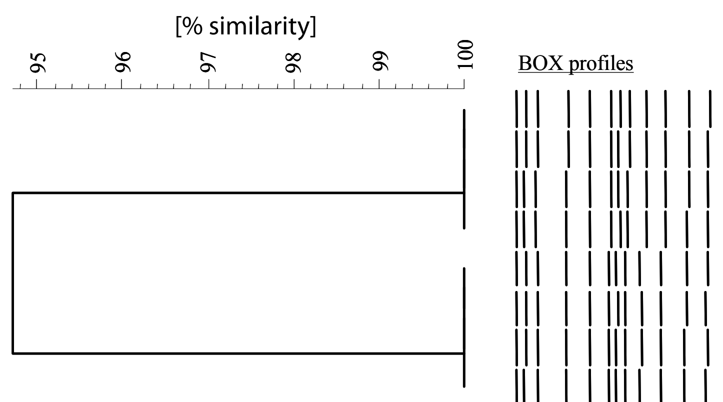


Figure C.1: UPGMA dendrogram of BOX fingerprints for actinobacterial isolates belonging to phylotype 2. All isolates were medium antagonists.

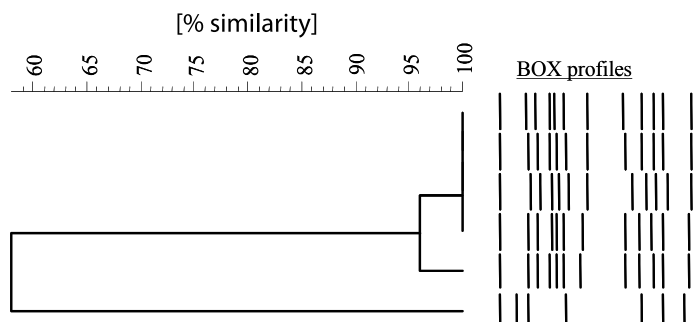


Figure C.2: UPGMA dendrogram of BOX fingerprints for actinobacterial isolates belonging to phylotype 7. All isolates were strong antagonists.

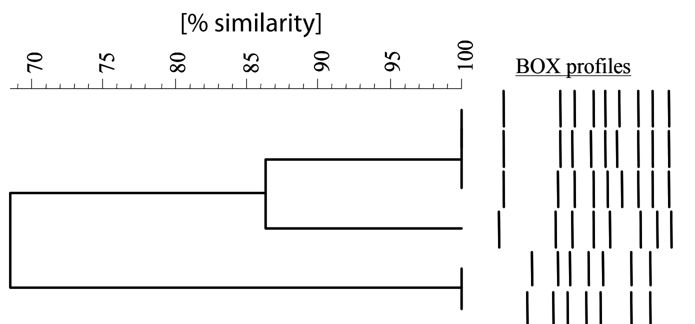


Figure C.3: UPGMA dendrogram of BOX fingerprints for actinobacterial isolates belonging to phylotype 38. All isolates were medium antagonists.

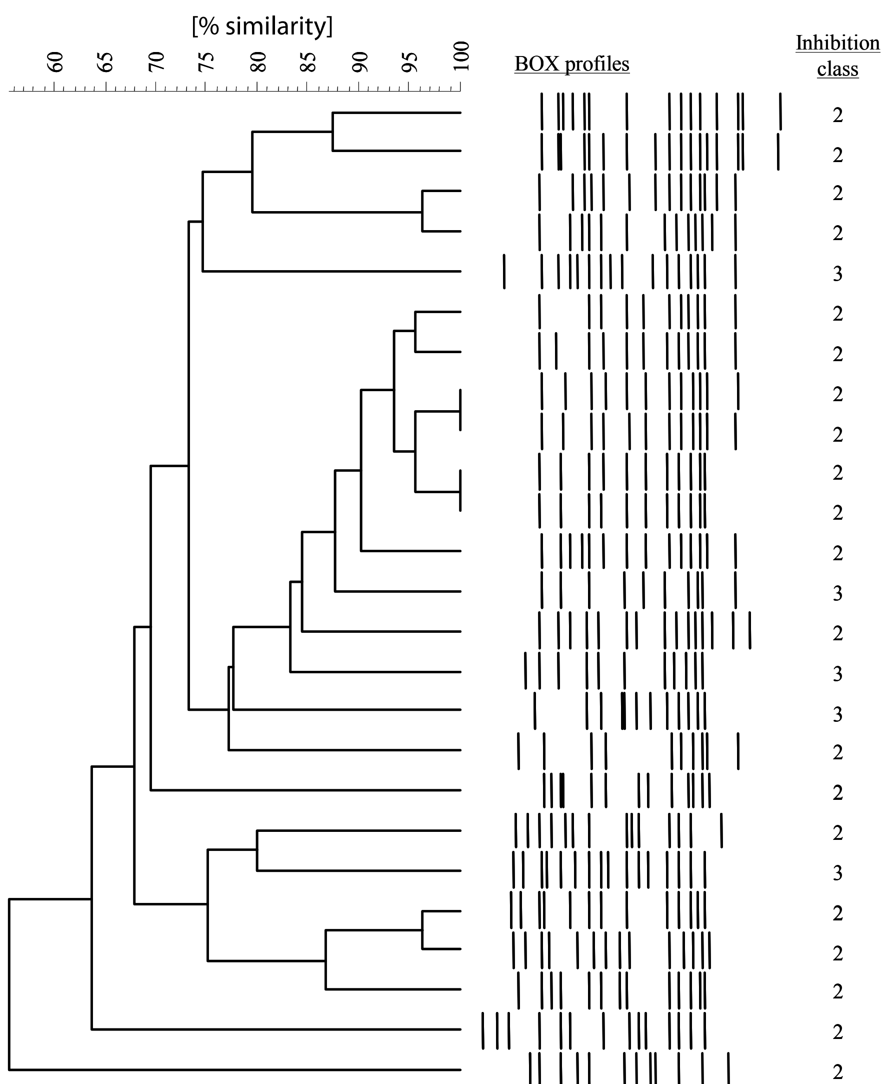


Figure C.4: UPGMA dendrogram of BOX fingerprints for actinobacterial isolates belonging to phylotype 102. Inhibition classes of the isolates are shown.

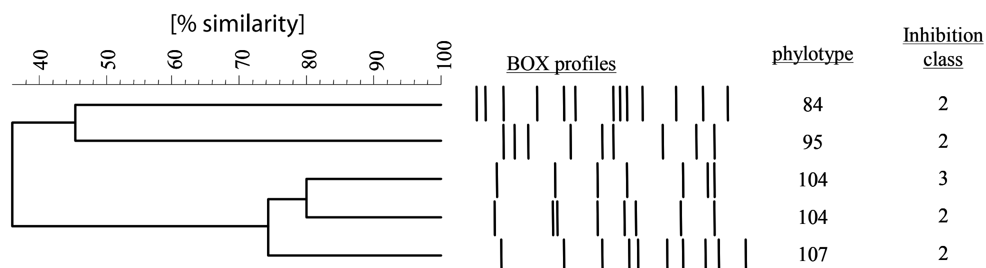


Figure C.5: UPGMA dendrogram of BOX fingerprints for actinobacterial isolates belonging to phylotypes 84, 95, 104 and 107. Inhibition classes for each isolate are indicated.

Supplementary fungal InterLINE-PCR dendrograms

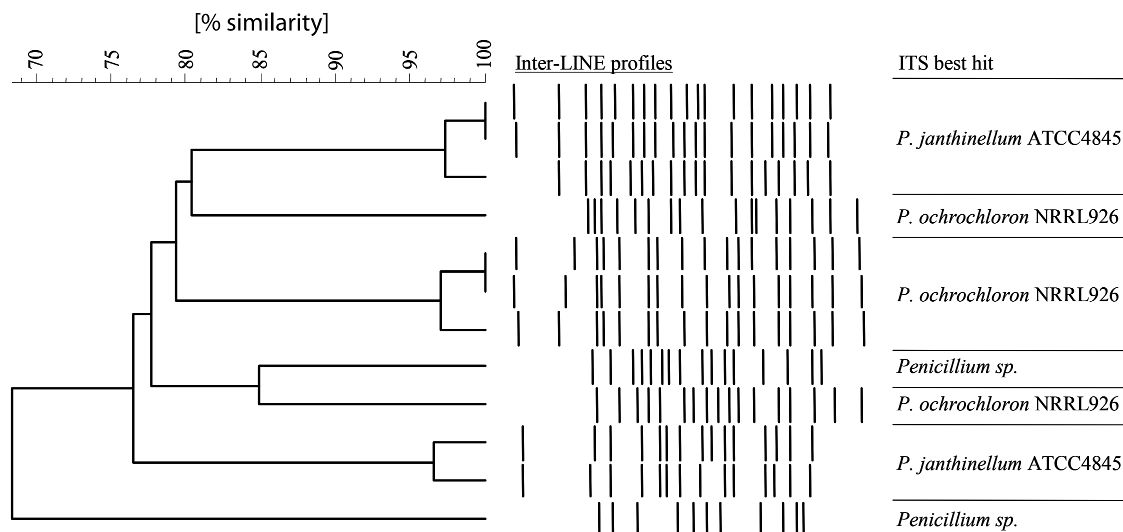


Figure C.6: UPGMA dendrogram of Inter-LINE fingerprints for all *Penicillium* isolates. Vertical bars indicate 90% similarity between isolates. Best hits using the NCBI BLAST tool are shown. P-values for all best hits were $P = 0$.

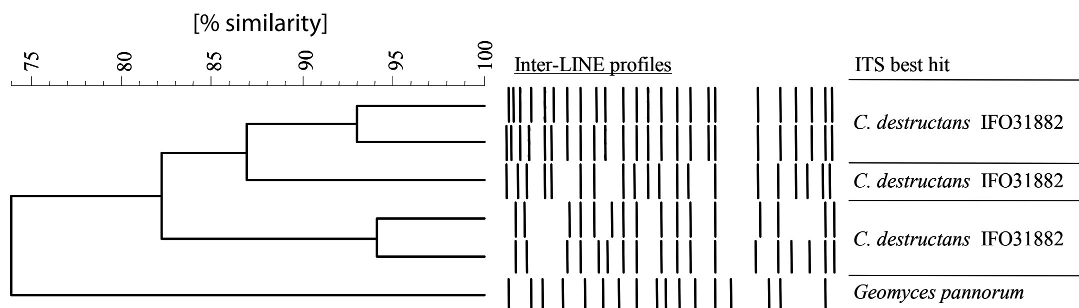


Figure C.7: UPGMA dendrogram of Inter-LINE fingerprints for all *Cylindrocarpon destructans* and *Geomyces pannorum* isolates. Vertical bars indicate 90% similarity between isolates. Best hits using the NCBI BLAST tool are shown. For *Geomyces pannorum* no close hit deposited in any culture collection could be found. P-values for all best hits were $P = 0$.