The Antagonistic Mechanisms Employed by *Trichoderma harzianum* and their Impact on the Control of the Bean Rust Fungus *Uromyces appendiculatus*

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Zusammenfassung

Die Pilzart *Trichoderma harzianum* ist bekannt als Antagonist gegenüber einem breiten Spektrum an phytopathogenen Pilzen. Einige Isolate von *T. harzianum* sind in Form von kommerziellen Präparaten erhältlich und lassen sich so in integrierten oder ökologischen Pflanzenproduktionssystemen einsetzen. Um die Entwicklung anderer Pilze zu kontrollieren bzw. zu hemmen, nutzt *T. harzianum* verschiedene antagonistische Mechanismen wie Konkurrenz, Parasitismus, Antibiose oder die Induktion von Resistenzen in Pflanzen.

Ziel dieser Dissertation war es, Profile über das antagonistische Potential von sechs *T. harzianum*-Isolaten zu erstellen, von denen fünf aus kommerziellen Produkten stammten. Darüber hinaus sollten die von ihnen genutzten antagonistischen Mechanismen evaluiert werden. Hierbei lag das Hauptaugenmerk auf der Kontrolle des Bohnenrosterregers *Uromyces appendiculatus*.

Die sechs *T. harzianum*-Isolate wurden hinsichtlich ihres Wachstumsverhaltens, ihrer Konkurrenzkraft und mykoparasitischen Aktivität, der Produktion extrazellulärer Enzyme mit lytischer Funktion und der Absonderung gasförmiger und nicht-gasförmiger Metabolite mit antifungaler Wirkung untersucht. Die daraus resultierenden Profile enthalten Informationen bezüglich der Wachstumsrate und des Sporulationspotentials der *T. harzianum*-Isolate sowie ihrer Kompetitivität und parasitischen Aktivität gegenüber *Botrytis cinerea*, *Pythium ultimum* und *Rhizoctonia solani*. Die lytische Aktivität der abgesonderten Zellulase, Glukanase, Chitinasen und Proteasen wurde auf festen Agarmedien und in Flüssigkultur festgestellt. Darüber hinaus wurde der antibiotische Einfluss von Metaboliten, die in flüssiges Kulturmedium abgegeben wurden, und der Einfluss von gasförmigen Metaboliten auf verschiedene Phytopathogene ermittelt.

Diese experimentell *in vitro* gewonnenen Daten vermitteln einen interessanten Eindruck über die Eigenschaften und Möglichkeiten der *T. harzianum*-Isolate. Dabei wird die große Variabilität innerhalb der Art *T. harzianum* herausgestellt. Diese macht es notwendig, umfangreiche Untersuchungen durchzuführen, um einzelne Isolate zu finden, die den Anforderungen genügen, die von der Wissenschaft, aber auch durch die praktische Nutzung in Form kommerzieller Produkte an sie gestellt werden.

Sporensuspensionen und Kulturfiltrate der sechs *T. harzianum*-Isolate wurden hinsichtlich ihrer Fähigkeit evaluiert, Infektionen von Bohnenblatt-Scheiben durch *U. appendiculatus* zu reduzieren sowie die Keimung und das Keimschlauchwachstum von Uredosporen des Bohnenrosterregers zu verhindern. Die Wirkung von protektiven Behandlungen mit *T. harzianum* reichte von keinem sichtbaren Effekt bis zu einer

Verringerung der Anzahl an Rostpusteln von über 50%. Zwischen dem Wirkungsgrad der Sporensuspensionen in den Blattscheiben-Experimenten und dem Wirkungsgrad von Kulturfiltraten der entsprechenden *T. harzianum*-Isolate in Keimtests ließen sich interessante Übereinstimmungen feststellen. Dies weist darauf hin, dass die antifungalen Effekte von Sporensuspension und Kulturfiltrat des jeweiligen *T. harzianum*-Isolats zumindest teilweise durch die gleichen sekundären, antibiotisch wirkenden Metabolite verursacht werden.

Die Wirkung von Sporensuspensionen und Kulturfiltraten der effektivsten *T. harzianum*-Isolate T12 und T_U gegenüber dem Bohnenrosterreger konnten in Gewächshausexperimenten bestätigt werden. Auch hier ließ sich die Wirkung einer protektiven Behandlung der Blattflächen von Bohnenpflanzen mit den Agenzien durch Antibiose erklären. Zusätzlich wurde die Induktion systemischer Resistenz in angrenzenden Fiederblättern festgestellt. Diese wurde insbesondere durch das Isolat T_U ausgelöst.

Durch das Benetzen von Bohnensaatgut mit Sporensuspensionen und die Applikation von *T. harzianum*-Sporen in das Substrat wurde keine Erhöhung der Widerstandsfähigkeit gegenüber dem Bohnenrost erreicht. Stattdessen kam es zu einem verringerten Wachstum der so behandelten Pflanzen.

Abschließend wurden Profile der sekundären Metabolite, die von den sechs *T. harzianum*-Isolaten produziert worden waren, erstellt. Die Analyse dieser Profile hatte die Identifikation solcher Substanzen zum Ziel, die möglicherweise für die antibiotischen Effekte der Kulturfiltrate verantwortlich waren. Dabei wurden mehrere Substanzen gefunden, deren molekulare Massen mit denen von bekannten sekundären Metaboliten von *T. harzianum* übereinstimmten. Diese wurden im Hinblick auf ihren potentiellen Einfluss insbesondere auf die Interaktion zwischen dem produzierenden *T. harzianum*-Isolat und dem Bohnenrosterreger *U. appendiculatus* evaluiert.

Trichoderma harzianum, Bohnenrost, Antibiose

Abstract

The fungal species *Trichoderma harzianum* is well-known for its antagonism towards a wide range of phytopathogenic fungi. Several strains of *T. harzianum* have been formulated into preparations, which are commercially available for use in integrated and ecological plant production systems. To exert its activity in terms of controlling or inhibiting the development of other fungi, *T. harzianum* employs several antagonistic mechanisms like competition, parasitism, antibiosis, or the induction of plant resistance.

The aim of this thesis was to create profiles of the antagonistic potential of six *T. harzianum* strains, five of them being isolated from commercial products, and to evaluate the antagonistic mechanisms employed by them with emphasis on the control of the bean rust fungus *Uromyces appendiculatus*.

The six *T. harzianum* strains were tested for their growth performance, their competitive and mycoparasitic activity, their production of extracellular enzymes with lytic function, and the release of volatile and non-volatile metabolites with antifungal activity. The resulting profiles contain information on mycelial growth rate and sporulation potential of the *T. harzianum* strains, on their competitiveness and their parasitic activity towards *Botrytis cinerea*, *Pythium ultimum*, and *Rhizoctonia solani*. The lytic activity of secreted cellulase, glucanase, chitinases, and proteases was determined on solid agar and in liquid culture medium. Furthermore, the antibiotic activity of non-volatile metabolites secreted into the liquid culture medium as well as the impact of volatile metabolites on various phytopathogens was assessed.

These data give an interesting insight into the properties and *in vitro* capabilities of the *T. harzianum* strains and emphasize the high variability within the species *T. harzianum*, which makes it necessary to perform extensive screenings to find single strains that sufficiently meet the needs of scientists or users of commercially formulated products.

Spore suspensions and culture filtrates of the six *T. harzianum* strains were evaluated regarding their ability to reduce infection of bean leaf discs by *U. appendiculatus* and to inhibit germination and germ tube growth of bean rust uredospores. Efficacy levels of protective *T. harzianum* treatments ranged from ineffectiveness to a reduction of the number of rust pustules of more than 50%. An interesting correlation between the efficacy levels of *Trichoderma* spore suspensions in leaf disc assays and that of the respective culture filtrates in germination tests was found. This points to the fact that the antifungal effects of spore suspension and culture filtrate of

the same *T. harzianum* strain are at least partly caused by the same secondary metabolites with antibiotic properties.

The bean rust controlling efficacy of spore suspensions and culture filtrates of the most effective *T. harzianum* strains T12 and T_U could be confirmed in greenhouse experiments, if the agents were applied as a protective treatment to the leaf surface of bean plants. This activity was consequently explained by antibiosis. Moreover, induction of systemic resistance in adjacent leaflets was observed especially for strain T_U. Application of *T. harzianum* spores to the seed or into the substrate failed to increase bean plant resistance and yielded negative growth responses.

Last but not least, secondary metabolite profiles of the six *T. harzianum* strains were generated and analysed aiming at the identification of compounds potentially responsible for the antifungal effects of *T. harzianum* culture filtrates. Several compounds with molecular masses that corresponded to those of known secondary metabolites of *T. harzianum* were found and evaluated with respect to their potential impact especially on the interaction between the producing *T. harzianum* strain and the bean rust fungus *U. appendiculatus*.

Trichoderma harzianum, bean rust, antibiosis

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List of Abbreviations

%	Percent
℃	Degree Celsius
µl	Microlitre
µm	Micrometre
AUGC	Area under the germination curve
AUGGC	Area under the germ tube growth curve
AUGPC	Area under the growth progress curve
AUIGC	Area under the inhibited growth curve
B.c.	*Botrytis cinerea*
BCA	Biological control agent
CF	Culture filtrate(s)
cfu	Colony forming unit(s)
cm	Centimetre
cm^2	Sqare centimetre
d	Day
Da	Dalton
EA	Ethyl acetate
FOL	*Fusarium oxysporum* f. sp. *lycopersici*
FOP	*Fusarium oxysporum* f. sp. *phaseoli*
g	Gram
G.spp.	*Gliocladium* species
h	Hour
HPLC	High performance liquid chromatography
HX	Hexane
l	Litre
m/z	Mass-to-charge ratio
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
P.u.	*Pythium ultimum*
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Negative decade logarithm of hydrogen ion concentration
psi	Pound per square inch
R.s.	*Rhizoctonia solani*
rH	Relative humidity
rpm	Revolutions per minute
T.h.	*Trichoderma harzianum*
T.spp.	*Trichoderma* species
TIC	Total ion current
V	Volt
v/v	Volume per volume

Abbreviations for the *Trichoderma harzianum* strains used in this study:

T12	taken from the fungal collection of the Institute of Plant Diseases and Plant Protection (Leibniz Universität Hannover, Germany)
T-22	isolated from TRIANUM-P (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands)
T39	isolated from TRICHODEX (Makhteshim-Agan Ltd., Tel Aviv, Israel)
T_R	isolated from TRI 003 (Plantsupport, Grootebroek, The Netherlands)
T_S	isolated from TRICHOSAN (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany)
T_U	isolated from UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand)

1. General Introduction

A wide range of microorganisms and naturally produced substances like plant-derived botanicals or antibiotic metabolites of microbial origin are classified as biopesticides. Despite the fact that the biopesticide share represents only little more than one percent of the total world pesticide market, organisms and compounds with biopesticidal activity are increasingly recognized as valuable components of plant protection systems (COPPING and MENN, 2000). Although biopesticides have several disadvantages compared to chemical pesticides, e.g. inconsistent field performance, limited shelf-life, and possibly higher economical costs, those critical aspects may be attenuated by a number of advantages which result from the usage of biological pesticides: (1) a strongly reduced activity or toxicity towards non-target organisms, (2) an optimised pesticide resistance management due to a broader range of applicable pesticidal agents, (3) the possibility of combining conventional and biological means of disease control, thereby reducing the output of synthetic chemicals, (4) greater acceptance by the consumer which is proven by increasing sales of organically produced food, and (5) an easier and less expensive registration process for biopesticides, at least in the US (COPPING and MENN, 2000).

The genus *Trichoderma* is well-known for the biopesticidal activity of a large number of strains from several *Trichoderma* species (HARMAN and BJÖRKMAN, 1998) as well as of many secondary metabolites produced by these strains (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al.*, 2005). In fact, *Trichoderma* spp. are soil-borne, saprophytically living fungal organisms (KLEIN and EVELEIGH, 1998), but many strains of several *Trichoderma* species have developed mechanisms of antagonistic activity towards other microorganisms (HJELJORD and TRONSMO, 1998). The parasitism of *Trichoderma* spp. on other fungi and the secretion of a “lethal principle” with antibiotic properties are known since 75 years (WEINDLING, 1932, 1934). Today, the array of known mechanisms of *Trichoderma* antagonism comprises (1) competition for space and nutrients (ELAD, 1996; SIVAN and CHET, 1989), (2) mycoparasitic activity (CHET *et al.*, 1998) and (3) antibiosis (HOWELL, 1998), (4) the degradation of enzymes which enable phytopathogens to exploit plant tissue (ELAD and KAPAT, 1999; KAPAT *et al.*, 1998) or (5) the degradation of substances of plant origin necessary for the elicitation of phytopathogenic activity (HOWELL, 2002), and (6) the induction of plant resistance against fungal, bacterial, or viral diseases (HARMAN *et al.*, 2004).

The diversity of these mechanisms is nicely demonstrated by several publications of HOWELL from the past 25 years. He found that the infection of cotton plants with

Pythium ultimum could be reduced by specific strains of *T. virens* and explained this activity with (1) parasitism, after observing parasitic interactions between antagonist and phytopathogen (HOWELL, 1982) and (2) antibiosis, after observing inhibition of *Pythium* pathogenic activity by mycoparasitic-deficient mutants of *T. virens* (HOWELL, 1991). The concept of *P. ultimum* biocontrol due to (3) systemic resistance induced by *T. virens* through elicitation of terpenoid synthesis in cotton roots was drawn into question, when *T. virens* strains that did not induce terpenoid synthesis were found to retain biocontrol efficacy (HOWELL, 2002). After mutants deficient for each of the above mentioned mechanisms still were able to control *P. ultimum* on cotton, it was demonstrated that the disease reducing effect was based on (4) the degradation of specific compounds from plant root exudates by *T. virens*, which otherwise would have induced germination of *P. ultimum* sporangia (HOWELL, 2002).

In general, single mechanisms, but also each possible combination of the mechanisms mentioned above may confer disease-reducing activity to antagonistic *Trichoderma* strains (HARMAN, 2000).

During the last 30 years, a vast number of *Trichoderma* isolates from diverse habitats were screened for their antagonistic potential yielding a great amount of scientific publications and few commercial products (HARMAN and BJÖRKMAN, 1998; MONTE, 2001). Extensive screenings were mostly done *in vitro* and promising candidates tested later on *in vivo*. Unfortunately, strains having an interesting profile of antagonistic activity *in vitro* may be much less effective in field experiments due to numerous environmental conditions that are not sufficiently simulated in the laboratory (HANNUSCH and BOLAND, 1996; HARMAN, 2006). Nonetheless, screening of *Trichoderma* strains *in vitro* and profiling the features of those strains demonstrating activity against phytopathogenic target fungi still is a valuable way of getting accustomed to promising *Trichoderma* strains.

Because *Trichoderma* spp. are soil-borne fungi, antagonistic strains were successfully applied as biopesticides or biological control agents (BCAs), as they are termed more often, mostly against soil-borne phytopathogens like *Fusarium* spp. (SIVAN and CHET, 1989), *Pythium ultimum* (HOWELL, 2002), *Rhizoctonia solani* (PAULA JÚNIOR *et al*., 2007), or nematodes (SHARON *et al*., 2001). Nevertheless, some strains have also been used as agents of foliar pathogen control e.g. against *Botrytis cinerea* (ELAD, 1996), *Sphaerotheca fusca* (ELAD *et al.,* 1998), or *Crinipellis perniciosa* (SANOGO *et al*., 2002). Only few publications report the control of rust fungi by means of *Trichoderma* spp. (GOVINDASAMY and BALASUBRAMANIAN, 1989; KAPOORIA and SINHA, 1969; LEVINE *et al*., 1936; SALLAM, 2001; SINHA and BAHADUR, 1974; TOSI and ZAZZERINI, 1994; ZADE *et al*., 2005).

The life cycle of the biotrophic and partly heteroecious rust fungi consists of up to five different spore stages, which infect the host quickly after landing on it, develop an intercellular mycelium within the host plant's tissue, and give rise to pustules containing populations of spores of the next life stage. In the life cycles of most rust fungi, one of the spore stages develops repeatedly, which leads to mass reproduction of the fungal organism through multiple generations. For *Uromyces appendiculatus* (Pers.) Unger, an autoecious, macrocyclic rust fungus causing serious rust epidemics on edible dry bean *Phaseolus vulgaris* L. in regions with extensive bean production in North, Middle, and South America and several African countries, mass reproduction takes place at the uredospore stage, which causes the typical disease symptoms of spot-like, brown rust pustules (DE JESUS JUNIOR *et al*., 2001; HABTU and ZADOKS, 1995; MCMILLAN *et al*., 2003; MMBAGA *et al*., 1996).

As the majority of rust mycelial growth takes place endophytically below the epidermal cells (HEATH, 1997), the control of rust fungi with mycoparasites can only take place after appearance of the rust symptoms. When the rust pustules rupture through the epidermal cell layers, the rust fungus re-appears at the leaf surface and its spores can be attacked by parasites, thereby decreasing the number of viable propagules and controlling the subsequent spread of the disease (SAKSIRIRAT and HOPPE, 1990).

If the initial infection of the host shall be controlled, microbial antagonists producing antifungal metabolites are more feasible (ANDREWS, 1992) due to inhibition of the germination of landed rust spores or by interfering with the growth of emerging germ tubes and with the formation of necessary infection structures (BAKER *et al*., 1983, 1985; YUEN *et al*., 2001).

Successful control of rust diseases by *Trichoderma* spp. was mostly explained by the activity of secondary metabolites with antifungal properties produced by the used strains. Living *Trichoderma* propagules (GOVINDASAMY and BALASUBRAMANIAN, 1989; KAPOORIA and SINHA, 1969; SALLAM, 2001), sterile fluids of germinated *Trichoderma* spore suspensions (GOVINDASAMY and BALASUBRAMANIAN, 1989; SINHA and BAHADUR, 1974), and filtrates of liquid *Trichoderma* cultures (ZADE *et al*., 2005) were shown to effectively reduce rust infection of plant tissue and thereby decrease disease severity. Parasitic activity of *Trichoderma* strains on rust pustules was observed less often (LEVINE *et al*., 1936; TOSI and ZAZZERINI, 1994).

To the author's knowledge, nothing is known about *Trichoderma*-mediated control of *U. appendiculatus*.

Repeated evaluations of fungicides registered for use against bean rust continuously proved them to be reliable agents of disease control (GENT *et al.*, 2001; STUMP *et al.*, 2000). Since no fungicide resistance of *U. appendiculatus* was reported until now, the need to search for other means of bean rust control is not as urgent as in other pathosystems (FRAC, 2006). Nevertheless, resistance towards fungicides has been observed in other rust fungi (COOK, 2001; DIRKSE *et al.*, 1982) and may therefore develop in bean rust as well. But even without the immediate necessity of broadening the spectrum of available bean rust controlling agents in terms of fungicide resistance management, the replacement of synthetic chemicals by BCAs bearing reduced risks of environmental pollution and for the consumer's health has its very own ethic value (RICARD and RICARD, 1997).

The overall objective of the thesis presented here was to evaluate the antagonistic potential of six strains of *Trichoderma harzianum* Rifai, five of them being isolated from commercial preparations, with emphasis on bean rust control by means of living propagules and of their secondary metabolites with antifungal activity. Four distinct sets of *in vitro* assays, greenhouse experiments, and chemical analyses were conducted to gather data on the following four objectives:

(1) To create profiles on the general antagonistic properties of six *T. harzianum* strains by *in vitro* evaluation of their growth performance, their competitive and mycoparasitic activity, their production of extracellular enzymes with lytic activity, and the release of volatile and non-volatile metabolites with antifungal activity.
(2) To evaluate the antagonistic activity of *T. harzianum* spore suspensions and the antifungal potential of sterile culture filtrates on the infectiousness of *U. appendiculatus* in leaf disc assays and on the process of germination of bean rust uredospores.
(3) To confirm the observed activity of those *T. harzianum* strains with the greatest efficacy against *U. appendiculatus* including aspects of resistance inducing and plant growth affecting properties of the selected *T. harzianum* strains in greenhouse experiments.
(4) To analyse the secondary metabolite profiles of the six *T. harzianum* strains with the aim of identifying those compounds responsible for the antibiotic effect of particular *T. harzianum* strains on *U. appendiculatus*.

Concerning the limited knowledge on *Trichoderma*-mediated control of rust fungi in the scientific literature, the author hopes, that the work presented here may serve as a kind of basic reference for future approaches in the field of controlling rust diseases by means of the biopesticide aka biological control agent *Trichoderma harzianum*.

2. Antagonistic Profiles of *Trichoderma harzianum* Strains from Commercial Biocontrol Products

2.1 Introduction

2.1.1 Overview on Trichoderma *antagonism*

The worldwide occurring fungal genus *Trichoderma* spp. comprises a group of about 89 named species (SAMUELS, 2006), some of them with great economical importance. Examples include *T. reesei* as a producer of industrially used cellulases (JUHÁSZ *et al*., 2004), *T. aggressivum* as a competitor of the commercial mushroom *Agaricus bisporus* (SAMUELS *et al*., 2002), *Trichoderma* species as toxin-producing indoor molds (KUHN and GHANNOUM, 2003), and some strains even acting as human pathogens (KREDICS *et al*., 2003).

A very prominent feature of several *Trichoderma* species is the antagonism exerted by these fungi on plant pathogens. *T. virens*, *T. koningii*, and mostly *T. harzianum* represent the group of species used as biological control agents (BCAs) in scientifical as well as practical approaches (BENITEZ *et al*., 2004; CHET, 1987; HARMAN, 2000) to control fungal diseases of plants on nearly every plant organ. As *Trichoderma spp.* are soil-borne microorganisms, the majority of diseases controlled by them infect the lower parts of the plant like roots, root crown, and stem base (HJELJORD and TRONSMO, 1998). Moreover, successful attempts have been made to control fungal diseases of upper parts of the plant like stem (O'NEILL *et al*., 1996), leaves (ELAD, 2000a), blossoms (ESCANDE *et al*., 2002; TRONSMO and YSTAAS, 1980), and fruits (HARMAN *et al*., 1996).

First observations of the antagonistic properties of *Trichoderma* spp. were done by WEINDLING. He described parasitism (1932) and the production of a "lethal principle", a substance with antifungal acitivity (1934) of *T. lignorum* against *Rhizoctonia solani*, two of the most important features of BCAs with regard to antagonism. In 1936, WEINDLING and EMERSON extracted the "lethal principle" responsible for the antibiotic interaction from a *Trichoderma* culture filtrate.

Since then, especially from the beginning of the 80s of the 20th century, *Trichoderma* research increased (ELAD *et al*., 1980, 1983; HOWELL, 1982; TRONSMO and YSTAAS, 1980). Until today, an uncountable number of scientific papers concerning biology and ecology as well as biocontrol by means of *Trichoderma* species has been published worldwide (HARMAN and KUBICEK, 1998; KUBICEK and HARMAN, 1998). A massive amount of molecular biological studies has further increased the knowledge on

Trichoderma spp. (KUBICEK *et al*., 2001; SZEKERES *et al*., 2005; VITERBO *et al*., 2002), making it one of the most studied fungal BCAs (VINALE *et al*., 2006).

The most abundant *Trichoderma* species, *T. harzianum*, is a species complex comprising a large group of worldwide occurring strains with very diverse properties (KUBICEK *et al*., 2003). Depending on the strains and characteristics studied, this complex can be split up in three to five subspecific groups (GRONDONA *et al*., 1997; HERMOSA *et al*., 2004).

Specific *T. harzianum* strains have competitive abilities against other fungi (HOWELL, 2003), especially due to their rhizosphere competence (AHMAD and BAKER, 1987). They are known as fungal hyperparasites (BENHAMOU and CHET, 1993, 1997; CORTES *et al*., 1998; ELAD *et al*., 1983) and producers of lytic enzymes (KREDICS *et al*., 2005; MARKOVICH and KONONOVA, 2003; VITERBO *et al*., 2002) as well as of secondary metabolites with antibiotic properties (GHISALBERTI and SIVASITHAMPARAM, 1991; SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al*., 2005). They can inhibit pathogenic action by degrading the pathogen's pathogenicity enzymes (ELAD and KAPAT, 1999; KAPAT *et al*., 1998) or by inducing systemic resistance in plants (BIGIRIMANA *et al*., 1997; HARMAN *et al*., 2004).

2.1.2 Competitive abilities

The ability of *Trichoderma* spp. to quickly grow under diverse climatic conditions, to occupy free space and to use free nutrients is important to suppress the saprophytic growth of soil-borne plant pathogens (NAAR and KECSKES, 1998; SIMON and SIVASITHAMPARAM, 1989). Generally, *Trichoderma* strains have a quick growing mycelium (MANCZINGER *et al*., 2002a), which sporulates profusely, increasing its population and facilitating further spread. A broad range of lytic enzymes allows the degradation of diverse molecules to gain nutrients (MANCZINGER *et al*., 2002a). Moreover, the ability to grow alongside the developing root, known as rhizosphere competence, enhances the biocontrol abilities of *Trichoderma* strains against soil-borne plant pathogens (AHMAD and BAKER, 1987).

Results from screenings on the climatic requirements for optimal growth of *Trichoderma* strains have been published (DANIELSON and DAVEY, 1973; EASTBURN and BUTLER, 1991; KREDICS *et al*., 2004; TRONSMO and DENNIS, 1978). It was shown that selection of *Trichoderma* strains for specific climatic conditions, e.g. cold-tolerant strains, is possible (ANTAL *et al*., 2000; KÖHL and SCHLÖSSER, 1988; TRONSMO and YSTAAS, 1980).

Dual culture experiments conducted under defined conditions in Petri dishes provide information on the antagonistic potential of single *Trichoderma* strains against selected pathogens (ANTAL *et al.*, 2000; ORTIZ and ORDUZ, 2000; SIVAKUMAR *et al.*, 2000). It has to be noted that direct transfer of these results to the very diverse habitats outside of the laboratory is not possible, due to the partially low predictive value of such results (HANNUSCH and BOLAND, 1996; HARMAN, 2006).

As competitive action is mostly accompanied by other antagonistic events, like parasitism, antibiosis, or resistance induction in the host plant, biocontrol was seldomly explained by competition alone (HOWELL, 2002; SIVAN and CHET, 1989; ZIMAND *et al.*, 1995).

2.1.3 Parasitic interaction

WEINDLING (1932) was the first to observe and describe parasitic *Trichoderma* spp. – pathogen interactions in dual culture experiments. He microscopically observed the typical coiling of *Trichoderma* hyphae around the attacked pathogen's hyphae and the subsequent coagulation of the host's protoplasm. Many parts of the processes during the parasitic interaction between *Trichoderma* strain and pathogen have now been observed in more detail (BENHAMOU and CHET, 1993, 1997; ELAD *et al.*, 1983; GUPTA *et al.*, 1999; INBAR *et al.*, 1996).

Confronted with a fungal target organism, a mycoparasitic *Trichoderma* strain grows towards it by chemotactic hyphal branching (LU *et al.*, 2004). It attaches to the host's mycelium via a lectin-mediated recognition mechanism (INBAR and CHET, 1992) and starts growing alongside the hyphae of the pathogen or coils around it (BENHAMOU and CHET, 1993; ELAD *et al.*, 1983; GUPTA *et al.*, 1999; INBAR *et al.*, 1996). Contact to the pathogen is further increased by the antagonist by appressoria- or hook-like structures (GUPTA *et al.*, 1999; INBAR *et al.*, 1996). Penetration of *Trichoderma* hyphae into the pathogen's hyphae may occur (ELAD *et al.*, 1983; GUPTA *et al.*, 1999; INBAR *et al.*, 1996). The interaction of a hyperparasitic *Trichoderma* strain with the target organism leads to granulation and vacuolisation of the protoplasm of attacked cells (INBAR *et al.*, 1996; WEINDLING, 1932), perforation of cell walls (ELAD *et al.*, 1983; INBAR *et al.*, 1996), loss of turgor, and collapse of cells (BENHAMOU and CHET, 1993; GUPTA *et al.*, 1999).

Trichoderma spp. – pathogen interactions do not necessarily involve all of these steps, as for example coiling around or penetration into the hyphae of the host is not always observed. Moreover, bursting and collapsing of mycelial structures can take place even without physical contact between *Trichoderma* spp. and pathogen by means of lytic

enzymes or secondary metabolites with antibiotic activity released by the *Trichoderma* strain (GUPTA *et al*., 1999).

In dual culture experiments of mycoparasitic *Trichoderma* strains and target fungi, the *Trichoderma* strain often stops the growth of the pathogen and subsequently grows over the mycelium of the target fungus (HARAN *et al*., 1996; MUKHERJEE and RAGHU, 1997; REY *et al*., 2001). It was found that mycoparasitic activity of *Trichoderma* spp. on plant pathogenic fungi does not only depend on the antagonist strain, but on the host fungus, too, because *Trichoderma* spp. may react in very different ways to different host fungi. For example, the same *T. harzianum* strain produced different chitinolytic enzymes when confronted with *Rhizoctonia solani* or *Sclerotium rolfsii*, leading to parasitic overgrowth of the former pathogen while the latter one was hardly overgrown (HARAN *et al*., 1996). In a successful mycoparasitic interaction, only the *Trichoderma* strain should be able to grow out of such a zone of overgrowth, if samples are placed on fresh medium, indicating the killing of the target (MUKHERJEE and RAGHU, 1997; REY *et al*., 2001).

Generally, parasitation of soil-borne (BENHAMOU and CHET, 1993; ELAD *et al*., 1983; INBAR *et al*., 1996; MUKHERJEE and RAGHU, 1997; REY *et al*., 2001) and foliar (BRADATSCH, 2006; GUPTA *et al*., 1999) pathogens is possible.

2.1.4 Production of lytic enzymes

Together with antibiotic secondary metabolites, lytic enzymes produced by *Trichoderma* spp. are the biochemical backbone for the antagonism of these beneficial fungi against fungal pathogens (MANCZINGER *et al*., 2002a). Some *Trichoderma* enzymes, especially chitinases, show the strongest antifungal activity known in nature (LORITO *et al*., 1993, 1998). By this, *Trichoderma* spp. seem specialized in not only attacking chitinous structures of hyphae, but also of conidia, chlamydospores, and sclerotia (LORITO *et al*., 1998).

The impact of *Trichoderma* spp. on the mycelium of the pathogen during the antagonistic and especially parasitic interaction is increased by the action of lytic enzymes as reviewed by KREDICS *et al*. (2005), MARKOVICH and KONONOVA (2003), and VITERBO *et al*. (2002). Chitinases (HARAN *et al*., 1996; HARMAN *et al*., 1993; KUBICEK e*t al*., 2001; LORITO *et al*., 1993, 1994b), glucanases (LORITO *et al*., 1994a, 1994b; THRANE *et al*., 1997), and proteases (ELAD and KAPAT, 1999; GEREMIA *et al*., 1993; MANCZINGER *et al*., 2002b; SZEKERES *et al*., 2004), isolated over the last two decades, are important parts of the *Trichoderma* toolbox regarding antagonism against plant pathogenic fungi. These enzymes lyze fungal cell wall components (CHET *et al*., 1998) or degrade the pathogen's pathogenicity enzymes (ELAD and KAPAT, 1999; KAPAT *et al*., 1998). Other enzymes may

degrade metabolites from plant exudates that stimulate the germination of spores of plant pathogenic fungi (HOWELL, 2002). Moreover, some enzymes have bacteriolytic properties (MANCZINGER, 2002b) and may thereby increase the competitive abilities of *Trichoderma* strains otherwise inhibited by bacteria (NAAR and KECSKES, 1998).

The effectiveness of lytic enzymes is enhanced by synergism with other molecules of fungal origin, e.g. other lytic enzymes (LORITO *et al.*, 1993, 1994a) or secondary metabolites with antibiotic activity (LORITO *et al.*, 1996; SCHIRMBÖCK *et al.*, 1994). This synergism may be the reason, why a *Trichoderma* strain can be a potent BCA, although the activities of its enzymes are not sufficient to control a fungal pathogen if tested seperately. Also, these enzymes may synergistically enhance the effect of fungitoxic compounds, thereby reducing the amount of fungicides needed for sufficient pathogen control (THRANE *et al.*, 1997; LORITO *et al.*, 1994b), or the effect of antibiotic metabolites of bacterial origin (WOO *et al.*, 2002).

Finally, lytic enzymes are subject to catabolite repression. Synthesis of chitinolytic enzymes, induced by carbon starvation, fungal cell walls, or pure chitin, is repressed by easy to digest carbon sources like glucose (VITERBO *et al.*, 2002). Synthesis of glucanases can be inhibited if glucose is available (THRANE *et al.*, 1997). Proteolytic enzyme expression is induced by the presence of fungal cell walls, but does only take place under de-repression conditions, meaning the absence of primary nitrogen sources like glutamine and ammonia (OLMEDO-MONFIL *et al.*, 2002).

2.1.5 Secondary metabolites with antibiotic activity

More than 100 secondary metabolites with antibiotic activities, produced by different *Trichoderma* strains, are known today (SIVASITHAMPARAM and GHISALBERTI, 1998). Next to lytic enzymes, they are the second large group of molecules beneficial to the antagonistic action of *Trichoderma* strains against microorganisms (MANCZINGER *et al.*, 2002a). Their production is dependent on the *Trichoderma* strain, environmental parameters as pH or temperature, and the colonized substrate (SIVASITHAMPARAM and GHISALBERTI, 1998; VIZCAÍNO *et al.*, 2005). These anitibiotic metabolites can largely be divided into three groups: compounds having a significant vapour pressure, which means that they can become volatile, water soluble metabolites, and the large group of amphipathic peptaibols, being able to interact with plasma membranes (GHISALBERTI and SIVASITHAMPARAM, 1991).

The first two metabolite groups of volatile and non-volatile antibiotics are derived from diverse metabolic pathways. Their production within the vast amount of *Trichoderma* strains does not show any kind of chemotaxonomical relationship between species.

Because different antibiotic metabolites may be produced from otherwise closely related strains of the same species and quite similar antibiotic metabolite profiles may be produced by strains belonging to different species, taxonomic studies on the basis of these biochemical markers would not give conclusive results (GHISALBERTI and ROWLAND, 1993). Such variability makes it necessary, to individually evaluate the antimicrobial spectrum of a given strain (VIZCAÍNO *et al*., 2005).

In a broader sense, the simple reference to *T. harzianum* as "the BCA species" is imprecise, because *T. harzianum* strains without any effect against fungal target organisms are known, although most *Trichoderma* strains used as BCAs belong to this species (GHISALBERTI and SIVASITHAMPARAM, 1991).

The volatile pyrone antibiotic 6-*n*-pentyl-2*H*-pyran-2-one, also termed 6-pentyl-*α*-pyrone (6PAP), produced by several *Trichoderma* species (CLAYDON *et al*., 1987, CUTLER *et al*., 1986), which is responsible for the characteristic coconut aroma of many *Trichoderma* strains (COLLINS and HALIM, 1972; HOWELL, 1998), is rated as the best characterized and most important *Trichoderma* antibiotic (VINALE *et al*., 2006). For example, the antagonistic success of several *Trichoderma* strains against *Gaeumannomyces graminis* var. *tritici* was correlated with the production of 6PAP (GHISALBERTI *et al*., 1990). Nevertheless, the mechanism by which most antibiotic metabolites act is still insufficiently established (SONG *et al*., 2006) and remains to be elucidated (VINALE *et al*., 2006). Moreover, the production of antibiotic metabolites in liquid media *in vitro* does not inevitably mean that these metabolites are also produced in soil (GHISALBERTI and SIVASITHAMPARAM, 1991). Therefore, simple Petri dish experiments, undertaken to screen for hopeful BCA candidates with strong antibiotic activity, should be accompanied by plant – pathogen interaction assays (HARMAN, 2006).

Peptaibols and related peptaibiotics are peptide antibiotics solely of fungal origin (DEGENKOLB *et al*., 2003). They are linear, amphipathic polypeptides composed of 5-20 amino acids containing the non-proteinogenic amino acids *α*-aminoisobutyric (Aib) acid or isovaline (Iva) generally produced in microheterogenous mixtures (SZEKERES *et al*., 2005). Since the first peptaibol alamethicin was isolated from *T. viride* culture filtrates (REUSSER, 1967), more than 300 molecules belonging to this class of peptides have been found and listed in the Peptaibol Database (WHITMORE *et al*., 2003). 190 of them were isolated from *Trichoderma* species, 54 being of *T. harzianum* origin (PEPTAIBOL DATABASE, 2007). A common feature of the amphipathic peptaibols, which result from non-ribosomal biosynthesis (WIEST *et al*., 2002), is the ability to form voltage-gated ion-channels through plasma membranes (SANSOM, 1993), thereby causing membrane leakage and metabolic disorders within the cell (EPAND and VOGEL, 1999; LORITO *et al*., 1996). The spectrum of

impact includes antibacterial, antiviral, antimycoplasmic, and antifungal activity as reviewed by KRAUSE *et al*. (2006) and SZEKERES *et al*. (2005).

Synergistic effects between the action of lytic enzymes and antibiotic metabolites were shown (LORITO *et al*., 1996; SCHIRMBÖCK *et al*., 1994). By degrading cell wall components, lytic enzymes facilitate the contact of antibiotic metabolites with the plasma membrane, increasing membrane leakage. Moreover, peptaibols may inhibit the activity of enzymes with synthethase function, thus preventing re-synthesis of the degraded cell wall components (LORITO *et al*., 1996).

The positive effect of antibiotic metabolites produced by *Trichoderma* spp. in terms of antagonism may be accompanied by an unwanted effect of plant growth reduction (OUSLEY *et al*., 1994). It was shown that the strongest producers of antibiotic metabolites, thereby being the strongest antagonists, may exert the most pronounced negative effect on plant growth (GHISALBERTI *et al*., 1990; HOWELL and STIPANOVIC, 1984). Especially the group of volatile pyrone antibiotics including 6PAP, produced by many *Trichoderma* strains, has this effect (GHISALBERTI *et al*., 1990; LUMSDEN *et al*., 1990; NASEBY *et al*., 2000).

In terms of biological control, little research was done concerning volatile secondary metabolites with antibiotic activity (DENNIS and WEBSTER, 1971b; BRUCE *et al*., 1984, 2000). Generally, volatile organic compounds should give a competitive edge to the producing organism towards other microorganisms (GHISALBERTI and SIVASITHAMPARAM, 1991), for example if the released volatile metabolites are able to affect mycelial growth and protein synthesis of target fungi (HUMPHRIS *et al*., 2002). Thereby, such volatile organic compounds including rather simple alkanes, alkoholes, aldehydes, and ketones (BRUCE *et al*., 2000) may be an important factor in the evolution of microorganisms with regard to community, population, and functional dynamics (HUMPHRIS *et al*., 2002).

Gas chromatographic analyses revealed that the profile of produced volatile metabolites depended on the producing *Trichoderma* strain, culture age, and the nutrient content of the medium (BRUCE *et al*., 2000; WHEATLEY *et al*., 1997). Enhanced production of 6PAP as a response of the producing *T. harzianum* strain to the presence of *Botrytis cinerea* as well as the partial degradation of the antibiotic metabolite by the pathogen could be detected (COONEY and LAUREN, 1998).

2.1.6 Trichoderma harzianum *strains*

The *T. harzianum strains* T-22 and T39, used in the study reported here together with four other *T. harzianum strains*, are among the most successful BCAs in terms of worldwide distribution of the respective commercial products (HARMAN, 2000; VINALE *et al*., 2006). T-22, one of the most studied *Trichoderma* strains (HARMAN *et al*., 2004), is distributed throughout the world under several brand names and mainly applied against *Fusarium* spp., *Pythium* spp., and *Rhizoctonia* spp. (HARMAN and BJÖRKMAN, 1998). The antagonistic mechanisms of this strain include competitive abilities by rhizosphere competence (HARMAN, 2000), parasitic action against plant pathogenic fungi (HARMAN and BJÖRKMAN, 1998), the production of few antibiotic metabolites (VINALE *et al*., 2006) as well as the induction of resistance (HARMAN, 2000; HARMAN *et al*., 2004). Positive growth responses occurred in many but not all of more than 500 recorded cases (HARMAN, 2006).

In scientific research projects, the main targets for T39, which originally was isolated from a cucumber fruit (Elad *et al*., 1993), have been *B. cinerea* (ELAD, 2000a, 2000b; ELAD *et al*., 1993; ELAD and KAPAT, 1999; KAPAT *et al*., 1998; MOYANO *et al*., 2003; ZIMAND *et al*., 1995) and mildew pathogens (ELAD, 2000a, 2000b; ELAD *et al*., 1998). Mycoparasitism or antibiosis are thought not to be among the mechanisms of action of T39 (ELAD, 1996; ELAD and KAPAT, 1999). Contrasting these results, VINALE *et al*. (2006) isolated T39 metabolites with antifungal activity from culture filtrates and dual culture assays. Competition for nutrients (ZIMAND *et al*., 1995), induction of resistance (ELAD, 2000a; ELAD *et al*., 1998), and the degradation of the pathogenicity enzymes of the necrotrophic *B. cinerea* by proteases (ELAD and KAPAT, 1999; KAPAT *et al*., 1998) undoubtedly contribute to the antagonistic activity of T39. Neither T39 nor T-22 produced the antibiotic metabolite 6PAP in the studies of VINALE *et al*. (2006).

For the remaining four *T. harzianum* strains, three of them isolated from commercial preparations, not much information on their antagonistic characteristics could be found. According to the product descriptions, the three commercial strains shall be used as a preventative measure against soil-borne diseases like *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., and *Sclerotium* spp. The non-commercial strain T12 was successfully used against *R. solani* (PAULA JÚNIOR and Hau, 2007; PAULA JÚNIOR *et al*., 2007).

In this study, the antagonistic features of these six strains were tested against *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycoperisci*, *Fusarium oxysporum* f. sp. *phaseoli*, *Pythium ultimum*, and *Rhizoctonia solani*. Mycelial growth and sporulation, competitive ability, parasitic interactions, and the production of lytic enzymes and secondary metabolites with antibiotic activity were assessed for all six *T. harzianum* strains to gain a profile of the antagonistic abilities of each strain.

2.2 Material and methods

2.2.1 Fungal strains

Six *T. harzianum* strains were used in this study, five of them isolated from commercial preparations: T-22 from TRIANUM-P (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands), T39 from TRICHODEX (Makhteshim-Agan Ltd., Tel Aviv, Israel), and the strains from TRI 003 (Plantsupport, Grootebroek, The Netherlands), TRICHOSAN (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), and UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand). The abbreviations T_R, T_S, and T_U will be used throughout this work for the latter three strains, respectively. The non-commercial strain T12 from the fungal collection of the Institute of Plant Diseases and Plant Protection (IPP; Leibniz Universität Hannover, Germany) was originally obtained as strain T000 from the Institute of Phytopathology and Applied Zoology (IPAZ; Justus-Liebig-University Gießen, Germany).

The used pathogens were isolates of *B. cinerea*, *F. oxysporum* f. sp. *lycopersici* (FOL), *F. oxysporum* f. sp. *phaseoli* (FOP), *P. ultimum*, and *R. solani* AG-4 taken from the fungal collection of the IPP.

2.2.2 General culture conditions

All fungal strains were maintained on PDA (Merck KGaA, Darmstadt, Germany) at 24°C. All Petri dishes used in the experiments had a diameter of 90 mm, were filled with 10 ml PDA, and were singly sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA), unless otherwise stated.

2.2.3 Determination of mycelial growth and sporulation potential of **Trichoderma harzianum** *strains*

Mycelial plugs with a 5-mm diameter were cut from the growing margin of three days old cultures of the *T. harzianum* strains and put overhead onto fresh PDA in the centre of the Petri dish. The mean radial growth per colony was calculated after 24, 48, 72, and 96 h by measuring the radius of the growing mycelium twice with an angle of 180 degrees between measurements. The experiment consisted of four replications per strain.

Spore suspensions were produced by scraping off sporulating mycelium from PDA cultures and suspending it in sterile A. dest. To remove mycelial fragments from the suspensions, they were filtered through 595 Schleicher & Schuell filter paper (Whatman International Ltd., Kent, England). Spore suspensions were adjusted to

1 x 10^4 spores / ml. Ten µl of each *Trichoderma* spore suspension were pipetted onto PDA and cultured for 24 h at 24°C. Petri dishes were not sealed with Parafilm. After 24 h, the length of ten hyphae emerged from the spores was measured with a binocular. The experiment consisted of four replications per spore suspension.

To determine sporulation potential of the *T. harzianum* strains, two media with differing amounts of nutrients were used. Mycelial plugs with a diameter of five mm were cut out of the growing margin of three days old cultures and put overhead onto PDA (39 g PDA / l) and 1/3-PDA (13 g PDA + 10 g Agar-Agar / l), because carbon starvation is known to increase sporulation (AGOSIN *et al.*, 1997). Petri dishes were not sealed with Parafilm to avoid self-inhibition of sporulation by the production of volatile secondary metabolites. As light is a known inducer of *Trichoderma* sporulation (BETINA and FARKAŠ, 1998), Petri dishes were taken out of the incubator every second day for five minutes. After 7 and 14 days of incubation, the mycelium of four replications per *Trichoderma* strain was scraped off from the agar plate and suspended in 50 ml A. dest. Dilutions were made and numbers of spores counted in a haemocytometer.

2.2.4 Competitive interaction

From the growing margin of three days old cultures of the six *T. harzianum* strains and of *B. cinerea*, *P. ultimum*, and *R. solani*, 5-mm plugs were transferred overhead to fresh PDA. Each *Trichoderma* – pathogen combination had four replications. A *Trichoderma* mycelial plug was placed at the border of the Petri dish, whereas the mycelial plug of the pathogen was put into the centre. The space between the pathogen and *T. harzianum* was 35 mm, whereas the free space to the other side of the Petri dish was 40 mm.

After 24, 48, 72, and 96 h, three growth measurements of the mycelia were done: (1) growth of *T. harzianum* directed towards the pathogen, (2) growth of the pathogen directed towards *T. harzianum*, and (3) growth of the pathogen into the free space.

The amount of mycelial growth inhibition of the pathogens by *T. harzianum* was calculated according to the mathmetical model $I = [(M_f - M_i) / M_f] * 100$, where I = mycelial growth inhibition, M_i = influenced mycelial growth of the pathogen, and M_f = free mycelial growth of the pathogen (ORTIZ and ORDUZ, 2000).

The experiment was conducted at 16°C and 24°C.

2.2.5 Parasitic interaction

From the growing margin of three days old cultures of the six *T. harzianum* strains and of *B. cinerea*, *P. ultimum*, and *R. solani*, 5-mm plugs were transferred overhead to fresh PDA. Each *Trichoderma* – pathogen combination had four replications. Mycelial plugs were placed at opposite sides of the Petri dish with a space of 75 mm between them.

Petri dishes were stored at 24°C. Five days after the mycelia got into contact with each other, quadrate plugs were cut from the zone of interaction that had developed. These plugs containing the mycelia of pathogen and antagonist were put onto PDA. The number of plugs that showed mycelial growth of the pathogens was recorded.

2.2.6 Production of lytic enzymes on solid substrates

The ability of the six *T. harzianum* strains to produce lytic enzymes was tested on water agar to which specific substrates were added. The tests for the production of endo-1,4-ß-glucanase (cellulase), endo-1,3-ß-glucanase, chitinases, and proteases were performed on 1% water agar amended with 0.1% ACZL-HE-cellulose, 0.1% ACZL-pachyman (both Megazyme, Bray, Ireland), 0.1% crab shell chitin (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), and 5% skimmed milk, respectively.

Mycelial plugs with a 5-mm diameter were cut from the growing zone of three days old *Trichoderma* cultures and incubated on the four media for varying periods of time. Because the growth of *T. harzianum* on water agar containing the AZCL-substrates or crab shell chitin did not lead to the production of easy to assess radial zones of substrate degradation, cellulolytic, glucanolytic, and chitinolytic activity was measured after 14, 28, and 21 days, respectively, by giving marks ranging from “0” to “5” meaning substrate degradation of “0%” to “100%”.

Activity of proteases was assessed after three days by recording the development of clearing zones.

2.2.7 Antibiotic effects of secondary metabolites produced in liquid culture

Culture filtrates were produced by cultivation of the six *T. harzianum* strains for ten days in PDB (Becton Dickinson GmbH, Heidelberg, Germany). For each strain, 250 ml PDB were initially inoculated with five mycelial plugs with a diameter of one centimetre. Incubation took place at room temperature (22°C) on a horizontal shaker at 85 rpm. To get rid of the major parts of the mycelium, liquid cultures were filtered through 595 Schleicher & Schuell filter paper. Afterwards cultures were sterile filtrated through Schleicher & Schuell filters

with pore sizes of 0.8 µm and 0.2 µm. Sterile culture filtrates were incorporated into PDA gaining culture filtrate agar with concentrations of 1, 3, 5, 10, 15, and 25%.

Mycelial plugs with a 5-mm diameter were cut from the growing margin of all pathogens' mycelia and put onto the culture filtrate agar media with four replications per combination of pathogen, culture filtrate, and culture filtrate concentration. The effect of the culture filtrates on mycelial growth was determined by calculation of the mean radial growth per colony. After 24, 48, 72, 96, and 120 h, radial growth of the growing mycelium was measured twice with an angle of 180 degrees between measurements.

2.2.8 Lytic enzyme activity in culture filtrates

The presence of lytic enzymes secreted into the PDB medium by the six *T. harzianum* strains was detected and estimated by adding five mg AZCL-HE-cellulose, AZCL-pachyman (both Megazyme, Bray, Ireland), chitin azure (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), or skimmed milk powder to one ml culture filtrate each.

The activity of cellulase, endo-1,3-ß-glucanase, and chitinases was visually estimated by the amount of substrate lysis and release of the blue dye from the chromogenic substrates. Marks ranging from 0 (no lysis of substrate particles) to 5 (complete lysis) were given. The presence of proteinases was detected by the lysis (yes or no) of the skimmed milk powder.

2.2.9 Production of volatile metabolites with antibiotic activity

The production of volatile secondary metabolites with antibiotic activity by the six *T. harzianum* strains was measured in a dual culture assay with the pathogens *B. cinerea*, *P. ultimum*, and *R. solani*. From the growing margin of three days old cultures of the six *T. harzianum* strains, 5-mm plugs were transferred overhead to fresh PDA, placed in the centre of 90-mm Petri dishes containing ten ml PDA, and cultured for specific periods of time. Repetitions of this procedure, always using three days old cultures of all *Trichoderma* strains, led to *Trichoderma* cultures with the age of 24 h, 7, 14, 21, and 28 days, which were used in the experiments with *R. solani*. The experiments with *B. cinerea* and *P. ultimum* were done only with 24 h and 7 days old cultures of the *T. harzianum* strains.

When the cultures of the *Trichoderma* strains reached these ages, 5-mm mycelial plugs were cut from the growing margin of three days old cultures of the pathogens and placed as well in 90-mm Petri dishes containing 10 ml PDA. While *B. cinerea* and

R. solani mycelial plugs were placed in the centre of the Petri dishes, the *P. ultimum* mycelial plugs were placed at the edge with respect to the greater speed of mycelial growth of *P. ultimum*.

For each *Trichoderma* strain – culture age – pathogen combination, replicated four times, the lids of the four Petri dishes containing the antagonist and the lids of the four Petri dishes containing the pathogen were discarded and the remaining bottoms were put onto each other in such a way, that the antagonist grew overhead of the pathogen with a layer of air dividing the mycelia from each other.

The antibiotic effect of volatile secondary metabolites was measured as the decrease in radial growth of the pathogens. Two kinds of controls were replicated four times: (1) pathogen – pathogen combinations by use of cultures of the pathogens having the respective age of 24 h, 7, 14, 21, or 28 days and (2) the pathogen combined with an empty second Petri dish. The second control was used to have a control in case of self-inhibition within the pathogen – pathogen combinations.

2.2.10 Statistical analysis

Hyphal and mycelial growth of the *T. harzianum* strains at different temperatures were statistically evaluated by contrast tests. Analyses of sporulation data, mycelial growth inhibition indices in competition studies, and areas under the inhibited growth curves (AUIGC), computed for the assay on the antibiotic effect of culture filtrates, were performed using Tukey's all-pair comparisons, which were most suitable to get information on all pairwise comparisons of interest. Areas under the growth progress curves in the assay on volatile *T. harzianum* metabolites with antibiotic activity were compared with the control by Dunnett's many-to-one comparisons. All analyses were performed with SAS version 8.02 (Statistical Analyses Systems Institute, Cary, NC, USA).

In all figures and tables, the variability is given by the standard error.

2.3 Results

2.3.1 Mycelial growth and sporulation potential of Trichoderma harzianum *strains*

It took several hours for the excised mycelium to regain its normal speed of growth, but from 24 h on, the mycelial growth of all *Trichoderma* strains led to linearly developing growth radii (data not shown).

The growth rate of all *T. harzianum* strain – temperature combinations was calculated. Increasing temperature from 16 to 24°C led to an increase of the *Trichoderma* growth rates, followed by a plateau of optimal temperature for mycelial growth between 24 and 28°C. Increase of temperature above 28°C to 32°C reduced mycelial growth per hour of the strains T12, T_R, and T_S, while T-22, T39, and T_U grew on with unchanged growth rate (Tab. 2.1).

Tab. 2.1. Growth rates of *Trichoderma harzianum* strains at temperatures between 16 and 32°C

	Mycelial growth [mm / h]				
Strain	16°C	20°C	24°C	28°C	32°C
T12	0.38 ± 0.005 bc[a]	0.52 ± 0.017 c	0.77 ± 0.012 bc	0.76 ± 0.043 bc	0.50 ± 0.008 c
T-22	0.51 ± 0.005 a	0.73 ± 0.012 a	0.98 ± 0.012 a	1.02 ± 0.009 a	1.03 ± 0.010 a
T39	0.32 ± 0.004 c	0.50 ± 0.009 c	0.78 ± 0.010 b	0.80 ± 0.010 b	0.80 ± 0.010 b
T_R	0.43 ± 0.005 ab	0.59 ± 0.006 b	0.70 ± 0.020 c	0.72 ± 0.020 c	0.54 ± 0.012 c
T_S	0.35 ± 0.006 c	0.56 ± 0.010 bc	0.76 ± 0.020 bc	0.83 ± 0.017 b	0.57 ± 0.010 c
T_U	0.17 ± 0.003 d	0.41 ± 0.013 d	0.47 ± 0.006 d	0.49 ± 0.005 d	0.50 ± 0.008 c

[a] Within each column, numbers followed by the same letter do not differ significantly according to pair-wise contrast tests.

Pairwise contrast tests at each temperature level revealed significant differences between growth rates of *Trichoderma* strains at the given temperatures of 16 to 32°C. At all temperatures, T-22 had the highest growth rate, while T_U showed the slowest mycelial growth. The European strains T12, T_R, and T_S as well as T39 from Israel showed rather similar growth responses to the different temperatures, with few differences according to their climatic requirements. Of these four strains, T_R was most cold-tolerant with the highest growth rate at 16°C. T39 on the other hand, growing little less than the European strains at 16°C, was the only one of these four strains whose growth did not significantly decrease at temperatures over 28°C.

Contrast tests that corresponded to the increase in growth rate from 16 to 24°C, the plateau between 24 and 28°C, and the decrease in growth rate at temperatures higher than 28°C, had a better fit to the data of T12, T_R, and T_S than contrasts matching only the growth rate increase from 16 to 24°C and a plateau for temperatures higher than 24°C. For *Trichoderma* strains T-22, T39, and T_U, this was exactly the other way round. Consequently, pairwise contrast tests between temperature levels of 28 and 32°C yielded significant *p*-values of $p < 0.001$ for strains T12, T_R, and T_S, but not for T-22, T39, and T_U. These significant differences are not indicated in Tab. 2.1 for reasons of clearness.

Of each *Trichoderma* spore suspension, 10-µl droplets were pipetted onto PDA medium and the length of the emerging hyphae was measured with a binocular after 24 h. Thereby, the two separate effects of germination speed and speed of hyphal growth both contributed to the overall length of the germination hyphae (Fig. 2.1). Because these two effects are not easily divided from one another, results have to be interpreted by using the information from the mycelial growth experiments.

During the first 24 h, T-22 and T_U produced longer hyphae at all three temperatures 20, 24, and 28°C than the other strains. Based on contrast tests, these differences between T-22 and T_U on one hand and T12, T39, T_R, and T_S on the other were statistically significant.

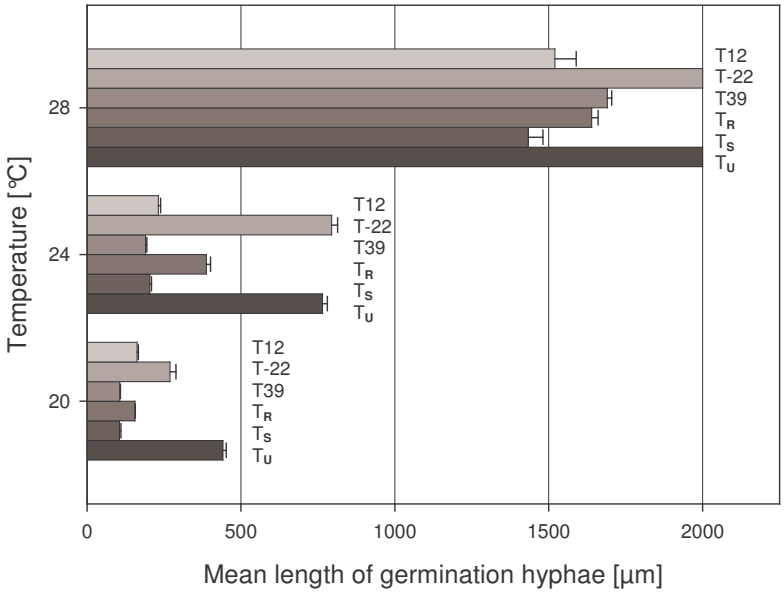


Fig. 2.1. Length of *Trichoderma harzianum* germination hyphae after 24 h growth at 20, 24, and 28°C.

At 20°C, T_U produced the longest germination hyphae within 24 h. Compared to T-22, this difference was nullified at 24°C, where the germ tubes grown from T-22 spores were even slightly longer than that of T_U. At 28°C, no difference could be observed between T-22 and T_U, because growth became too branched for simple measurements of hyphal length, but it can be assumed that T-22 might have produced a longer germ tube than T_U in regard to its very high mycelial growth rate at that temperature.

No clear results could be obtained at 32°C for any of the strains, because of the strong growth and branching of all strains' hyphae. In contrast, no germ tube growth was observed with the binocular after 24 h at 16°C with the exception of T-22 with a mean germ tube length of less than 20 µm (data not shown).

The data of sporulation (Tab. 2.2) of the *Trichoderma* strains showed that increasing time of cultivation led to an increase in the amount of harvested spores. This increase was statistically significant for T12, T39, T_R, and T_U on PDA and for T-22 and T_S on 1/3-PDA. Because starvation acts as an inducer of sporulation for some strains, while others sporulate better on rich media, using 1/3-PDA medium increased as well as decreased the number of produced spores depending on the *Trichoderma* strain. Within the first week, using 1/3-PDA led to significant increases in sporulation for T12 and T_S and significant decreases for T-22 and T_U. After the second week of cultivation, an increase in sporulation was significant only for T_S. On the other hand, T-22, T39, and T_U showed significant reductions in spore numbers compared to normal PDA medium.

Tab. 2.2. Spore numbers produced by *Trichoderma harzianum* mycelia after cultivation for 7 and 14 days at 24°C in 90-mm Petri dishes

	Cultivation time	
	7 days	14 days
Strain	PDA	
T12	$3.3 * 10^8 \pm 3.2 * 10^7$ c[a]	$1.1 * 10^9 \pm 1.7 * 10^8$ b
T-22	$1.1 * 10^9 \pm 8.0 * 10^7$ ab	$1.5 * 10^9 \pm 2.6 * 10^7$ b
T39	$1.5 * 10^9 \pm 2.0 * 10^8$ a	$2.8 * 10^9 \pm 1.7 * 10^8$ a
T_R	$4.2 * 10^7 \pm 1.3 * 10^7$ c	$5.5 * 10^8 \pm 1.3 * 10^7$ c
T_S	$7.5 * 10^7 \pm 9.4 * 10^6$ c	$1.7 * 10^8 \pm 2.2 * 10^7$ c
T_U	$8.4 * 10^8 \pm 7.1 * 10^7$ b	$1.4 * 10^9 \pm 8.8 * 10^7$ b
	1/3-PDA	
T12	$1.1 * 10^9 \pm 7.1 * 10^7$ a	$1.5 * 10^9 \pm 1.5 * 10^8$ a
T-22	$1.1 * 10^8 \pm 2.2 * 10^7$ d	$6.5 * 10^8 \pm 4.2 * 10^7$ b
T39	$1.0 * 10^9 \pm 7.0 * 10^7$ ab	$1.5 * 10^9 \pm 7.3 * 10^7$ a
T_R	$3.2 * 10^8 \pm 3.4 * 10^7$ cd	$3.7 * 10^8 \pm 5.0 * 10^7$ b
T_S	$6.0 * 10^8 \pm 6.1 * 10^7$ bc	$1.3 * 10^9 \pm 1.8 * 10^8$ a
T_U	$3.1 * 10^8 \pm 2.2 * 10^7$ cd	$4.3 * 10^8 \pm 4.2 * 10^7$ b

[a] Within each column and medium, numbers followed by the same letter do not differ significantly according to Tukey's all-pair comparisons.

Comparisons of the *Trichoderma* strains showed great differences with regard to sporulation potential. T12, T-22, T_U, and especially T39 produced far more spores than T_R and T_S on PDA. On 1/3-PDA, T12, T39, and T_S were sporulating the most. While T12 and T_R produced rather equal numbers of spores on both media and T_S spore numbers strongly increased on 1/3-PDA, T-22, T39, and T_U sporulated much weaker on the nutrient-poor 1/3-PDA than on normal PDA.

2.3.2 Competitive interaction

Dual culture experiments between the six *T. harzianum* strains and three pathogens were conducted at 2 different temperatures. Fig. 2.2 to 2.4 show the radial growth of the two competing mycelia in each pathogen – antagonist combination. The growth of the *T. harzianum* strains is displayed as increasing curves, while the growth of the pathogens is displayed as decreasing curves, with the corresponding scales for *T. harzianum* and pathogen growth on the left and right side of the graphs, respectively. No standard error bars are included in the graphs, because all standard errors had values below one mm. The bars of these small standard errors would have lain within the dots and triangles of the respective data points and spoiled their shape.

2.3.2.1 Competitive interaction with **Botrytis cinerea**

At 16°C, the *Trichoderma* strains and *B. cinerea* grew towards, but mostly did not grow into one another (Fig. 2.2). Further growth of both interacting fungi was inhibited. Especially in the cases of T_S and T_U, even after five days, the distance between both interacting fungi still was two and five mm, respectively (Fig. 2.2e and 2.2f). With the exception of T_R, the radial growth of *B. cinerea* was inhibited about 50%. Mycelial growth of T_R was stronger than that of the other *Trichoderma* strains under the given temperature (Fig. 2.2d). Therefore, T_R inhibited *B. cinerea* by 64%. Although T_U grew rather slow at 16°C, it was able to stop mycelial growth of *B. cinerea* over a distance of 7.5 mm (Fig. 2.2f). The involvement of antibiotic metabolites especially in this interaction may explain these observations.

At 24°C, T_R inhibited *B. cinerea* growth by nearly 76% (Fig. 2.2d) followed by T12, T-22, T39, and T_S with inhibition values of about 70%. T_U also grew slower at 24°C than the other strains and inhibited *B. cinerea* only by 58%. Within the five days of observation, T12 and T_R were the only *T. harzianum* strains that invaded the *B. cinerea* mycelium by more than one mm (Fig. 2.2a and 2.2d).

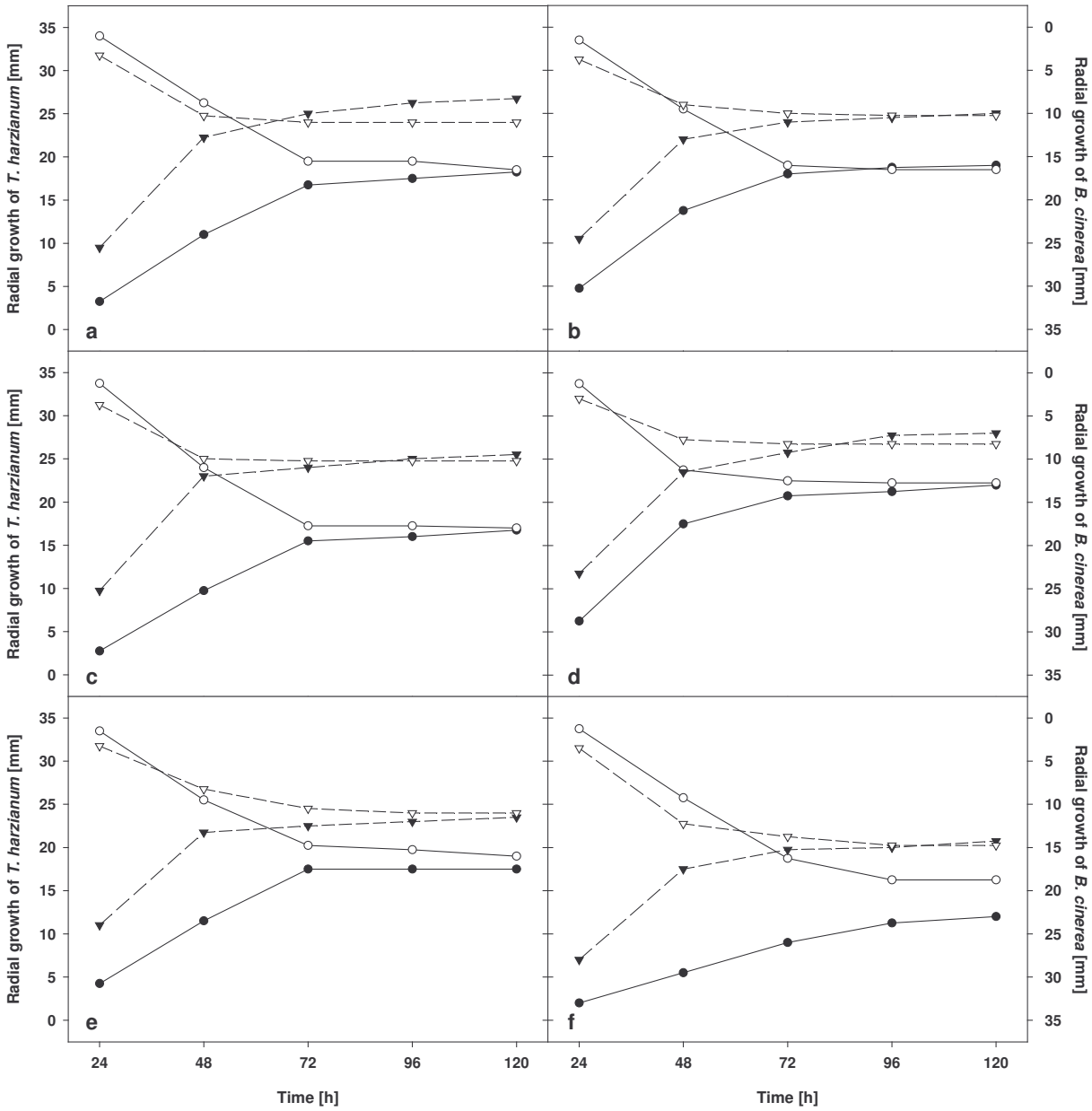


Fig. 2.2. Radial growth of *Botrytis cinerea* (○, ▽), if confronted with strain T12 (a), T-22 (b), T39 (c), T_R (d), T_S (e), or T_U (f) of *Trichoderma harzianum* (●, ▼) at 16°C (●, ○) and 24°C (▼, ▽).

2.3.2.2 Competitive interaction with Pythium ultimum

At 16°C, the very quickly growing *P. ultimum* reached growth radii between 23 and 29 mm for T_S and T_U, respectively (Fig. 2.3e and 2.3f). The mycelia of pathogen and antagonist got in contact during the time period between 24 and 48 h. After 48 h, no further growth of *P. ultimum* was observed, while the *T. harzianum* strains invaded the mycelium of the pathogen and constantly grew within it, thereby devastating the mycelial structures of *P. ultimum*. The quickest growing strain, T-22, also showed the greatest parasitic activity (Fig. 2.3b). Within little more than 96 h, T-22 had completely overgrown the *Pythium* mycelium and reached the mycelial disk that started the growth of the pathogen. Even after 120 h, the slowest strain at 16°C, T_U, still was 12 mm away from this inoculation point of *P. ultimum* (Fig. 2.3f).

At 24°C, *P. ultimum* growth was stopped at little smaller growth radii compared to the 16°C level by three *Trichoderma* strains, T-22, T_R, and T_U (Fig. 2.3b, 2.3d, and 2.3f). With the exception of T39 and T_R (Fig. 2.3c and 2.3d), *P. ultimum* growth stopped after only 24 h at growth radii of 23 to 25 mm. For T39 and T_R, *Pythium* mycelia reached radii of 27 to 28 mm. *Trichoderma* growth within the *Pythium* mycelium developed quicker than at 16°C.

Antibiotic interactions were not as pronounced as in the experiment with *B. cinerea*. For both temperatures it was just weakly visible, that T_S and T_U stopped the growth of *P. ultimum* just millimetres before the antagonist grew over the pathogen's mycelium (Fig. 2.3e and 2.3f).

At both temperatures, the growth rate of the *T. harzianum* strains was nearly the same before and after contact was made to the mycelium of *P. ultimum*. This is indicated by the linearity of the graphs in Fig. 2.3. In a few cases, a little lag period in *T. harzianum* growth could be seen directly after contact was made between antagonist and pathogen. But afterwards, *T. harzianum* strains regained their normal growth rate.

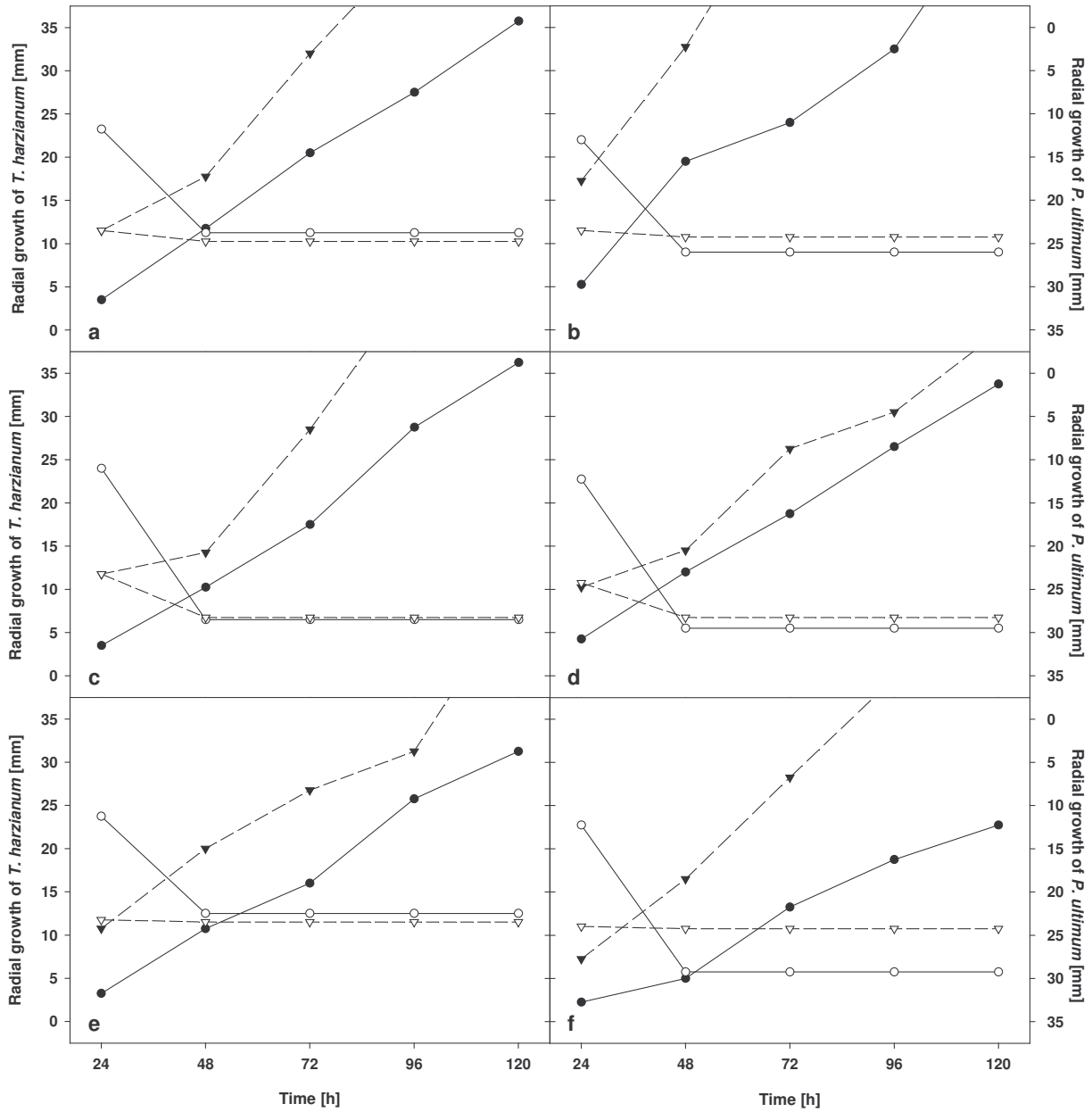


Fig. 2.3. Radial growth of *Pythium ultimum* (○, ▽), if confronted with strain T12 (a), T-22 (b), T39 (c), T_R (d), T_S (e), or T_U (f) of *Trichoderma harzianum* (●, ▼) at 16°C (●, ○) and 24°C (▼, ▽).

2.3.2.3 Competitive interaction with Rhizoctonia solani

As *T. harzianum* and *R. solani* have nearly the same speed of growth, they got into contact to one another after 15 to 20 mm of free growth with 17,5 mm as the half distance (Fig. 2.4). This is the case for both temperatures, but the time period until contact was made differed with ca. 72 h at 16°C and little less than 48 h at 24°C. Exceptions to this were T_R at 16°C and T_U at both temperature levels (Fig. 2.4d and 2.4f).

At 16°C, T_R stopped the growth of *R. solani* after 48 h at a radius of 12 mm, itself being 6 mm apart from the mycelium of the pathogen (Fig. 2.4d). T_R closed this gap within the following 40 h at a much lower growth rate as observed during the first 48 h. T_U also stopped the growth of the *R. solani* mycelium before contact between antagonist and pathogen was made (Fig. 2.4f). At 16°C, *R. solani* growth stopped after 72 h at a radius of 18 mm, with a distance of 7 mm to the T_U mycelium. After 120 h, this gap was still 4 mm in size. At 24°C, *R. solani* growth stopped after 48 h at a radius of 16 mm, while T_U was still 3 mm apart from it. Both observations may be interpreted by the production of secondary metabolites with antifungal activity against *R. solani* by T_R and T_U.

In all cases in which the antagonist and the pathogen came in contact, only *T. harzianum* showed further growth. In contrast to the results for *P. ultimum* (Fig. 2.3), the speed of growth within and on top of the *R. solani* mycelium was slowed down compared to free growth of the *T. harzianum* mycelium.

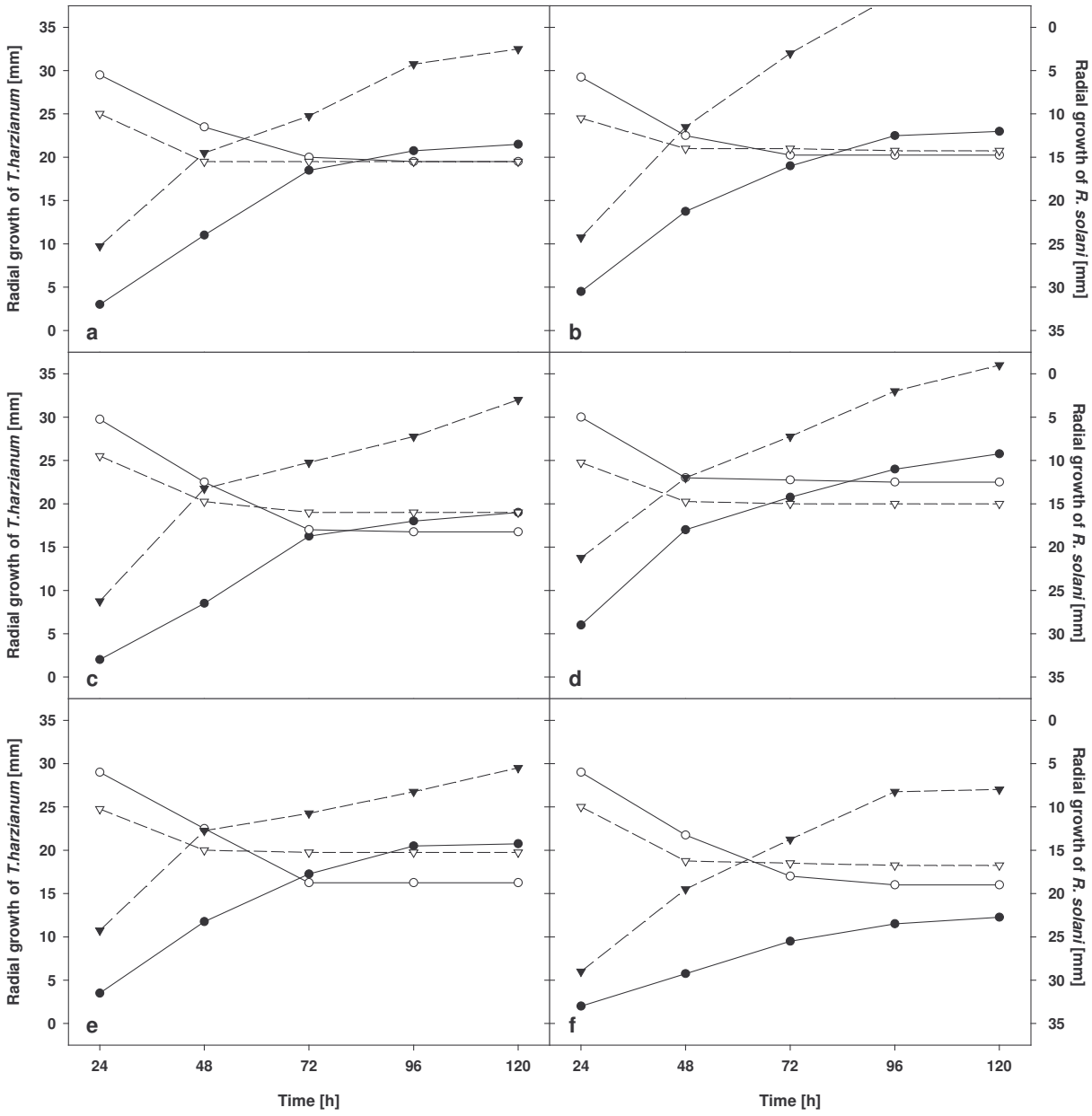


Fig. 2.4. Radial growth of *Rhizoctonia solani* (○, ▽), if confronted with strain T12 (a), T-22 (b), T39 (c), T_R (d), T_S (e), or T_U (f) of *Trichoderma harzianum* (●, ▼) at 16°C (●, ○) and 24°C (▼, ▽).

2.3.2.4 Inhibition index

For all interactions, inhibition indices were calculated based on the following formula: $I = [(M_f - M_i) / M_f] * 100$. Fig. 2.5 shows the development of the inhibition indices of all pathogen – antagonist interactions at 16 and 24°C. If the radius of the pathogen's mycelial growth was greater towards the *Trichoderma* mycelium than into the free space after 24 h, the inhibition index gave negative values, which can be seen for a few curves, starting at radial growth inhibition values below zero. Because mycelial growth of both fungi was un-inhibited in nearly all pathogen – antagonist combinations during the first 24 h, this observation can be explained by simple variation in the speed of mycelial growth of the pathogen to the free and the to-be-occupied-by-*Trichoderma* side of the Petri dish.

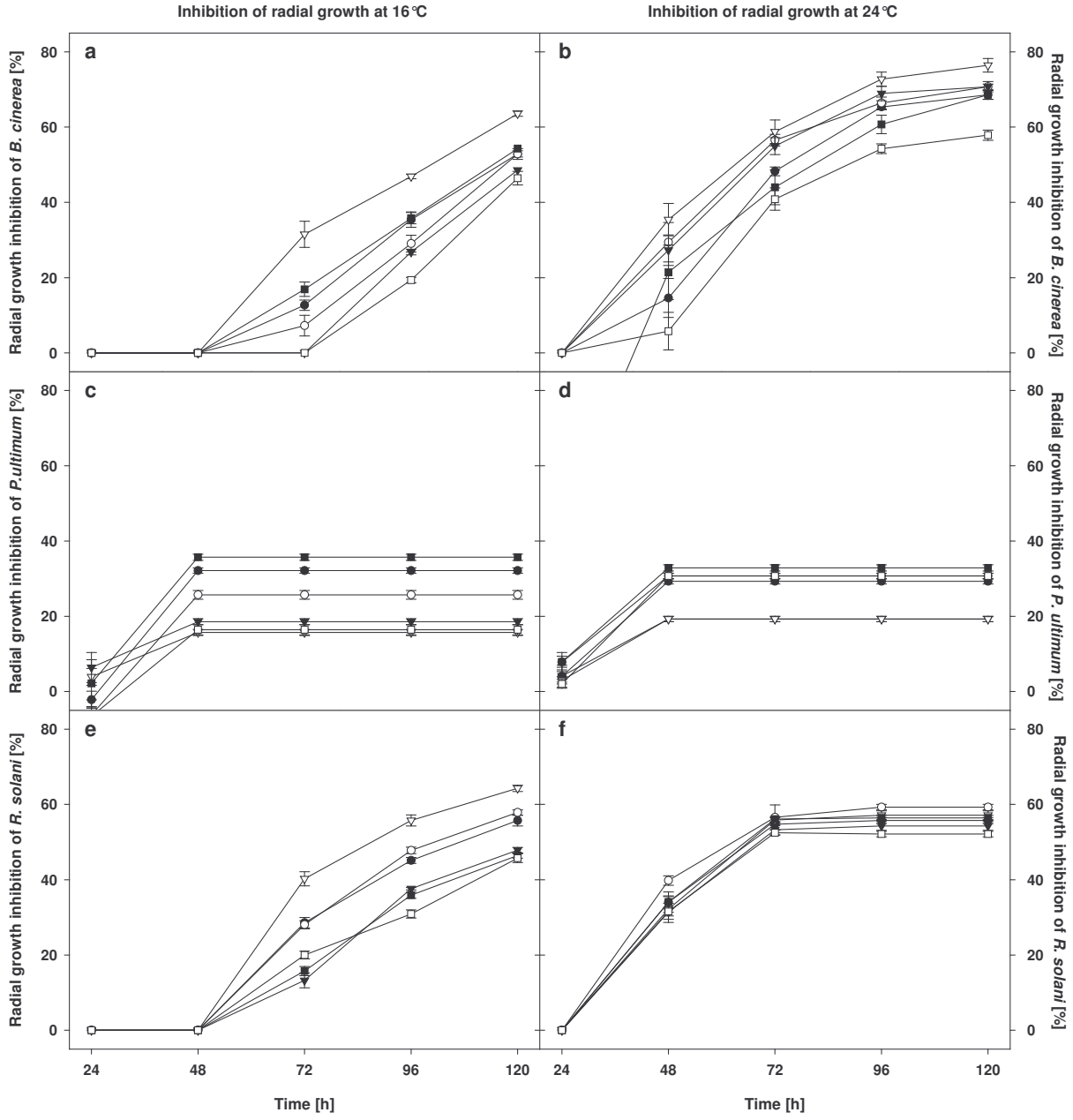


Fig. 2.5. Radial growth inhibition of *Botrytis cinerea* (a & b), *Pythium ultimum* (c & d), and *Rhizoctonia solani* (e & f) at 16 and 24°C, if confronted with *Trichoderma harzianum* T12 (●), T-22 (○), T39 (▼), T_R (▽), T_S (■), or T_U (□).

Generally, the inhibition index increased as long as the pathogens' freely growing mycelium increased and was fixed when reaching a diameter of 35 mm, which was the maximum space between pathogen and antagonist. During the time of observation this did not happen for *B. cinerea* (Fig 2.5a and 2.5b), while *P. ultimum* filled the available space in the Petri dish within 48 h (Fig. 2.5c and 2.5d). *R. solani* did not reach the capacity of the Petri dish at the 16°C level (Fig. 2.5e), but at 24°C, the inhibition indices of the interaction between *R. solani* and the antagonist strains were fixed after 96 h (Fig. 2.5f).

For the statistical analysis of these indices, the 'fixed values' were used if available. If not, the values reached after 120 h as the greatest observed inhibition indices were taken for analysing the inhibition potential of the six *T. harzianum* strains (Tab 2.3).

Tab. 2.3. Radial growth inhibition caused by *Trichoderma harzianum* against *Botrytis cinerea* after 120 h, *Pythium ultimum* after 48 h, and *Rhizoctonia solani* after 120 and 96 h of co-culture at the two temperature levels of 16 and 24°C, respectively

	Inhibition index [%]					
	B. cinerea		*P. ultimum*		*R. solani*	
Isolat	16°C	24°C	16°C	24°C	16°C	24°C
T12	52.9 ± 1.4 bc[a]	68.6 ± 1.2 b	32.1 ± 0.7 a	29.3 ± 0.7 a	55.7 ± 1.4 b	55.7 ± 0.8 ab
T-22	52.9 ± 0.8 bc	70.7 ± 0.7 b	25.7 ± 1.2 b	30.7 ± 0.7 a	57.9 ± 0.7 b	59.3 ± 0.7 a
T39	48.6 ± 0.7 cd	70.7 ± 1.4 b	18.6 ± 0.8 c	19.3 ± 0.7 b	47.9 ± 0.7 c	54.3 ± 1.2 ab
T_R	63.6 ± 0.7 a	76.4 ± 1.8 a	15.7 ± 0.8 c	19.3 ± 0.7 b	64.3 ± 0.8 a	57.1 ± 1.2 ab
T_S	54.3 ± 1.5 b	68.6 ± 1.2 b	35.7 ± 0.8 a	32.9 ± 0.8 a	46.5 ± 0.7 c	56.4 ± 0.7 ab
T_U	46.4 ± 1.8 d	57.9 ± 1.4 c	16.4 ± 1.4 c	30.7 ± 0.7 a	45.7 ± 1.2 c	52.1 ± 0.7 b

[a] Within each column, numbers followed by the same letter do not differ significantly according to Tukey's all-pair comparisons, performed for all treatments at once.

2.3.2.5 Effects of antagonist and temperature

The most effective strain in terms of inhibition of *B. cinerea* was T_R. At both temperatures it slowed down and finally stopped the growth of the pathogen before any hyphal contact was established. Such an effect could also be observed for other strains, especially T_S and T_U. While the antibiotic effect of T_S was a bit weaker than that of T_R, T_U was much less effective due to its slow mycelial growth, although this strain clearly produced metabolites with strong antifungal activity, acting over a distance of more than 10 mm as could be observed at the 16°C level.

T39 and T_R were least effective in inhibiting the growth of *P. ultimum*, because these two strains allowed further *Pythium* growth after initial hyphal contact. During the first hours after contact, the two mycelia merged into one another, until the antagonist finally

stopped further development of the pathogen's mycelium. T_U again was growing too slowly at 16°C, to effectively compete with *P. ultimum*.

Strains T-22, T_R, and T_U were able to stop *R. solani* before their mycelia got in contact. This antibiotic interaction was more pronounced at 16°C than 24°C and made them the most effective competitors at the lower temperature, with the exception of T_U. Antibiosis could not be attributed to the interaction of T12, T39, and T_S with the pathogen. Despite its antibiotic effect, T_U was the weakest competitor due to its slow growth.

Results of the statistical analyses concerning the effect of temperature on the competitive interaction between the *T. harzianum* strains and the pathogens are not included in Tab. 2.3 for reasons of clearness. Increasing the temperature from 16 to 24°C led to significantly greater inhibition of *B. cinerea* by all *Trichoderma* strains, while the growth of *P. ultimum* was significantly stronger inhibited only by T_U. T39, T_S, and T_U reduced the growth of *R. solani* to a greater extent at 24°C than at 16°C. There was only one case of a significantly decreased inhibition due to higher temperature: T_R was less effective against *R. solani* at the higher temperature of 24°C.

2.3.3 Parasitic interaction

After five days of interaction, T12, T_S, and T_U had killed the mycelium of all three tested pathogens in each case. *B. cinerea* was able to grow out of the zone of interaction with T-22 and T39 in 5 out of 16 and 1 out of 16 cases, respectively. T39 did not kill *R. solani* in 3 out of 16 cases. T_R showed the weakest performance in parasitizing and killing the mycelia of the pathogens. It was not successful in 7, 16, and 4 out of 16 cases for *B. cinerea*, *P. ultimum*, and *R. solani*, respectively.

2.3.4 Production of lytic enzymes on solid substrates

The *Trichoderma* strains were grown on water agar amended with dyed enzyme-specific substrates, crab shell chitin, or skimmed milk to prove the production of extracellular lytic enzymes by degradation of the substrates. Strongest mean degradation of cellulose was 100, 85, and 70% for T39, T-22, and T12, respectively. T_R, T_U, and T_S degraded cellulose to a lesser extent of 55, 45, and 20%, respectively. Degradation of the ß-glucan AZCL-pachyman was not detected with any of the *Trichoderma* strains after four weeks of incubation. Within three weeks, crab shell chitin was only degraded by T_U, T12, and T_R by 70, 50, and 15%, respectively.

Measurement of degradation of the proteins of skimmed milk was done using the relation of clearing zone to radial mycelial growth. Strongest lytic activity was found for T39, T_U, and T12, followed by T_S, T_R, and T-22 with 86, 81, 77, 59, 41, and 19%, respectively.

2.3.5 Lytic enzyme activity in culture filtrates

The dyed enzyme-specific substrates AZCL-HE-cellulose, AZCL-pachyman and chitin azure as well as skimmed milk powder were given into fresh *Trichoderma* culture filtrates, and the degree of substrate particle lysis was determined.

The strongest producer of cellulases was T39 with 100% lysis of the substrate within three days followed by T12 and T_R with 60% and 70% lysis after 3 days, respectively, and 80% for both strains after seven days. T_U and T_S culture filtrates showed less activity in degrading the cellulose with 15 and 20%, respectively, after three days and 60 and 55%, respectively, after seven days. No cellulase activity was observed for the T-22 culture filtrate.

AZCL-pachyman, the substrate for a endo-1,3-ß-glucanase, was degraded by all culture filtrates. The most effective culture filtrate was that of T_R with 90% degradation after seven days, followed by T_U, T-22, T_S, T39, and T12 with degradation levels of 80, 70, 65, 60, and 55%, respectively.

Generally, no chitinase activity could be observed for the culture filtrates.

T12, T_S, and T_U culture filtrates showed protease activity towards skimmed milk powder. Mixing it into the filtrates led to agglutination first and lysis afterwards.

2.3.6 Antibiotic effects of secondary metabolites produced in liquid culture

B. cinerea, *P. ultimum*, *R. solani*, FOL, and FOP were grown on PDA amended with increasing concentrations of *T. harzianum* culture filtrates ranging from 1 to 25%. Generally, mycelial growth of the pathogens decreased with increasing culture filtrate concentration, but there were huge differences concerning the effect of different culture filtrates on a certain pathogen or concerning the effect of a certain culture filtrate on different pathogens (Fig. 2.6).

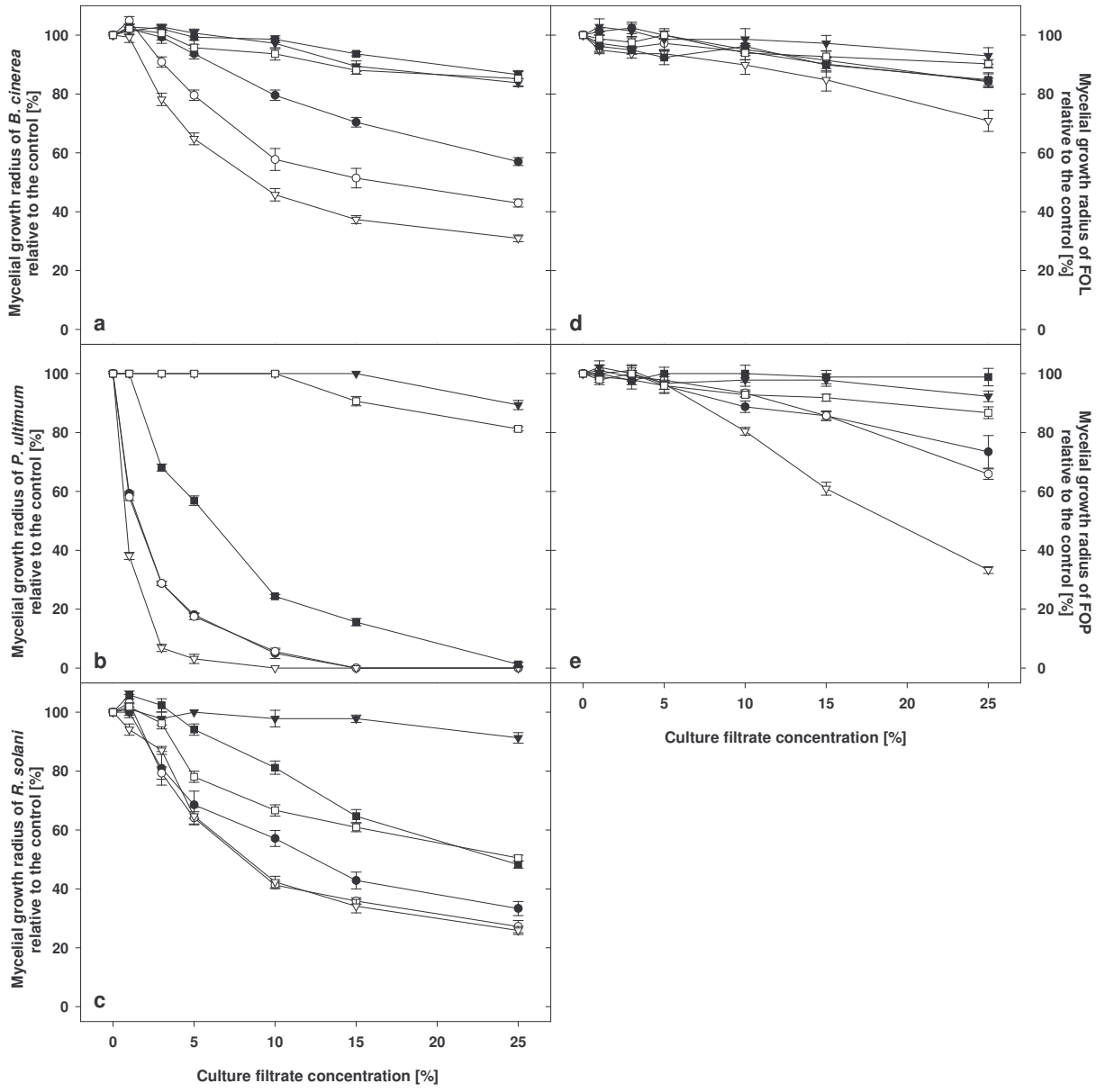


Fig. 2.6. Radial growth of *Botrytis cinerea* after 96 h (a), *Pythium ultimum* after 48 h (b), *Rhizoctonia solani* after 48 h (c), *Fusarium oxysporum* f. sp. *lycopersici* after 120 h (d), and *Fusarium oxysporum* f. sp. *phaseoli* after 120 h (e) on PDA amended with increasing culture filtrate concentrations of *Trichoderma harzianum* T12 (●), T-22 (○), T39 (▼), T_R (▽), T_S (■), or T_U (□).

The growth of *P. ultimum* was most strongly reduced by the *Trichoderma* culture filtrates, its growth completely inhibited if 10 or 15% of T_R, T-22, and T12 or 25% of T_S were incorporated into the medium (Fig. 2.6b). All culture filtrates with the exception of T39 had a rather strong effect against *R. solani* with growth reductions ranging from 50 to 70% if 25% culture filtrate were used in the medium (Fig. 2.6c). Only T_R, T-22, and T12 showed a marked effect against *B. cinerea* (Fig. 2.6a).

The *Fusarium* isolates showed a greater tolerance against the antibiotic effect of the secondary metabolites in the culture filtrates. FOL reached over 70% of its normal growth even if the PDA medium was amended with 25% culture filtrate (Fig. 2.6d). FOP was a little less tolerant, with the T_R culture filtrate being the only one to show a marked effect of 70% growth reduction at the highest culture filtrate concentration (Fig. 2.6e).

In most cases of *B. cinerea*, *P. ultimum*, and *R. solani*, 50% of the maximum growth reduction was reached at low culture filtrate concentrations between 1 and 5% (Fig. 2.6a, 2.7b, and 2.7c). Contrasting these results, the *Fusarium* species showed only little growth reduction before the culture filtrate concentration reached 10% (Fig 2.6d and 2.6e).

The areas under the curves demonstrating inhibited growth (AUIGC) due to increasing culture filtrate concentrations, thereby indicating strong inhibition by low AUIGC-values, were calculated. Based on these AUIGC-values, an ANOVA including all antagonist – pathogen combinations was computed (Tab. 2.4).

Tab. 2.4. Areas under the inhibited growth curves of *Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Fusarium oxysporum* f. sp. *phaseoli* cultivated on PDA amended with *Trichoderma harzianum* culture filtrates for 96, 48, 48, 120, and 120 h, respectively

	AUIGC [%*%]				
Strain	*B. cinerea*	*P. ultimum*	*R. solani*	FOL	FOP
T12	1941 ± 17 c[a]	285 ± 7 b	1376 ± 32 b	2339 ± 19 bc	2184 ± 25 b
T-22	1557 ± 36 b	284 ± 8 b	1199 ± 14 a	2298 ± 17 b	2181 ± 22 b
T39	2336 ± 20 d	2447 ± 8 d	2427 ± 5 e	2439 ± 22 c	2426 ± 15 cd
T_R	1246 ± 18 a	132 ± 5 a	1189 ± 25 a	2147 ± 34 a	1766 ± 21 a
T_S	2385 ± 17 d	781 ± 16 c	1875 ± 33 d	2287 ± 29 b	2481 ± 36 d
T_U	2294 ± 23 d	2336 ± 15 d	1711 ± 10 c	2359 ± 23 bc	2319 ± 25 c

[a] Within each column, numbers followed by the same letter do not differ significantly according to Tukey's all-pair comparisons.

Tukey's all-pair comparisons showed that the T_R culture filtrate was most effective against all pathogens, while T39 gave the least pronounced effects against them, with the exception of T_S being less effective against FOP. Overall, T12 and T-22 were less effective than T_R, but more effective than T_S and T_U.

2.3.7 Production of volatile metabolites with antibiotic activity

B. cinerea, *P. ultimum*, and *R. solani* were grown in the presence of *T. harzianum* mycelia of varying ages. If the *Trichoderma* mycelium was only 24 h old, it steadily grew on for the next two to three days until the available medium in the Petri dish was overgrown. After *T. harzianum* had been cultivated for one or more weeks, it did not produce further amounts of mycelium, though sporulation was visible.

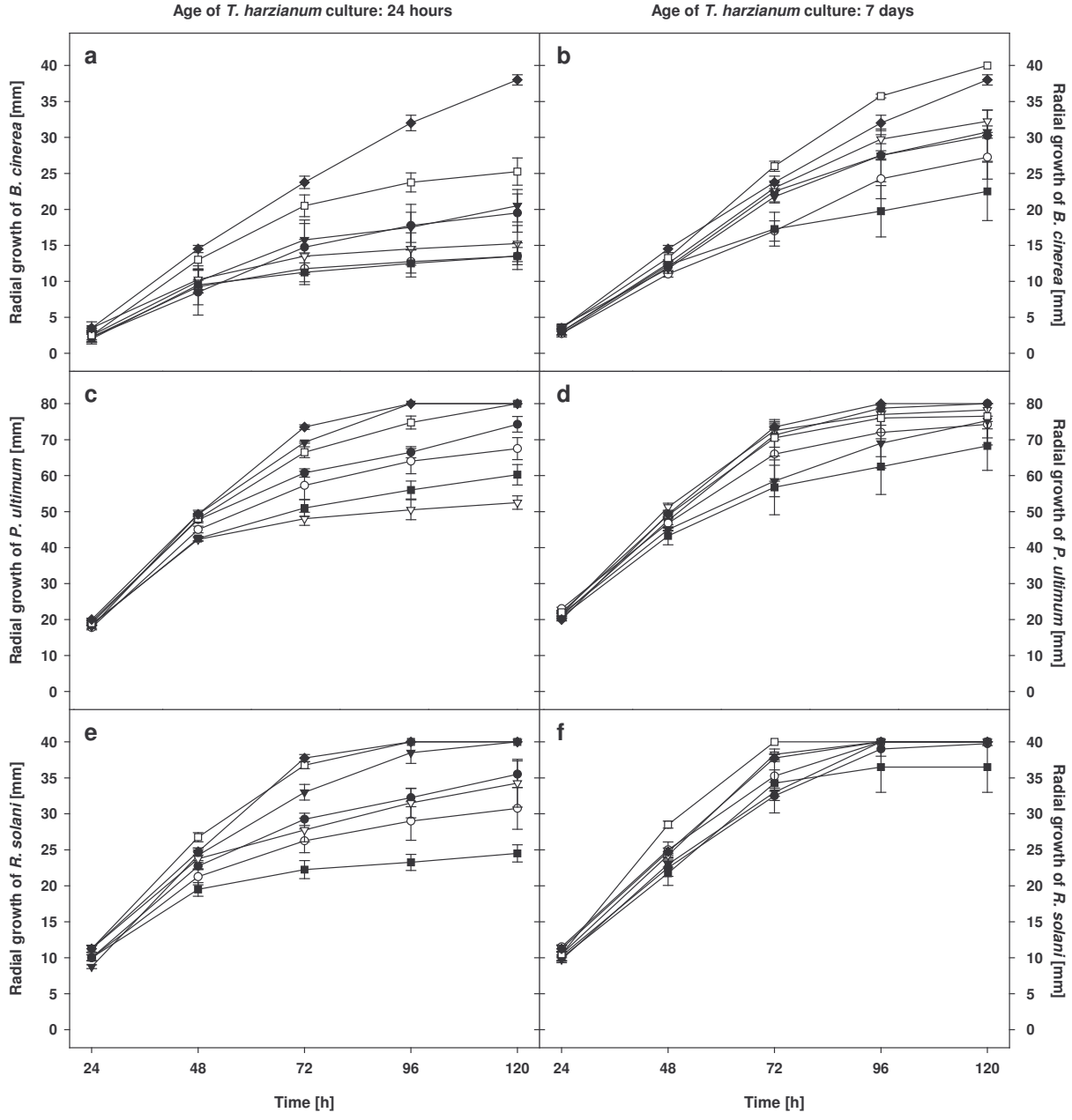


Fig. 2.7. Radial growth[a] of *Botrytis cinerea* (a & b), *Pythium ultimum* (c & d), and *Rhizoctonia solani* (e & f) confronted with 24 hours and 7 days old mycelia of *Trichoderma harzianum* T12 (●), T-22 (○), T39 (▼), T_R (▽), T_S (■), T_U (□), or without confrontation (◆). [a] The position of the mycelial plugs of the pathogens in the Petri dishes allowed radial growth of 80 mm for *P. ultimum* and 40 mm for *B. cinerea* and *R. solani*.

If the pathogens were confronted with the *T. harzianum* strains grown 24 h in advance, the radial growth of mycelia of the pathogens was slowed down in most cases (Fig. 2.7a, 2.7c, and 2.7e). The growth of the pathogens was much less affected, if the *Trichoderma* mycelia were seven days old when confrontation took place (Fig. 2.7b, 2.7d, and 2.7f).

To measure the amount of growth reduction due to the presence of the *T. harzianum* strains and their respective volatile metabolites, the areas under the growth progress curves (AUGPC) were calculated and statistically analysed. Strong growth inhibiting effects are indicated by low AUGPC-values (Fig. 2.8).

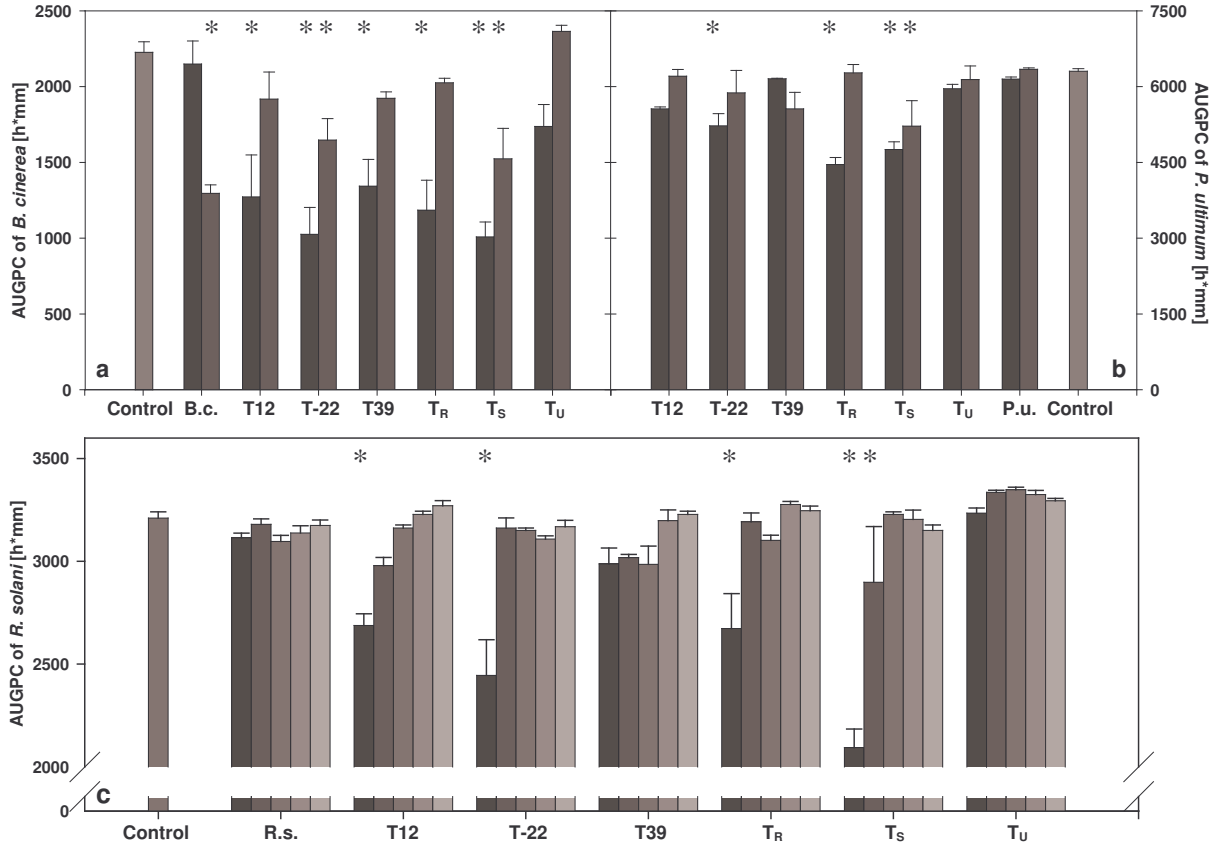


Fig. 2.8. AUGPC of *Botrytis cinerea* (a), *Pythium ultimum* (b), and *Rhizoctonia solani* (c) confronted with its own mycelium (B.c., P.u., R.s.) or with *Trichoderma harzianum* mycelia (T12, T-22, T39, T_R, T_S, T_U) of differing ages; a, b: 24 h (▬), 7 days (▬); c: 24 h (▬), 7 days (▬), 14 days (▬), 21 days (▬), 28 days (▬). Significant differences to the control treatment without confrontation are indicated by *.

With the exception of T_U, all 24 h old *Trichoderma* strains significantly reduced the AUGPC of *B. cinerea* (Fig. 2.8a). Additionally, T-22 and T_S significantly affected the *B. cinerea* growth, if seven days old. *P. ultimum* radial growth was only slowed down significantly by 24 h old T-22 and T_R, as well as T_S of both ages (Fig. 2.8b). Growth reduction of *R. solani* mycelium was significant for 24 h old strains T12, T-22, and T_R (Fig. 2.8c). Again, T_S mycelia with ages of 24 h and seven days at the beginning of the confrontation experiment led to a significant decrease of the *R. solani* AUGPC. No significant AUGPC reduction was observed, if *T. harzianum* mycelia were 14 to 28 days of age when the experiment started.

The most effective producer of volatile metabolites with antibiotic properties in terms of reducing the radial growth of the tested plant pathogenic fungi was T_S. 24 h as well as seven days old T_S mycelia significantly inhibited the growth of all pathogens tested (Fig. 2.8). The great standard error observed in the confrontation assay of seven days old mycelium of T_S and *R. solani* results from strong differences in the four replications (Fig. 2.8c). In three out of four replications, T_S was less effective against *R. solani*, while the growth reduction of *R. solani* in the fourth replication was as strong as in the confrontation assay with 24 h old T_S. Repeating the experiment led to the same result of a prolonged effect of seven days old T_S mycelium on *R. solani* growth in two out of four cases (data not shown).

T_U was the only *Trichoderma* strain that showed no effect on any of the pathogens (Fig. 2.8). The most strongly influenced pathogen was *B. cinerea* with growth reductions in 7 out of 12 confrontation assays: 5 out of 6 and 2 out of 6 confrontations for 24 h and seven days old *Trichoderma* mycelia, respectively, led to significant decreases in *B. cinerea* growth (Fig. 2.8a).

Besides slowing down mycelial growth, the volatile antifungal metabolites had another effect, not measured in this assay: The mycelium of the pathogens did not only grow slower but it also was thinner. This was the case especially for the mycelial growth of *P. ultimum*, which kept up its radial growth but lost much of its cottony character.

2.4 Discussion

The aim of this study was to gain profiles of the used six *T. harzianum* strains. The *in vitro* experiments were conducted to characterize their competitive, parasitic, and antibiotic properties. Although successful biological control in *in vitro* assays does not guarantee a successful in-field performance, it may help to divide interesting candidates from those with little or no antagonistic abilities.

2.4.1 Mycelial growth and sporulation

At each temperature level, the mycelial growth of the *T. harzianum* strains developed linearly with a constant growth rate. These growth rates differed up to more than 100% between *Trichoderma* strains. T-22, which was obtained by protoplast fusion with the intention to produce a superior strain in terms of rhizosphere competence (HARMAN, 2000), grew by far the quickest, while the Thailand-strain T_U had only half the growth rate of the former strain over the entire temperature range. Compared to one another, the strains originating from Europe T12, T_R, and T_S as well as T39 from Israel had rather equal growth rates over the temperature range of 16 to 28°C. But at 32°C, all European strains showed reduced growth, while T-22, T39, and T_U, especially the latter two strains originating from warmer climatic zones, showed even little increase in mycelial growth speed. This gives emphasis to the importance of selecting *Trichoderma* strains not only with respect to their antagonistic performance, but also to their climatic demands (ANTAL *et al.*, 2000, HANNUSCH and BOLAND, 1996).

Looking at the germination of *T. harzianum* spores and the subsequent growth of the germination hyphae, again T-22 and T_U stood out against T12, T39, T_R, and T_S. After 24 h, these two strains had by far developed the longest hyphae. This could have been expected for the very quickly growing T-22, but the result of T_U was surprising. The Thailand-strain T_U, having only about 50% of the growth rate of T-22 and being rather sensitive to lower temperatures, produced germination hyphae with a 40% greater length within 24 h at 20°C. This suggests that the simple cracking of the spore shell and subsequent hyphal growth do not underly the same factors influencing these events. While growth processes depend on the speed of the fungal metabolism, the time necessary for the opening of the spore shell may be determined by physico-chemical aspects of the spore shell's architecture. The longer the observed time period, the greater was the impact of hyphal growth, which means the impact of the fungal metabolism. Because the metabolism was strongly influenced by temperature, with increasing hyphal

growth as response to increasing temperature, the impact of germination speed on the result got weaker if temperature increased from 20 to 28°C.

It can be hypothesized that T-22 produced the longest hyphae during 24 h at 28°C and that the difference between the length of the T_U hyphae and that of the four other strains decreased, because all these strains have greater hyphal / mycelial growth rates than T_U.

While mycelial growth is elementary to increase the metabolically active population, sporulation is important for further increase of population density and survival of the fungus. Thereby, starvation may act as an inducer of sporulation (AGOSIN *et al.*, 1997), but can also decrease the amount of produced spores, depending on the *T. harzianum* strain. This is of special concern for the industrial production of active *Trichoderma* propagules used for commercial *Trichoderma* preparations (AGOSIN and AGUILERA, 1998).

2.4.2 Growth inhibition, parasitism, and antibiosis

The competitive interactions between the six *T. harzianum* strains and a certain pathogen were rather similar with little variation in terms of additionally occurring antibiosis. In contrast, great differences could be observed between the reactions of *T. harzianum* in general to the different pathogens *B. cinerea*, *P. ultimum*, and *R. solani*.

The growth of *P. ultimum* was not only stopped by the contact with the antagonist, but the *Trichoderma* strains grew into and exploited the pathogen's mycelium nearly without reducing their growth rates. As the PDA medium as well as the *Pythium* cell wall consist partly of cellulose, the antagonist can use the same enzyme to degrade both molecular structures. Synthesis of 1,3-ß-glucanases, necessary to degrade the second important structural component of the *Pythium* cell wall (BENHAMOU and CHET, 1997), most likely took place and increased the mycoparasitic activity of *T. harzianum*.

The mycelium of *R. solani*, consisting of cells with chitinous walls, makes it necessary for *T. harzianum* to synthesize other enzymes than glucanases (BENHAMOU and CHET, 1993). As the synthesis of chitinases and the lysis of the respective molecular bonds may be more time consuming than the degradation of cellulose, this may explain the reduction of the growth rate of *T. harzianum*.

Most interestingly, all *Trichoderma* strains were able to stop the growth of *B. cinerea*, but had huge problems in growing into and parasitizing the pathogen's mycelium. Although the *B. cinerea* mycelium consists of chitin like *R. solani*, it may induce different chitinases or it may fail to induce particular enzymes as observed by HARAN *et al.* (1996) for the interaction of *T. harzianum* with *S. rolfsii*. Generalization of such results is

not possible, considering that other *Trichoderma* strains successfully parasitzed *S. rolfsii* (MUKHERJEE and RAGHU, 1997).

The observed inability of the *T. harzianum* strains, tested in this study, to parasitize *B. cinerea* may also be explained by an antibiotic interaction of *B. cinerea* with *T. harzianum*. The growth rates of all *Trichoderma* strains were reduced before contact to *B. cinerea* was established, indicating that *B. cinerea* produced a metabolite that inhibited the growth and parasitic activity of *T. harzianum* (RUBEZHNIAK *et al.*, 1995). On the other hand, the growth of *B. cinerea* stopped as well, leaving a gap between the mycelia of both competing fungi. Especially the interaction with T_R and T_U led to broader gaps, which were slowly closed by mycelial growth of the antagonists. Therefore, it can be hypothesized that both interacting fungi produced antifungal metabolites, but that *T. harzianum* was able to detoxify the antibiotic produced by *B. cinerea*, while *B. cinerea* was not, although the ability of *B. cinerea* to degrade the *T. harzianum* metabolite 6PAP has been demonstrated (COONEY and LAUREN, 1998).

The impact of antifungal metabolites of T_R and T_U was also visible in the interaction with *R. solani*. Confronted with *P. ultimum*, however, T_R was unable to stop the growth of the pathogen until both fungi had merged several millimetres into one another. Wheter secondary metabolites of a certain *Trichoderma* strain act as antibiotics, is therefore depending on the target fungus (VIZCAÍNO *et al.*, 2005). The development of greater inhibition zones at lower temperature levels is in accordance with TRONSMO and DENNIS (1978), possibly due to an increased production of the inhibitory metabolites or because diffusion of those metabolites was less affected by temperature than the mycelial growth rate.

Summarizing the results, it can be said that all three antagonistic mechanisms were involved in the interactions: (1) competition for space and nutrients, in which the fast growing *T. harzianum* strains had the greatest impact, (2) parasitism, if mycelia of antagonist and pathogen got in contact to one another and if the antagonist was able to enzymatically degrade the pathogens' hyphae, and (3) antibiosis, if the antogonist produced some diffusible metabolite(s) that stopped the growth of the pathogen prior to direct hyphal contact or enhanced the parasitic action of *T. harzianum* by additionally weakening the pathogen.

Of these three types of interaction, competition combined with antibiosis resulted in an inhibition of the pathogens' mycelial growth. The inhibition index, calculated by comparing the inhibited growth with the potential free and uncompeted growth of the pathogens, gave a measure to find out the most effective inhibitor.

Due to its comparatively quick mycelial growth at 16°C and the antibiotic effect of its secondary metabolites against *B. cinerea* and *R. solani*, T_R was most effective against

these fungi, with only T-22 being even a little more effective against *R. solani* at 24°C because of its very quick growth. Although antibiosis is not that clear-cut in the interaction of T_S and T_U with *P. ultimum*, it is noteworthy that T_S, most probably stopping the mycelial growth of *P. ultimum* even before hyphal contact was established, was the most effective strain against this pathogen.

T_U on the other hand, although producing some metabolite(s) that affected all three pathogens, had a too slow mycelial growth especially at 16°C to effectively compete with the pathogens.

The results from the competition / inhibition study led to the following conclusion: Generally, the most effective competitors are those which occupy the available space and exploit the available nutrients the quickest and stop the growth of the competing pathogen in advance of hyphal contact by the production of antifungal metabolites.

After hyphal contact was established, most *Trichoderma* strains killed the pathogens' mycelia within five days since first hyphal contact. These results are consistent with the findings of MUKHERJEE and RAGHU (1997) and REY *et al*. (2001). The pathogen was able to survive for the given time span in a few cases, but it is very likely that a longer time period given for the antagonistic action of *T. harzianum* on the pathogens would have led to the death of the pathogens' mycelia in even more cases.

To inhibit the growth of a fungal pathogen does not necessarily mean to kill it instantly. This was demonstrated by the inability of T_R to effectively parasitize the pathogens it was confronted with. Although this strain strongly inhibited *B. cinerea* and *R. solani*, these fungi were able to grow out of the zone of interaction in more cases than after interaction with the other *Trichoderma* strains. Moreover, if no inhibition takes place, successful control of a pathogen is very unlikely, as proven by T_R in its interaction with *P. ultimum*, where the pathogen survived the interaction in all cases.

2.4.3 Production of lytic enzymes on solid substrates and in culture filtrates

Cellulases and proteases were produced by all *T. harzianum* strains, but results differed in terms of amount or activity of enzymes. T39, known to be an abundant producer of proteases (ELAD and KAPAT, 1999), showed the strongest degradation of skimmed milk. T39 also was the strongest producer of cellulases. T-22, which also showed cellulase activity, was weakest in degrading skimmed milk proteins. Interestingly, only three strains were able to degrade crab shell chitin, namely T_U, T12, and T_R. For T39, this is no surprise, as this strain is known to be a weak mycoparasite (ELAD, 1996). But for the mycoparasitic strain T-22 (HARMAN and BJÖRKMAN, 1998), this result was unexpected.

The highest overall enzymatic activity was shown by T12. This strain degraded cellulose, chitin, and proteins by 70, 50, and 77%, respectively. This is interesting in comparison to the results from the competitive and parasitic interaction studies. In the first ones, T12 never was the strongest competitor, but it performed far better than the weakest strains. Moreover, next to T_S and T_U it was the only strain to kill all pathogens' mycelia within the given time span of five days.

The inability of all *Trichoderma* strains to degrade the glucan AZCL-pachyman, was observed even a second time, when this part of the experiment was repeated. The azurine-dyed glucan seemed to be not sufficient to induce the synthesis of glucanolytic enzymes by the *T. harzianum* strains if cultivated on water agar amended with this chromogenic substrate.

The culture filtrates, produced on the basis of a medium containing glucose and cellulose, mostly showed strong cellulolytic and glucanolytic activity. No explanation could be found, why the T-22 culture filtrate completely lacked cellulase activity. As the synthesis of chitinases is repressed by glucose (VITERBO *et al.*, 2002), it is not surprising that no chitinase activity could be detected. Synthesis of proteases is repressed by primary nitrogen sources like ammonia and glutamine (OLMEDO-MONFIL *et al.*, 2002). These compounds most probably are part of the PDA medium, which is produced of glucose and a 'potato infusion'. The reason for protease activity in culture filtrates of T12, T_S, and T_U might be a decrease of the primary nitrogen level during the culture of these strains to a de-repression level that allowed protease synthesis (OLMEDO-MONFIL *et al.*, 2002). In contrast to results of THRANE (1997), glucanase synthesis was not repressed by glucose.

2.4.4 Antibiotic effects of secondary metabolites produced in liquid culture

All pathogens were inhibited by increasing concentrations of the culture filtrates of *T. harzianum*, but great differences could be observed between pathogens and the antagonist strains. The greater the growth rate of the pathogen, the stronger it was inhibited. Being able to detoxify antifungal substances, as for example *B. cinerea* can degrade 6PAP (COONEY and LAUREN, 1998), gives those fungal species an advantage towards other competing microorganisms that lack such capability. Additionally, having more time to degrade an antibiotic metabolite due to slower growth of the own mycelium, may help *B. cinerea* and *F. oxysporum* to keep up normal development of their mycelia, even if in contact with *T. harzianum* metabolites. Similar results were obtained by DENNIS and WEBSTER (1971a) and SCARSELLETTI and FAULL (1994), who showed that

F. oxysporum was less affected by *T. harzianum* metabolites than other target fungi like *R. solani*.

With regard to the amount of more than 100 known secondary metabolites with antibiotic properties produced by *Trichoderma* spp. (SIVASITHAMPARAM and GHISALBERTI, 1998), it is very unlikely that the six *T. harzianum* strains used in this study produced the same antifungal substance just in differing quantities. T_S and T_U culture filtrates for example only differed slightly with regard to their effect on all tested pathogens except *P. ultimum*. T12 and T-22 partly differed (*B. cinerea*, *R. solani*), but partly did not (*P. ultimum*, FOP).

Most probably, *Trichoderma* strains produce mixtures of substances whereby the observed antifungal effects may be the result of the whole mixture as well as it may be primarily caused by only one substance in the mixture (SIVASITHAMPARAM and GHISALBERTI, 1998). Some metabolites may exert strong effects against single pathogens (T_S vs. *P. ultimum*), while others may have general growth inhibiting properties (T_R vs. all).

T39 grew in the medium in the same way as the other strains did, but produced no or nearly no metabolites with antifungal activity, as it is known for this strain (ELAD, 1996). Significant antifungal activity of T39 metabolites as reported by VINALE *et al*. (2006) could not be confirmed. From the ineffectiveness of the T39 culture filtrate in these assays, it can be concluded that the antibiotic effect of the culture filtrates was not caused by degradation products from the nutrient solution.

One more conclusion can be drawn from the ineffectiveness of strain T39. The antibiotic effect of the culture filtrates incorporated in the PDA medium was not caused by lytic enzymes. Despite the T39 culture filtrate showed the strongest cellulose activity, it was inactive against the mycelium of *P. ultimum* growing on and within this agar.

As chitinases were not detected in any culture filtrate, the effect of the culture filtrate agars on the other four tested pathogenic fungi cannot be explained by the action of chitinases lysing the fungal cell walls. Concerning the endo-1,3-ß-glucanase, T_R was the strongest producer, which fits to the result of the culture filtrate agar tests. On the other hand, the differences between the six *T. harzianum* strains were rather low regarding the glucanolytic activity of the culture filtrates, but large concerning their antibiotic activity. Finally, it is very unlikely that extracellular enzymes which are bound into an agar matrix, can easily exert their lytic activity.

The result of T_R vs. *P. ultimum* demonstrated that secondary metabolites with antibiotic activity are not produced under all culture conditions (SIVASITHAMPARAM and GHISALBERTI, 1998). Although the T_R culture filtrate was extremely effective against *P. ultimum*, T_R was ineffective to stop the growth of the same pathogen in the competition / inhibition study.

2.4.5 Production of volatile metabolites with antibiotic activity

Volatile metabolites exhibiting antibiotic activity towards microorganisms are important if they provide the producing fungus with an advantage over his competitors (GHISALBERTI and SIVASITHAMPARAM, 1991; HUMPHRIS *et al.*, 2002). The production of volatile metabolites with fungal growth inhibiting properties was strongest within the first days of growth of *T. harzianum*. The older the cultures of *T. harzianum* got, the weaker was their effect in terms of mycelial growth inhibition of the pathogens confronted with the *Trichoderma* cultures.

It can be concluded that the mycelium produced the active metabolites within the first few days of its growth. The data do not suggest that sporulation was responsible for the production of volatile metabolites with antifungal properties, because sporulation took place mostly at the end of the first and the beginning of the second week. Only one week old mycelium of strain T_S led to significant inhibition of the pathogens' mycelial growth, although this effect was lower compared to T_S mycelium with an age of only 24 h.

Summarizing the above, it can be hypothesized that the production of volatile metabolites with antibiotic activity accounts for a part of the antagonistic power especially of strain T_S.

2.4.6 Concluding remarks

The results of the various experiments conducted for this study revealed some tremendous differences in the profiles of 'available' antagonistic mechanisms of the used *T. harzianum* strains, although they are partly recommended for the control of the same diseases. Generally, biological control against plant pathogens can be gained by competition, parasitism, or antibiosis, including every possible combination of these antagonistic mechanisms. Moreover, parasitism-related hydrolytic enzymes and antibiosis-related metabolites may act synergistically, thus reducing the necessary amount of each component to a level without antagonistic property if applied alone. Last but not least, resistance induction or interactions that only take place in the presence of antagonist, pathogen, and plant add up to the wide spectrum of actions and events that may lead to what is called 'biological control' (HARMAN, 2006).

Concerning this, it is impossible to finally evaluate a *Trichoderma* strain solely on the basis of the results from this study. But if a well characterized strain successfully controls a certain pathogen in the field or fails to control it, the profile resulting from such a characterizing study may answer the instantly appearing question: "Why?"

Tab. 2.5. Profiles of antagonistic activity of *Trichoderma harzianum* strains: Mycelial growth rate, sporulation potential, competitive and parasitic activity

	Growth rate [mm / h] at optimal temp. [°C][a]		Sporulation potential [spores / 90-mm Petri dish][b]		Inhibition index [%][c]						Parasitic activity [%][d]		
					B.c.		P.u.		R.s.				
Strain	Rate	Temp.	14 d on 1/3-PDA	14 d on PDA	16°C	24°C	16°C	24°C	16°C	24°C	B.c.	P.u.	R.s.
T12	0.77	24	$1.5 * 10^9$	$1.1 * 10^9$	53	69	32	29	56	56	100	100	100
T-22	1.03	32	$6.5 * 10^8$	$1.5 * 10^9$	53	71	26	31	58	59	69	100	100
T39	0.80	32	$1.5 * 10^9$	$2.8 * 10^9$	49	71	19	19	48	54	94	100	81
T_R	0.72	28	$3.7 * 10^8$	$5.5 * 10^8$	64	76	16	19	64	57	56	0	75
T_S	0.83	28	$1.3 * 10^9$	$1.7 * 10^8$	54	69	36	33	46	56	100	100	100
T_U	0.50	32	$4.3 * 10^8$	$1.4 * 10^9$	46	58	16	31	46	52	100	100	100

[a] Mycelial growth rates of the *T. harzianum* strains at the strain-specific optimal growth temperature; data taken from Tab. 2.1.

[b] Spore numbers produced by *T. harzianum* mycelia after cultivation for 14 days at 24°C in 90-mm Petri dishes on 1/3-PDA and PDA medium; data taken from Tab. 2.2.

[c] Radial growth inhibition caused by the *T. harzianum* strains against *Botrytis cinerea* (B.c.) after 120 h, *Pythium ultimum* (P.u.) after 48 h, and *Rhizoctonia solani* (R.s.) after 120 and 96 h of co-culture at the two temperature levels of 16 and 24°C, respectively; data taken from Tab. 2.3.

[d] Parasitism of the *T. harzianum* strains on *B. cinerea* (B.c.), *P. ultimum* (P.u.), and *R. solani* (R.s.). Parasitic activity values were calculated as the percentage of successful killings of the target pathogen by *T. harzianum* in 16 cases.

Tab. 2.6. Profiles of antagonistic activity of *Trichoderma harzianum* strains: Lytic enzyme activity and production of secondary metabolites with antibiotic activity

	Lytic enzyme activity on solid medium [%][a]				Lytic enzyme activity in culture filtrates [%][b]				Mycelial growth inhibition by antibiotic metabolites secreted into PDB medium [%][c]					Mycelial growth inhibition by volatile metabolites [%][d]		
Strain	CE	GL	CH	PR	CE	GL	CH	PR	B.c.	P.u.	R.s.	FOL	FOP	B.c.	P.u.	R.s.
T12	70	0	50	77	80	55	0	yes	23	89	45	6	13	43	12	16
T-22	85	0	0	19	0	70	0	no	38	89	52	8	13	54	17	24
T39	100	0	0	86	100	60	0	no	7	2	3	2	3	40	2	7
T_R	55	0	15	41	80	90	0	no	50	95	52	14	29	47	29	17
T_S	20	0	0	59	55	65	0	yes	5	69	25	9	1	55	25	35
T_U	45	0	70	81	60	80	0	yes	8	7	32	6	7	22	5	0

[a] Lytic activity of cellulase (CE), endo-1,3-ß-glucanase (GL), chitinases (CH), and proteases (PR) produced by *T. harzianum* strains on solid agar media measured by visual estimation of enzyme-specific substrate degredation.

[b] Lytic activity of cellulase (CE), endo-1,3-ß-glucanase (GL), chitinases (CH), and proteases (PR) produced by *T. harzianum* strains in liquid culture measured by visual estimation of enzyme-specific substrate degredation.

[c] Effect of antibiotic metabolites secreted by the *T. harzianum* strains into PDB medium on the mycelial growth of *Botrytis cinerea* (R.s.), *Pythium ultimum* (P.u.), *Rhizoctonia solani* (R.s.), *Fusarium oxysporum* f. sp. *lycopersici* (FOL), and *Fusarium oxysporum* f. sp. *phaseoli* (FOP) if cultivated on PDA amended with increasing concentrations of the *T. harzianum* culture filtrates. Values representing the reduction of the phytopathogens' unaffected AUIGC of 2500%*% were calculated on the basis of AUIGC data from Tab. 2.4.

[d] Effect of volatile metabolites of the *T. harzianum* strains produced on solid PDA on the mycelial growth of *Botrytis cinerea* (R.s.), *Pythium ultimum* (P.u.), and *Rhizoctonia solani* (R.s.), if cultivated in the presence of 24 h old mycelia of the *T. harzianum* strains. Values representing the reduction of the phytopathogens' AUGPC were calculated on the basis of AUGPC data from Fig. 2.8.

3. *In Vitro* Assays on the Control of the Bean Rust Fungus *Uromyces appendiculatus* by Means of Spore Suspensions and Culture Filtrates of *Trichoderma harzianum*

3.1 Introduction

Several species of the fungal genus *Trichoderma* are well-known antagonists of other fungi (HJELJORD and TRONSMO, 1998). First observations of such interactions were done by WEINDLING (1932, 1934), who reported mycoparasitism and antibiosis, two major mechanisms involved in the antagonism of *Trichoderma* spp. Today, many of the events taking place during antagonistic relationships between *Trichoderma* strains and fungal target organisms are known: competition for space and nutrients (ELAD, 1996; SIVAN and CHET, 1989) or for plant exudates (HOWELL, 2002), chemotactic hyphal branching of the antagonist towards the target fungus (LU *et al*., 2004), attachment via a lectin-mediated recognition mechanism (INBAR and CHET, 1992), hyphal coiling around the target's hyphae and mycoparasitic activity (BENHAMOU and CHET, 1993), production of lytic enzymes (KREDICS *et al*., 2005; MARKOVICH and KONONOVA, 2003; VITERBO *et al*., 2002) as well as secondary metabolites with antibiotic properties (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al*., 2005) and the synergism between these classes of molecules (LORITO *et al*., 1996; SCHIRMBÖCK *et al*., 1994). Moreover, *Trichoderma* strains may induce systemic resistance in plants by activating the plants own defense mechanisms against potential attacks from plant pathogens including fungi, bacteria and viruses (HARMAN *et al*., 2004). Hence, several *Trichoderma* strains are known as feasible biological control agents (BCAs) (HARMAN and BJÖRKMAN, 1998; MONTE, 2001).

Because *Trichoderma* species are soil-borne organisms with a need of moisture to colonize any habitat, most part of scientific research done during the last 30 years in terms of biological plant disease control concerned their use as antagonists of soil-borne pathogens (HJELJORD and TRONSMO, 1998). Nonetheless it was shown, that *Trichoderma* spp. are able to act as BCAs against foliar pathogens as well. The diverse antagonistic mechanisms facilitate the control of pathogens attacking upper parts of the plant like stem (O'NEILL *et al*., 1996), leaves (ELAD, 2000a), blossoms (ESCANDE *et al*., 2002; TRONSMO and YSTAAS, 1980), and fruits (HARMAN *et al*., 1996). Successful

utilization of a specific antagonistic mechanism is thereby strongly dependent of the biology and pathogenicity of the target fungus.

Botrytis cinerea, for example, is a necrotrophic fungus that synthesizes enzymes with lytic activity on components of the plant cell wall and outer layers of the leaf like pectolytic enzymes and cutinase. Moreover, its spores need nutrients available on the leaf surface to germinate (ELAD, 1996). *T. harzianum* strain T39 was very effective in reducing the germination of *B. cinerea* spores by competition regarding the availability of nutrients and in reducing the pathogen's infectiousness by producing proteases, that lyzed the pathogenicity enzymes produced by *B. cinerea* (ELAD and KAPAT, 1999; KAPAT *et al.*, 1998). *B. cinerea* spore germination was also inhibited by means of chitinolytic (LORITO *et al.*, 1993) and glucanolytic (LORITO *et al.*, 1994) enzymes isolated from *T. harzianum* strain P1.

Fungi, that produce an abundant mycelium on the surface of plant organs like powdery mildews (BRADATSCH, 2006; ELAD *et al.*, 1998), *Botryodiplodia theobromae* (GUPTA *et al.*, 1999), or *Crinipellis perniciosa* (SANOGO *et al.*, 2002), were antagonized by *Trichoderma* spp. through parasitism. Moreover, inhibition of the pathogen's development was explained by *Trichoderma*-mediated induction of plant resistance (ELAD *et al.*, 1998).

Rust fungi only produce a single germination hypha on the leaf surface, which penetrates through a stoma and gives rise to an intercellular mycelium within the leaf. Therefore, rust infection is more easily controlled by antibiosis than by parasitism or competition (ANDREWS, 1992). According to this, the antagonism of *Trichoderma* spp. against rust fungi other than bean rust was explained by an antifungal effect of secondary metabolites produced by the *Trichoderma* strain resulting in inhibition of rust spore germination or germ tube elongation (GOVINDASAMY and BALASUBRAMANIAN, 1989). Such effects were reported for rust spores confronted with living *Trichoderma* propagules (GOVINDASAMY and BALASUBRAMANIAN, 1989; KAPOORIA and SINHA, 1969; SALLAM, 2001), the sterile supernatant of germinated spore suspensions (GOVINDASAMY and BALASUBRAMANIAN, 1989; SINHA and BAHADUR, 1974), and filtrates of 15-days-old liquid cultures (ZADE *et al.*, 2005).

Dispersal of rust spores could also be reduced by *Trichoderma* spp. growing over rust pustules or parasitizing rust spores. But this interaction was described less often (LEVINE *et al.*, 1936; TOSI and ZAZZERINI, 1994) as well as the inability of rust hyphae to penetrate the leaf surface through stomata due to a competitive interaction with fungal structures of *Trichoderma* sp. present on the leaf surface (SALLAM, 2001).

The bean rust fungus *Uromyces appendiculatus* is of worldwide importance as a yield-reducing disease of *Phaseolus vulgaris* L., potentially causing yield losses up to 50% (BERGER *et al.*, 1995; DE JESUS JUNIOR *et al.*, 2001; VENETTE and JONES, 1982). Heavy

epidemics occur especially in the tropics and subtropics, because of the climatic conditions favouring the spread and infectiousness of *U. appendiculatus* (STAVELY, 1991). Control of the bean rust fungus is achieved by application of several disease management measures like cultural practices, cultivation of rust-resistant varieties, and the use of protectant and systemic fungicides (MCMILLAN *et al*., 2003). Efficacy levels of fungicides in terms of reducing rust disease severity reach over 90% (GENT *et al*., 2001; STUMP *et al*., 2000).

In a few cases, the possibility of controlling the bean rust disease by fungal or bacterial antagonists was scientifically investigated throughout the last 25 years (BAKER *et al*., 1983, 1985; GRABSKI and MENDGEN, 1985, 1986; SAKSIRIRAT and HOPPE, 1990; YUEN *et al*., 2001). Bacterial antagonists were successful due to the production of antibiotic metabolites that negatively affected rust spore germination (BAKER *et al*., 1983, 1985; YUEN *et al*., 2001), while the antagonistic fungus *Verticillium lecanii* grew parasitically on bean rust uredia and uredospores (GRABSKI and MENDGEN, 1985, 1986; SAKSIRIRAT and HOPPE, 1990). To the author's knowledge, no information on the control of *U. appendiculatus* by means of *Trichoderma* spp. is available.

In this study the ability of *T. harzianum* to control the bean rust fungus in leaf disc assays was investigated to answer the question by which antagonistic mechanism such control is achieved.

3.2 Material and methods

3.2.1 Fungal strains

Six *T. harzianum* strains were used in this study, five of them isolated from commercial preparations: T-22 from TRIANUM-P (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands), T39 from TRICHODEX (Makhteshim-Agan Ltd., Tel Aviv, Israel), and the strains from TRI 003 (Plantsupport, Grootebroek, The Netherlands), TRICHOSAN (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), and UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand). The abbreviations T_R, T_S, and T_U will be used throughout this work for the latter three strains, respectively. The non-commercial strain T12 from the fungal collection of the Institute of Plant Diseases and Plant Protection (IPP; Leibniz Universität Hannover, Germany) was originally obtained as strain T000 from the Institute of Phytopathology and Applied Zoology (IPAZ; Justus-Liebig-University Gießen, Germany).

Uredospores of the bean rust fungus *U. appendiculatus* were taken from the fungal collection of the IPP.

3.2.2 General culture conditions

All *Trichoderma* strains were maintained on PDA (Merck KGaA, Darmstadt, Germany) at 24°C in Petri dishes with a diameter of 90 mm, which were filled with 10 ml PDA and singly sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA).

U. appendiculatus was maintained by inoculating bean plants, harvesting freshly produced uredospores, and storing them at -20°C. Uredospores taken from the freezer and used in the experiments were usually not older than 4 to 8 weeks.

3.2.3 Production of **Trichoderma harzianum** *spore suspensions and culture filtrates*

Spore suspensions were produced by scraping off sporulating mycelium from PDA cultures and suspending it in sterile A. dest. To remove mycelial fragments from the suspensions, they were filtered through 595 Schleicher & Schuell filter paper (Whatman International Ltd., Kent, England). Spore suspensions were adjusted to the desired concentration.

Culture filtrates were produced by cultivation of the six *T. harzianum* strains for ten days in PDB (Becton Dickinson GmbH, Heidelberg, Germany). For each strain, 250 ml PDB were initially inoculated with five mycelial plugs with a diameter of one cm. Incubation took place at room temperature (22°C) on a horizontal shaker at 85 rpm. To

get rid of the major parts of the mycelium, liquid cultures were filtered through 595 Schleicher & Schuell filter paper. Afterwards, cultures were sterile filtrated through Schleicher & Schuell filters with pore sizes of 0.8 µm and 0.2 µm.

3.2.4 Leaf disc assays

Bean plants of cultivar 'Maja' (Hild Samen GmbH, Marbach, Germany) were sown in seed trays and cultivated for ten to twelve days in the greenhouse until primary leaves had fairly developed. At the time of harvest of primary leaves, trifoliate leaves had just started to grow. Leaf discs with a diameter of 20 mm were cut out of harvested primary leaves with a cork borer. Two leaf discs per 60-mm Petri dish were positioned upside down on 1% water agar.

In all leaf disc assays, each treatment consisted of ten Petri dishes and application of sterile A. dest. served as the control treatment. Leaf discs were inoculated with rust spore suspensions with concentrations of 1 x 10^4 spores / ml a defined period of time after they had been treated with *T. harzianum* spore suspensions or culture filtrates. Rust spore suspensions were amended with few droplets of Tween 20 for better distribution of the spores within the suspension.

Six different kinds of assays were carried out. Generally, *Trichoderma* treatments were applied to leaf discs directly after they had been placed in Petri dishes. Before and after inoculation with bean rust spores, Petri dishes were cultivated at 24°C and 85% rH with a light period of 16 h in a climate cabinet. All spore suspensions, culture filtrates, and control treatments were applied to the leaf discs using 25-ml pump spray bottles. Spraying one time was sufficient to apply the spore suspensions to the whole surface of a leaf disc which had a size of 3.14 cm². By spraying one time, circa 100 µl liquid were applied.

Disease severity was assessed with a binocular by counting uredial rust pustules that had developed ten to twelve days after rust inoculation. Mean rust pustule numbers per Petri dish were calculated.

3.2.4.1 Assay on the effect of spore suspensions of six Trichoderma harzianum strains on bean rust severity

Spore suspensions of all *T. harzianum* strains with a concentration of 5 x 10^6 spores / ml were applied to leaf discs. Inoculation with a bean rust spore suspension took place four days after inoculation with *T. harzianum*. This assays was repeated two times. Both results are shown.

3.2.4.2 Assay on the effect of spore suspensions with increasing concentrations of two Trichoderma harzianum strains on bean rust severity

Spore suspensions of *T. harzianum* strains T12 and T_U with concentrations of 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6, and 5 x 10^7 spores / ml were applied to leaf discs. Inoculation with a bean rust spore suspension took place four days after inoculation with *T. harzianum*.

3.2.4.3 Assay on the effect of suspensions of alive or autoclaved spores with increasing concentrations on bean rust severity

Suspensions of alive and autoclaved spores of *T. harzianum* strains T12 and T_U with concentrations of 5 x 10^5, 5 x 10^6, and 5 x 10^7 spores / ml were applied to leaf discs. Inoculation with a bean rust spore suspension took place four days after inoculation with *T. harzianum*.

3.2.4.4 Assay on the effect of co-inoculation of bean rust and Trichoderma harzianum spore suspensions or spore suspension supernatants on bean rust severity

Spore suspensions of *T. harzianum* strains T12 and T_U with a concentration of 5 x 10^6 spores / ml were applied to leaf discs four days prior to rust inoculation. Additionally, a mixed suspension of *T. harzianum* and rust spores with concentrations of 5 x 10^6 and 1 x 10^4 spores / ml, respectively, was applied at the same day (day zero).

These procedures were carried out a second time with supernatants of *T. harzianum* spore suspensions after *Trichoderma* spores had been removed from the suspensions by centrifugation at 10,000 rpm for five minutes.

3.2.4.5 Assay on the effect of culture filtrates with increasing concentrations of two Trichoderma harzianum strains on bean rust severity

Culture filtrates of T12 and T_U were diluted to 10, 25, and 50%. Filtrates with these concentrations, the undiluted culture filtrates (100%), and sterile A. dest. that served as

the control treatment were applied to leaf discs and allowed to dry for one to two hours. Inoculation with rust spores took place 24 h later.

3.2.4.6 Assay on the effect of protective and curative treatment with culture filtrates of two Trichoderma harzianum *strains on bean rust severity*

Undiluted culture filtrates of T12 and T_U, and sterile A. dest. that served as the control treatment were applied to leaf discs 24 h before or 24 h after bean rust inoculation. In both cases, leaf disc surfaces were allowed to dry for one to two hours directly after treatment application.

3.2.5 Re-isolation of Trichoderma harzianum *colony forming units (cfu) from bean leaf discs*

Single leaf discs with a size of 3 x 3 cm were cut out of primary bean leaves and put onto water agar in 60-mm Petri dishes. Spore suspensions of all *T. harzianum* strains with a concentration of 5 x 10^6 spores / ml and sterile A. dest. as control treatment were applied directly after leaf discs had been prepared. Each treatment consisted of 40 leaf discs. Five leaf discs of each treatment were cultivated for 2, 4, 7, 10, 14, 17, and 21 days before re-isolation of *T. harzianum* was carried out. From five leaf discs per treatment, *T. harzianum* cfus were re-isolated directly after spore suspensions had been applied.

Re-isolations were done by mechanically grinding the leaf material in 10 ml of sterile A. dest. This suspension was diluted 250-fold, and 100 µl of the resulting suspension were spread onto *Trichoderma*-selective medium (ELAD *et al*., 1981). Developing cfu were counted.

3.2.6 Germination tests

Sterile culture filtrates of all *T. harzianum* strains were incorporated into 1% water agar gaining culture filtrate agar with concentrations of 1, 3, 5, 10, 15, and 25%. Moreover, culture filtrates of T12 and T_U were incorporated into 1% water agar with concentrations of 5 and 1%, respectively, after filtrates had been heated up to 40, 60, 80, or 100°C for 10 minutes, or had been autoclaved at 121°C. This agar was poured into 60-mm Petri dishes. Sterile A. dest., added to 1% water agar at the same concentrations like the culture filtrates served as the control.

Per Petri dish, 50 µl of a bean rust spore suspension with a concentration of 5 x 10^4 spores / ml were spread over the culture filtrate agar and allowed to germinate for 24 h at 24°C. Afterwards, the percentage of germinated spores was visually determined

for 100 spores by use of a light microscope. Germ tube length was calculated as the mean length of ten measured germ tubes per Petri dish. Each treatment consisted of four replications.

The inhibition of germination and subsequent germ tube growth by *T. harzianum* culture filtrates was observed microscopically by putting autoclaved cellophane foil (Alba Einmachhaut, Gehring & Neiweiser GmbH + Co. KG, Bielefeld, Germany) onto water agar amended with 3, 5, and 10% culture filtrate of strains T12 and T_U and applying the bean rust spore suspension onto this cellophane membrane. After 24 h, pieces were cut out of the cellophane and transferred to object slides. Microscopical examination by interference microscopy and photographic documentation were done with an Axiophot photomicroscope (Carl Zeiss MicroImaging GmbH, Göttingen, Deutschland).

3.2.7 Statistical analysis

The variables measured in this study were (1) number of rust pustules per leaf disc, (2) the number of germinated rust spores, and (3) the length of the rust spore germ tubes. Percent reduction in the number of rust pustules and percent reduction in the number of germinated rust spores were computed. Moreover, the impact of increasing *T. harzianum* culture filtrate concentrations of the different *T. harzianum* strains on rust spore germination and germ tube elongation was assessed by computing areas under the germination curves (AUGC) and areas under the germ tube growth curves (AUGGC).

Because of the high variation of germination and germ tube growth data (as well as AUGC and AUGGC data) between culture filtrate treatments and control, which occurred in the germination tests, those data were subjected to natural logarithm transformation before they were analysed statistically.

All analyses were performed using procedures in SAS version 8.02 (Statistical Analysis Systems Institute, Cary, NC, USA). Analyses of variance were performed for most experimental data with two exceptions: (1) Analysis of covariance was applied to the data from the assay on the effect of increasing culture filtrate concentrations on bean rust severity and (2) *T. harzianum* cfu re-isolation data were analysed using weighted linear regressions.

Mean separations were done by Bonferroni-adjusted t-tests or by statistical methods for multiple comparisons like Duncan's multiple range tests or contrast tests. Testing methods are specified where results are shown.

In all figures and tables, the variability is given by the standard error.

3.3 Results

3.3.1 Leaf disc assays

Six different assays were performed on leaf discs which had been cut out of primary bean leafs and transferred into Petri dishes filled with water agar. The aim of these assays was to assess the effect of spore suspensions and culture filtrates of all six *T. harzianum* strains or of strains selected because of their superior efficacy.

3.3.1.1 Effect of spore suspensions of six Trichoderma harzianum *strains on bean rust severity*

Application of *T. harzianum* spore suspensions to leaf discs prior to inoculation with bean rust spores led to significant decreases in the number of developing uredial rust pustules depending on the *T. harzianum* isolates (Tab. 3.1).

Tab. 3.1. Effect of *Trichoderma harzianum* spore suspensions applied four days in advance of bean rust inoculation on the number of developing uredial rust pustules on leaf discs

	Uredial pustules per leaf			
	Experiment 1		Experiment 2	
Treatment	Number [mean]	Control [%][a]	Number [mean]	Control [%]
T12	30.8 ± 2.0 ab[b]	48.3	35.1 ± 3.8 ab	41.9
T-22	58.6 ± 5.3 d	1.5	62.0 ± 3.6 d	0.0
T39	44.5 ± 4.8 c	25.2	43.1 ± 4.8 bc	28.7
T_R	58.9 ± 5.9 d	1.0	56.5 ± 5.0 d	6.5
T_S	43.1 ± 4.7 bc	27.6	44.8 ± 4.0 c	25.9
T_U	29.3 ± 3.3 a	50.8	25.3 ± 2.8 a	58.2
A. dest.	59.5 ± 4.5 d	---	60.4 ± 3.1 d	---

[a] Percent reduction in number of uredial pustules as compared to the number on A. dest. treated leaf discs.
[b] Figures within one column followed by the same letter do not differ significantly according to Duncan's multiple range test, p=0.05.

Especially isolate T_U reduced the mean number of pustules per leaf disc about 51 and 58%, followed by T12 with 48 and 42% rust pustule reduction achieved in two independent experiments. Efficacy of T39 and T_S reached control values between 25 to 29%, while T-22 and T_R had no effect on bean rust infection.

3.3.1.2 Effect of spore suspensions with increasing concentrations of two **Trichoderma harzianum** *strains on bean rust severity*

Increasing the concentration of the most effective isolates T12 and T_U from 5 x 10^3 to 5 x 10^7 spores / ml led to decreasing rust pustule numbers per leaf disc (Fig. 3.1). The falling functions y = A – exp (r * x) with A = 51.2 and r = 0.45 for T12 and y = A * exp(–r * x) with A = 50.0 and r = 0.13 for T_U were fitted to the mean pustule numbers at each *Trichoderma* concentration. In these functions, x = logarithmic spore concentration (+1) and y = mean number of uredial rust pustules per leaf disc. Resulting R^2-values for the models of the effect of increasing T12 and T_U spore concentrations on bean rust infection were R^2 = 98.8% and R^2 = 98.7%, respectively.

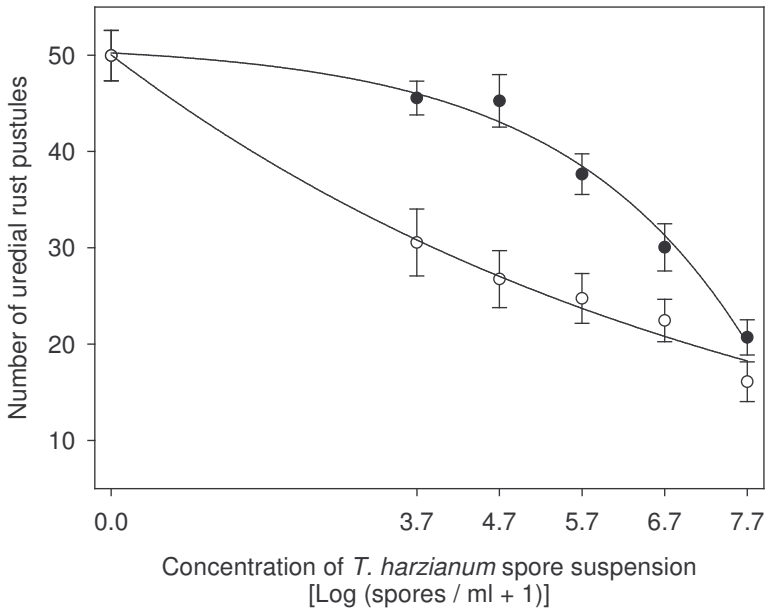


Fig. 3.1. Effect of increasing concentrations of applied *Trichoderma harzianum* spore suspensions of T12 (●) and T_U (○) on the number of developing uredial rust pustules per leaf disc after inoculation with bean rust.

The functions reflect the greater effect of T_U on rust infection over the complete range of applied *Trichoderma* spore concentrations. While the mean pustule number is reduced only by higher T12 concentrations, the curve displaying the effect of increasing T_U concentrations is exponentially falling. This demonstrates that T_U showed higher efficacy than T12 especially at the lower concentrations of 5 x 10^3 and 5 x 10^4 spores / ml.

Contrast tests statistically proved that T_U did reduce the number of rust pustules significantly even at the lowest concentration of 5 x 10^3 spores / ml, while 5 x 10^5 spores of T12 had to be applied to significantly reduce bean rust infection on leaf discs. These results were consistent, when experiments were repeated (data not shown).

3.3.1.3 Effect of suspensions of alive or autoclaved spores with increasing concentrations on bean rust severity

Autoclaving *Trichoderma* spores of T12 did not eliminate their effect on rust infection (Fig. 3.2). Although slightly higher, the percent reduction of uredial pustules by living T12 spores was not significantly different from autoclaved spores. Autoclaving spores of T_U eliminated their ability to reduce rust spore infections. Only application of the highest concentration of 5×10^7 spores per millilitre autoclaved *Trichoderma* spore suspension was sufficient to control the infection with *U. appendiculatus* by 19%.

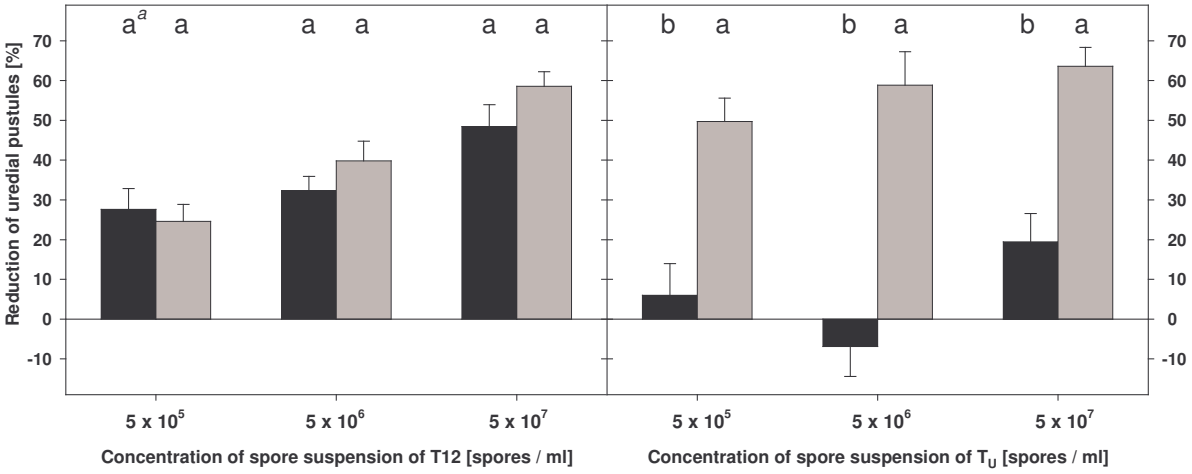


Fig. 3.2. Effect of increasing concentrations of autoclaved (■) and living (▭) *Trichoderma harzianum* spores of T12 and T_U applied to leaf discs on the number of developing uredial rust pustules per leaf disc after inoculation with bean rust. [a] The paired bars, marked by the same letter do not differ significantly according to pairwise Bonferroni-adjusted t-tests.

3.3.1.4 Effect of co-inoculation of bean rust and Trichoderma harzianum *spore suspensions or spore suspension supernatants on bean rust severity*

If *Trichoderma* spores were co-inoculated in a combined treatment with bean rust spores, a marked decrease in bean rust control could be observed for both isolates T12 and T_U in comparison to the standard *Trichoderma* treatment four days prior to rust inoculation (Tab. 3.2). Nevertheless, spores of T12 and T_U applied in combination with *U. appendiculatus* inoculation were capable of controlling bean rust infection by 17.7 and 14.8%, respectively. Application of supernatants of the spore suspensions after removal of the *Trichoderma* spores by centrifugation led to a significant loss of efficacy of the *Trichoderma* treatments. Only the T_U supernatant showed a minor reducing effect on bean rust infection.

Tab. 3.2. Effect of *Trichoderma harzianum* spore suspensions and spore suspension supernatants of strains T12 and T_U applied in combination with bean rust spores or four days in advance of bean rust inoculation on the number of developing uredial rust pustules on leaf discs

	Uredial pustules per leaf			
	Co-Inoculation d0[a]		Inoculation d4	
Treatment	Number [mean]	Control [%][b]	Number [mean]	Control [%]
T12 suspension	49.7 ± 2.1 a[c]	17.7	33.4 ± 2.7 a	39.5
T12 supernatant	60.2 ± 2.0 b	0.3	51.8 ± 3.3 b	6.3
T_U suspension	51.5 ± 3.5 a	14.8	26.7 ± 2.4 a	51.8
T_U supernatant	54.9 ± 3.7 ab	9.1	50.5 ± 3.6 b	8.9
A. dest.	60.4 ± 1.9 b	---	55.3 ± 2.5 b	---

[a] Leaf discs were inoculated with bean rust by co-inoculation on the same day ‘d0’ or 4 days after treatment ‘d4’.
[b] Percent reduction in number of pustules as compared to the number on A. dest. treated leaf discs.
[c] Figures within one column followed by the same letter do not differ significantly according to Duncan’s multiple range test, p=0.05.

3.3.1.5 Effect of culture filtrates with increasing concentrations of two **Trichoderma harzianum** *strains on bean rust severity*

Application of T12 and T_U culture filtrates prior to bean rust inoculation reduced the number of rust pustules that developed per leaf disc after bean rust inoculation. This inhibition of infection increased with increasing culture filtrate concentration (Fig. 3.3).

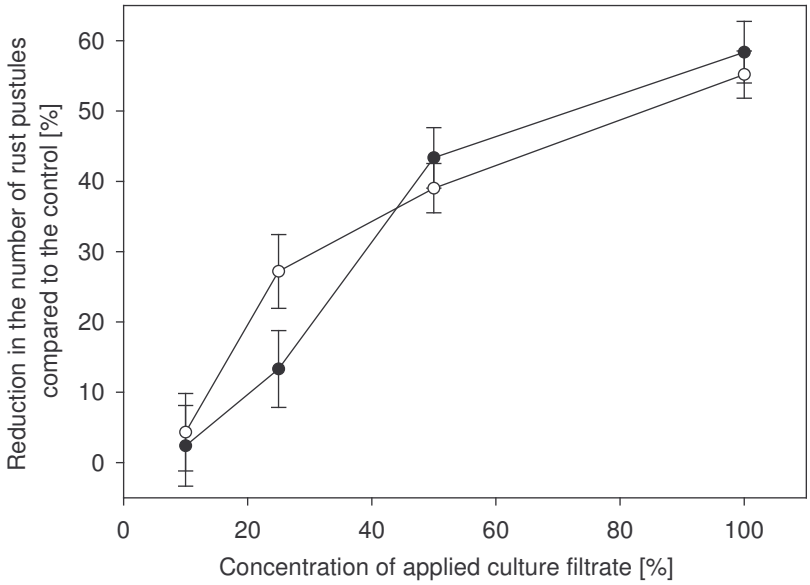


Fig. 3.3. Effect of increasing *Trichoderma harzianum* culture filtrates concentrations of strains T12 (●) and T_U (○) on the number of developing uredial rust pustules on leaf discs when applied one day before inoculation with bean rust.

An analysis of covariance was performed and revealed that there was no significant difference between T12 and T_U, if the whole range of applied culture filtrate concentrations of 10 to 100% was taken into account. By contrast tests, it was statistically demonstrated that a concentration of 50% of the T12 culture filtrate was needed to significantly reduce the number of bean rust pustules, while 25% of the T_U culture filtrate were enough in terms of significantly reducing the number of rust pustules per leaf disc.

3.3.1.6 *Effect of protective and curative treatment with culture filtrates of two* **Trichoderma harzianum** *strains on bean rust severity*

The protective application of undiluted culture filtrates of *T. harzianum* strains T12 and T_U 24 h before inoculation with *U. appendiculatus* reduced the number of rust pustules that developed per leaf disc to the same extent as in the previous experiment on increasing *T. harzianum* culture filtrate concentrations with control values of 55 to 60%. The numbers of rust pustules were significantly lower compared to the numbers that developed after curative culture filtrate treatment and compared with both control treatments (Tab. 3.3).

Tab. 3.3. Effect of *Trichoderma harzianum* culture filtrates applied as protective or curative treatment 24 h before or after bean rust inoculation on the number of developing uredial rust pustules on leaf discs

Treatment		Number [mean]	Control [%][a]
protective	T12	28.7 ± 2.9 a[b]	60.3
	T_U	32.2 ± 3.0 a	55.4
	A. dest.	72.2 ± 3.2 b	---
curative	T12	69.0 ± 2.9 b	12.1
	T_U	73.5 ± 2.6 b	6.3
	A. dest.	78.4 ± 2.0 b	---

[a] Percent reduction in number of uredial pustules as compared to the number on leaf discs from the corresponding 'protective' or 'curative' control treatment.
[b] Numbers followed by the same letter do not differ significantly according to Tukey's all-pair comparisons.

Curative treatment of bean leaf discs with T12 and T_U culture filtrates did not have any effect on the numbers of rust pustules compared to the 'protective' control treatment. A minor, but insignificant reduction of rust pustules compared to the 'curative' control treatment, which resulted in the highest rust pustule numbers, could be noticed.

3.3.2 Re-isolation of Trichoderma harzianum *from leaf disc surfaces*

At the dilution level used in this experiment, no colony forming units (cfu) of *Trichoderma* spp. were isolated from control leaf discs. Re-isolation of *T. harzianum* strains revealed rather similar population dynamics on the surface of bean leaf discs of strains T12, T_R, and T_S (Fig. 3.4a, 3.4d, and 3.4e). Following a lag period of four days with minor decreases or increases in cfu numbers, population density increased between days four and seven, reaching a first peak after one week at 3×10^5 to 3.5×10^5 cfu per leaf disc. Thereafter, population density declined within the second week, but increased again to a maximum of 4×10^5 to 4.5×10^5 cfu per leaf disc within the third week.

The population of strain T-22 developed in a different manner, increasing steadily with an initial peak in population density two to three days earlier than T12, T_R, and T_S (Fig. 3.4b).

Population dynamics of T39 and T_U showed a slower development (Fig. 3.4c and 3.4f). During the first 10 to 14 days after *Trichoderma* spore application, population densities of both strains declined. Thereafter, a small increase in the number of cfu re-isolated from leaf disc surfaces was observed, but cfu numbers stayed even lower than the number of spores initially applied. Finally, population density decreased again. No second peak was observed within the third week.

Linear regressions weighted with the reciprocal variance highlight the overall trends in population dynamics. Increases or decreases in population densities of T12, T_R, and T_S were not statistically significant, while the decreases of T39 and T_U, and the distinct increase in population density of T-22 were significant with p-values of $p < 0.001$ for all three strains.

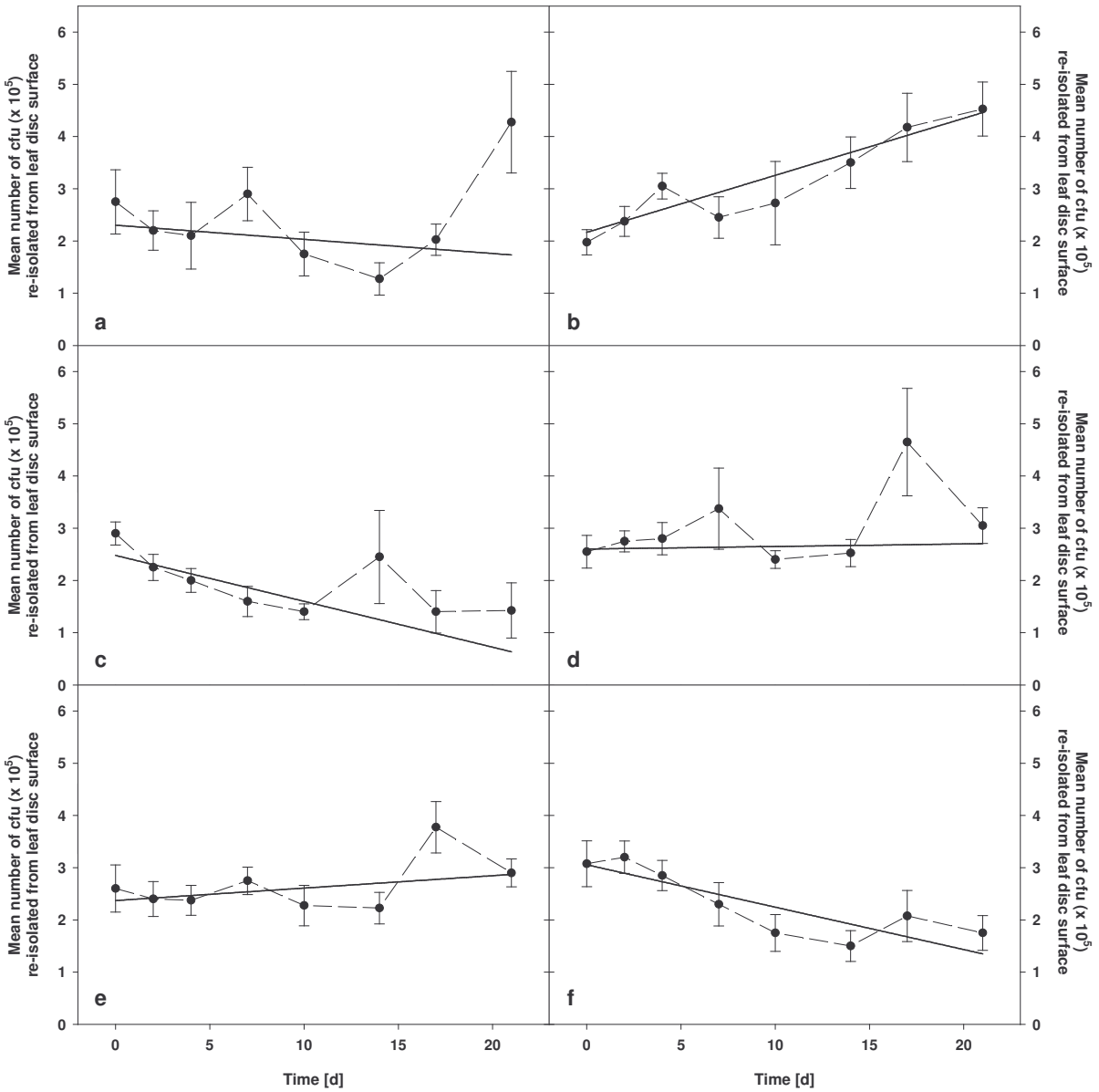


Fig. 3.4. Population dynamics of *Trichoderma harzianum* strains T12 (a), T-22 (b), T39 (c), T_R (d), T_S (e), and T_U (f) during the first three weeks after inoculation of bean leaf discs with *Trichoderma harzianum* spore suspensions. Line plots (- - -) and weighted linear regression curves (——) are shown.

3.3.3 Germination tests

If bean rust spores were spread on water agar, 80 to 90% had germinated 24 h later and had developed germ tubes with a mean length of about 850 µm. If rust spore germination took place on water agar amended with low concentrations of culture filtrates of *T. harzianum* strains T12 and T_U, pronounced reductions in the numbers of germinated rust spores as well as a very strong decrease in germ tube growth were observed microscopically (Fig. 3.5).

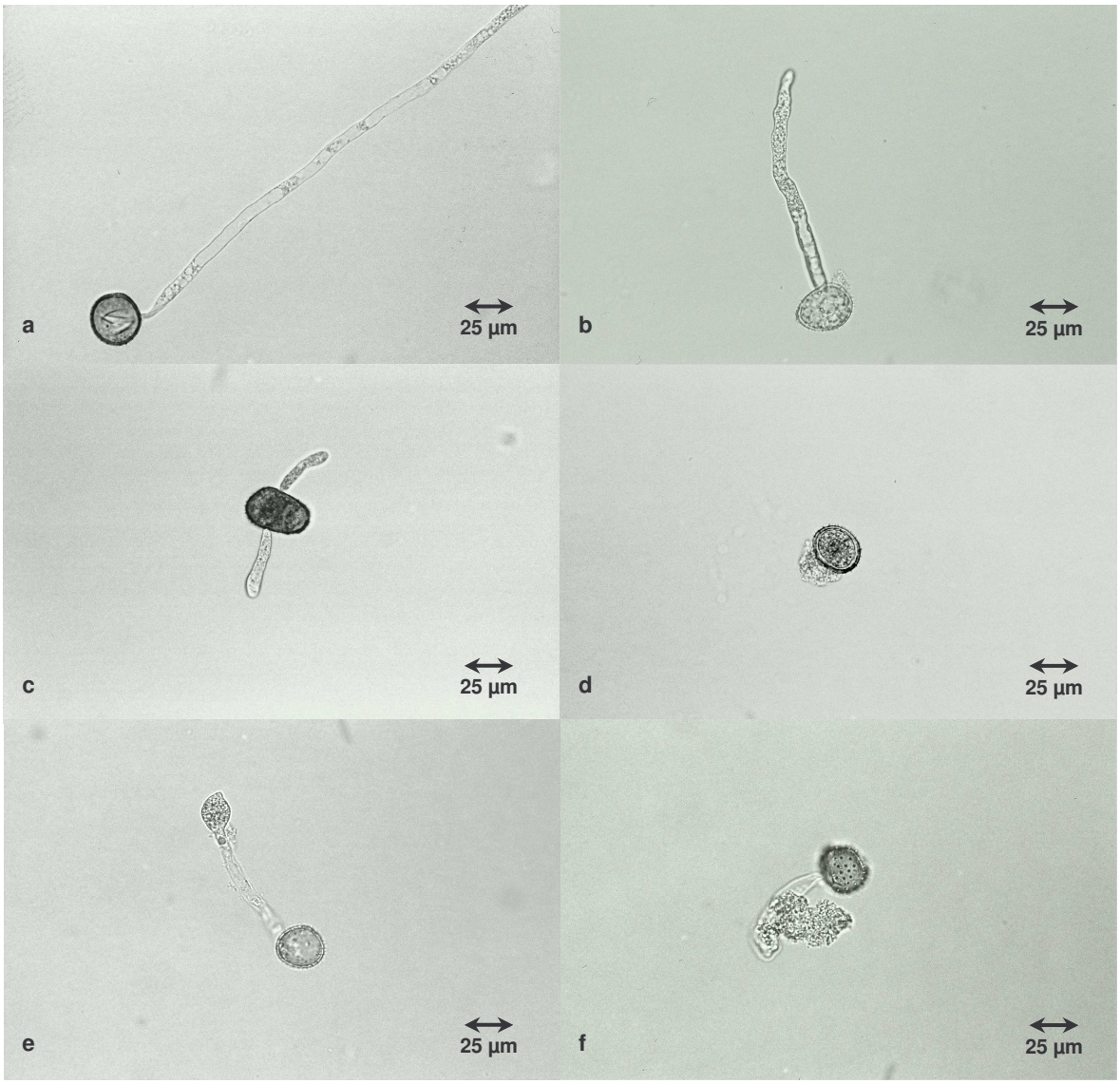


Fig. 3.5. Effect of *Trichoderma harzianum* culture filtrates on bean rust spore germination and germ tube growth after 24 h. Uredospores germinated on water agar (a), on water agar amended with 3% (b), 5% (c), and 10% (d) of T12 culture filtrate, or with 3% (e) and 5% (f) of T_U culture filtrate.

Fig. 3.5a shows the growth of a bean rust germ tube on water agar after 24 h, whereas Fig. 3.5b to 3.5d as well as Fig. 3.5e and 3.5f exemplify the effects of adding culture filtrates of *T. harzianum* strains T12 and T_U, respectively, to the water agar. Germ tubes were shortened and the protoplasm was granulated (Fig. 3.5b). The bursting of germ tubes could be observed (Fig. 3.5f) as well as the leakage of protoplasm directly from the germinating spore (Fig. 3.5d).

Next to these general antibiotic effects, the culture filtrates of T12 and T_U influenced the process of germination in individual ways: Spores partly produced double germ tubes if confronted with the T12 culture filtrate (Fig. 3.5c) and some germ tubes developing on water agar amended with the T_U culture filtrate differentiated into appressoria-like structures (Fig. 3.5e) (HEATH, 2007; WYNN, 1976). Both observations were never made with uredospores germinating on pure water agar.

The numbers of germinating uredospores (Fig. 3.6a) and the length of germ tubes (Fig. 3.6b) decreased with increasing concentrations of *T. harzianum* culture filtrates. The T_U culture filtrate was most effective, allowing only 22% germination of uredospores if 1% culture filtrate was added to the water agar. The culture filtrates of T12, T-22, T39, and T_S differed only slightly from each other, but the T_R filtrate showed a marked lower efficacy in inhibiting germination. Overall, germ tube growth was stronger affected by *Trichoderma* culture filtrates than rust spore germination. T_U and T12 showed the strongest germ tube growth inhibition, while the effect of T_R and T_S was least pronounced.

Increasing concentrations of A. dest. added to 1% water agar showed no effect on germination, but lead to slightly increasing germ tube length. This most probably was due to the agar consistency becoming softer and easier to penetrate for the germ tubes.

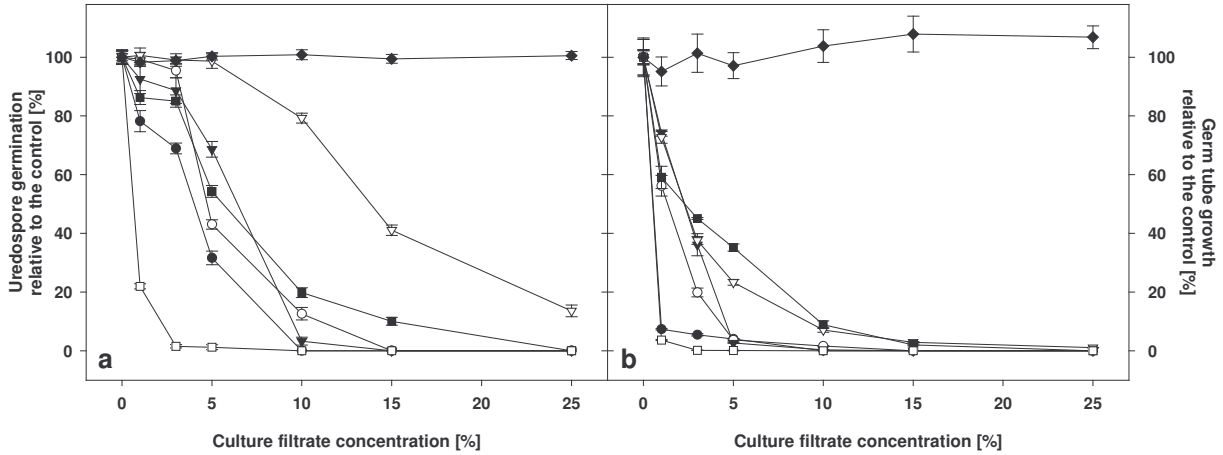


Fig. 3.6. Effect of increasing concentrations of A. dest. (◆) or culture filtrates of *Trichoderma harzianum* strains T12 (●), T-22 (○), T39 (▼), T_R (▽), T_S (■), and T_U (□) incorporated into water agar on germination (a) and germ tube growth (b) of bean rust spores.

The areas under the curves, that show the inhibiting effects on germination (AUGC) and on germ tube growth (AUGGC) over the range of the culture filtrate concentrations, were computed and provided measures for comparing the effects of the different *T. harzianum* culture filtrates (Tab. 3.4).

Tab. 3.4. Effects of increasing culture filtrate concentrations of *Trichoderma harzianum* incorporated into water agar on the area under the germination curve (AUGC) and the area under the germ tube growth curve (AUGGC) of bean rust spores

	Germination		Germ tube growth	
Treatment	AUGC [%*%]	Reduction [%][a]	AUGGC [%*%]	Reduction [%][b]
T12	416 ± 10.7 b[c]	83.4	86 ± 0.7 b	96.7
T-22	604 ± 17.7 c	75.8	195 ± 6.3 c	92.5
T39	623 ± 21.2 c	75.1	244 ± 7.5 d	90.6
T_R	1517 ± 18.8 e	39.3	379 ± 5.5 e	85.4
T_S	714 ± 20.3 d	71.4	412 ± 9.2 f	84.1
T_U	90 ± 4.3 a	96.4	56 ± 0.5 a	97.8
A.dest.	2499 ± 16.2 f	---	2598 ± 60.6 g	---

[a] Percent reduction in area under the germination curve as compared with the A. dest.
[b] Percent reduction in area under the germ tube growth curve as compared with the A. dest.
[c] Figures within one column followed by the same letter do not differ significantly according to Duncan's multiple range test, p=0.05.

Over the complete range of culture filtrate concentrations, the T_U culture filtrate reduced germination and germ tube growth the strongest by 96.4 and 97.8%, respectively, followed by the T12 culture filtrate with reduction of germination by 83.4% and of germ tube growth by 96.7%. The effects of both culture filtrates differed significantly from the control, from each other, and from the other four strains' culture filtrates.

Except for the T_R culture filtrate, which reduced uredospore germination markedly less than the other ones, no dramatic differences in germination and germ tube growth affected by the remaining culture filtrates were observed. Nonetheless, most differences between effects of culture filtrates were statistically significant.

Heat treatment of the culture filtrates from strains T_U and T12 only slightly influenced their efficacy in terms of inhibiting bean rust spore germination and germ tube growth (Tab. 3.5).

Tab. 3.5. Effect of heated *Trichoderma harzianum* culture filtrates (CF) of strains T12 and T_U incorporated into water agar on germination and germ tube growth of bean rust spores

	Effect of 5% T12 culture filtrate			
Heat treatment	Germinated spores [%]	Control [%][a]	Germ tube length [µm]	Control [%][b]
40℃ CF	25.3 ± 2.7 a[c]	67.3	28.0 ± 0.9 a	96.7
60℃ CF	22.3 ± 1.3 a	71.2	28.8 ± 2.5 a	96.6
80℃ CF	22.8 ± 3.1 a	70.6	27.8 ± 0.5 a	96.7
100℃ CF	22.8 ± 1.8 a	70.6	28.8 ± 2.0 a	96.6
121℃ CF	62.0 ± 1.6 b	19.7	41.0 ± 2.5 b	95.2
40℃ A. dest.	77.3 ± 1.9 c	---	852.5 ± 52.0 c	---

	Effect of 1% T_U culture filtrate			
Heat treatment	Germinated spores [%]	Control [%]	Germ tube length [µm]	Control [%]
40℃ CF	31.8 ± 0.5 a	65.9	36.3 ± 1.3 a	95.5
60℃ CF	32.8 ± 1.0 a	64.8	39.0 ± 1.9 a	95.1
80℃ CF	31.0 ± 1.1 a	66.7	33.0 ± 3.7 a	95.9
100℃ CF	31.8 ± 1.0 a	65.9	38.5 ± 1.3 a	95.2
121℃ CF	34.0 ± 0.7 a	63.4	38.3 ± 4.4 a	95.2
40℃ A. dest.	93.0 ± 0.7 b	---	797.0 ± 46.0 b	---

[a] Percent reduction in number of germinated rust spores.
[b] Percent reduction in germ tube length.
[c] Figures within one column followed by the same letter are not significantly different according to multiple contrast tests; p=0.05.

Addition of 5% T12 culture filtrate or 1% T_U culture filtrate to water agar reduced germination about 70 and 66%, respectively, while germ tube elongation was reduced about 96.6 and 95.5%, respectively. Heating the culture filtrates to temperatures up to 100℃ did not change their effects on germination and germ tube growth.

Autoclaved (at 121℃) T12 culture filtrate, added to water agar, showed a marked decrease in the inhibition of bean rust spore germination and a slight decrease in the inhibition of germ tube elongation, both efficacy changes statistically significant based on multiple contrast tests. Autoclaving the T_U culture filtrate did not change its ability to control rust spore germination and germ tube elongation.

3.4 Discussion

Rust fungi produce no epiphytic mycelium during their uredial stage with the exception of a germ tube, which emerges from the uredospore and grows on the leaf surface towards a stoma. Formation of an appressorium over this opening is followed by hyphal penetration through the stoma (HEATH, 1997). For the bean rust fungus *U. appendiculatus*, the process from germination to infection takes less than 24 h, so that the epiphytic hyphal part of the pathogen becomes irrelevant.

During the following ten to fourteen days, *U. appendiculatus* develops within the leaf. It produces intercellular mycelium, forms haustoria for gaining nutrients, and eventually builds a uredium below the epidermal cell layers of the abaxial leaf surface. By breaking through the epidermis, the bean rust fungus returns to the leaf surface with an opened uredium, presenting thousands of spores ready to be taken to a new host by wind or rain (STAVELY, 1991).

From the landing of the uredospore to the take-off of a new generation of uredial rust spores, there are only two stages that can be attacked by microbial beneficials. First, microorganisms producing antifungal substances may inhibit the germination of the rust spore, germ tube elongation, and appressorium formation. Second, microbial beneficials may parasitize on the newly developed rust pustule. While parasitism on rust uredia was mainly attributed to fungal hyperparasites like *V. lecanii* (GRABSKI and MENDGEN, 1985, 1986; SAKSIRIRAT and HOPPE, 1990), antibiotic metabolites inhibiting bean rust spore germination were produced by bacteria of diverse genera (BAKER *et al*., 1983, 1985; YUEN *et al*., 2001). Generally, to prevent the initial infection is of greater interest than solely to reduce the subsequent spread of the disease.

Some strains of the fungal BCA *T. harzianum* are known to produce antifungal metabolites (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al*., 2005). Therefore, this study investigated the ability of *T. harzianum* to inhibit the infection of bean leaves by *U. appendiculatus*.

The main result is that selected *T. harzianum* strains are able to reduce infection of leaf discs under laboratory conditions. Thereby, great differences between the six *T. harzianum* strains were observed, ranging from ineffectiveness of T-22 and T_R to strong effects of T12 and T_U with 40 to 50% and 50 to 60% reduction of the number of rust pustules, respectively. If compared to results obtained in dual culture assays against plant pathogens like *Botrytis cinerea*, *Pythium ultimum*, or *Rhizoctonia solani*, in which T-22 and T_R very effectively controlled the target fungi (Chapter 2), the findings for the activity against bean rust emphasize that selection of the most effective strain for each individual pathosystem is a prerequisite for successful application of *Trichoderma* strains in

biological control (GRONDONA *et al.*, 1997). In the present study, promising bean rust control above 50% disease reduction was only achieved with one out of five commercial *T. harzianum* strains, none of them recommended as BCA against rust fungi.

The results of the leaf disc assays implicate that simply applying fungal cells to the surface of the leaf has no effect on the bean rust fungus in terms of competition for space or nutrients, as can be seen in the cases of T-22 and T_R. *U. appendiculatus*, which feeds on nutrients from its own uredospore during the infection period, was still able to reach the stomata of the bean leaf. Parasitism on the rust spore directly after it landed on the leaf or parasitism on the germ tube by *T. harzianum* is very unlikely due to the very short period of time during which the rust fungus can be attacked at these structures. Hence, the reduction of rust pustules, developing after bean rust inoculation on bean leaf discs treated with *T. harzianum* spore suspensions, is most probably due to antibiosis.

Although a great number of metabolites with antifungal properties synthesized by *Trichoderma* spp. is known (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al.*, 2005), no information is available concerning the effect of any of these metabolites against rust fungi. Therefore, only few conclusions on the nature of the antifungal metabolites of the *T. harzianum* strains, applied during this study, showing activity against *U. appendiculatus* can be drawn from the results of the experiments.

Although the spore suspensions were produced by washing-off the *T. harzianum* spores from the mycelium, the antifungal metabolites are not of mycelial origin. Otherwise, the supernatants used in the assay on the effect of co-inoculation of bean rust and *T. harzianum* preparation on disease severity should have been effective, too. A little effect was observed for the supernatant of T_U, a strain known to produce antifungal metabolites during culture on PDA (Chapter 2). But the effect was much lower than if T_U spores were applied to the leaf discs.

The T12 spore suspension is negatively affecting infectiousness of the uredospore even after autoclaving. This result suggests that the secondary metabolites of T12 responsible for the major part of the effect on *U. appendiculatus* are heat stable antibiotics contained in the T12 spore and released when the spore is destroyed while autoclaved or when the living spore germinates. Other metabolites, which probably are not heat stable may account for the minor, statistically insignificant reduction of bean rust control in comparison to the non-autoclaved T12 spore suspension.

The T_U spore suspension lost its efficacy when autoclaved. A reason for this may be that T_U metabolites are not heat stable, or that they are not released from the spore itself but produced during the process of germination. As autoclaving killed the spores and prevented germination, antifungal metabolites could not be produced.

Compared with the results of the spore suspension assays, germination tests done with *T. harzianum* culture filtrates incorporated into water agar showed very similar results. While spore suspensions of T_U were more effective than T12, followed by T39 and T_S, T-22 and T_R, reduction of germination and germ tube growth was highest for culture filtrates of T_U and T12 followed by T-22, T39, T_S, and T_R. With the exception of T-22, results from spore suspension and culture filtrate experiments seem to be correlated with each other, if spore suspension effects in terms of rust control are compared with AUGC and AUGGC values resulting from germination tests. Therefore, it is proposed that the active antifungal metabolites released by germinating *T. harzianum* spores onto the surface of leaf discs are also produced by *T. harzianum* strains if grown in liquid culture.

The reason for the T-22 spore suspension being completely ineffective in repeated leaf disc assays is unknown. Because re-isolation of T-22 from the leaf disc surface resulted in cfu numbers similar or even higher than for the other *T. harzianum* strains, it can be hypothesized that T-22 spores do not contain the same antifungal metabolites, which are produced by T-22 in liquid culture (VINALE *et al.*, 2006).

Population development on leaf discs showed an initial lag period after application of *Trichoderma* spore suspensions with minor decreases or increases in population density within the first four days. This was influenced by germination and the beginning of mycelial development on one side and by the death of *Trichoderma* spores on the other. Following this period, the influence of mycelial growth got stronger until the scarce amount of nutrients available on the leaf surface was mostly used up after the first week, which caused the following decrease in population density. After the second week, leaf quality declined. This led to an increase of available nutrients and subsequently to a new increase in the amount of living propagules of *T. harzianum* on the surface of the leaf discs.

T-22 mycelium developed a little quicker and especially T_U mycelium slower than T12, T_R, and T_S, which is in correspondence with their growth properties on PDA. The decrease in population density of T39 shows some similarity to results obtained by FREEMAN *et al.*, 2004. Despite these findings, a better performance compared to the other strains on the leaf surface could have been expected with regard to the fact that this strain was isolated from TRICHODEX, a commercial preparation used for the biological control of plant pathogens attacking the phylloplane (ELAD, 1996, 2000; ELAD *et al.*, 1998).

Experiments with the culture filtrates gave some more insight into the nature of the active antibiotic metabolites. Results from previous experiments showed enzymatic activities of cellulase and glucanase, but not of chitinases in the culture filtrates (Chapter 2). This is in accordance with published results about culture filtrates from glucose-based media (VITERBO *et al.*, 2002). Enzyme activity in the culture filtrates did not

match with the results from bean rust uredospore germination tests. *T. harzianum* culture filtrates that proved to be rather ineffective in terms of inhibition of rust spore germination, showed strong cellulolytic and glucanolytic activity. Moreover, heat treatment including autoclaving could only reduce efficacy of culture filtrates to a minor extent.

For the T12 culture filtrate as for the T12 spore suspension, efficacy in terms of rust inhibition was somewhat reduced by autoclaving. Again, this strengthens the hypothesis of T12 producing a mixture of antifungal metabolites with a heat stable main component and others that degrade if autoclaved. Production of a mixture of secondary metabolites with antibiotic activity is in common with other findings for strain T12 (Chapter 2) as well as it is known for *T. harzianum* strains T-22 and T39 (VINALE *et al*., 2006) and also for other *Trichoderma* spp. (DENNIS and WEBSTER, 1971b).

Whatever metabolites are responsible for the rust inhibiting effect of *T. harzianum* strain T_U, these compounds seem to be heat stable if produced in culture filtrate, but instable if T_U spores act as source. An explanation mentioned above could be, that the antibiotic metabolite is not contained and passively released by T_U spores, but actively produced while germination takes place.

Possibly, several of the questions appearing above could have been answered through experiments performed with supernatants of pre-germinated *T. harzianum* spore suspensions. For example, testing such supernatants after autoclaving would have given additional insight to whether the antifungal metabolites active on the leaf discs are heat stable or not. Unfortunately, several attempts to germinate *T. harzianum* spores in watery suspensions failed. This is in contrast to results of GOVINDASAMY and BALASUBRAMANIAN (1989), who showed an antibiotic effect of the supernatant of pre-germinated *T. harzianum* spore suspensions.

Last but not least, undiluted culture filtrates of T12 and T_U showed strong disease reducing effects of 55 to 60% if applied to leaf discs prior to inoculation with bean rust. On the other hand, applying those culture filtrates as a curative treatment 24 h after bean rust inoculation had no effect on bean rust germlings, which had already successfully penetrated through the stomata and infected the leaf tissue. The number of rust pustules that developed after curative culture filtrate treatment was as high as after the ‘protective’ control treatment, but a little lower than after the ‘curative’ control treatment. Regarding the latter treatment, few additional rust infections could take place after ‘curative’ application of A. dest. Applying the culture filtrates of *T. harzianum* strains T12 and T_U after bean rust inoculation could only protect the bean leaf disc against those additional infections, but had no curative effect. This demonstrates that the antifungal metabolites present in the culture filtrates of these two *Trichoderma* strains did not enter the bean leaf tissue.

Trichoderma spp. are successful BCAs, their efficacy as disease controlling, resistance inducing, and plant growth promoting beneficial microorganisms well-known (HARMAN, 2006). They have been widely used as antagonists of soil-borne diseases, but single strains could also be established for several years as commercial BCAs of foliar pathogens (ELAD, 2000a).

Despite the successful application against a broad spectrum of important diseases, only very few attempts have been made to control rust fungi by means of *Trichoderma* spp. (GOVINDASAMY and BALASUBRAMANIAN, 1989; ZADE *et al*., 2005). The present study is one of the first to show the disease reducing effect of spore suspensions as well as culture filtrates against a rust fungus and the only one presenting results of *T. harzianum* being antagonistic against the bean rust fungus *U. appendiculatus*. Although not all *T. harzianum* strains showed bean rust inhibiting activity, the efficacy of the selected strains T12 and T_U in terms of disease reduction due to inhibition of germination and germ tube growth is very promising. The antibiotic effect of *T. harzianum* strains T12 and T_U has been proven in greenhouse experiments, too (Chapter 4). Therefore, specific isolates of *T. harzianum* may be useful to improve disease management systems as well as *Trichoderma* metabolites with antifungal activity may provide an additional tool for managing fungicide resistance.

Although numerous publications document the good performance of fungicidal compounds from diverse chemical classes against rust fungi (GENT *et al*., 2001; MUELLER *et al*., 2005; STUMP *et al*., 2000), few reports state that fungicide resistant isolates of rust species may develop (COOK, 2001; DIRKSE *et al*., 1982).

To avoid loosing fungicides that are effectively controlling rust fungi today, all options to reduce the possible development of fungicide resistance should be included into disease management systems. *T. harzianum* strains producing antifungal compounds provide such an option.

4. Mechanisms of *Trichoderma*-mediated Bean Rust Control: Antibiosis and Induced Resistance

4.1 Introduction

The bean rust fungus *Uromyces appendiculatus* is of worldwide importance as a yield-reducing disease of *Phaseolus vulgaris* L., potentially causing yield losses up to 50% (BERGER *et al*., 1995; DE JESUS JUNIOR *et al*., 2001; VENETTE and JONES, 1982). Heavy epidemics occur especially in the tropics and subtropics, because of the climatic conditions favouring the spread and infectiousness of *U. appendiculatus* (STAVELY, 1991). Control of the bean rust fungus is achieved by application of several disease management measures like cultural practices, cultivation of rust-resistant varieties, and the use of fungicides (MCMILLAN *et al*., 2003). Significantly reduced disease incidence was reported for repeated evaluations of fungicides from different chemical groups (GENT *et al*., 2001; STUMP *et al*., 2000). Nevertheless, publications document that fungicide resistant races of rust species may develop against varying fungicidal compounds (COOK, 2001; DIRKSE *et al*., 1982; FRAC, 2006). Therefore it is necessary to utilize all components available within an integrated bean production system to gain optimal disease and resistance management (MCMILLAN *et al*., 2003), like alternating fungicides with different modes of action (MUELLER *et al*., 2004).

Researchers were able to elucidate several means of bean rust control other than chemical fungicides, especially the application of microbial antagonists or substances with resistance inducing properties. The possibility of controlling bean rust by fungal or bacterial antagonists was investigated throughout the last 25 years (BAKER *et al*., 1983, 1985; GRABSKI and MENDGEN, 1985, 1986; SAKSIRIRAT and HOPPE, 1990; YUEN *et al*., 2001). Bacterial antagonists negatively affected rust spore germination by secretion of antibiotic metabolites (BAKER *et al*., 1983, 1985; YUEN *et al*., 2001), while the antagonistic fungus *Verticillium lecanii* grew parasitically on bean rust uredia and uredospores (GRABSKI and MENDGEN, 1985, 1986; SAKSIRIRAT and HOPPE, 1990).

Resistance against *U. appendiculatus* was successfully induced in bean plants by chemical agents like BTH (benzo-[1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester) or others (DANN and DEVERALL, 1995; SIEGRIST *et al*., 1997; TYIHÁK *et al*., 1989), elicitors of fungal origin (HÜMME *et al*., 1978), or due to pre-inoculation with fungal organisms (DANN and DEVERALL, 1995; TAKAHASHI *et al*., 1985; YARWOOD, 1956).

To the author's knowledge, no information regarding the control of *U. appendiculatus* by means of *Trichoderma* spp. is available although this fungal genus is widely recognized for its antagonistic behaviour against fungal pathogens (HARMAN, 2006). While interacting with foliar pathogens, *Trichoderma* spp. employ various antagonistic mechanisms. Competition for nutrients (ZIMAND *et al*., 1995), mycoparasitic activity (BRADATSCH, 2006; GUPTA *et al*., 1999; SANOGO *et al*., 2002), inhibition of the pathogen due to degradation of its lytic enzymes (KAPAT *et al*., 1998), or antibiosis (GOVINDASAMY and BALASUBRAMANIAN, 1989) may take place on the leaf surface, depending on the physiological characteristics of both pathogen and antagonist strain.

Moreover, *Trichoderma* strains may induce systemic resistance in plants by activating the plants own defence mechanisms against potential attacks of plant pathogens including fungi, bacteria, and viruses (HARMAN *et al*., 2004). *Botrytis cinerea* and *Colletotrichum lindemuthianum* disease symptoms were significantly reduced through *Trichoderma*-mediated resistance induction in bean plants when grown in soil amended with *T. harzianum* (BIGIRIMANA *et al*., 1997, DE MEYER *et al*., 1998). As a side effect of root application of *T. harzianum*, plant growth promotion is reported quite often in the scientific literature (HARMAN, 2000).

Rust fungi that only produce a single germination hypha on the leaf surface, which penetrates through a stoma and gives rise to an intercellular mycelium within the leaf, are more easily controlled by antibiosis than by parasitism or competition (ANDREWS, 1992). According to this, the antagonism of *Trichoderma* spp. against rust fungi other than bean rust was explained by an antifungal effect of secondary metabolites produced by the *Trichoderma* strain resulting in inhibition of rust spore germination or of germ tube elongation (GOVINDASAMY and BALASUBRAMANIAN, 1989). Such effects were reported for bean rust spores confronted with living *Trichoderma* propagules (GOVINDASAMY and BALASUBRAMANIAN, 1989; KAPOORIA and SINHA, 1969; SALLAM, 2001), the sterile fluid from germinated spore suspensions (GOVINDASAMY and BALASUBRAMANIAN, 1989; SINHA and BAHADUR, 1974), and filtrates of 15-days-old liquid cultures (ZADE *et al*., 2005). No case of increased plant resistance elicited by these *Trichoderma* treatments was reported.

In prior experiments, the antibiotic effects exerted by *T. harzianum* spore suspensions and culture filtrates on the bean rust fungus *U. apppendiculatus* in leaf disc assays and germination tests were shown (Chapter 3). In the subsequent study reported here, the ability of two *T. harzianum* strains to control bean rust in greenhouse trials was investigated. Emphasize was given to the question, whether reduction of disease severity could be explained solely by an antibiotic effect of spore suspension or culture filtrate treatments, or if induction of systemic resistance added up to the observed level of disease control.

4.2 Material and methods

4.2.1 Fungal strains

Two *T. harzianum* strains were used in this study: The non-commercial strain T12 from the fungal collection of the Institute of Plant Diseases and Plant Protection (IPP; Leibniz Universität Hannover, Germany), originally obtained as strain T000 from the Institute of Phytopathology and Applied Zoology (IPAZ; Justus-Liebig-University Gießen, Germany), and the isolated strain from the commercial preparation UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand). The abbreviations T12 and T_U will be used throughout this work for the two strains, respectively.

Uredospores of the bean rust fungus *U. appendiculatus* were taken from the fungal collection of the IPP.

4.2.2 General culture conditions

All *Trichoderma* strains were maintained on PDA (Merck KGaA, Darmstadt, Germany) at 24°C in Petri dishes with a diameter of 90 mm, which were filled with 10 ml PDA and singly sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA).

U. appendiculatus was maintained by inoculating bean plants, harvesting freshly produced uredospores, and storing them at -20°C. Uredospores taken from the freezer and used in the experiments usually were not older than 4 to 8 weeks.

4.2.3 Production of **Trichoderma harzianum** *spore suspensions and culture filtrates*

Spore suspensions were produced by scraping off sporulating mycelium from PDA cultures and suspending it in sterile A. dest. To remove mycelial fragments from the suspensions, they were filtered through 595 Schleicher & Schuell filter paper (Whatman International Ltd., Kent, England). Spore suspensions were adjusted to the desired concentration.

Culture filtrates were produced by cultivation of the two *T. harzianum* strains for ten days in PDB (Becton Dickinson GmbH, Heidelberg, Germany). For each strain, 250 ml PDB were initially inoculated with five mycelial plugs with a diameter of one cm. Incubation took place at room temperature (22°C) on a horizontal shaker at 85 rpm. To get rid of the major parts of the mycelium, liquid cultures were filtered through 595 Schleicher & Schuell filter paper. Afterwards, cultures were sterile filtrated through Schleicher & Schuell filters with pore sizes of 0.8 µm and 0.2 µm.

4.2.4 Trials with spore suspension or culture filtrate treatment of leaves

Single bean seeds of cultivar 'Speedy' (Hild Samen GmbH, Marbach, Germany) were directly sown into 0.88-litre pots containing 300 g substrate (Archut Fruhstorfer Erde, P-Typ; Hawita Gruppe GmbH, Vechta, Germany). Bean plants were reared for 21 to 25 days in the greenhouse until the first trifoliate leaf had largely developed. Before treatments with *T. harzianum* spore suspensions or culture filtrates started, plants were transferred to climate chambers, where the experiments took place under defined climatic conditions. Trials with culture filtrates were carried out at 24°C and relative humidity values of 55 to 60%, while spore suspension experiments were conducted at 24°C and 85 to 90% rH to allow *T. harzianum* spores to germinate or at least to keep spores alive on the leaf surface.

Spore suspensions and culture filtrates were applied 1, 3, 5, and 7 day(s) prior to bean rust inoculation to the right leaflet of the first trifoliate leaf with a 25-ml pump spray bottle. The left leaflet was treated with sterile A. dest. (Fig. 4.1). The control plants were treated with sterile A. dest. on both lateral leaflets four days before rust inoculation.

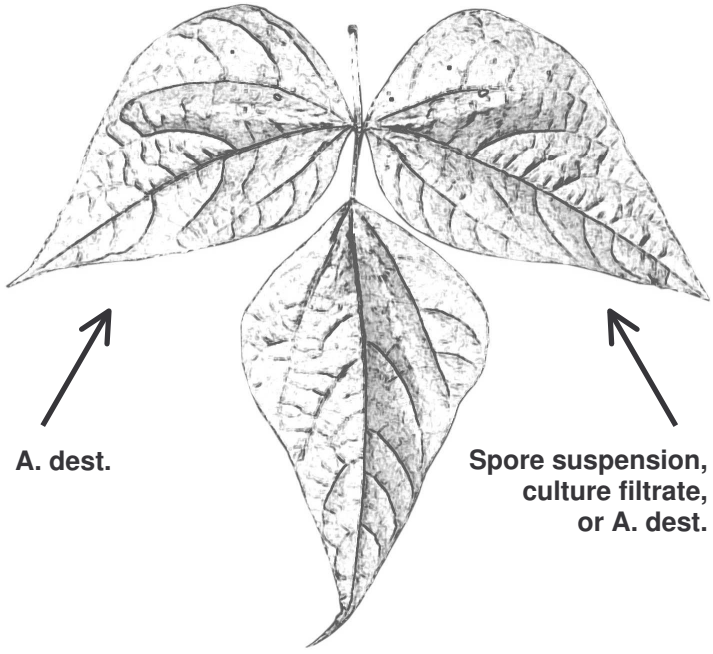


Fig. 4.1. Scheme of spore suspension, culture filtrate, and A. dest. application (by T. Karrasch).

4.2.5 Trials with spore suspension treatment of seeds or substrate

The seed treatment part of this trial was done by bathing bean seeds of cultivar 'Speedy' (Hild Samen GmbH, Marbach, Germany) in spore suspensions of *T. harzianum* strains T12 and T_U with concentrations of 5×10^7 spores / ml for three minutes. Afterwards, they were singly sown into 0.88-litre pots containing 300 g substrate (Archut Fruhstorfer Erde, P-Typ; Hawita Gruppe GmbH, Vechta, Germany). The substrate had been moistened prior to sowing with 100 ml of sterile A. dest.

For the substrate treatment part, bean seeds were bathed in sterile A. dest. for three minutes and singly sown into 0.88-litre pots containing 300 g substrate, that had been pre-treated with 100 ml of *T. harzianum* spore suspensions of strains T12 and T_U with

concentrations of 3 x 10^5 spores / ml. These treatments led to *T. harzianum* spore numbers of either 1 x 10^5 spores attached to the seed or 3 x 10^7 spores distributed within the substrate, meaning a concentration of 1 x 10^5 spores / g substrate. The control treatment consisted of bean seeds bathed in sterile A. dest. sown into substrate moistened with 100 ml of sterile A. dest.

Fifteen bean seeds were used for each treatment. Due to negative effects of the *T. harzianum* treatments, bean seeds partly did not germinate or growth of bean plants was negatively influenced. Therefore, ten of fifteen plants, which had developed well, were selected for bean rust inoculation. Rearing of bean plants took place in a climate chamber at 24°C and 55 to 60% rH. At the end of the trial, mean leaf size of the lateral leaflets of the first trifoliate leaf and the dry matter of the plants were determined.

4.2.6 Inoculation

Rust spore suspensions with a concentration of 1 x 10^5 spores / ml were produced in sterile A. dest. amended with few droplets of Tween 20 for better distribution of the spores within the suspension. Before inoculation, all plants were transferred into a foliage tunnel in the greenhouse. Inoculation was performed with a 25-ml pump spray bottle. The bean rust spore suspension was applied to right and left leaflet of the first trifoliate leaf of each plant until run-off. After inoculation, the greenhouse table was flooded with water and the foliage tunnel closed to assure very high relative humidity for 24 h, necessary for rust germination and infection of bean plants.

Thereafter, bean plants were taken out of the foliage tunnel and cultured for 12 to 14 days until disease symptoms had fully developed. Disease severity was measured with a LemnaTec Scanalyzer (LemnaTec GmbH, Würselen, Germany).

4.2.7 Statistical analysis

The variables measured in this study were (1) percentage of diseased leaf area, (2) leaf size, and (3) plant dry weight. Procedures in SAS version 8.02 (Statistical Analysis Systems Institute, Cary, NC, USA) were used to perform analyses of variance and mean separations by (1) Dunnett's many-to-one comparisons for diseased leaf area data of foliar spore suspension and culture filtrate treatments 1 day before bean rust inoculation, (2) contrast tests for all data from foliar treatment experiments, and (3) Tukey's all-pair comparisons for data from the seed and substrate treatment experiment.

Diseased leaf area values relative to the control were calculated for graphic presentation of data in Fig 4.2 and 4.3. The variability is given by the standard error.

4.3 Results

4.3.1 Spore suspension experiment

No statistically significant difference was detected in the *T. harzianum* spore suspension experiment regarding mean disease levels on right and left lateral leaflets of leaves treated with sterile A. dest., according to t-test comparisons. Hence, data from right and left leaflet of control plants were pooled. Control of bean rust by the *T. harzianum* spore suspension treatment was calculated in relation to these mean disease severities.

The mean disease severity on control plants of the spore suspension experiment was 8.7%. When *T. harzianum* spore application took place one day prior to rust inoculation, T_U was more effective than T12 with diseased leaf areas relative to the control of 41 and 60%, respectively, on the treated right leaflets (Fig. 4.2). These differences to the control treatment were statistically significant according to Dunnett's many-to-one comparisons, which (for statistical reasons) were conducted only for the treatments one day before rust inoculation. With extending time interval between spore application and rust inoculation, the diseased leaf area increased. Statistically, this trend was not significant based on linear contrast tests.

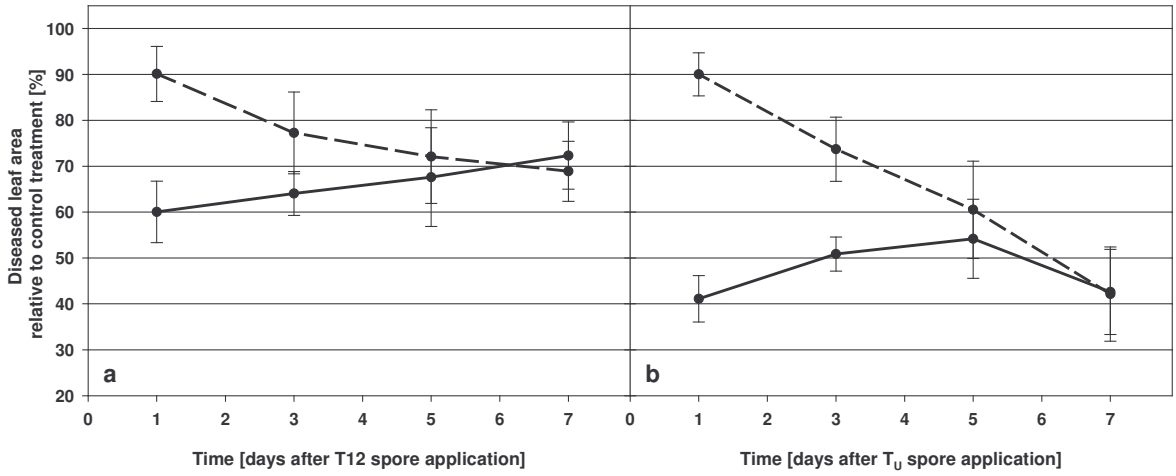


Fig. 4.2. Effect of time between application of *Trichoderma harzianum* T12 (a) or T_U (b) spore suspension and rust inoculation on disease severity on untreated (- - -) and treated (——) leaflets.

On the non-*Trichoderma*-treated left leaflet, the diseased area decreased with extending time interval between spore application and rust inoculation, indicating resistance induction. Minimum diseased leaf area of 42 and 69 % of the control for T_U and T12, respectively, resulted from the longest resistance induction interval of seven days between *T. harzianum* spore application and bean rust inoculation. For T_U, but not for T12, this effect superimposed the direct antibiotic effect of the spore suspension on the right

lateral leaflet. In this case, resistance induction, also taking place in the *Trichoderma*-treated right leaflet, compensated for the declining efficacy of the direct antibiotic effect of the spore suspension. Contrast test-derived *p*-values for these trends were $p = 0.052$ for T12 and $p < 0.001$ for T_U, indicating that reduction in disease severity due to induced resistance was statistically significant for T_U and nearly so for T12.

4.3.2 Culture filtrate experiment

Again, data from right and left lateral leaflet of control plants were pooled, and bean rust control through application of culture filtrates of *T. harzianum* strains T12 and T_U was calculated in relation to these mean disease severities.

The mean disease severity on the control plants in the culture filtrate experiment was 10.7%. When culture filtrate application took place one day prior to inoculation of bean leaves, T12 was more effective than T_U with diseased leaf areas relative to the control of 42 and 75%, respectively, on the treated right leaflets (Fig. 4.3). Only the effects of application of T12 and T_U culture filtrates one day before rust inoculation were (for statistical reasons) compared with the control by Dunnett's many-to-one comparisons and proved to be statistically significant.

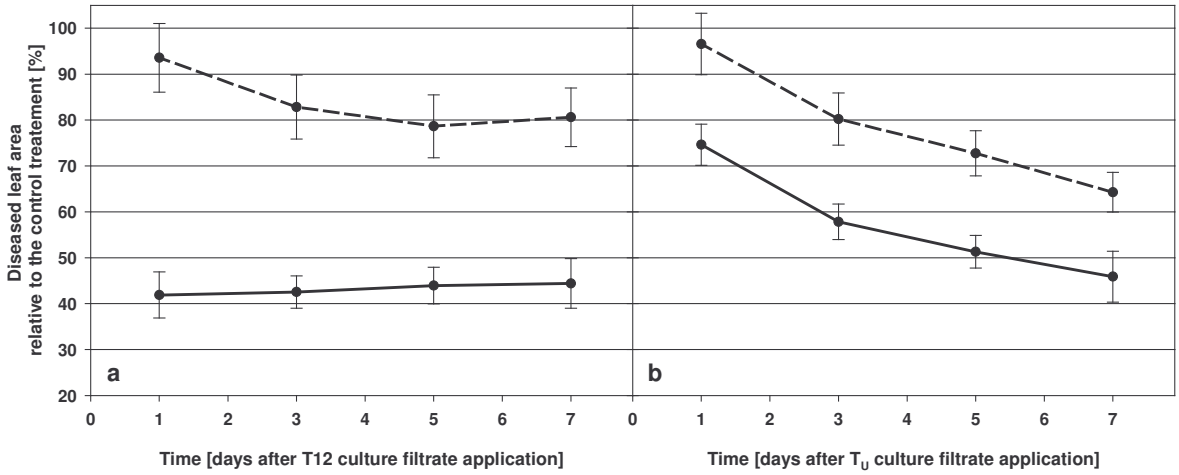


Fig. 4.3. Effect of time between application of *Trichoderma harzianum* T12 (a) or T_U (b) culture filtrate and rust inoculation on disease severity on untreated (- - -) and treated (——) leaflets.

With longer time intervals between T12 culture filtrate application and rust inoculation, the effect of the culture filtrate did not change significantly according to linear contrast tests. The level of resistance induced on the left leaflet was rather low with a minimum diseased leaf area of 79%. The trend of induced resistance getting stronger with increasing time interval between treatment with T12 culture filtrate and rust inoculation was apparent, but failed to be statistically significant with a *p*-value of $p = 0.079$.

Treatment with T_U culture filtrate resulted in a strong resistance induction. With extending time interval, the diseased leaf area decreased, reaching a minimum diseased leaf area of 64% of the control treatment when rust inoculation took place seven days after culture filtrate treatment. As resistance induction took place in the right leaflet as well, the diseased leaf area relative to the control decreased from 75 to 46% with extending time interval. Again, such an effect was not visible for T12. Linear contrast tests proved decreases in disease severity over time to be significant on both leaflets with *p*-values of $p < 0.001$.

4.3.3 Substrate and seed treatment experiment

In general, no positive effect of substrate or seed treatment with *T. harzianum* strains T12 and T_U in terms of disease reduction or growth promotion was observed (Tab. 4.1). Neither did the treatments affect disease severity due to induced resistance against *U. appendiculatus*, nor did they lead to increased plant growth. While the treatment of the substrate with T_U had no significant effect on the growth of bean plants, T12 substrate and seed treatments, and especially the bathing of seeds in the T_U spore suspension negatively influenced the growth of the bean cultivar 'Speedy'. Although not significant for all parameters, reduction of plant growth as a consequence of *T. harzianum* treatment was clearly observable, even after ten normally developed plants out fifteen bean plants had been selected for inoculation with bean rust and all further analyses.

Tab. 4.1. Effect of substrate or seed treatment with *Trichoderma harzianum* spore suspensions on bean rust disease severity, leaf size of the first trifoliate leaf, and dry weight of the shoot

Treatment	Disease severity [%]	Leaf size [cm²]	Dry weight [g]
T12 substrate	10.66 ± 1.03 a[a]	39.77 ± 2.44 bc	1.37 ± 0.07 ab
T12 seed	11.50 ± 0.93 a	38.95 ± 1,42 c	1.39 ± 0.05 a
T_U substrate	10.29 ± 0.95 a	47.08 ± 2.40 ab	1.54 ± 0.08 a
T_U seed	10.05 ± 0.67 a	33.16 ± 1.80 c	1.09 ± 0.08 b
Control	10.89 ± 0.65 a	49.19 ± 0.98 a	1.61 ± 0.07 a

[a] Within each column, numbers followed by the same letter do not differ significantly according to Dunnett's many-to-one comparisons.

4.4 Discussion

4.4.1 Antibiosis

In the scientific literature, only few reports on *Trichoderma*-mediated control of rust fungi are available. The majority of them explain reduction in rust disease severity with an antibiotic effect of living propagules of *Trichoderma* spp. (GOVINDASAMY and BALASUBRAMANIAN, 1989; KAPOORIA and SINHA, 1969; SALLAM, 2001; Chapter 3) or with antibiotic metabolites present in the supernatant of germinated spore suspensions (GOVINDASAMY and BALASUBRAMANIAN, 1989; SINHA and BAHADUR, 1974) or culture filtrates (ZADE *et al.*, 2005; Chapter 3). These findings are in common with the knowledge about *Trichoderma* spp. producing secondary metabolites with antibiotic properties (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al.*, 2005) as well as with the general statement that the short epiphytic phase of rust fungi makes an antibiotic interaction more likely than disease control by competition or parasitism (ANDREWS, 1992). Therefore, inhibition of germination and hyphal growth of the bean rust fungus by antibiotic metabolites is far more likely to take place during the 24 h infection period of *U. appendiculatus* if challenged with *T. harzianum*.

The observation of antibiosis being one out of two forces responsible for the control of *U. appendiculatus* by *T. harzianum* in the greenhouse is in common with results derived from leaf disc assays performed with six different *T. harzianum* strains (Chapter 3). Strains T12 and T_U proved to be the most effective strains in terms of inhibition of uredospore germination and disease reduction and, therefore, were selected for the greenhouse trials. In the greenhouse studies reported here, many results from the previous laboratory trials were confirmed.

It seems unlikely that the mycelial production of antibiotic metabolites reduced the bean rust severity. If mycelial production of antibiotic metabolites had taken place during the growth of the mycelium on the leaf surface, the level of disease should have decreased with increasing time interval between *Trichoderma* spore application and bean rust inoculation. As spore suspensions were most effective one day after application, it seems that spores release antibiotic metabolites during their germination. Another possibility could be a strong production of extracellular metabolites by the hypha while emerging from the spore. Leaf disc assays proved that both explanations may be correct. While T12 spores release heat stable metabolites with antifungal properties against *U. appendiculatus*, the metabolites, which confer antibiotic activity to T_U, seem to be produced during the process of the germination hypha emerging from the spore (Chapter 3).

The results for the culture filtrates support the hypothesis of an antibiotic interaction. In contrast to the spore suspensions, the T_U culture filtrate was much less effective than the T12 culture filtrate. This may be due to metabolites in the T12 culture filtrate acting as surfactants. This property of the T12 culture filtrate could be observed during its application. The leaf surface was not only wetted by droplets, but covered with a film of the filtrate. Additionally, these surfactant-like compounds may have increased the adhesion of the culture filtrate to the leaf surface. Likewise, appropriate adjuvants used with commercial foliar fungicides improve coverage and retention of fungicides on treated plant parts (GENT *et al.*, 2003).

Lacking such properties, the T_U culture filtrate was less evenly distributed over the leaf surface and partly washed off during rust inoculation. This also explains the reduced efficacy of the T_U culture filtrate in the greenhouse experiments compared to the results of leaf disc assays conducted with culture filtrates of T_U and T12. In these, T_U was as effective as T12 with reduced numbers of rust pustules on leaf discs of about 55% (Chapter 3). In contrast to the greenhouse experiments, run-off and loss of the efficacy of the T_U culture filtrates did not occur in the leaf disc assays. Adding a spreader / sticker – component to the T_U culture filtrate should therefore enhance its efficacy.

4.4.2 Induced resistance

With increasing time period between application of the *Trichoderma* treatments and bean rust inoculation, the disease severity on the non-*Trichoderma*-treated left lateral leaftlet decreased slightly for spore suspension and culture filtrate treamtents of *T. harzianum* strain T12 and strongly for strain T_U. The translocation of the antibiotic metabolites from the inoculated right lateral leaflet to the non-inoculated left lateral leaflet can most likely be excluded as an explanation for this effect, because a curative effect of the application of at least *T. harzianum* culture filtrates was not observed in leaf disc assays (Chapter 3). This indicates that the responsible antifungal metabolites are not taken up into the bean leaf tissue. Hence, the *Trichoderma* treatments seem to induce systemic resistance.

For each *Trichoderma* strain, the curves showing disease severity on the untreated left leaflet are very similar in shape, depending on the time period between application of spore suspension or culture filtrate to the right leaflet of the first trifoliate leaf and inoculation of bean plants.

T12 spore suspensions and culture filtrates induced only low levels of systemic resistance as seen in the low decrease of disease severity on the left leaflet. In both cases, the direct antibiotic effect of the treatments was not notably changed over time by locally induced resistance in the right leaflet. Contrasting these results, T_U spore

suspensions and culture filtrates induced a stronger systemic resistance in the left leaflet. Moreover, locally induced resistance in the right leaflet superimposed and enhanced the antibiotic effect on the right leaflet.

Finally, the very similar abilities of spore suspensions and culture filtrates to induce resistance suggest that resistance had been elicited by some secondary metabolites released from the spores or growing *Trichoderma* hyphae and present in the culture filtrates of T12 and especially T_U.

Within this scenario, enzymes with cellulolytic activity are likely candidates for the role of elicitor molecules inducing resistance to *U. appendiculatus* in bean plants. This hypothesis is based on a row of evidences. First, mycelia of T12 and T_U were shown to produce cellulase (endo-1,4-ß-glucanase) on water agar media in which chromogenic cellulose (AZCL-HE-cellulose; Megazyme, Bray, Ireland) was the only substrate (Chapter 3). It is very likely that a spore suspension gained by washing off *Trichoderma* spores from a mycelium grown on a glucose/cellulose-based medium contains active cellulase. Second, germinating *T. reesei* spores released enzymes with cellulolytic activity (CHAUDHARY and TAURO, 1982). Third, culture filtrates of T12 and T_U showed cellulolytic activity (Chapter 3). And fourth, a cellulase from *T. longibrachiatum* activated plant defence mechanisms and induced resistance in the cotyledons of melon (*Cucumis melo*) against the powdery mildew pathogen *Sphaeroteca fuliginea* (MARTINEZ *et al*., 2001) as well as a crude cellulase of *T. viride* was capable of activating a defence-related signalling cascade (PIEL *et al*., 1997). Thus, an involvment of this class of enzymes seems to be reasonable.

On the other hand, T12 was less effective in inducing resistance than T_U, but produced more cellulase in liquid potato dextrose broth (PDB) and on solid agar medium (Chapter 2). Therefore, other metabolites with resistance eliciting capability may be important as well. Even autoclaved *Trichoderma* mycelial extracts from PDB culture were shown to increase plant resistance (CHANG *et al*., 1997). Therefore, further experimental work is needed to prove the hypothesis of *T. harzianum* cellulase being an elicitor molecule able to induce systemic resistance within the pathosystem of *U. appendiculatus* and *P. vulgaris*.

No induction of systemic resistance against *U. appendiculatus* could be observed after applying *Trichoderma* spore suspensions of strains T12 and T_U to substrate or bean seed. Although diverse strains of *Trichoderma* spp. are able to elicit resistance in plants against fungal, bacterial, or even viral diseases (HARMAN *et al*., 2004), to the author's knowledge, there is no publication available reporting *Trichoderma*-induced resistance to rust diseases. The present study provides evidence that resistance induction against the bean rust fungus is possible if *T. harzianum* agents are applied to the leaf surface.

Application of strains T12 and T_U to seed or substrate was, however, not effective in this respect.

In nearly all cases, in which systemic resistance was induced to *U. appendiculatus*, elicitors were applied to the leaf surface and not to the root system (DANN and DEVERALL, 1995; HÜMME *et al*., 1978; TAKAHASHI *et al*., 1985; TYIHÁK *et al*., 1989; YARWOOD, 1956). Only SIEGRIST *et al*. (1997) demonstrated that seed treatment with chemical elicitors induced resistance to foliar pathogens like *U. appendiculatus*. It remains to be elucidated if *T. harzianum* strains, known to have resistance inducing abilities like T39 (ELAD, 2000a) or T-22 (HARMAN, 2000; HARMAN *et al*., 2004), would activate defence mechanisms against *U. appendiculatus* when applied to the root system of bean plants.

4.4.3 Effect of leaf age on bean rust infection and induced resistance

The numbers of rust pustules developing after inoculation as well as the sporulation potential of those pustules tend to decrease with increasing leaf age (IMHOFF *et al*., 1982; JENNINGS *et al*., 1990; MELCHING *et al*., 1988). Because all plants used in the presented experiments had the same age at the time of inoculation regardless of the preceding *Trichoderma* treatment, an effect of plant age as the reason for reduced bean rust severity compared to the control can be excluded.

Literature states that older leaves react more sensible to resistance eliciting signals and express stronger resistance reactions than young leaves (HEIL, 1999; HERBERS *et al*., 1996; HERMS and MATTSON, 1992; VAN LOON, 1997). Moreover, it takes a period of several days for the induced resistance to become systemic. The time period that elapsed in the experiments between first *Trichoderma* treatments and inoculation was sufficient to observe the induction of systemic resistance especially in the non-treated left leaflets of bean plants. This means that the strongest resistance induction was observed in those leaves, which were youngest when treated with spore suspensions or culture filtrates of *T. harzianum*. No inhibition of this induced resistance due to a younger leaf age, which resulted from the increasing time period before bean rust inoculation, could be observed.

4.4.4 Effect of Trichoderma harzianum *on plant growth*

After the plant growth-promoting effect of *T. harzianum* had been reported for the first time (BAKER *et al*., 1984), numerous reports followed, presenting further evidence for the positive effect of *Trichoderma* spp. on plant growth (CHANG *et al*., 1986, HARMAN, 2000; KLEIFELD and CHET, 1992; WINDHAM *et al*., 1986). On the other hand, few publications document that it is not possible to generalize the observed plant growth-promoting effect for every plant species – *Trichoderma* strain combination (GERLAGH *et al*., 1999; LUMSDEN *et al*., 1990). OUSLEY *et al*. (1993, 1994) demonstrated that plant growth may decrease if *Trichoderma* spp. are applied to the substrate. In their interpretation of the results, they followed GHISALBERTI *et al*. (1990) who showed that the particular *T. harzianum* strain, which produced the greatest amount of antifungal pyrone compounds making it the most potent antagonist of *Gaeumannomyces graminis* var. *tritici*, had also the most detrimental effect on plant growth.

In the present study, the negative effect on plant growth, that would have been even more pronounced if no selection of rather well developed bean plants had been carried out, is in common with the aforementioned observations and interpretations. Strains producing high amounts of secondary metabolites, which may be desirable in terms of disease control, may exert negative effects on plant growth. Especially the antifungal metabolite 6-pentyl-α-pyrone (6PAP), which is responsible for the typical coconut-aroma of many *Trichoderma* cultures (COLLINS and HALIM, 1972), is known to inhibit plant growth (CLAYDON *et al*., 1987; CUTLER *et al*., 1986; LUMSDEN *et al*., 1990). For T_U, especially for its culture filtrate, such coconut-odour was clearly noticeable. Therefore, the inhibition of plant growth by T_U may arise from the production of this secondary metabolite. No coconut-smell could be detected for solid or liquid T12 cultures.

In more general terms it can be stated that each single *Trichoderma* strain – pathogen – plant species or even plant genotype combination (HARMAN, 2006) has to be evaluated carefully, if application of living propagules of a certain *Trichoderma* strain is considered, because generalization concerning the type of interaction between *Trichoderma* and plant species is not possible.

5. Compounds with Potential Antifungal Activity against *Uromyces appendiculatus* Isolated from Six *Trichoderma harzianum* Strains

5.1 Introduction

Trichoderma spp. are ubiquitous soil-borne fungal organisms with strains adapted to the diverse habitats of all climatic zones (KLEIN and EVELEIGH, 1998). Next to being important saprophytes (KUBICEK-PRANZ, 1998), they are known as antagonists of soil-borne phytopathogens like *Fusarium* spp. (SIVAN and CHET, 1989), *Pythium ultimum* (BENHAMOU and CHET, 1997), *Rhizoctonia solani* (PAULA JÚNIOR *et al*., 2007), or nematodes (SHARON *et al*., 2001), and fungal pathogens attacking upper plant parts like *Botrytis cinerea* (ELAD, 1996), *Sphaerotheca fusca* (ELAD *et al.,* 1998), or *Crinipellis perniciosa* (SANOGO *et al*., 2002).

Antagonistic activities of *Trichoderma* spp. comprise competition (SIVAN and CHET, 1989), parasitism (BENHAMOU and CHET, 1997), and antibiosis (GHISALBERTI *et al*., 1990). Quick mycelial growth and rhizosphere competition (AHMAD and BAKER, 1987) accompanied by the secretion of lytic enzymes (VITERBO *et al*., 2002) and secondary metabolites with antibiotic activity (SIVASITHAMPARAM and GHISALBERTI, 1998) are the physiological backbone to the antagonistic strength of *Trichoderma* spp.

Scientists evaluate and try to utilize the antagonistic features of *Trichoderma* spp. since the early 1930s, when WEINDLING (1932) described "*Trichoderma lignorum* as a parasite of other soil fungi" and earned his degree of Doctor of Philosophy in Plant Pathology for his work on "The lethal principle of *Trichoderma lignorum* in its action on *Rhizoctonia solani*" (1933). The "lethal principle" was characterized as a "deadly substance […] excreted into the surrounding medium by the young hyphae" of *T. lignorum* (1934).

Almost fourty years later, the first extensive studies on the antagonistic activity of *Trichoderma* spp. caused by the production of non-volatile and volatile metabolites with antibiotic activity (DENNIS and WEBSTER, 1971a, 1971b) as well as a study on the parasitic interaction between fungal pathogens and strains of *Trichoderma* spp. producing or non-producing secondary metabolites with antibiotic activity (DENNIS and WEBSTER, 1971c) were published.

Another twenty years later, GHISALBERTI and his colleagues were the first to provide reviews on the secondary metabolites of *Trichoderma* spp. (1991, 1993), followed by the

most comprehensive review until today (SIVASITHAMPARAM and GHISALBERTI, 1998), in which more than 120 structures of secondary metabolites are displayed and their biological activities are described.

Recently, the major secondary metabolites of the two commercial *T. harzianum* strains T-22 and T39 were described (VINALE *et al*., 2006).

A large review on peptaibols and related peptaibiotics, being a specific class of *Trichoderma* metabolites, is available (SZEKERES *et al*., 2005). Moreover, the web-based, freely accessible Peptaibol Database (WHITMORE *et al*., 2003), allows searches for peptaibol sequences and structures. Today, the database contains sequences of 317 peptaibols, 190 of them being produced by *Trichoderma* spp. including 54 peptaibols of *T. harzianum* (PEPTAIBOL DATABASE, 2007).

Secondary metabolites of *Trichoderma* spp. were shown to have antibacterial (BRÜCKNER and KOZA, 2003; SONG *et al*., 2006), antiviral (YUN *et al*., 2000), antimycoplasmic (LECLERC *et al*., 2001), and antifungal (DENNIS and WEBSTER, 1971a, 1971b; GHISALBERTI *et al*., 1990; LORITO *et al*., 1996; SCHIRMBÖCK *et al*., 1994; SONG *et al*., 2006) activity. In some cases, e. g. for the peptaibols, the mechanisms by which the antibiotic compound interferes with cellular structures or metabolic pathways have been elucidated, but for many other molecules only the general activity against some target organism(s) is known (SIVASITHAMPARAM and GHISALBERTI, 1998).

Antibiotic activities against target fungi by single metabolites (ANEJA *et al*., 2005), metabolite mixtures (VINALE *et al*., 2006), or synergistic action of secondary metabolites and lytic enzymes (LORITO *et al*., 1996; SCHIRMBÖCK *et al*., 1994) include reduction in fungal spore germination and germ tube elongation (SCHIRMBÖCK *et al*., 1994) and inhibition of mycelial growth (DENNIS and WEBSTER, 1971a, 1971b, 1971c; Chapter 2). The infection of plants with varying species of rust fungi could be reduced by treatment with conidial spores (GOVINDASAMY and BALASUBRAMANIAN, 1989; Chapters 3 and 4), the fluid of pre-germinated spore suspensions (GOVINDASAMY and BALASUBRAMANIAN, 1989; SINHA and BAHADUR, 1974), and culture filtrates (ZADE *et al*., 2005; Chapters 3 and 4) of diverse *Trichoderma* strains.

Until now, no secondary metabolites of *Trichoderma* spp. that reduce infectiousness of rust fungi by inhibiting rust spore germination or germ tube growth are known. The experimental work reported here comprises basic research in the field of isolation and identification of secondary metabolites of *T. harzianum* with antifungal activity towards the bean rust fungus *Uromyces appendiculatus*. Because this study has just been started, only preliminary results are shown. Possible future approaches are discussed.

5.2 Material and methods

5.2.1 Fungal strains

Six *T. harzianum* strains were used in this study, five of them isolated from commercial preparations: T-22 from TRIANUM-P (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands), T39 from TRICHODEX (Makhteshim-Agan Ltd., Tel Aviv, Israel), and the strains from TRI 003 (Plantsupport, Grootebroek, The Netherlands), TRICHOSAN (Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany), and UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand). The abbreviations T_R, T_S, and T_U will be used throughout this work for the latter three strains, respectively. The non-commercial strain T12 from the fungal collection of the Institute of Plant Diseases and Plant Protection (IPP; Leibniz Universität Hannover, Germany) was originally obtained as strain T000 from the Institute of Phytopathology and Applied Zoology (IPAZ; Justus-Liebig-University Gießen, Germany).

Uredospores of the bean rust fungus *U. appendiculatus* were taken from the fungal collection of the IPP.

5.2.2 General culture conditions

All *Trichoderma* strains were maintained on PDA (Merck KGaA, Darmstadt, Germany) at 24°C in Petri dishes with a diameter of 90 mm, which were filled with 10 ml PDA and singly sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA).

U. appendiculatus was maintained by inoculating bean plants, harvesting freshly produced uredospores, and storing them at -20°C. Uredospores taken from the freezer and used in the experiments were usually not older than 4 to 8 weeks.

5.2.3 Production of **Trichoderma harzianum** *culture filtrates*

Culture filtrates (CF) were produced by cultivation of the six *T. harzianum* strains for ten days in PDB (Becton Dickinson GmbH, Heidelberg, Germany). For each strain, 250 ml PDB were initially inoculated with five mycelial plugs with a diameter of one cm. Incubation took place at room temperature (22°C) on a horizontal shaker at 85 rpm. To get rid of the major parts of the mycelium, liquid cultures were filtered through 595 Schleicher & Schuell filter paper (Whatman International Ltd., Kent, England). Afterwards, cultures were sterile filtrated through Schleicher & Schuell filters with pore sizes of 0.8 µm and 0.2 µm.

5.2.4 Assay on antibiotic activity of culture filtrate extracts

Ten ml of the culture filtrates of *T. harzianum* strains T12 and T_U were extracted with equal amounts of either ethyl acetate (EA; Diagonal GmbH & Co KG, Münster, Germany) or hexane (HX; Diagonal GmbH & Co KG, Münster, Germany) at 24°C using a horizontal shaker at 250 rpm for 20 min. After separation of the organic and the watery fraction, the solvents were evaporated using the rotary evaporator RV05 (Janke & Kunkel GmbH & Co KG, IKA-Werk, 79219 Stauffen). The dried organic fraction was dissolved in 10 ml sterile A. dest. and the watery fraction was adjusted to the original volume of 10 ml.

Each fraction of the four culture filtrate – solvent combinations was incorporated into 1% water agar gaining solid media amended either with the EA- or HX-soluble or with the EA and HX-insoluble metabolites of *T. harzianum* strains T12 and T_U with a concentration of 15%. This agar was poured into 60-mm Petri dishes.

Per Petri dish, 50 µl of a bean rust spore suspension with a concentration of 5×10^4 spores / ml were applied and allowed to germinate for 24 h at 24°C. Afterwards, the percentage of germinated spores was visually determined for 100 spores by use of a light microscope. Germ tube growth was assessed qualitatively. Each combination of *T. harzianum* strain and solvent fraction consisted of four replications.

5.2.5 Analysis of potential secondary metabolites of Trichoderma harzianum *with antifungal activity against* Uromyces appendiculatus

5.2.5.1 Chemicals

For the extraction of culture filtrates, ethyl acetate (Diagonal GmbH & Co KG, Münster, Germany) was used. A. bidest., methanol (HPLC grade; Fisher Scientific GmbH, Schwerte, Germany), and acetonitrile (HPLC grade; VWR International GmbH, Darmstadt, Germany) were used in the HPLC-MS.

5.2.5.2 Analytical equipment

EA-extracts were evaporated to dryness with the rotational vacuum concentrator RVC 2-25 (Martin Christ GmbH, Osterode, Germany).

The HPLC system consisted of the ProStar 410 HPLC AutoSampler, two ProStar 210 solvent delivery modules providing a binary system (both Varian Deutschland GmbH, Darmstadt, Germany), and the column oven Jetstream II Plus (TECHLAB GmbH, Erkerode, Germany). A Polaris C18-Ether column (100 x 2 mm i.d.; 3 µm particle size) and a guard column MetaGuard of the same material (both Varian Deutschland GmbH, Darmstadt, Germany) were used for analytical HPLC.

Mass spectrometry was performed with the 500-MS LC ion trap coupled to an electrospray ionization chamber (Varian Deutschland GmbH, Darmstadt, Germany).

5.2.5.3 Extraction of organic compounds from culture filtrates followed by HPLC-MS

Fourty ml of the culture filtrates of all six *T. harzianum* strains and 40 ml of the PDB medium, which served as the control, were extracted two times with equal volumes of EA at 24°C using a horizontal shaker at 250 rpm for 20 min. The organic fractions of both extractions were combined, evaporated to dryness at 40°C, taken up in one ml of 50:50 (v/v) methanol/water, and filtered through a 0.2-µm Opti-Flow TF filter with a diameter of 13 mm (Wicom Germany GmbH, Heppenheim, Germany). Per *T. harzianum* strain, four samples were produced by the described method and stored at -18°C.

Before HPLC-MS analysis was performed, all 7 x 4 extracts were brought to room temperature. 10-µl samples were subjected to chromatography by the automated sampling device ProStar 410 HPLC AutoSampler. Samples were eluted with a linear gradient of 95% A. bidest. and 5% acetonitrile (A) / methanol (B) as follows: 0 to 2.20 min: 10% B; 2.20 to 25.0 min: from 10% B to 98% B; 25.0 to 30.0 min: 98% B. Separation took place at 40°C at a flow rate of 0.2 ml / min.

The eluate was nebulized in an electrospray ionization (ESI) chamber with drying gas pressure of 20 psi and nebulizer gas pressure of 50 psi. Gas temperature was reduced from 350 to 250°C with increasing proportion of methanol in the eluate. For the detection of positive $[MH]^+$ ions, needle and shield voltage were 5000 and 600 V, respectively. The capillary voltage was 50 V. Mass spectral data in the range of 100 to 1000 m/z were collected in positive ionization mode. Per second, 5000 Da were scanned and three consecutive scans were averaged.

MS data were transformed into chromatograms using MS Data Review version 6.9 (Varian Deutschland GmbH, Darmstadt, Germany) and later on converted into netCDF data format.

5.2.5.4 Data analysis

Data from positive ionization mode were processed as follows: Minor differences in retention times of different chromatograms were corrected by peak alignment performed with the freely available software XCMS version 1.5.2 (SMITH *et al.*, 2006), which was run under R package version 2.4.0. The resulting data were normalized (using a PERL-script written by P. Karlovsky), to compensate for differences in the concentrations of the extracts and/or loading volumes.

The final outputs of the analysis of positive ionization data were tables representing peaks defined by mass-to-charge ratio, retention time, and assigned normalized signal

intensity calculated by the integration of peaks in extracted ion chromatograms for each sample. All peaks were evaluated and excluded from further analysis if at least one of the following criteria was met: (1) peaks were present in the PDB medium, (2) peaks had integrated values smaller than 50,000 TIC, or (3) peaks were present in less than three (of four) *T. harzianum* strain extracts.

Finally, medians were calculated for the four samples of each *T. harzianum* culture filtrate.

5.2.5.5 Comparison of compounds detected by HPLC-MS with known secondary metabolites of Trichoderma harzianum

By use of ChemSketch version 10.0 (Advanced Chemistry Development Inc., Toronto, Canada), the molecular masses of 135 secondary metabolites of *Trichoderma* spp. and the closely related *Gliocladium* spp. were calculated based on their structure formulae displayed in several publications (ANEJA *et al*., 2005; CORLEY *et al*., 1994; GHISALBERTI and ROWLAND, 1993; GHISALBERTI and SIVASITHAMPARAM, 1991; GHISALBERTI *et al*., 1990; SIVASITHAMPARAM and GHISALBERTI, 1998; VINALE *et al*., 2006).

These mass data of known secondary metabolites of *Trichoderma* and *Gliocladium* strains were compared with the masses of compounds detected by HPLC-MS in the EA-extracts of culture filtrates of the six *T. harzianum* strains used in this study. The term “potential secondary metabolites” is used in this study for compounds which masses resemble those of known secondary metabolites of *T. harzianum*.

5.2.6 *Statistical analysis*

Analysis of variance and mean separation by Dunnett’s many-to-one comparisons for bean rust spore germination data from the assay on antibiotic activity of culture filtrate extracts was performed using procedures in SAS version 8.02 (Statistical Analysis Systems Institute, Cary, NC, USA). The variability is given by the standard error.

5.3 Results

5.3.1 Antibiotic activity of culture filtrate extracts

Culture filtrates of *T. harzianum* strain T12 and T_U were extracted with ethyl acetate (EA) and hexane (HX). The antibiotic activity of the organic EA- or HX-soluble metabolites and the activity of the remaining watery fraction were tested in a bean rust uredospore germination assay (Tab. 5.1).

Germination was influenced neither by the HX-fraction of T12 nor of T_U, indicating that HX was not adequate to extract the secondary metabolites responsible for the antibiotic activity against bean rust from the culture filtrates.

Germination was significantly reduced by the EA-fraction of T12 as well as by the remaining watery fraction. This indicates that at least two metabolites are responsible for the antibiotic effect of T12 against the bean rust fungus, one of which is soluble in EA, while the other is not.

A little effect of the EA-extract of T_U was observed, too, indicating that the EA-soluble metabolites from the T_U culture filtrate had only minor effects on the germination of the bean rust uredospores.

Tab. 5.1. Effect of ethyl acetate and hexane extracts of *Trichoderma harzianum* culture filtrates on germination and germ tube growth of bean rust uredospores

		Ethyl acetate		Hexane	
Strain	Fraction	Germination [%][a]	Germ tube length	Germination [%]	Germ tube length
T12	organic	61.8 ± 3.9 *	very short	75.3 ± 1.9 *	long
	watery	59.5 ± 2.7 *	very short	1.0 ± 0.7 *	nearly zero
T_U	organic	70.0 ± 1.7 *	slightly shortened	82.0 ± 1.2	long
	watery	0.0 ± 0.0 *	zero	1.5 ± 1.2 *	nearly zero
Control	---	82.8 ± 1.2	long	82.8 ± 1.2	long

[a] Percent germination of bean rust spores. Figures within one column followed by * are significantly different from the control according to Dunnett's many-to-one comparisons; p=0,05.

5.3.2 *Potential secondary metabolites of* Trichoderma harzianum *with antifungal activity against* Uromyces appendiculatus

Since signals from positive ionization mode are usually higher and MS literature is mostly based on positive ESI data, this study is also focussed on positive ionization data, which are presented as results and discussed later on.

When culture filtrates of all six *T. harzianum* strains were extracted with EA and the organic EA-fractions were analysed by HPLC-MS, a total number of 221 signals specific for the *T. harzianum* culture filtrates were detected (Tab. 5.2). Molecular mass-to-charge ratios ranged from 73 to 1000 m/z and retention times from 92 to 2010 scan numbers with ca. one scan per second.

Tab. 5.2. Basic data derived from the analysis of HPLC-MS results

	Basic data
Number of metabolites isolated from EA extracts of culture filtrates	221
Number of metabolites found in the culture filtrate of six strains	2
Number of metabolites found in the culture filtrate of five strains	4
Number of metabolites found in the culture filtrate of four strains	2
Number of metabolites found in the culture filtrate of three strains	2
Number of metabolites found in the culture filtrate of two strains	19
Number of metabolites found in the culture filtrate of single strains	192
Lowest metabolite mass [in m/z]	73
Highest metabolite mass [in m/z]	1000
Lowest retention time [in scan numbers]	92
Highest retention time [in scan numbers]	2010
Lowest total ion current [TIC]	1.16×10^6
Highest total ion current [TIC]	8.86×10^8

Of these 221 signals, 192, 19, 2, 2, 4, and 2 were present in the filtrates of single, two, three, four, five, or all six *T. harzianum* strain cultures, respectively. Of the 192 putative compounds produced by single strains, 29, 45, 21, 33, 34, and 30 were only found in the culture filtrates of T12, T-22, T39, T_R, T_S, and T_U, respectively (Tab. 5.3). Overall, rather equal numbers of EA-soluble compounds between 39 and 50 were found in the culture filtrates of T12, T-22, T39, T_R, T_S, and T_U.

Medians of detectable metabolite concentrations measured as total ion current (TIC) ranged from 1.16×10^6 to 8.86×10^8 (Tab. 5.2). For the 29 compounds present in the culture filtrates of more than one strain, highest amounts were found in the culture filtrate of T12, T-22, T39, T_R, T_S, and T_U, in 5, 2, 6, 10, 3, and 3 cases, respectively. Overall, the 1 to 2-fold and 2 to 5-fold differences in signal intensities were observed in the culture filtrate of one strain compared to the intensities originating from the filtrates of the other strain cultures in 20 and 9 cases, respectively (Tab. 5.3).

Tab. 5.3. Numbers of signals (putative compounds) detected in the culture filtrates of one or more *Trichoderma harzianum* strains

	T12	T-22	T39	T_R	T_S	T_U
Putative compounds in the CF of a particular strain	50	50	44	46	47	39
Putative compounds in the CF of a single strain	29	45	21	33	34	30
Signals in the CF of a particular strain with highest intensity compared to other strains	5	2	6	10	3	3
Signals in the CF of a particular strain with 1 to 2-fold higher intensity compared to other strains	4	2	2	8	1	3
Signals in the CF of a particular strain with 2 to 5-fold higher intensity compared to other strains	1	0	4	2	2	0
Putative compounds in the CF of a particular strain, which masses resemble those of known secondary metabolites of *Trichoderma* spp.	16	9	11	8	9	7
Putative compounds in the CF of a particular strain, which masses resemble those of known secondary metabolites of *T. harzianum*	6	3	3	4	4	2
Putative compounds in the CF of more than one strain, which masses resemble those of known secondary metabolites of *T. harzianum*[a]	3	1	3	2	2	0
Putative compounds in the CF of a single strain, which masses resemble those of known secondary metabolites of *T. harzianum*	3	2	0	2	2	2

[a] Four compounds were found in the CF of more than one strain: three of them in the CF of two strains and one compound in the CF of five strains.

In the culture filtrates of T12, T-22, T39, T_R, T_S, and T_U, 16, 9, 11, 8, 9, and 7 putative compounds were found, respectively (Tab. 5.3), the masses of which resemble those of known secondary metabolites of *Trichoderma* spp., according to ANEJA *et al*. (2005), CORLEY *et al*. (1994), GHISALBERTI and ROWLAND (1993), GHISALBERTI and SIVASITHAMPARAM (1991), GHISALBERTI *et al*. (1990), SIVASITHAMPARAM and GHISALBERTI (1998), and VINALE *et al*. (2006). In these publications, a total of 135 secondary metabolites of *Trichoderma* spp. and *Gliocladium* spp. are listed. Of these, 12 and 18 are known to be produced by several *Trichoderma* species including *T. harzianum* or solely by *T. harzianum* strains, respectively, whereas 65 were isolated from cultures of *Trichoderma* species other than *T. harzianum* (Tab. 5.4). Six metabolites were produced by *Trichoderma* spp. and *Gliocladium* spp., while 34 metabolites were only detected in *Gliocladium* spp. cultures. These results are based on the species names mentioned in the primary literature (SIVASITHAMPARAM and GHISALBERTI, 1998).

Tab. 5.4. Number of known secondary metabolites of *Trichoderma* spp. and *Gliocladium* spp. found in the literature and potentially found in the culture filtrates in this work

	Producing organism[a]				
	T.h.	T.h. & T.spp.	T.spp. - T.h.	T.spp. & G.spp.	G.spp. - T.spp.
Number of known secondary metabolites described in the literature	18	12	65	6	34
Number of known secondary metabolites with masses corresponding to those found in the culture filtrates	8	5	21	3	8
Number of putative compounds found in the culture filtrates with masses corresponding to those from the literature	10	5	28	3	7

[a] Metabolites produced only by *T. harzianum* (T.h.), *T. harzianum* and other *Trichoderma* spp. (T.h. & T.spp.), other *Trichoderma* spp. than *T. harzianum* (T.spp. – T.h.), *Trichoderma* spp. and *Gliocladium* spp. (T.spp. & G.spp.), and only by *Gliocladium* spp. (G.spp – T.spp.).

Of the 30 secondary metabolites known from cultures of *T. harzianum*, 13 had molecular masses, which corresponded to the masses of molecules found in the culture filtrates of the six *T. harzianum* strains. Overall, 15 different putative substances were detected in the culture filtrates, the masses of which corresponded to the 13 *T. harzianum* metabolites listed in the literature (Tab. 5.4). Four of these 15 compounds were found in the culture filtrates of more than one *T. harzianum* strains. Of the remaining nine compounds, which masses resembled those of known secondary metabolites of *T. harzianum*, 3, 2, 0, 2, 2, and 2 were solely found in the culture filtrates of strain T12, T-22, T39, T_R, T_S, and T_U, respectively (Tab. 5.3).

Tab. 5.5 lists all 15 molecules, their masses and retention times, the amounts found in the culture filtrates of the respective *T. harzianum* strain, and the name of the potential secondary metabolites which have the same masses as that of the detected molecules. Fig. 5.1 to 5.4 show all structures of those cited secondary metabolites of *T. harzianum*.

Tab. 5.5. Mass spectrometry signals detected in the culture filtrates of the six *Trichoderma harzianum* strains, which masses resemble those of known secondary metabolites.

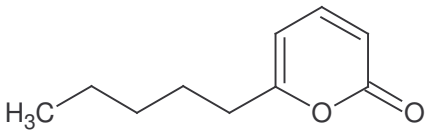
No.	Mass [Da]	Retention [sec][a]	T12[b]	T-22	T39	T_R	T_S	T_U	Potential secondary metabolite[c]
1	112	142	---	---	---	2.1	4.6	---	Uracil
2	164	1240	---	---	---	---	---	7.1	6-pent-1-enyl-*α*-pyrone
3	164	174	---	---	---	1.9	---	---	6-pent-1-enyl-*α*-pyrone
4	166	1269	---	---	---	---	---	126.5	6-pentyl-*α*-pyrone
5	204	100	11.6	4.1	7.2	13.9	6.9	---	Dehydroxy harzianolide
6	204	1198	16.9	---	---	---	---	---	Dehydroxy harzianolide
7	220	1129	40.7	---	---	---	---	---	T39butenolide
8	222	1206	19.1	---	---	---	---	---	Harzianolide
9	240	1093	11.0	---	23.0	---	---	---	Cyclonerodiol
10	281	1524	---	---	---	---	116.7	---	Harzianopyridone
11	281	171	11.8	---	11.0	---	---	---	Harzianopyridone
12	281	213	---	32.8	---	---	---	---	Harzianopyridone
13	282	1525	---	---	---	---	15.2	---	Koninginin E, koninginin B, or seco-koninginin
14	300	953	---	12.3	---	---	---	---	Harziandione
15	400	1063	---	---	---	85.9	---	---	Harzianum A

[a] Retention time of the $[MH]^+$-ion.

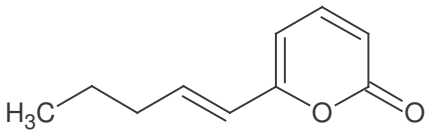
[b] Detected intensities of metabolic signals in the respective culture filtrate in millions counts [TIC].

[c] Potential secondary metabolites according to the mass-to-charge ratios of detected signals and the masses of known secondary metabolites of *T. harzianum*.

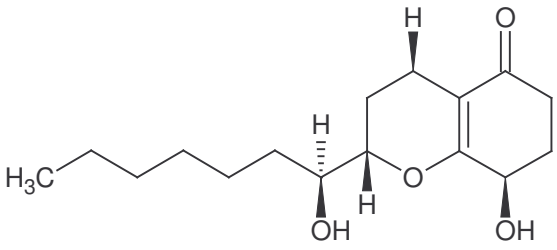
Pyrones



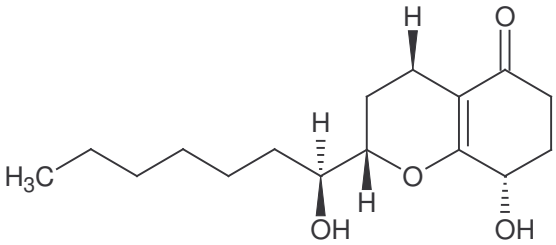
4: 6-pentyl-*α*-pyrone
166 Da



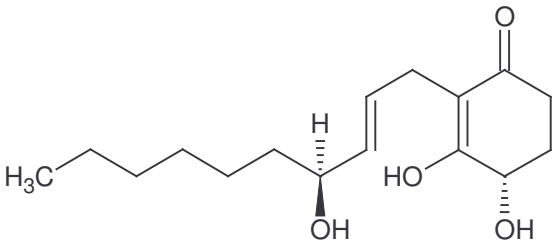
2 / **3**: 6-pent-1-enyl-*α*-pyrone
164 Da



13: Koninginin B
282 Da



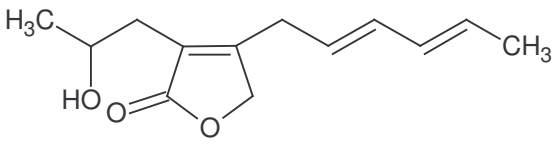
13: Koninginin E
282 Da



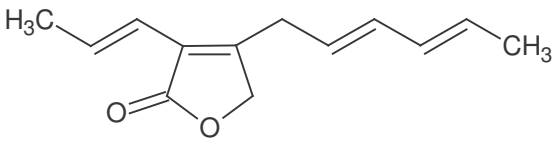
13: Seco-koninginin
282 Da

Fig. 5.1. Pyrone compounds potentially present in the culture filtrates of examined *Trichoderma harzianum* strains. Structur formulae drawn with ChemSketch version 10.0 (Advanced Chemistry Development Inc., Toronto, Canada).

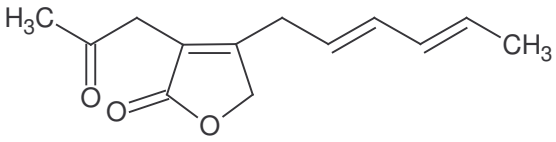
Butenolides



8: Harzianolide
222 Da



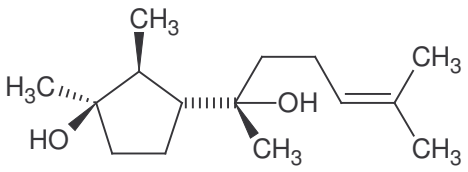
6: Dehydroxy harzianolide
204 Da



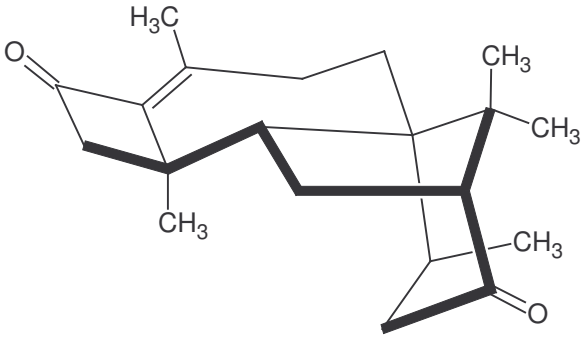
7: T39butenolide
220 Da

Fig. 5.2. Butenolide compounds potentially present in the culture filtrates of examined *Trichoderma harzianum* strains. Structur formulae drawn with ChemSketch version 10.0 (Advanced Chemistry Development Inc., Toronto, Canada).

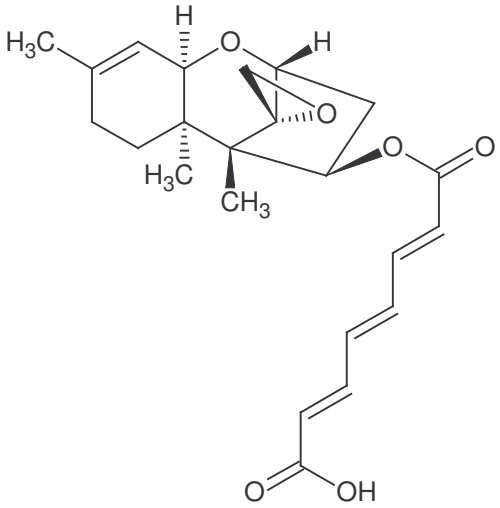
Terpenoids



9: Cyclonerodiol
240 Da



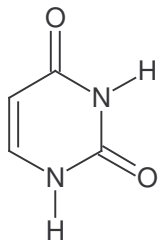
14: Harziandione
300 Da



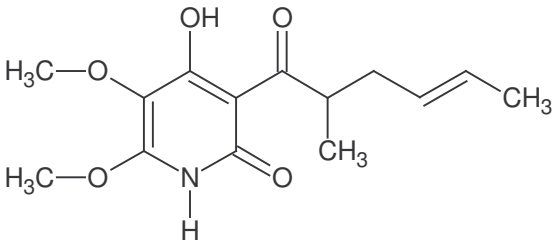
15: Harzianum A
400 Da

Fig. 5.3. Terpenoid compounds potentially present in the culture filtrates of examined *Trichoderma harzianum* strains. Structur formulae drawn with ChemSketch version 10.0 (Advanced Chemistry Development Inc., Toronto, Canada).

Others



1: Uracil
112 Da



10 / **11** / **12**: Harzianopyridone
281 Da

Fig. 5.4. Other compounds potentially present in the culture filtrates of examined *Trichoderma harzianum* strains. Structur formulae drawn with ChemSketch version 10.0 (Advanced Chemistry Development Inc., Toronto, Canada).

5.4 Discussion

Like many other fungal species, *Trichoderma* spp. produce secondary metabolites with antibiotic activity (SIVASITHAMPARAM and GHISALBERTI, 1998). The inhibition of mycelial growth of target fungi confronted with developing mycelium of the antagonist or with sterile filtrated fluids from liquid cultures of *T. harzianum* strains is a clear indication for an antibiotic interaction due to the presence of antibiotic metabolites (Chapters 2 and 3).

Several aspects have to be considered with regard to production and activity of such metabolites. Although many *Trichoderma* strains are known to produce secondary metabolites with antifungal activity, this is not observed for all strains (DENNIS and WEBSTER, 1971a; GHISALBERTI *et al*., 1990).

For example, the well-known antagonist of *Botrytis cinerea*, *T. harzianum* T39, is most often cited to have no antibiotic activity towards its target *B. cinerea* (ELAD, 1996; ELAD and KAPAT, 1999). In contrast to this statement, VINALE *et al*. (2006) reported the production of three metabolites, harzianolide, T39butenolide, and harzianopyridone, when T39 was cultivated in liquid culture for 31 days at 25°C. These compounds displayed antifungal activity towards *Gaeumannomyces graminis* var. *tritici*, *Pythium ultimum*, and *Rhizoctonia solani*. On the other hand, T39 produced only T39butenolide and harzianopyridone, but no harzianolide during an interaction study with *R. solani* on solid agar medium at 25°C (VINALE *et al*., 2006).

Questions arise from these findings concerning the effect of the environmental conditions under which the growth of a particular *Trichoderma* strain takes place, e.g. type of substrate or temperature, on the production of certain metabolites. Additionally, the spectrum or amount of antifungal metabolites produced within a *Trichoderma* strain – phytopathogen – interaction depends on the target fungus, while reactions of the target towards particular compounds also vary (COONEY and LAUREN, 1998; VINALE *et al*., 2006).

Hence, observing no antibiotic interaction between T39 and *B. cinerea* may be due to environmental parameters, due to *B. cinerea* not inducing the production of antibiotic metabolites by T39, or due to *B. cinerea* being tolerant or resistant towards these metabolites, as well as being able to degrade them (COONEY and LAUREN, 1998).

The profile of secondary metabolites produced by a single *Trichoderma* strain may differ strongly from metabolic profiles of other strains. Not only do different *Trichoderma* species produce different metabolites (GHISALBERTI and SIVASITHAMPARAM, 1991; SIVASITHAMPARAM and GHISALBERTI, 1998), but even the profiles of strains from the same species can be very different (GHISALBERTI *et al*., 1990; VINALE *et al*., 2006). Some metabolites like 6PAP are produced by a greater number of *T. harzianum* isolates (CLAYDON *et al*., 1987; COONEY and LAUREN, 1998; GHISALBERTI *et al*., 1990;

SCARSELLETTI and FAULL, 1994), while others produce metabolites solely reported for single strains, like T22azaphilone and T39butenolide produced by T-22 and T39, respectively (VINALE *et al*., 2006).

Moreover, some metabolites are reportedly being produced by spores (MERLIER *et al*., 1984) or during sporulation (BODO *et al*., 1985), or on the contrary during phases of growth decline (LUMSDEN *et al*., 1990). *Trichoderma* strains may loose their ability to produce a certain secondary metabolite (BREWER *et al*., 1987) or gain the ability to produce new secondary metabolites after mutagenesis (GRAEME-COOK and FAULL, 1991). Last but not least, single spore cultures of the same field isolate of *T. koningii* were reported to display varying production of the antibiotic 6PAP (WORASATIT *et al*., 1994).

Summarizing the above, it can be concluded that the observation of antibiosis between *Trichoderma* spp. and a target fungus, resulting from the activity of particular secondary metabolites, is a common interaction, but it may be reproducible only for a defined pair of fungal strains under defined physiological and environmental parameters.

When the contract of this PhD work ended, the analysis of secondary metabolites of *T. harzianum*, that confer the antibiotic activity against the bean rust fungus to the antagonistic strains, had just started. Thus, there was no time left to carry out fragmentation studies with the most interesting HPLC peaks, to perform preparative chromatographic separation of the extracted metabolites from the *T. harzianum* culture filtrates, to evaluate the biological activity of separated fractions against the bean rust fungus, and subsequently to elucidate the structures of relevant metabolites by NMR and other techniques. Therefore, it was only possible to speculate on the effect of the compounds in the culture filtrates, which masses resemble those of known secondary metabolites of *T. harzianum* (Tab. 5.5), termed “potential secondary metabolites” in this work.

In the culture filtrates of the six *T. harzianum* strains, 221 metabolites were detected that definitely were of fungal origin. Two were found in all six culture filtrates, while 192 could only be detected in the culture filtrates of single strains. In each of the six culture filtrates, 39 to 50 compounds were found, 21 to 45 originating from the filtrates of individual strains.

While each of these individual metabolites may confer antibiotic activity to the particular strain producing it, it cannot be excluded that metabolites detected in the culture filtrates of more than one strain are responsible for part of the observed antifungal effects. To limit the number of metabolites potentially responsible for the observed antibiotic activitiy especially against the bean rust fungus, only those compounds with molecular masses which resemble the masses of known secondary metabolites of *T. harzianum*

referenced in ANEJA *et al*. (2005), CORLEY *et al*. (1994), GHISALBERTI and ROWLAND (1993), GHISALBERTI and SIVASITHAMPARAM (1991), GHISALBERTI *et al*. (1990), SIVASITHAMPARAM and GHISALBERTI (1998), and VINALE *et al*. (2006) were used to evaluate the profiles of compounds found in the culture filtrates of the six examined *T. harzianum* strains.

When interpreting the findings of potential secondary metabolites with a focus on their biological activity against the bean rust fungus *U. appendiculatus*, direct conclusions can only be drawn from the effect of the culture filtrates on germination and germ tube growth of bean rust uredospores (Chapter 3). It remains to be determined whether the metabolites detected in the culture filtrates are also present in the fluid of germinated spore suspensions.

As seen for *T. harzianum* strain T-22, results from assays performed with spore suspensions or culture filtrates may differ considerably, indicating that metabolites produced by mycelia in liquid culture are not necessarily released from *Trichoderma* spores or produced during the process of germ tube development (Chapter 3).

Similar results from leaf disc assays with spore suspensions and culture filtrates of strains T12 and T_U in terms of antifungal activity against *U. appendiculatus* suggest that the same metabolites were responsible for the effects of the two treatments by each strain. Comparison of the chemical composition of culture filtrates and the fluid of germinated *T. harzianum* spores, will be needed to test this hypothesis.

Nonetheless, with the exception of T-22, the efficacy of spore suspensions in leaf disc assays was reflected by results from germination experiments conducted on water agar amended with culture filtrates of the remaining five *T. harzianum* strains (Chapter 3). Therefore it is hypothesized that the compounds found in the T12 culture filtrate potentially have a greater antifungal effect on *U. appendiculatus* than metabolites from culture filtrates of T-22, T39, T_R, and T_S. Moreover, compounds found in the culture filtrate of T12 and of other *T. harzianum* strains as well, seem to be less important, as long as T12 did not produce them in much higher concentrations.

Because the EA-extract of the T_U culture filtrate showed only little antifungal activity against bean rust, the compounds found in the culture filtrate of T_U are of minor significance, too.

Harzianolide (**8**) and two related compounds, dehydroxy harzianolide (**5**, **6**) and T39butenolide (**7**) were potentially found in the T12 culture filtrate. Moreover, compounds having the same masses as cyclonerodiol (**9**) and harzianopyridone (**11**) were detected. With the exception of cyclonerodiol, which has plant growth regulating properties (GHISALBERTI and ROWLAND, 1993), these secondary metabolites all have antifungal

activity. Regarding their potential activity against *U. appendiculatus*, compound **5** can be neglected because it was present in the culture filtrate of the ineffective strain T_R in even greater amounts. Considering that the production of dehydroxy harzianolide was neither reported for T-22 nor T39, it can be doubted that compound **5** really is dehydroxy harzianolide.

Compounds **9** and **11** were only found in the culture filtrates of T12 and T39. T39 is known to produce harzianopyridone (VINALE *et al*., 2006). Hence, compound **11** probably is harzianopyridone and is produced by both T12 and T39. The potential harzianopyridone can only be responsible for part of the effect of T12 on bean rust, because rather equal amounts of compound **11** were found in the culture filtrates of both strains. On the other hand, the presence of harzianopyridone might explain the effect of T39 in leaf disc as well as germination assays (Chapter 3).

If compound **11** really is harzianopyridone, then compounds **10** and **12** present in the culture filtrates of T_S and T-22, respectively, cannot be the same compound. This is further supported by the fact, that harzianopyridone was not reported for T-22 (VINALE *et al*., 2006). For T_S, no information on its secondary metabolite profile is available in the literature, which is also the case for T_R and T_U.

In case it is not cyclonerodiol, compound **9** may also be responsible for part of the efficacy of T39. Considering that it was detected in the T39 culture filtrate at a more than 2-fold higher concentration than in the culture filtrate of T12, its contribution to the effect of the T12 culture filtrate against the bean rust fungus seems to be rather small.

Three compounds (**6**, **7**, **8**) were found solely in the culture filtrate of T12 and may therefore have the greatest effect on *U. appendiculatus*. These compounds potentially are harzianolide (**8**) and the related compounds dehydroxy harzianolide (**6**) and T39butenolide (**7**). If compounds **7** and **8** are T39butenolide and harzianolide, respectively, questions arise why these compounds were not found in the culture filtrate of T39, because this strain produced both of them if cultivated for 31 days in PDB (VINALE *et al*., 2006). Maybe the short cultivation period of ten days during the experiments reported here was not sufficient for T39 to produce these compounds.

Interestingly, three compounds (**5**, **12**, **14**) were found for T-22 not reported by VINALE *et al*. (2006), but three metabolites reported by them, two anthraquinones and T22azaphilone, were not found in the experiments of this study. Both experiments differed strongly with regard to the cultivation period allowed for growth of T-22: ten days in the study reported here compared to 31 days in their experiments. Nonetheless, compounds **12** and **14**, which are solely found in the culture filtrate of T-22, may be responsible for the effect of the T-22 culture filtrate on germination and germ tube growth of *U. appendiculatus*.

The four compounds found in the culture filtrate of T_R (**1**, **3**, **5**, **15**) are of no interest with regard to bean rust control, because T_R was almost ineffective against the bean rust fungus. However, with respect to the very strong antibiotic effect of the T_R culture filtrate against *Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, and also against two *Fusarium* species (Chapter 2), the potential pyrone compound (**3**) and the potential trichothecene harzianum A (**15**), both being *T. harzianum* metabolites with known antifungal activity (CLAYDON *et al.*, 1987; CORLEY *et al.*, 1994), are very interesting. Of all six *T. harzianum* strains, T_R was the only strain with considerable activity against the *Fusarium* species (Chapter 2). Compounds **3** and **15**, which were only found in the culture filtrate of T_R, may be responsible for this effect.

Compared to the T_R culture filtrate, the 2-fold amount of compound **1**, which has the same mass as uracil, was detected in the culture filtrate of T_S. Because no antibiotic activity of this pyrimidine was reported (SIVASITHAMPARAM and GHISALBERTI, 1998), it seems to be unlikely that this compound confers antifungal activity against *U. appendiculatus* or other target fungi to the producing *T. harzianum* strain.

Three more compounds were found in the culture filtrate of T_S (**5**, **10**, **13**), with the same masses as dehydroxy harzianolide (**5**), harzianopyridone (**10**), and as a group of three similar antifungal metabolites (GHISALBERTI and ROWLAND, 1993): koninginin B, koninginin E, and seco-koninginin (**13**). As already discussed, compound **5** certainly confers no activity against the bean rust to the producing organism, and compound **10** most likely is not harzianopyridone, but still may be active in terms of antibiosis. Also the potential koninginin-compound (**13**) may be responsible at least for part of the effect of T_S on the bean rust fungus.

The T_U culture filtrate showed strong rust controlling activity (Chapter 3), but the resulting EA-extract did not. Therefore, compounds **2** and **4** cannot be responsible for the observed effect. Only in the T_U culture filtrate a compound with a mass corresponding to the mass of the best-known antifungal secondary metabolite of *T. harzianum* 6-pentyl-*α*-pyrone (6PAP) could be found. 6PAP is well-known for its coconut-aroma (COLLINS and HALIM, 1972) and its volatile character (CLAYDON *et al.*, 1987; COONEY and LAUREN, 1998; GHISALBERTI *et al.*, 1990). T_U culture filtrates had a distinct coconut-smell. Therefore it seems very likely that compound **4** is 6PAP. Confusingly, PDA cultures of T_U were the only ones that showed absolutely no antifungal activity due to any kind of volatile metabolite against *B. cinerea*, *P. ultimum*, or *R. solani* (Chapter 2). Maybe the amount of 6PAP produced by strain T_U when cultured on solid PDA medium was too low to exert any growth inhibiting effect towards the challenged phytopathogenic fungi.

The fact that no compounds with the same mass as 6PAP were found in the culture filtrates of T12, T_R, and T_S makes it apparent that the antifungal activity demonstrated by

these strains *in vitro* (Chapters 2 and 3) is based on other antifungal metabolites with volatile and non-volatile character. Strains T39 and T-22 are already known not to produce 6PAP (VINALE *et al*., 2006).

Concerning all the above, the potential harzianolide compounds (**6**, **7**, **8**) and the potential harzianopyridone (**11**) may be of special interest with regard to the antifungal activity of the T12 culture filtrate against the bean rust fungus. The same applies to compounds **12** and **14**, compounds **9** and **11**, as well as compounds **10** and **13** found in the culture filtrate of strains T-22, T39 and T_S, respectively. A test on the effect of the EA-extracts of the culture filtrates of these *T. harzianum* strains on the germination of *U. appendiculatus* would provide some additional information on the impact of their respective metabolites.

The ineffectiveness of the organic EA-extract of T_U on uredospore germination compared to the highly effective watery fraction demonstrates that the secondary metabolites which contribute the major part to the antifungal activity of this strain, can only be analysed following extraction with other solvents than HX or EA. Especially the highly antibiotic peptaibols (SZEKERES *et al*., 2005) have to be extracted with polar solvents like *n*-butanol. It is possible that peptaibols are responsible for the antibiotic activities of the T_U strain and its culture filtrate observed in Chapters 2, 3, and 4. Furthermore, also the T12 culture filtrate may contain peptaibols, because organic as well as watery fraction remaining after EA-extraction showed very similar antifungal activity against bean rust, indicating that only part of the responsible metabolites are described here.

In general, the results from most experiments in the previous chapters support the hypothesis that mixtures of secondary metabolites are the key element to the varying antifungal effects observed for the broad range of *T. harzianum* strain – target fungus – combinations presented in this thesis. Therefore it is necessary to repeat the present study with other solvents and to perfom preparative purification and identification of the potential metabolites conferring the antibiotic activity to the *T. harzianum* strains. Moreover a comparison of the secondary metabolite profiles of culture filtrates with the profiles of germinated spore suspension fluids would be helpful. These analyses can answer the question whether the effects of *T. harzianum* spore suspension and culture filtrate treatments on the bean rust fungus *U. appendiculatus* are caused by the same secondary metabolites.

6. Final Discussion

6.1 The diversity of antagonistic mechanisms and their impact on *Trichoderma*-mediated biological control

Scientific research on the antagonism of *Trichoderma* spp. towards other fungal organisms has revealed a range of very different antagonistic mechanisms responsible for *Trichoderma*-mediated plant disease control: (1) competition for space and nutrients (ELAD, 1996; SIVAN and CHET, 1989), (2) mycoparasitic activity (CHET *et al*., 1998) and (3) antibiosis (HOWELL, 1998), (4) the degradation of enzymes which enable phytopathogens to exploit plant tissue (ELAD and KAPAT, 1999; KAPAT *et al*., 1998) or (5) the degradation of substances of plant origin necessary for the elicitation of phytopathogenic activity (HOWELL, 2002), and (6) the induction of plant resistance against fungal, bacterial, or viral diseases (HARMAN *et al*., 2004).

It is possible to evaluate single mechanisms by *in vitro* interaction studies of specific combinations of antagonist and phytopathogen (Chapter 2), but generalization of these results for other *Trichoderma* strains, other pathogens, or other environmental conditions is nearly impossible (GHISALBERTI and ROWLAND, 1993; HANNUSCH and BOLAND, 1996; HARAN *et al*., 1996; Chapter 2).

The major part of this work focussed on the experimental evaluation of the antagonistic mechanisms employed by six *Trichoderma harzianum* strains. Within this broad conception, emphasis was given to the elucidation of the mechanisms responsible for the successful antagonism of *T. harzianum* strains T12 and T_U towards the bean rust fungus *Uromyces appendiculatus*.

This final chapter intends to give a conclusive discussion of the antagonistic mechanisms that were observed during the numerous experiments by which *T. harzianum* – pathogen – interactions were investigated.

6.1.1 Competition

In vivo, rhizosphere competence is a very important property of *Trichoderma* strains (AHMAD and BAKER, 1987; HARMAN, 2000). Attachment of developing *Trichoderma* mycelium to the root serves a double purpose: The *Trichoderma* strain grows in the vicinity of a source of nutrients and thereby is directly attached to the plant organ to be protected against infection by soil-borne phytopathogens. If a *Trichoderma* strain is able to

compete for this space and the secreted root-exudates, it may be a useful competitive BCA (SIVAN and CHET, 1989). The same is true for the application of *Trichoderma* spp. onto the leaf surface, where nutrients are scarce. If a *Trichoderma* strain can use those trace amounts of nutrients better or quicker than other fungal organisms, it may have the potential to outcompete foliar pathogens (ELAD, 1996).

In vitro experiments performed on nutrient agar can demonstrate the competitive ability of *Trichoderma* strains under rather artificial conditions, which in most cases can only imprecisely simulate the nutrient supply and the environmental conditions occurring *in vivo*.

In spite of these constraints, some interesting differences between the six *T. harzianum* strains used in this study and tremendous variations in the reactions of each respective *T. harzianum* strain towards the different pathogens were observed. This did not only demonstrate the varying potential of individual strains to compete with a particular pathogen, but also the great impact of the pathogen itself on the development of the interaction (Chapter 2).

In terms of bean rust control, no competition for space was noticed. Because bean rust spores do not need external nutrient supply to germinate and infect the bean leaf, competition for nutrients was also not responsible for the observed bean rust control (Chapters 3 and 4).

6.1.2 Parasitism

Parasitic interactions between *Trichoderma* strains and phytopathogenic fungi have often been observed microscopically (BENHAMOU and CHET, 1993, 1997, ELAD *et al*., 1983; GUPTA *et al*., 1999; INBAR *et al*., 1996; WEINDLING, 1932). A common event during such interactions is the coiling of the *Trichoderma* hyphae around the hyphae of the pathogen, thereby increasing the contact between its own hyphae and the pathogen's ones. The secretion of lytic enzymes and antibiotic metabolites leads to degradation of cell wall components and de-regulation of the metabolism of the affected pathogen's cells (LORITO *et al*., 1996).

Again, the reactions of varying *Trichoderma* strains towards a particular pathogen may differ and the reaction of a particular *Trichoderma* strain towards different pathogens may range from strong parasitism to failure in activity. In the *in vitro* experiments, most of the six *T. harzianum* strains were able to grow over and exploit the mycelia of *Pythium ultimum* and *Rhizoctonia solani*, but showed nearly no parasitic activity against *Botrytis cinerea*, possibly due to the antibiotic activity of the pathogen (Chapter 2).

Some variations were observable concerning the parasitic efficacy of the *Trichoderma* strains against the former two pathogens. The general mycelial growth rate of the *T. harzianum* strains also largely determined the speed of parasitism of the pathogenic mycelia. This led to differences in the speed of killing of the pathogens within the zones of mycelial interaction and overgrowth. In one case (T_R vs. *P. ultimum*), complete ineffectiveness was observed, highlighting the dependence of effective parasitism on the specific combination of antagonist strain and pathogen (Chapter 2).

Due to the very short period of bean rust germ tube growth before entering the intercellular spaces of the bean leaf, any parasitic interaction between *Trichoderma* strains and *U. appendiculatus* prior to infection, is absolutely unlikely. Because *T. harzianum* does not enter the intercellular spaces below the epidermal cell layer, a parasitic interaction within the leaf can be excluded (Chapter 3).

6.1.3 Antibiosis

Several hundred secondary metabolites produced by *Trichoderma* spp. are known (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al.*, 2005). These metabolites are most likely produced with the purpose of increasing the competitiveness of the producing *Trichoderma* strain towards other microorganisms (GHISALBERTI and SIVASITHAMPARAM, 1991; SZEKERES *et al.*, 2005). Although not associated with positive effects in all cases (LUMSDEN *et al.*, 1990; OUSLEY *et al.*, 1993, 1994) or from every perspective (RICARD and RICARD, 1997), antibiotic metabolites may confer an increase in biopesticidal activity to the producing BCA strain.

In correspondence to the two antagonistic mechanisms discussed above, the occurrence of antibiotic activity of *Trichoderma* strains largely depends on the strain itself, the growth conditions, and on the target fungus the *Trichoderma* strain is interacting with (SIVASITHAMPARAM and GHISALBERTI, 1998; VINALE, 2006). The ability to produce specific antibiotic metabolites may get lost (BREWER *et al.*, 1987) or may be gained by mutagenesis (GRAEME-COOK and FAULL, 1991), and the level of antibiotic production may vary between propagules of the same strain (WORASATIT *et al.*, 1994).

The culture filtrates of all six *T. harzianum* strains showed antifungal activity against a variety of phytopathogens, but great differences between combinations of different strains with the same phytopathogen were found. The antibiotic activity of a particular strain depended on the varying pathogens, but the variations between the *T. harzianum* strains were relatively clear-cut, allowing to rank the strains from the least effective T39 to the most effective T_R, nearly independent of the target fungi (Chapter 2).

Nonetheless, the antibiotic activity of a specific strain may be dramatically dependent on the pathogen. This was demonstrated by the T_R culture filtrate being by far the least effective in terms of inhibiting germination of bean rust uredospores (Chapter 3). This was a surprise considering the great efficacy of the T_R culture filtrate against all other target fungi (Chapter 2). On the other hand, these results perfectly fit to the results obtained in the assays on bean rust control by T_R spore suspensions. Both, spore suspension and culture filtrate of T_R were more or less ineffective against *U. appendiculatus* (Chapter 3).

Protective treatments with spore suspensions or culture filtrates of strains T12 and T_U before bean rust inoculation showed strong activity against *U. appendiculatus* in leaf disc assays, but no curative effect was observed when culture filtrates were applied after inoculation with bean rust uredospores (Chapter 3). This indicates that uptake of the rust inhibiting metabolites into the leaf tissue did not occur or that uptake was too low to negatively affect rust pustule development.

The efficacy of *T. harzianum* spore suspensions and culture filtrates was confirmed in greenhouse experiments. The antifungal effect of the applied *T. harzianum* agents was only slightly weakened when the time period between leaf treatment with T12 and T_U spore suspensions or culture filtrates and bean rust inoculation increased from one day to one week (Chapter 4). Resistance of the compounds, which are responsible for the antifungal activity of the *T. harzianum* agents against bean rust, towards heat, drought, and UV-irradiation is a critical factor regarding their long-term disease control efficacy and reliability. Therefore, the obtained results are promising.

Some evidence on the identity of the antibiotic compounds found in the culture filtrate where gained by the analysis of the profiles of ethyl acetate soluble secondary metabolites of the six *T. harzianum* strains. Some conclusions could be drawn in terms of including or excluding potential metabolites from the list of those compounds that might be involved in the control of the bean rust fungus by antibiosis. Based on the presented results, the best-known *T. harzianum* antibiotic 6PAP did not essentially contribute to the antifungal activity of *T. harzianum* against *U. appendiculatus*, while harzianolide and the two related compounds dehydroxy harzianolide and T39butenolide potentially produced by T12 are likely candidates for the antibiotic interaction (Chapter 5). Definite identification of the responsible metabolites in future approaches may gain powerful means of bean rust control derived from *T. harzianum* cultures.

6.1.4 Induced resistance

The induction of plant resistance towards fungal, bacterial, and viral diseases was observed for several *Trichoderma* strains (HARMAN *et al*., 2004). Because the experimental work in the greenhouse was focussed on only two *T. harzianum* strains (Chapter 4) which had been selected as being the most effective ones with regard to reducing bean rust infection in leaf disc assays and inhibiting germination and germ tube growth (Chapter 3), statements concerning the potential of *T. harzianum* to induce resistance in bean plants against *U. appendiculatus* can only be made based on the two selected strains displaying the strongest antifungal activity against bean rust.

The spatial separation of the site of resistance elicitation on the plant from the site of pathogen inoculation is a prerequisite to demonstrate systemic resistance induction. This was ensured (1) by *Trichoderma* treatment and rust inoculation on separate leaflets of the same trifoliate leaf or (2) by *T. harzianum* spore application to seed or substrate and leaf inoculation with bean rust uredospores.

Induced resistance was apparent if rust inoculation took place several days after treatment of adjacent leaflets with spore suspensions as well as culture filtrates especially of *T. harzianum* strain T_U. The compounds responsible for this activity as well as the nature of the resistance reaction remain to be elucidated in future research.

The treatment of seed or substrate with spore suspensions of the *T. harzianum* strains having the most pronounced antibiotic activity against bean rust gained no systemic resistance against *U. appendiculatus*, but negatively affected the development of bean plants (Chapter 4). Such growth inhibition of bean plants has already been described (GERLAGH *et al*., 1999), illustrating the potential phytotoxicity of some antibiotic *Trichoderma* metabolites (CUTLER *et al*., 1986).

6.2 On the usefulness of *Trichoderma*-derived biopesticides with antiobiotic activity

In general, not every antagonistic mechanism that can be observed and which effects can be measured in laboratory experiments contributes to the biocontrol ability of an investigated *Trichoderma* strain against a specific pathogen under less controlled environmental conditions in field experiments (HANNUSCH and BOLAND, 1996; HARMAN, 2006). In this respect, the production of secondary metabolites with antibiotic activity depends on the *Trichoderma* strain, environmental parameters as pH or temperature, and the colonized substrate (SIVASITHAMPARAM and GHISALBERTI, 1998; VIZCAÍNO *et al*., 2005). Thus it is very likely that antibiosis did not contribute to the control of *B. cinerea* by *T. harzianum* strain T39 (ELAD, 1996; ELAD and KAPAT, 1999), although this strain possesses the ability to produce antifungal secondary metabolites *in vitro* (VINALE *et al*., 2006; Chapter 2).

Additionally, the synergism between lytic enzymes and antibiotics (LORITO *et al*., 1996; SCHIRMBÖCK *et al*., 1994), which enhances the antagonistic activity of *Trichoderma* strains, is crucial with regard to the rather low concentrations of the involved metabolites naturally synthesized by *Trichoderma* strains. If individual metabolites are applied artificially, the concentrations needed for any antibiotic activity are significantly higher than if a mixture of metabolites acting synergistically is used for the purpose of inhibiting the growth of target fungi (LORITO *et al*., 1996). *In vivo*, *Trichoderma* strains employ such mixtures. Even very low concentrations of particular metabolites may have great importance for particular activities within this concept of synergism.

Concerning the vast amount of known secondary metabolites with antibiotic activity (SIVASITHAMPARAM and GHISALBERTI, 1998; SZEKERES *et al*., 2005), the high number of *Trichoderma* strains that are known to produce antibiotics, and the purpose, these compounds serve in nature (GHISALBERTI and SIVASITHAMPARAM, 1991; SZEKERES *et al*., 2005), it is unlikely that *Trichoderma* strains not producing antibiotics are the ones which are adapted best to their habitats, where they have to compete with a multitude of other microorganisms (NAAR and KECSKES, 1998).

Looked upon in the light of these facts, the general classification of *Trichoderma* strains as “plant strengtheners” is put into question. Regarding the restrictive regulations for disease control in ecological plant production systems, the use of *Trichoderma* strains producing antibiotics may raise objections.

In summary it can be said that from not observing antibiotic activity within a specific interaction between *Trichoderma* spp. and another microorganism under specific

environmental conditions, no conclusion can be drawn about the general inability of the *Trichoderma* strain to produce secondary metabolites with antibiotic activity.

With respect to the ethics of biopesticide application, it might be desirable to achieve successful control of plant pathogens by other antagonistic mechanisms than antibiosis (RICARD and RICARD, 1997). But from the scientific as well as from the practical point of view, control of rust fungi by means of *Trichoderma* spp. can be realized best by antibiosis.

In those terms, the application of living propagules in form of spore suspensions does not differ from the application of the culture filtrates of *Trichoderma* strains. If the rust controlling capability of both agents relies on the presence of the same antibiotic metabolites, applying the living BCA does only have a higher psychological value, making it easier to accept the inconvenient truth that the application of compounds with antibiotic activity to control rust fungi is more or less inevitable.

6.3 Closing remarks

Apart from the necessity of screening for an appropriate *Trichoderma* strain, that satisfies the demands in terms of disease reduction, resistance induction, or plant growth promotion, its application as a biological control agent or biopesticide has to fit into the economical and ecological concept of an integrated disease management system. First of all, costs of formulation, production, and utilization have to be within economically feasible parameters. Furthermore, *Trichoderma* preparations must be able to replace at least single chemical fungicide applications by an acceptable efficacy.

Especially for the application of *Trichoderma* spore suspensions to the foliage of plants, this is very doubtful. In the experiments it was necessary to keep relative humidity artificially high for several days after *Trichoderma* spore application. At relative humidity values normally occurring in the greenhouse of 50 to 60% rH, germination of *T. harzianum* spores and release of their antifungal metabolites did not take place (data not shown).

Therefore, application of culture filtrates, which act independently of relative humidity, are much more reliable. Another advantage of solely using the metabolite responsible for the inhibition of bean rust infectiousness, is the possibility to purify the molecule and to formulate a preparation which has all the beneficial physico-chemical properties of a conventional fungicide and contains the naturally bio-synthesized secondary metabolite of the respective *Trichoderma* strain at a concentration that gives disease control levels comparable to commercial chemical fungicides. In the case of *U. appendiculatus*, this means control levels higher than 90% reduction of disease severity (GENT *et al.*, 2001; STUMP *et al.*, 2000).

More concerns are aroused with respect to the antibiotic effect of beneficial microorganisms than if the biological activity was due to competition or parasitism. With respect to these concerns, biopesticides based on microorganism-derived compounds have to be evaluated carefully for possible unwelcome side-effects, which may pose a threat to the user, the environment, or the consumer (COPPING and MENN, 2000).

This study does not give any answers to the questions arising from these considerations. But what definitely can be pointed out is this: Some strains of *T. harzianum* are able to control the bean rust fungus by means of their secondary metabolites. Chemical analysis of these metabolites will broaden the spectrum of substances known to control the bean rust fungus and possibly other rust fungi, too. Such metabolites derived from selected *T. harzianum* strains may be useful future tools for both disease and fungicide resistance management.

7. References

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