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UNIVERSITA' DEGLI STUDI DI SASSARI

DIPARTIMENTO DI SCIENZE AMBIENTALI AGRARIE E BIOTECNOLOGICHE AGRO-ALIMENTARI

SEZIONE DI MICROBIOLOGIA GENERALE ED APPLICATA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE MICROBICHE

Biodiversity of the genus *Trichoderma* and identification of marker genes involved in the antagonism between *Trichoderma* spp. and plant pathogenic fungi

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CICLO XX
2004-2007

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Biodiversity of the genus *Trichoderma* and identification of marker genes involved in the antagonism between *Trichoderma* spp. and plant pathogenic fungi

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1. Introduction

Trichoderma spp. (Division *Ascomycota,* Order *Hypocreales,* Family *Hypocreaceae*, Genus *Trichoderma*) live in soils all over the world, in all climatic zone, and are characterised by their rapid growth and resistance to noxious substances. Usually they exercise a dominant role in the ecosystem of soil fungi (Danielson and Davey, 1973; Widden, 1979; Widden and Abitbol, 1980; Roiger *et al.*, 1991; Wardle at al., 1993; Klein and Eveleigh, 1998), as they are important decomposers of woody and herbaceous materials (Rossman, 1996). Fast growth and the ability to employ a vast variety of nutrients make them also necrotrophic against many primary wood decomposers like white and brown rot fungi of the order Basidiomycetes (Lisboa de Marco *et al.*, 2004). More recently, selected species of the *Longibrachiatum* clade have also become known as opportunistic pathogens of immunocompromised mammals, including humans (Kredics *et al.*, 2003).

1.1 Morphology

Morphological description of *Trichoderma* is not any easy task to undertake. The number of useful morphological characters, such as colour or pattern is quite limited for defining different species of *Trichoderma*. In 1969 Rifai attempted a first serious approach in a systematic description of *Trichoderma* species assigning them into nine "aggregate species", after addition of morphological characters deriving from a teleomorph of *Trichoderma*. In the 80s Bissett performed a re-identification of Rifai's aggregate species, classifying as five sections, two of them being monophyletic (Bissett, 1991a,b). In contrast to Rifai, Bissett almost did not consider teleomorph characteristics of *Trichoderma*. Finally the work of Samuels provided detailed observations and comments on the utility of morphological characters to define species in *Trichoderma* (Samuels, 1996), creating the basis for a reliable morphologically-based description of *Trichoderma* species. For this reason also today identifications based on morphological characters remain the primary method for identification and verification of species in *Trichoderma*, assumed at least when taxa have been described adequately in the literature.

The different useful characters in distinguishing *Trichoderma* species can be colony character, growth rates in culture, conidia production, pigments, odours, conidiophore branching and aggregation (Figures 1, 2). Some species grow with distinctable colony characters, hence it is not easy to sufficiently describe them all and therefor being useful for identifaction. Different species exhibit usually different growth rates and are also distinguishable by the way they produce conidiophores (Bissett 1991a, b; Samuels et al., 2006). Some do produce conidia from effused conidiophores and some do it from conidiophore aggregated in pustules or fascicles. Some species can be described by their diffusable pigments, like the strains of section *Longibrachiatum* producing bright greenish

yellow ones by first isolation. Others show red or a total lack of pigments. *T. aureoviride* releases characteristic crystals to the culture media. Characteristic aromatic odours resembling coconut are produced commonly by strains of *T. viride*, and sometimes also by *T. atroviride* (Bissett 1991a, b; Samuels et al., 2006).

Assignment of *Trichoderma* strains to sections and species aggregates is usually based on their pattern of conidiophore branching and aggregation into pustules and fascicles. Section *Pachybasium* strains possess characterisitc compact pustules. They usually show a quite regular conidiophore branching with broad and straight or narrow and flexuous branches. Some species of the section *Pachybasium* show a characteristic conidiophore apex, that is ending in a sterile straight or coiled elongation (Samuels *et al.,* 2006). Phialide shape is mostly characterisitic for every section. In the case of section *Pachybasium* phialides are short and plump. Section *Longibrachiatum* phialides instead show elongated, langeniform or even cylindrical forms. Phialides of most species end in a elongated and narrow terminus. Some species of section *Longibrachiatum* possess so-called aphanophialides (Gams, 1971), that is subterminal conidiophoric cells that producing conidia through a short lateral neck. Variations in conidia dimension of is not very vast, but the advertant observer can notice consistent slight differences in size. The conidial surface and shape ranges from smooth, rough (*T. viride* clade) or ornamented to globose, ellipsoidal, obovoidal or short-cylindrical. *T. saturnisporum* and *T. ghanense* produce conidia with wing-like or bullate projections of the outer wall. In the end also the pigmentation of conidia can be characterisitc for some species, varying from different green shades to colourless, or more rarely grey and brown. Characters obtained from the frequently present chlamydospores usually don't help too much in distinguishing at the species level and even less useful are the few characters shown by

the vegetative hyphae (Bissett 1991a,b).

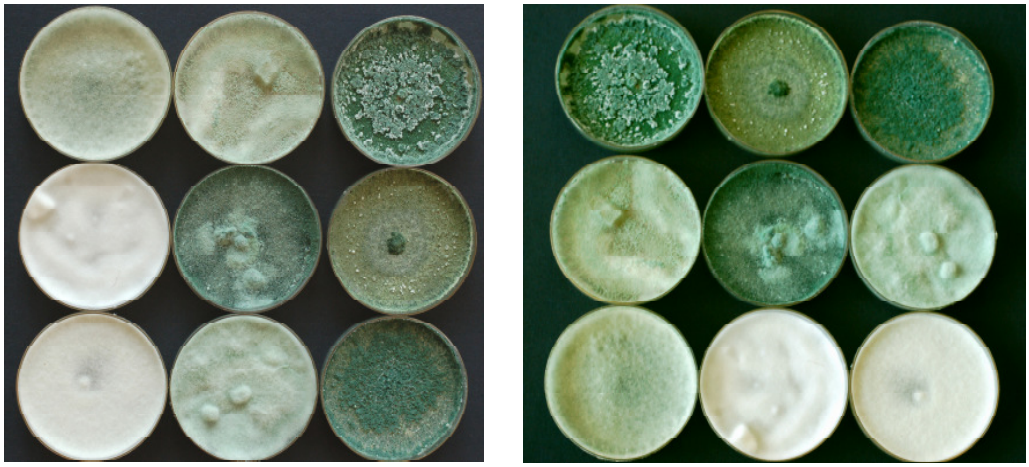

Figure 1:

Aspects of *Trichoderma* spp. in culture on potato dextrose agar (PDA)

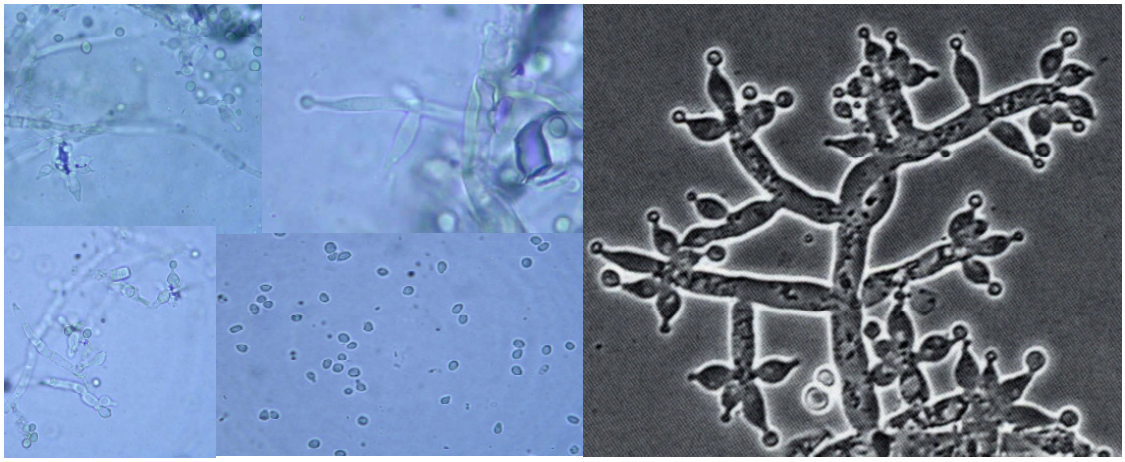

Figure 2:

Conidiophores of *Trichoderma* spp.

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1.2 Systematics, Taxonomy, Phylogeny

Trichoderma was described for the first time in 1794 by Persoon. As *Trichoderma* spp. imposed the problem of an accurate morphological characterisation, until the late 60s almost all strains were identified as *T. viride*, as proposed by Bisby in 1939 with his single species concept for *Trichoderma*. In contrast *T. viride* is a quite rarely found species, so that it has to be concluded that most of isolates at that time were other taxa and hence misidentified. In 1969 Rifai performed a revision of *Trichoderma* systematics in his thesis including also some *Hypocrea* species in his morphological considerations. He developed the concept of "aggregate" species. Rifai identified nine species aggregates, conceding that some of them contain more than one species which morphologically were not distinguishable. This concept, including more and more elaborated morphological characters, displayed the guideline for an accurate species description of *Trichoderma* for about 15 years. In the 80s and early 90s Bissett and Gams revised the aggregate species concept of Rifai by addition of more morphological characters and the inclusion of some species previously believed to belong to *Gliocladium*. Bissett's work ended up with the description of several defined taxa, divided into five sections: *Longibrachiatum, Pachybasium, Trichoderma, Saturnisporum* and *Hypocreanum* (Bissett, 1992). Molecular tools like DNA sequencing had their great breakthrough in the mid 90s and made available what was missing for so long – a great variety or number of characters applicable for the description of the taxa and their phylogeny (Kubicek *et al.*, 2003; Samuels, 1996). The sections described by Bissett were revised, but in general they were confirmed. The analyses were based on Random Amplified Polymorphic DNA (RAPD)-profiles, sequence analysis of the internal transcribed spacer ITS1 and 2 of rDNA and physiological analysis, and for the first time potential teleomorphs (*Hypocrea* spp.) were included where known and possible(Kullnig-Gradinger *et al.*, 2002;

Wuczkowski *et al.*, 2003). At the end, 89 taxa could be described, 14 holomorphs, 49 teleomorphs and 25 anamorphs. Section *Saturnisporum* was integrated to the monophyletic section *Longibrachiatum* including 10 taxa. *Longibrachiatum* represents a quite small section that is localised phylogenetically distant from the other sections. Sequence analysis of different genes confirmed that section *Pachybasium* is paraphyletic, leading to the subdivision of the section into two clades – *Pachybasium* A and *Pachybasium* B. The few taxa belonging to clade *Pachybasium* A are more closely related to taxa found in section *Trichoderma*, being now part as clade in this section. Clade *Pachybasium* B comprises several taxa - almost all the taxa that Bissett described for section *Pachybasium* - and remained section. Until now the name of one of the clades was not changed, leading easily to confusion. Continous work (Samuels, 2005; Druzhinia and Kubicek, 2005) on morphological description and research for suitable genes appliable to phylogenetic considerations led to the actual division of *Trichoderma*/*Hypocrea* spp. into 13 clades/sections (Figure 3) and further revision of the *Trichoderma*- and *T. koningii*- aggregate species (Samuels *et al.* 2006; Samuels, 2006) (Figure 4).

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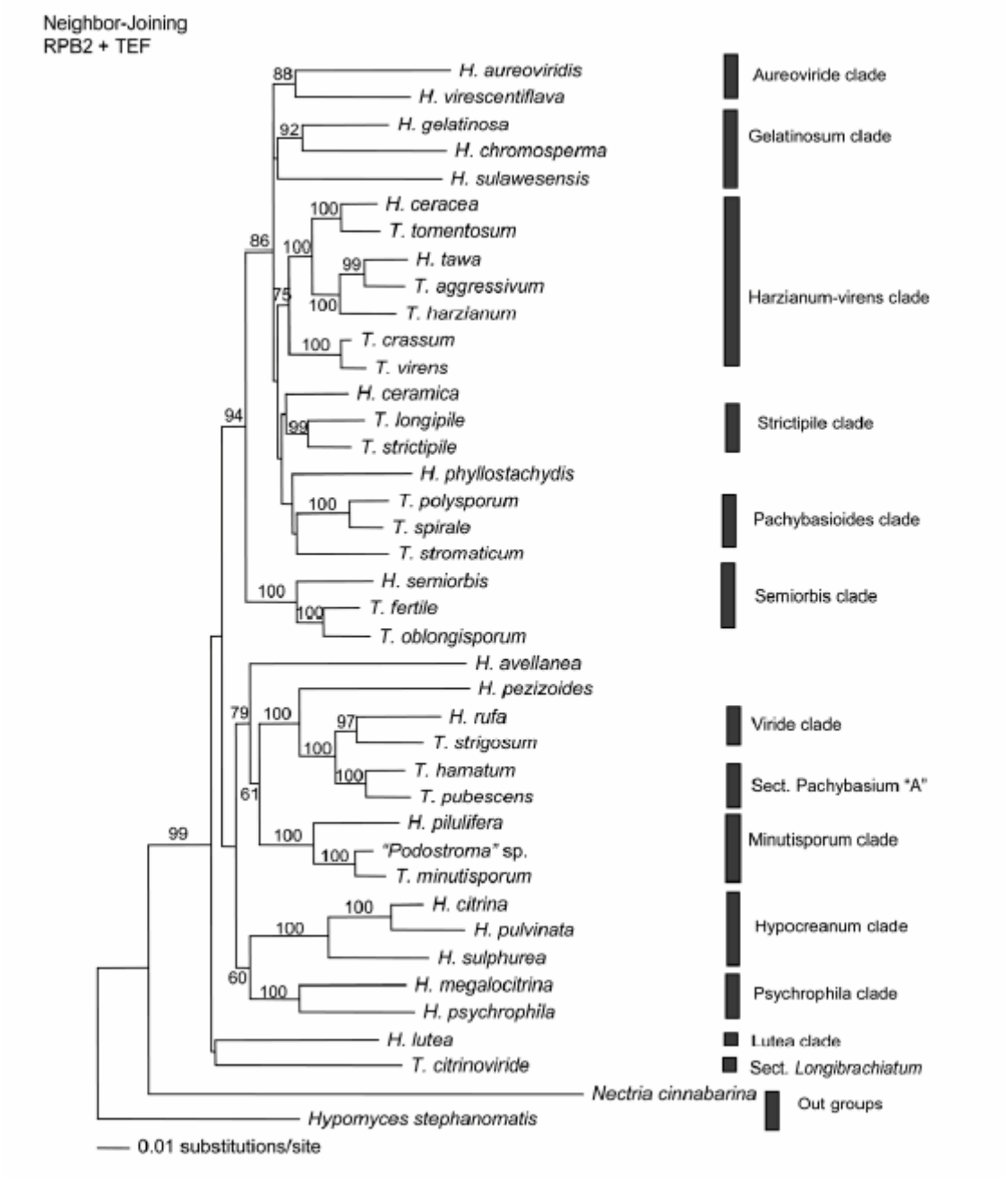

Figure 3:

Neighbour-joining tree with combined RPB and EF-1α data sets. Numbers at branches represent bootstrap values based on 1,000 replicates. (from Samuels, 1996:)

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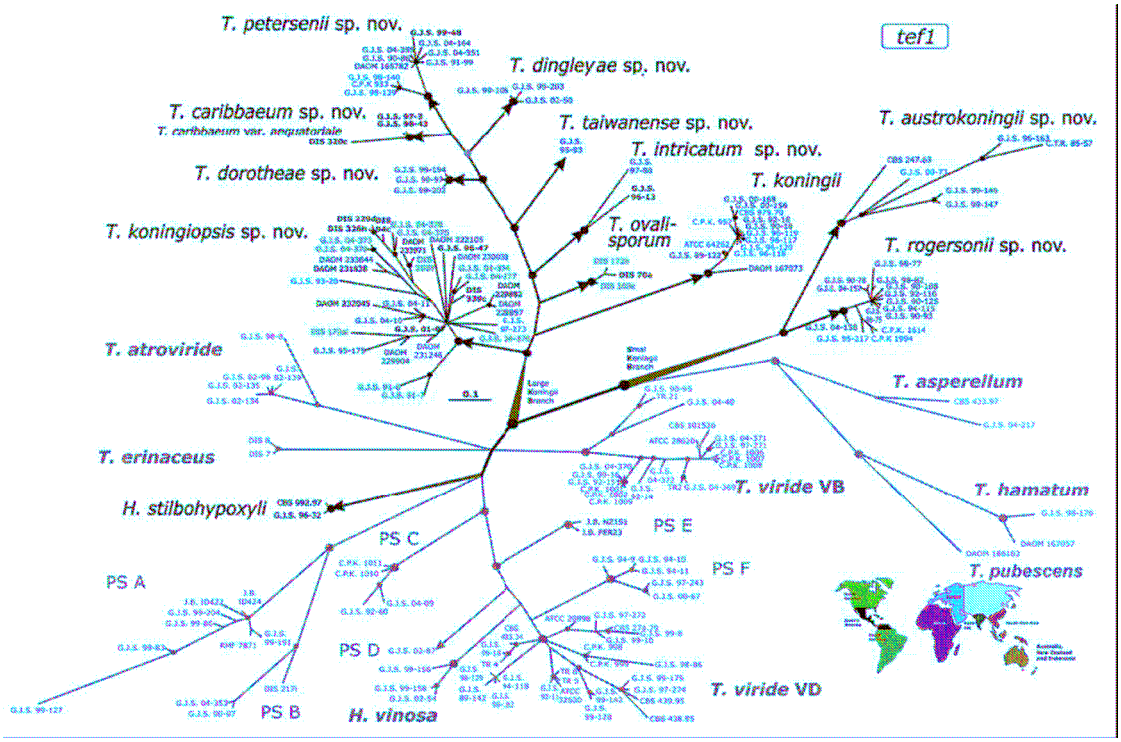

Figure 4:

Bayesian radial phylogram showing the structure of the *'Viride'* Clade as it was inferred based on sequences of two introns of *tef1*. Grey colour is used to separate specimens which are not discussed in this study but whose sequences were used to produce the multiple sequence alignment. Arrows indicate branches leading to species recognized within *T. koningii* aggregate species. In the highlighted part of the tree, grey filled circles at nodes indicate posterior probability coefficients higher than 0.90 as they were obtained after 5 million generations; black filled circles at nodes show support higher than 0.95. Font colours correspond to regions of sampling on the schematic map. Clades identified as "PS A–F" in the lower half of the tree represent undescribed phylogenetic species (from: Samuels *et al.*, 2006).

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1.3 Biocontrol

Modern agriculture is affected by different plant disease epidemics caused by the cultivation of one or a few crops creating an ecologically unbalanced system. The diseases are caused mainly by fungi, bacteria, viruses and nematodes that are mainly controlled by fungicides and pesticides. These chemical treatments select an increasing number of resistant strains and species that can be the basis for new break outs of the former "controlled/eliminated" disease. The nowadays informed costumer asks for agricultural products without chemical residues and a neat way of agriculture, that means without impact on men, on the environment or on the water quality. Agricultural industries are therefor looking for biologically and environmentally acceptable alternative methods of disease control, grouped in the name "biological control/biocontrol".

A definition of biological control was given by Cook and Baker in 1983: "Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man." A complete biological control program may range from choosing a fungicide which will be least harmful to beneficial microorganisms, the application of more resistant cultivars of the host or of less virulent variants of the pathogen, or microbial antagonistis to the plant disease pathogens.

The advantages of biological control methods is their possible use as part of an overall integrated disease management program to reduce the legal, environmental, and public safety hazards of chemicals. Applications usually are less frequent and much more safe in handling for the operating person. In addition, it may be a more economical

alternative to some fungicides. Some biological control measures can actually prevent, rather than limiting, economic damage to agricultural crops. Unlike most fungicides, biological controls are often very specific for a particular plant pathogen. Other helpful microorganisms, insects, animals, or people can go completely unaffected or disturbed by their use.

On the other hand biological control takes more intensive management and planning. It can take more time, it can require more record keeping, more patience, and sometimes more education or training. Successful use of biological control requires a greater understanding of the biology of both the pathogen and its antagonists. In some cases, biological control may be more costly than chemical treatment. Often, the results of using biological control are not as dramatic or quick as the results of fungicide use. The specificity of some biocontrol agents can represent also a disadvantage, requiring many targeted applications for a vast array of plant pathogens. Also abiotic and biotic factors like weather, disease pressure and competition from the indigenous microflora have a general impact on the biocontrol agent, probably causing less effective, inconsistent performances.

1.3.1 Biocontrol strategies

In the past, biocontrol strategies were developed on a "suck it and see" approach. Although this approach was successful, when control did break down, the reasons for the lack of control were not known. To address this problem it was important to develop biological control strategies based on a sound understanding of the biology of the plant pathogens and their natural enemies combined with the interactions between them and also with the plants themselves (Prabavathy *et al.*, 2005; Donzelli and Harman, 2001; Inbar and Chet, 1995; Kullnig *et al.*, 2000; Lorito *et al.*, 1996;, Ramot *et*

al., 2000; Carsolio *et al.*, 1999). Research focused on combining glasshouse and laboratory experiments with simulation modelling. The experimental work could provide information on the biology of the pathogens and their natural antagonists.

One interesting field to study is the phenomenon of disease-suppressive soils. Observed in many locations around the world, suppressive soils are those in which a specific pathogen does not persist despite favorable environmental conditions, the pathogen establishes but doesn't cause disease, or disease occurs but diminishes with continuous monoculture of the same crop species (Schrot and Hancock, 1982). Suppressive soils revealed as living laboratories where the complex interactions among microorganisms result in disease suppression and in addition they have proved to be sources of some important antagonists (Mazzola, 2002)

The difficulties in understanding the complex interactions of the approach to biological control by suppressive soils have led the researchers to instead introduce individual strains of microorganisms as biocontrol agents (Gielen *et al.*, 2004). Research has yielded some practical solutions to plant disease problems and resulted in the development of several commercially available biocontrol, bio-fungicide products. Although the mode of action is understood for relatively few biocontrol systems, research with specific antagonists has led to important discoveries about biocontrol mechanisms (Brunner *et al.*, 2005; Harman *et al.*, 2004; Yedidia *et al.*, 1999; Lorito *et al.*, 1996; Samuels, 1996).

1.3.2 Mechanisms of biocontrol agents

There are three main mechanisms by which one microorganism may limit the growth of another microorganism: antibiosis, mycoparasitism, and competition for resources.

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Antibiosis. Antibiosis is defined as inhibition of the growth of one microorganism by another as a result of diffusion of an antibiotic. Antibiotic production is very common among soil-dwelling bacteria and fungi, and in fact many of our most widely used medical antibiotics (e.g., streptomycin) are made by soil microorganisms. Antibiotic production appears to be important to the survival of microorganisms through elimination of microbial competition for food sources, which are usually very limited in soil (Velusamy *et al.*, 2006).

Unfortunately there is often little correlation between the ability of a microorganism to inhibit the growth of a pathogen in a Petri dish and its effectiveness in disease suppression in the field. Usually, large numbers of test microorganisms are screened in a plant bioassay for their ability to suppress disease, and those which are effective are studied further to figure out the mechanism of biocontrol (Vinale *et al.*, 2006).

Mycoparasitism. Another mechanism of biocontrol is destructive mycoparasitism. This refers to parasitism of a pathogenic fungus by another microorganism. It involves direct contact between the fungi resulting in death of the plant pathogen, and nutrient absorption by the parasite. The electron microscope has afforded us stunning views of mycoparasites coiled around the hyphal strands of pathogenic fungi. Mycoparasites produce cell wall-degrading enzymes which allow them to bore holes into other fungi and extract nutrients for their own growth (Inbar and Chet, 1992). But many so-called mycoparasites also produce antibiotics which may first weaken the fungi they parasitize.

Competition for resources. Microorganisms compete with each other for carbon, nitrogen, oxygen, iron and other micronutrients. Nutrient competition is likely to be the most common way by which one organism limits the growth of another, but demonstrating that this is actually responsible for biological control is quite challenging. (Jacobs and Gray, 2003). Occasionally, the specific object of competition is known. For

example, in most terrestrial habitats, microbial competition for the soluble form of iron, Fe^{3+}, is keen. Some fungi and bacteria produce very large molecules called siderophores which are efficient in chelating Fe^{3+}. Individual strains can have their own particular siderophores and receptors which can bind Fe^{3+} in such a way that the iron becomes inaccessible to other microorganisms, including pathogens. In some cases, siderophore production and competitive success in acquiring Fe^{3+} is the mechanism by which biocontrol agents control plant diseases (Benitez *et al.*, 2004).

1.3.3 Actual application fields

Research in the biocontrol field is making rapid progress in understanding the mechanisms of biocontrol, and several biocontrol products are now available for practical, widespread use in plant disease control. A particular intensive application of biocontrol agents has been developed for greenhouse systems where the intensive cultivation puts the workers at a continuous risk of fungicide exposure (Paulitz and Bélanger, 2001). As a consequence, many commercial fungicides are not applied creating a demand for alternative "healthy" treatments. In the defined area of the greenhouse, parameters like temperature and humidity are strictly reviewed and permit usually use of less inoculum than for treating a field. Therefore, biological control of plant diseases in greenhouses is a unique niche and attractive alternative to chemical pesticides. Biological control of greenhouse insects is already the predominant method in e.g. the United Kingdom, but the application of biological fungicides is still a developing market (Paulitz and Bélanger, 2001).

A large area of interest in biocontrol is the reduction of plant diseases caused by soil-borne and foliar plant pathogenic fungi. Roughly 70% of all the major crop diseases are caused by fungi, or the fungus-like Chromista (Deacon, 1997). Notorious examples are

species belonging to the genera *Rhizoctonia, Botrytis, Phytophthora, Pythium, Sclerotinia* and *Fusarium*. Most of the formulations of commercially available biocontrol products against plant pathogenic fungi contain the bacteria *Pseudomonas* and *Bacillus* or fungi belonging to the genus *Hypocrea/Trichoderma* (Paulitz and Bélanger, 2001).

The regulatory climate for biopesticides has improved, which has streamlined and accelerated the registration process. In the past years there has been a proliferation of companies interested in bringing new biocontrol products to the marketplace. Many of these companies are working with university researchers and other public sector scientists who have been encouraged to develop their biocontrol research to provide practical alternatives to chemical agents.

1.4 *Trichoderma* spp. and biocontrol

In the last years biocontrol of plant pathogens became more and more interesting and also feasible. The system offers also an alternative to chemical fungicides, bearing the risk of dependence by unidirectional orientation, pushing versus selection of resistant pathogenic strains and environmental pollution. Research on fungal species capable of controlling fungal plant pathogens led quickly to *Trichoderma* spp., the benefits of which in plant disease control are known since early 1930's (Weindling, 1934). As a consequence of the fact that application of the so-called biocontrol strains is hardly predictable, still in many cases a chemical treatment is the application of choice. In order to develop biocontrol agents that can compete with their chemical counterparts, studies are focused on obtaining a broad and merley complete knowledge of the biochemical events which determine the antagonistic interactions with the pathogen. (Prabavathy *et al.,* 2006; Harman *et al.*, 2004; Donzelli and Harman, 2001; Inbar and Chet, 1995; Lorito *et al.*, 1996) Characterisation and identification of proteins and

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pathways and their regulation involved in mycoparasitism would offer strategies to improve the reliability on *Trichoderma* as a biocontrol agent. There are several *Trichoderma* species as *T. harzianum, T. atroviride* and *T. asperellum* which seem particularly suitable for biocontrol applications.

Which factors made the genus *Trichoderma/Hypocrea* an exemplar candidate for biological control in the context of plant diseases caused by soil-borne fungi? The anamorphic fungal genus *Trichoderma* (*Hypocreales, Ascomycota*) is a cosmopolitan fungus that means that it can be more or less found in soils all over the world. In these soils *Trichoderma* is setting the tone regarding decompostion of wood and other forms of plant organic substrates (Samuels, 1996; Klein and Eveleigh, 1998). *Trichoderma* species have adapted to all kinds of climates (from tundra to tropics), making of them one of the most common fungi in the world. This overwhelming dominant role in the world of soil-fungi is probably due to their extreme flexible metabolic adaptation and their therefore quite aggressive competitive nature (Samuels, 1996; Klein and Eveleigh, 1998). All known *Trichoderma* species exhibit fast growth rates and produce high numbers of conidia that permit a vast dispersion. For many *Trichoderma* species, only the asexual status (anamorph) is known, hence they are classified as imperfect fungi. With molecular biology methods the number of teleomorphic *Hypocrea* that could be linked to their *Trichoderma* anamorphs is increasing (Gams and Bissett, 1998). As described in above chapters, classical morphological description of *Trichoderma/Hypocrea* is rather difficult due to the lacking number of morphological characters, but molecular phylogenetic markes facilitate a quite exact taxonomic identification of all the known species (Druzhinina and Kubicek, 2005).

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Many isolates/species of *Trichoderma* are capable to antagonise other fungi. Their repertoire goes from the above mentioned mechanisms like antibiosis and mycoparasistim to a forced and very straight competition for nutrients. But *Trichoderma* do not always only doing harm on fungal soil pathogenes, as for example *T. aggressivum* attack the commercially cultivated mushrooms like *Agaricus* and *Pleurotus* and is in this case a considerable pathogen (Seaby, 1998). The ability of *Trichoderma* spp. to metabolise even complex carbohydrates made them interesting for industry purposes as production of cellulases and hemicellulases by *T. reesei*/*H. jecorina* (Kubicek and Penttilä, 1998) or in the emerging fuel industry sector of bio-ethanol and bio-ethane production (Li *et al.*, 2007).

1.4.1 Biocontrol mechanisms of *Trichoderma/Hypocrea*

Since almost 80 years the beneficial effects of *Trichoderma/Hypocrea* species have been known and used in agriculture for fighting against soil-borne plant diseases (Weindling, 1934). Knowledge became more and more detailed over these years, leading to the detection/identification of several species particularly indicated in field application of disease control. An increasing number of new isolates exhibiting amazing biocontrol features are finding their way to commercially available formulations. Nowadays there are 15 products containing also *Trichoderma* species on the marketplace, the most known Trichodex™ (Makhteshim, Israel), Binab™ (Bio-Innovation, Sweden), Gliomix™ (Verdera Oy, Finland) and T-22G™ (Bio-Works, USA). Many of the products are not registered as biocontrol agents but only as growth promoters, in order to by-pass the restrictive standards that stipulate testing for their toxicology and their efficacy (Paulitz and Bélanger, 2001). Especially the prudential selection of a strain with a consistent performance in the field - including study possibilities in the laboratory or greenhouse

environment at one's hand - is more intricate as it seemed. For this reason research is focused on the study of the mechanisms *Trichoderma* employs to accomplish disease control. To achieve an optimal application of *Trichoderma/Hypocrea* for the control of plant diseases during cultivation and storage, a detailed understanding of the biocontrol agents' modes of action and their limitations is essential.

Antagonistic *Trichoderma* species "work" with a vast array of actions that contribute all together to their hight potential in biocontrol. They compete with the fungal pathogen for nutrients and space, they are able to break down noxious chemicals into innocuous components, they actively attack and destroy fungal pathogens and they enhance plant resistance by induction of plant systemic resistance.

Winning the competition race over all the other participants means mainly to be able to grow and to proliferate more quickly than the others. *Trichoderma* spp. can metabolize a broad spectrum of organic substrates, often difficult to decompose for other species (Lorito et al. 1996). They further produce many and long-living conidia that safeguard their survival in the long run (Hjeljord and Tronsmo, 1998). In addition they ensure their dominance in the respective soil, inhibiting the pathogen to grow by lowering the pH of the soil, producing antibiotics and by struggling the pathogen's spore germination with inhibiting metabolites (Benitez *et al.,* 2004). The *Trichoderma* species in their part seem to be less sensitive to similar attacks from the fungal pathogens. Ability to populate soil ecosystems as the dominant species by famishment of the enemy is a very effective tool as it is the most common cause for the death of microorganisms (Benitez *et al*, 2004).

Trichoderma/Hypocrea species which antagonise other fungi usually do attack them by actions of mycoparasitism (Figure 5). This requires a complex sequence of interactions

that can vary from species to species but roughly follow a common pattern. *Trichoderma/Hypocrea* antagonists are able to detect other fungi and then grow towards them; additionally the strains release extra-cellular enzymes as there are hydrolytic, cell-wall-degrading enzymes (Cortes *et al.,* 1998; Zeilinger *et al.*, 1999; Kullnig *et al.*, 2000) helping it to attach to the host, coil around the host hyphae, to form appressoria on the host surface and to start penetration (Inbar and Chet 1992; Rocha-Ramirez *et al.*, 2002). The next step in mycoparasitism of *Trichoderma/Hypocrea* spp. is the massive production of extracellular chitinases, glucanases, proteases and even antibiotic compounds like peptaibols (Schirmbock *et al.*, 1994; Lorito *et al.*, 1996; Szekeres *et al.*, 2005) that enable the mycoparasite to penetrate the cell wall and entry into the lumen of its host at thoses sites where it formed appressoria (Schirmbock *et al.*, 1994; Lorito *et al.*, 1996; Szekeres *et al.*, 2005; Inbar and Chet, 1992).

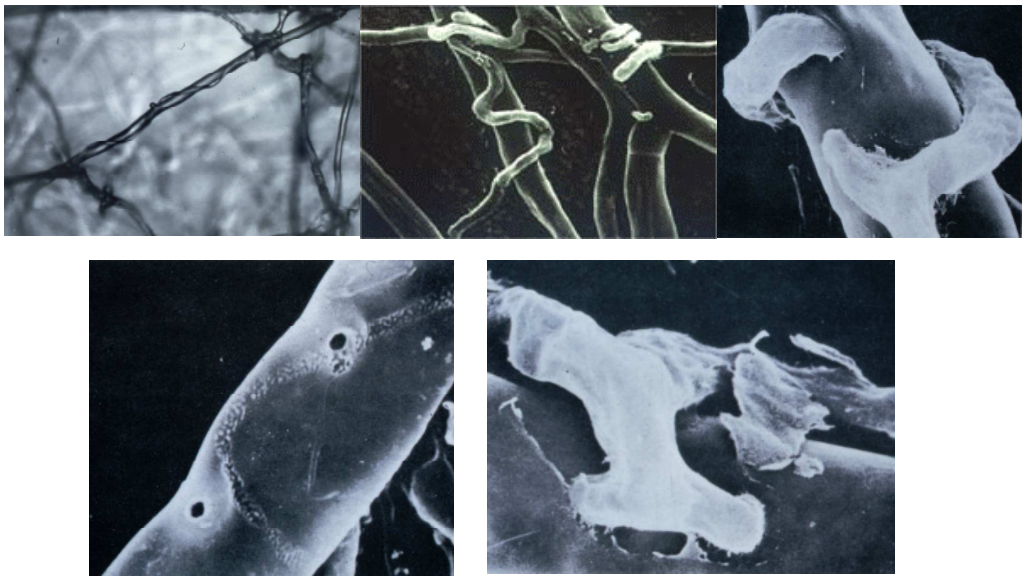

Figure 5:

Trichoderma spp. attacking *R. solani*, by directed growth, coiling around the hyphae, forming appressoira and penetrating the host.

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1.4.2 Lytic enzymes of *Trichoderma/Hypocrea*

One reason for the astonishingly remarkable fast and efficient exploitation of almost any organic compound and therefor nutrients of *Trichoderma/Hypocrea* species, is their enormous equipment of lytic enzymes. Organic matters consist of a wide range of sugars, homo- and heteropolysaccharides. Proteases do further help to damage other microorganisms which could compete with them. Several hydrolytic enzymes of different antagonistic *Trichoderma/Hypocrea* species have been cloned and their role and regulation in mycoparasitism was characterised. The complete sequencing of the *T. reesei/H. jecorina* genome provided finally the data for a detailed study on the genes encoding for those enzymes and on how they are regulated. Also the *H. atroviridis* genome is almost completely sequenced, creating a broad basis for further studies on genes involved in biocontrol mechanisms.

Chitinases. Chitin is one of the most abundant polymers in the biosphere and its degradation is involved in many biological processes. Chitinolytic enzymes are found among all kingdoms, e.g., protista, bacteria, fungi, plants, invertebrates and vertebrates (Cabib, 1987; Gooday, 1990; Sahai and Manocha, 1993). Lytic degradation of this (1-4)-β-linked homopolymer of N-acetyl-D-glucosamine by mycoparasitic fungi concern also the relationship between the fungus and the plant (St. Leger *et al.*, 1987; Mauch *et al.*, 1988).

Chitinolytic enzymes can be divided into exo- and endo-acting enzymes based on their reaction end products and catalytic mechanism. β-N-acetylglucosaminidases (NAGases, EC 3.2.1.52) catalyse the hydrolysis of terminal non reducing N-acetyl-D-glucosamine (GlcNAc) residues and transglycosilation reactions. Polymer chemistry industry uses

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these enzymes to synthesize regio- and stereo-selective polymers (Kobayashi *et al.*, 1997).

Additionally to the exo-acting NAGases, endo-β-N-acetylglucosaminidases (EC 3.2.1.96) exist, which catalyse the hydrolysis of the N, N'-diacetylchitobiosyl unit in high-mannose glycopeptides and glycoproteins containing the [Man(GlcNAc)2]Asn-structure, with one GlcNAc residue remaining attached to the protein and the rest of the oligosaccharide being released intact (Horsch *et al.*, 1997).

Chitinases (EC 3.2.1.14) catalyse random hydrolysis of N-acetyl-β-glucosaminide 1,4-β-linkages in chitin and chito-oligomers according to an endo-mechanism with (GlcNAc)2 and some (GlcNAc)3 as the only end products.

Glucanases. Chitin and β-1,3-glucan are the principal modules fungal cell walls are made of and *Hypocrea/Trichoderma* possess a range of chitinases and β-1,3-glucanases which do the main work in the degradation of the latter (Mahadevan and Tatum, 1967). It seems that - after breaking down the obstacle of the pathogens' cell wall – *Trichoderma* spp. are able to prevent the pathogen from germination or further growth by the release of β-1,3-glucanases that collaborate with chitinases and antibiotics (Benitez *et al.*, 2004). Some of the β-1,3-glucanases have been isolated and cloned so far, as *bgn13.1* (de la Cruz *et al.*, 1995) and *lam1.3* (Cohen-Kupiec *et al.*, 1999) from *H. lixii*, *glu78* from *H. atroviridis* (Donzelli *et al.*, 2001) and *Tv-bgn1* and *Tv-bgn2* from *H. virens* (Kim *et al.*, 2002). However an effective antagonist needs further glucanases to degrade other structurally important cell wall components like β-1.6-glucan, α-1,3-glucan, or faced with chitin as only carbon source. So far three β-1.6-glucanases from *H. lixii* (BGN16.1, BGN16.2, BGN16.3) have been purified and are believed to be essential in the fungal cell wall attack, rich of chitin (Montero *et al.*, 2005; de la Cruz and Llobell, 1999; Delgado-Jarana *et al.*, 2000). The working object of α-1,3-glucanases are glucose polymers linked by a-

1.3-glucosidic bounds. The α-1,3-glucanases can be divided into endo- (final degradation product is a small glucose polymer) and exo-glucanases (final degradation product is a glucose monomer). Studies on exo- α-1,3-glucanases *agn13.1* and *agn13.2* from *H. lixii* proved their role in mycoparasitism (Fuglsang *et al.*, 2000; Ait-Lahsen *et al.*, 2001; Sanz *et al.*, 2005).

Proteases. Several different extracellular proteases have been isolated from *Hypocrea* and *Trichoderma* (Antal *et al.*, 2001; Delgado-Jarana *et al.*, 2002; Williams *et al.*, 2003; Suarez *et al.*, 2004). They seem to be another important set of enzymes enabling a well-organised biocontrol reaction. It was shown that proteases of *H. lixii* could deactivate to some extend hydrolytic enzymes released from pathogens as *B. cinerea*, resulting in reduced germination activity by the pathogen (Kapat *et al.*, 1998; Elad and Kapat, 1999). Besides deactivation of plant pathogens' enzymes, proteases may be important for the mycoparasitic process by degrading the protein components of the host well wall. In order to better understand the way of reaction and involvment of protease in mycoparasitic interaction some proteases of *Trichoderma/Hypocrea* spp. have been cloned. *Prb1* protease of *H. atroviridis*, like its orthologues from *H. virens* and *T. hamatum* (Pozo *et al.*, 2004; Steyaert *et al.*, 2004), is a subtilisin-like serine protease and was shown to play its part in mycoparasitism of *R. solani* (Geremia *et al.*, 1993; Flores *et al.*, 1997; Cortes *et al.*, 1998; Olmedo-Monfil *et al.*, 2002). Expression experiments based on interaction with fungal cell walls, confrontation assays with pathogens, or carbon or nitrogen as limiting factors showed the upregulation of *pra1*- (trypsin-like serine protease), *papA*- (aspartic protease), *papB*- (vacuolar aspartic protease), and an extracellular aspartic protease (Suarez *et al.*, 2004, 2005; Delgado-Jarana *et al.*, 2002; Viterbo *et al.*, 2004).

Cellulases, Xylanases and other hydrolytic enzymes. The big family of cellulases, which are in their chemical class β-1.4-glucanases, consits of exo-(cellobiohydrolases) and endo- β-1,4-glucanases and β-glucosidases and their various isozymic forms. The industrial interest in *H. jecorina*, in order to easily produce cellulases or to express heterologous proteins, made the cellulytic system of this fungus to a well-studied object. As cellulose is not the major cell wall component of plant pathogenic fungi, the importance of this system in biocontrol was put aside for a long time. Recent studies applying *H. jecorina* mutants overexpressing cellulases showed a clear mycoparasitic effect on the oomycete *Pythium*, possessing cellulose in its cell wall, and even on *R. solani* (Schmoll and Kubicek, 2003). Migheli *et al.* (1998) overexpressed the cellulase egl1 in *T. longibrachiatum* and obtained transformants with increased biocontrol activities.

Xylanases like endoxylanases and β-1,4-xylosidases represent another set of industrial interesting enzymes mainly applied in the paper industry (Buchert *et al.*, 1998). These enzymes can degrade the heteropolysaccharides β-1,4-xylans that are composed of a backbone of β-1,4-linked xylopyranosyl residues with L-arabinose, D-glucuronic acid, ferulic acid and coumaric acid as their side groups (Kulakarni *et al.*, 1999). Like for other hydrolytic enzymes as galactosidases, pectinases or mannosidases, the role of these extracellular enzymes in mycoparasitism and hence in biocontrol is still not clear.

1.4.3 Trichoderma-induced defence related reactions of plants

Trichoderma/Hypocrea spp. as for example *T. harzianum/H. lixii* were shown to improve plant growth and root development (Harman *et al.*, 2004). The strains colonize the root surfaces as opportunistic, avirulent plant symbionts where production of different compounds induces the pathway of systemic resistance in plants (Harman *et al.*, 2004). The release of phytoalexins by the plant, an antimicrobial compound, was shown to be

increased in presence of *Trichoderma* spp. Additionally, proteome analysis of *H. lixii* identified homologues of the avirulence genes *Avr4* and *Avr9* from *Cladosporium fulvum*. The protein products of avirulence genes have been identified in a variety of fungal and bacterial plant pathogens. They usually function as race- or pathovar specific elicitors that are capable of inducing hypersensitive responses and other defense-related reactions in plant cultivars that contain the corresponding resistance gene (Harman *et al.*, 2004).

2. Aim of the study

As Mediteranean islands are known for their high divesity of vascular plants and a high level of endemism, they can provide basic insights into the relationship between geographical patterns and ecological processes. Studying the soil saprotrophic fungus *Trichoderma/Hypocrea* is of special interest to our working group which is active on the field of plant protection. The reason for this interest resides in its world-wide distribution, the continous on going identification of new isolates, and its high percentage of isolates being effective biocontrol agents.

The current study concerning *Trichoderma/Hypocrea* spp. in Sardinia tried to give answers to the following questions regarding these fungi: How is the diversity and distribution of the soil saprotrophic fungus *Tricoderma/Hypocrea* on the Sardinian island? Can we find many or even new species capable for efficient biocontrol on plant pathogenic fungi? Can we identify special genes involved in the mycoparasitic antagonism of *Trichoderma* spp. with plant pathogenic fungi?

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During the present PhD project, 482 *Trichoderma* spp. isolates were identified and characterised, and 222 of these isolates were successively tested on their biocontrol capacities by greenhouse experiments. A gene library was created by the Rapid Subtraction Hybridisation (RaSH) approach, permitting identification of genes especially expressed by *T. harzianum* during antagonism with the common plant pathogenic fungus *Rhizoctonia solani* Kuehn. For this purpose *T. harzianum* and *R. solani* mycelia were placed to interact with each other to provide the basis of cDNA material. In addition, the use of the available genome sequence of the closely related fungus *Hypocrea jecorina (T. reesei)* for sequence identification, allowed the identification of a high proportion of clones with high probabilities.

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3. Materials and Methods

3.1 Sample sites and sampling procedure.

Composite soil samples were collected in the period 2003-2006 at 15 sites in Sardinia, Italy (Figure 6). Soil samples (approximately 1000 g) were collected at the ends of two 20 m perpendicular transects and from the intersecting point at each site (Summerell *et al.,*1993), by using a weeding hoe to a depth of 5-15 cm. The location of transects was selected in a way avoiding the rhizosphere of big plants. The samples for each site were separated in two subsamples: one for the chemical analyses and the other for identification of *Trichoderma* spp.. The soil collected was stored in paper bags at +4°C after they were sieved, air dried and mixed thoroughly.

Figure 6:

Soil sampling sites included in this study.

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3.2 Characterisation of soil properties

Soil samples were passed through a 0.2-cm sieve to separate coarse fragments and soil. Soil fractions were analyzed for texture, i.e. percentage composition of sand, silt, and clay, by using the Bouyoucos mechanical method (Day, 1965). The percentage of soil organic carbon and soil organic matter in each sample was determined according to Nelson and Sommers (1996).

3.3 Strains – isolation and storage

Isolation of *Trichoderma* spp. The soil dilution plate method was used for isolation of *Trichoderma*. Approximately 200 g of soil from each soil sample were sieved through a stainless steel wire mesh (710 μm diam) and three 1-g sub-samples of soil were suspended in 99 ml 0.05% water agar, giving a 1:100 dilution; two successive dilutions (1:1000 and 1:2000) were prepared and aliquots of 1 mL of each suspension were evenly spread on 90-mm diam Petri plates containing *Trichoderma* selective medium (TSM, Elad *et al.,*1981), and on potato dextose agar (PDA; Merck & Co., Whitehouse Station, NY, USA) amended with streptomycin sulphate and oxytetracycline hydrochloride (100 + 100 μg mL^{-1}; Sigma, St. Louis, MO, USA). Plates were incubated under near ultra violet light with a 12 h photoperiod at approximately 25°C. Three plates per suspension were prepared, giving a total of 27 plates per sample and per substrate (TSM or PDA; Figure 7). After 7-10 days, all colonies resembling *Trichoderma* were transferred to PDA plates and incubated as above. *Trichoderma* colonies were subsequently subcultured using the single spore technique (Figure 8) and stored at 4°C until DNA extraction.

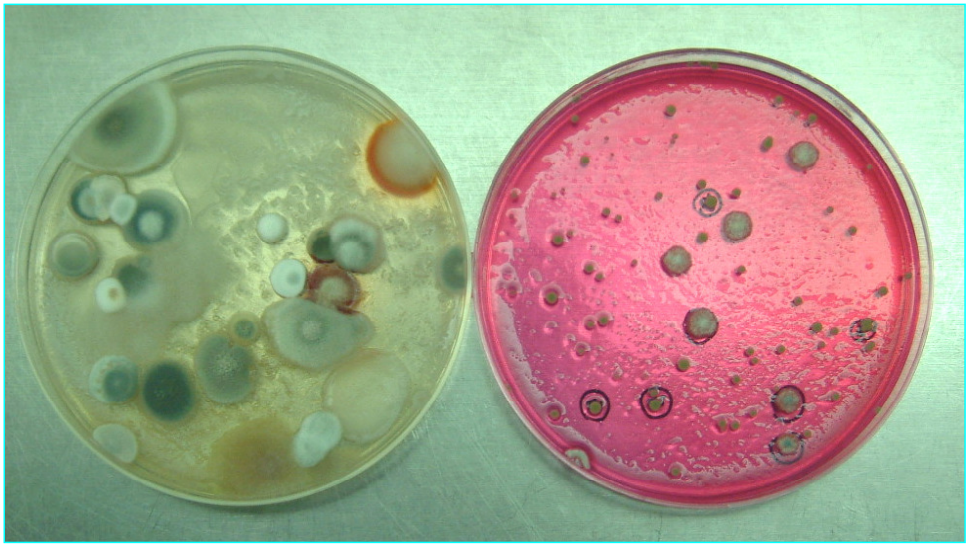

Figure 7:

Isolation of *Trichoderma* spp. on PDA and *Trichoderma*-selective medium.

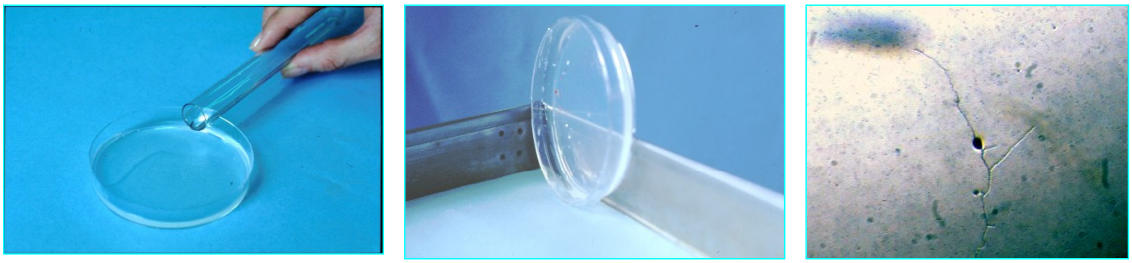

Figure 8:

Setting up pure cultures using the single spore technique.

Long-term storage. For long-term storage, plugs (0.5 cm diam) were cut from PDA cultures and kept in 50% glycerol at –80°C. All the isolates described in the present work are conserved in the fungal collection of the Centre for Biotechnology Development and Biodiversity Research, University of Sassari, Sardinia, Italy. Representative species have been deposited at CBS, Utrecht, The Netherlands

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3.4 Greenhouse experiments testing the pathosystem *Vicia faba/R. solani/Trichoderma.*

Greenhouse experiments. A total of 222 isolates, illustrating a representative number of isolates, were tested for their ability as antagonists against *R. solani* by greenhouse experiments. Using a sterile cork borer, plugs of 15mm in diameter were cut from 7-day-old cultures of *Trichoderma* spp. and *R. solani* grown at 25°C and potato dextrose agar (PDA, Difco, Becton Dickinson, Franklin Lakes, NJ). One plug of *Trichoderma* was paired with one plug of *R. solani*, by placing the mycelia in direct contact (Figure 9). Plug pairs were incubated in the dark at 25°C for 24 h and then transferred to the centre of plastic (4.5 cm in diameter, 55mL capacity, one plug per pot), and covered by a 2 cm layer of sterilised (121°C for 60 min on 2 successive days) potting mix (Humin-Substrat N17, Neuhaus, Germany) (Figure 10). For each treatment, three replicates (10 seeds for each replicate) were incubated on a bench in a glasshouse for 7 days before *Vicia faba* seeds (one seed per pot) were added. Pots were watered daily and the average temperature was 25–20°C (min 10–15°C, max 25–35°C). Seedling emergence was checked weekly after 7–21 days. After the last survey, the healthy plant stand was assessed.

After 21 days we were able to distinguish between antagonistic isolates that allow the test plant to grow, and non-antagonistic strains, which didn't lead to seed germination.

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Figure 9:

Paired agar plugs of *Trichoderma* spp. and *Rhizoctonia solani* mycelia that served as inoculum to infest sterilised substrates in the greenhouse experiments.

As controls test plants were sown in untreated sterile substrate and in substrate infested exclusively with *R. solani*.

Figure 10:

Infestation of sterilised substrate with agar plugs consisting of potential biocontrol *Trichoderma* isolates interacting with *Rhizoctonia solani*.

Disease index. For each host-pathogen combination the following parameters were taken into account: emergence, height and severity of infection – as recorded on the seedlings 21 days after the infection. A range of four classes of infection severity (from

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0 to 3; Figure 11) was defined on the basis of the extension of the symptoms on the seedlings and disease index was calculated as described by McKinney.

$$\sum \left[\frac{(\quad v \times n \quad)}{N \times V} \right] \times 100$$

v: valore di classe; n: numero di casi osservati per ciascuna classe; N: numero totale di casi osservati; V: valore massimo di classe

Figure 11:

Vicia faba plants exhibiting the different disease indices considered in final analysis of crops in the biocontrol trials in the greenhouse.

3.5 DNA isolation, M13-based RAPD-PCR, ITS-TEF sequencing

DNA isolation. Fungal genomic DNA was purified from fresh mycelium by following a standard method (Aljanabi and Martinez, 1997) as described: approximately 100 mg of fresh mycelium was homogenised in a 1.5 mL reaction tube using a pestel in 450 µL

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of extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2.0 mM EDTA pH 8.0, 400 μg mL^{-1} proteinase K, 2% sodium dodecyl sulfate). Samples were incubated for 1 h at 65°C. After adding 300 μl of saturated NaCl solution, samples were briefly vortexed and centrifuged at 10,000 x *g* for 30 min, the supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and then precipitated with one volume of isopropanol. The pellet was rinsed with 70% ethanol, resuspended in TE, pH 7.5 (Maniatis *et al.*, 1982) and stored at 4°C. All reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Species identification. Isolates were pre-screened by RAPD-PCR (Williams *et al.,*1990) applying the M13 primer (5'-GAGGGTGGCGGTTCT-3') as described by Turner *et al.* (1997). The final concentrations of the 25 μl-PCR reaction mix consisted in 1x REDTaq PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of primer M13, 1 unit of REDTaq™ DNA polymerase and 50-100 ng of template DNA. The PCR reaction was performed with a GeneAmp PCR Systems 9700 thermocycler (Applied Biolabs) as follows: an initial degradation step at 94°C for 2 min, followed by 40 cycles comprising 1 min at 94°C, 1 min at 37°C and 2 min at 72°C, to be then concluded by a final elongation step at 72°C for 10 min. PCR products were separated on a 1.5% Tris-Acetate-EDTA (TAE) agarose gel (5 V cm^{-1}, 50-60min), transferred to a UV-transilluminator (Gel Doc 2000; Biorad, CA, USA) where their images were photographed and recorded. Isolates from the same sample, and sharing a similarity of > 98 % (calculated as similarity index according to Nei and Li (1979), as given in Turner *et al.* (1997) were considered to be the same species, and only one of them was

further analysed. To this end, a fragment of the nuclear rRNA comprising the internal transcribed spacer regions 1 and 2 (ITS 1 and 2) and the 5.8S rRNA gene was amplified using primers its1 (TCCGTAGGTGAACCTGCGG) and its4 (TCCTCCGCTTATTGATATGC). PCR reaction mix was set up with following final concentrations: 1x REDTaq PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin), 0.2 mM dNTPs, 1µM each primer, 2 units of REDTaq DNA polymerase and 50-100 ng of template DNA. The polymerase chain reaction was carried out in a GeneAmp PCR Systems 9700 thermalcycler (Applied Biolabs) with 3 min at 95°C as initial degradation step, followed by 33 cycles composed of degradation at 95°C for 30 sec, annealing at 48°C for 45 sec, and elongation at 72°C for 60 sec. The run was concluded with a final elongation step at 72°C for 7 min in order to enable the enzyme to complete correctly all created fragments. PCR-amplified fragments were separated and detected as described above. In addition, the fourth large intron of the translation elongation factor 1-alpha (*tef1*) gene was amplified if necessary as described by Druzhinina *et al.* (2004) (primer: EF-728F and TEFLLErev). Automated sequencing was performed with an ABI Prism 3100 DNA Sequencer (Applied Biosystems, Norwalk, CT, USA) at the sequencing core facility C.R.I.B.I. – Bio Molecular Research at the University of Padova, Italy.

For species identification, ITS 1 and 2 sequences were subjected to analysis by *Trich*OKEY (http://www.isth.info/tools/molkey/index.php; Druzhinina *et al.,* 2005). In ambiguous cases, usually common for Section *Trichoderma*, the result was re-checked by analysis of the large intron of *tef1* gene sequence using sequence similarity search (SSS) against a database of type sequences implemented in *TrichoBLAST* (www.isth.info/tools/blast, Kopchinskiy *et al.,* 2005). For analysis of unusual ITS1 and

2 or *tef1* alleles, sequences were automatically aligned with GeneDoc 2.6.002, manually edited and inspected by eye. Potentially unique alleles were then confirmed by SSS against NCBI GenBank, and a database of fungal strains of Vienna University of Technology which currently contains more than 2700 *Hypocrea/Trichoderma* strains with more than 3300 sequences. A haplotype was considered to be unique if at least one allele (ITS1 and 2 or *tef1*) did not occur in any other strain isolated outside of Sardinia.

3.6 Rapid Subtraction Hybridisation – RaSH

Fungal culture conditions. All gene expression studies were carried out with a potent biocontrol strain of *T.*) *harzianum* (T 4.67), a *T. harzianum* strain incapable of biocontrol (T4.31) and *R. solani* strain AG-4. For the subtractive library experiments, T4.67 and *R. solani* were pre-grown as liquid cultures in PDB for 3 days at 25°C. Mycelia were washed 3 times with sterile water and transferred onto new medium in order to induct expression of genes involved in the interaction between antagonistic *Trichoderma* and *Rhizoctonia*. The experiment was set up as follows (Figure 12): washed mycelium of T4.67 was transferred on (i) agar plates of synthetic medium (Yedidia *et al.*, 1999) containing glucose [SM+glucose, consisting of (g l^{-1}): $(NH_4)_2NO_3$, 1.0), $MgSO_4x7H_2O$, 0.2, K_2HPO_4, 0.9, KCl, 0.15, $FeSO_4x7H_2O$, 0.01, $ZnSO_4x7H_2O$, 0.009, $MnSO_4xH_2O$, 0.007, glucose, 30] or (ii) agar plates of synthetic medium with no glucose but with a layer of washed *R. solani* mycelium (SM+*Rhizoctonia*). *Trichoderma* mycelia were separated from the solid media (SM+glucose and *SM+Rhizoctonia*) by a sterile sheet of cellophane (Model 583 gel dryer, BIO-RAD, CA, USA) that allowed the interaction and metabolic exchange between fungi and media. Cultures were incubated at 25°C.

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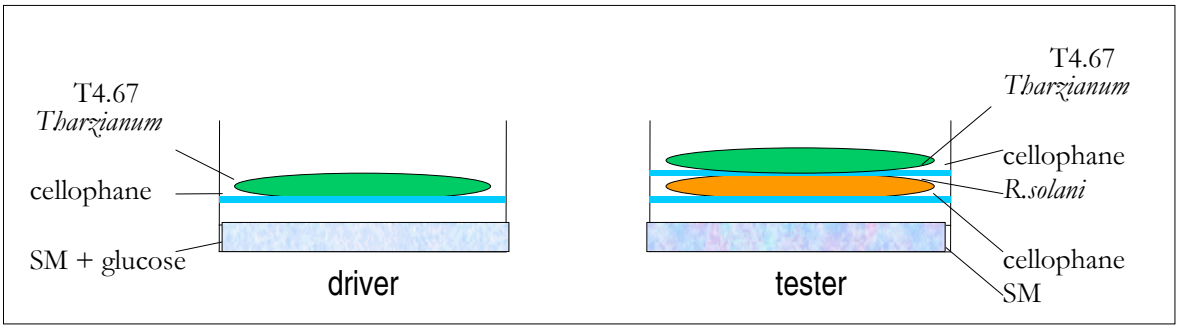

Figure 12:

Culture conditions of *Trichoderma* spp. and *R. solani* permitting interaction between the strains.

Bacterial culture conditions. Bacterial cultures of *Escherichia coli* DH5α or JM109 were grown at 37°C on Luria-Bertani (LB) medium as described by Sambrock *et al.* (1989) supplemented with ampicillin (100 µg L^{-1}; Sigma-Aldrich, Steinheim, Germany) in case of selection pressure after cloning.

RNA isolation. Mycelia of *T. harzianum* T4.67 were sampled after 1, 2, 3, 4, 6, 8, 12, and 24 hours of interaction with SM+glucose and SM+Rh, respectively, immediately flash-frozen in liquid nitrogen and stored at –80°C until RNA isolation. Fungal mycelia were ground to a fine powder under liquid nitrogen and total RNAs were isolated using the RNeasy Maxi Kit (N°75162, Qiagen, Hilden, Germany) following the manufacturers' instructions.

Adapter and primers. The following adapters XE-14 (5'-CTGATCACTCGACA-3'), XEA-13 (5'-CCAGGTCTCGAG-3'), XET-13 (5'-CCTGGTCTCGAG-3') and primers XEA-18 (5'-TGATCACTCGAGACCAGG-3'), XET-18 (5'-

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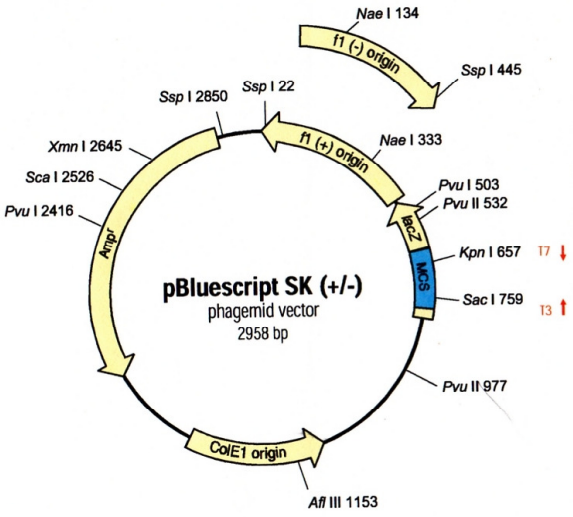
TGATCACTCGAGACCTGG-3') were applied for the construction of cDNA libraries. "RaSH" polymerase chain reaction was carried out with primers RaSH-F (5'-ACTCACTATAGGGCGATTG-3') and RaSH-R (5'-GGAATTCGATATCAAGCTTATC-3').

Plasmid vectors. The pBlueScript SK (+/-) plasmid vector (Stratagene, La Jolla, CA, USA) was used for subtraction library construction.



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Rapid Subtraction Hybridisation. Driver (*T. harzianum* T4.67 grown on SM+glucose) and tester (T 4.67 grown on SM+*Rhizoctonia*) RNAs, isolated after 1, 2, 3, 4, 6, 8, 12 and 24h, were pooled, respectively, and 1 µg of each RNA pool was transcribed into cDNA using the CreatorSMART library construction Kit (BectonDickinson, Biosciences Clontech, CA, USA) as described by the manufacturer. cDNAs were digested with *Eco*RII (New England Biolabs, Beverly, USA) at 37°C for 3h. Adapter ligation was then carried out with the TaKaRa DNA Ligation Kit Ver.2.1. (Otsu, Shiga, Japan) applying XE-14, XEA-13, XET-13 oligonucleotides. After incubation at 55°C for 3 min, the mix was cooled down slowly to room temperature (RT) and then to 8°C. Ligation of adapters was carried out at 4°C overnight. In order to amplify the digested cDNA material, PCR was performed applying primers XEA-18 and XET-18, that correspond to the adapter sequences. PCR was carried out 50 x 100 µL for each "adapter"-DNA: *Taq* polymerase reaction buffer (1x), $MgCl_2$ (2.5 mM), dNTP (0.2 mM), XEA-18 (1 µM), XET-18 (1 µM), *Taq* polymerase (1 unit, Promega, Madison, USA) and 1 µL of cDNA. PCR program was run as follows: initial denaturing step for 5 min at 72°C, followed by 25 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension step of 10 min at 72°C.

PCR products were purified using the Qiaquick PCR Purification Kit (N° 28104 Qiagen, Hilden, Germany). About 1 µg of amplified tester cDNA was digested by *Xho*I (New England Biolabs, Beverly, USA) at 37°C during 4 h and then applied to the hybridisation reaction. One-hundred ng of tester cDNA was mixed with 3 µg of the driver cDNA (ratio 1:30) in 10 µL of hybridisation solution (0.5 M NaCl, 50 mM Tris-HCl pH 7.5, 0.2% [w/v] SDS, 40% [v/v] formamide) and, after boiling for 5 min, incubated at 42°C for 48 h. The hybridisation mix was purified using the Qiaquick PCR

Purification Kit and adjusted to a final volume of 30 μL. Eight μL of this mixture was ligated with *Xho*I-digested pBlueScript SK (+/-) vector at 4°C over night using the Takara DNA Ligation Kit Ver. 2.1. 50 μL of competent *E. coli* DH5α cells were incubated with 8 μL of the ligation mix on ice for 30 min. Cells were then heat-shock treated at 42°C for 2min and incubated, after addition of 200 μL of LB broth, at 37°C for 1h. Transformation mix was then inoculated on LB agar plates, containing 100 μg mL^{-1} ampicillin, 80 μg mL^{-1} X-Gal and 0.5 mM IPTG and incubated at 37°C for 16h (Figure 13).

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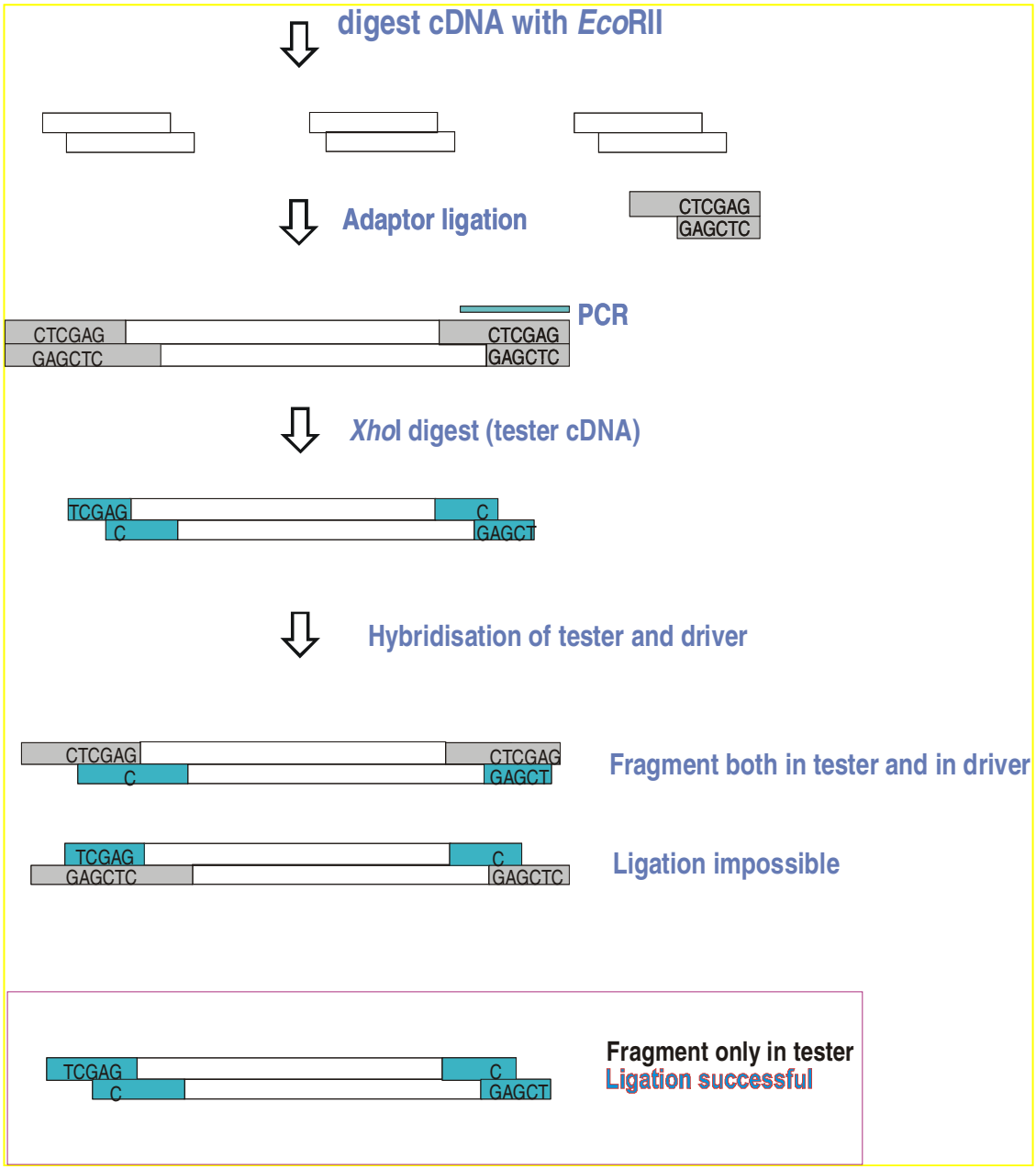

Figure 13:

Principles of the Rapid Subtraction Hybridisation technique.

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Colony screening and reverse Northern blot. Bacterial colonies grown on the selective medium were picked randomly and incubated in LB medium containing ampicillin (100 μg mL^{-1}) at 37°C for 4-6 h. Two μL of these liquid cultures served as template in PCR, by using RaSH primers, in order to amplify inserts. PCR was carried out as follows: each PCR mix of 50 μL contained *Taq* polymerase buffer (1x), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, and 1 U of *Taq* polymerase (Promega, Madison, USA). The PCR run had an initial denaturing step at 94°C for 1 min, followed by 30 cycles consisting of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C.

Ten μL of each PCR product were loaded twice, on two single 1.5% agarose gels, which were run at 5 V cm^{-1} for 30 min. The gels were photographed under UV-light (Gel Doc 2000, BioRad) and then prepared for transfer of the PCR fragments onto nylon membranes (Hybond-N, Amersham Biosciences, Freiburg, Germany). Gels were depurinated in 0.25 N HCl for 15 min, and then denatured in 0.5 N NaOH for 30 min, under gentle shaking respectively. Gel slots were sealed with melted agarose at the same concentration as the gels' one. Gels were transferred in 10x SSC for 90 min at 5 inches of Hg using a vacuum blotter (Vacuum Blotter Model 785, BioRad) following the manufacturer's protocol. After the transfer membranes were soaked in 2x SSC for 5 min and DNA was fixed on the membranes in a oven at 80°C for 30 min. In this way it was possible to screen each cloned insert with two different probes: (i) labelled cDNA of *T. harzianum* T4.67 grown on SM+g and (ii) labelled cDNA of T4.67 grown on SM+*Rhizoctonia*. Hybridisation and detection was carried out using the Gene Images CDP Star detection module (Amersham Biosciences, Freiburg, Germany). Blots were hybridised - overnight with gentle agitation - at 60°C with probe concentrations about

10 ng/mL of hybridisation buffer (5x SSC, 0.1% SDS, 5% dextrane sulfate, 1:20 dilution of liquid block). Blots were then washed with stringency wash solution 1 (1x SSC, 0.1% SDS) for 15 min at 60°C, followed by stringency wash solution 2 (0.5x SSC, 0.1% SDS) for 15 min at 60°C. Following the stringency washes, blots were incubated with gentle agitation for 1 hour at room temperature in a 1:10 dilution of liquid block agent in buffer A (100mM Tris-HCl, pH 9.5, 300 mM NaCl). Blots were incubated for 1 hour with the anti-fluorescein-AP conjugate (1:5000 dilution in buffer A supplemented with 0.5% BSA). Unbound conjugate was removed by washing 3 times for 10 min in sufficient volumes of 0.3% Tween 20 in buffer A. Any excess wash buffer was drained off and covered with detection reagent for 2-5 min. Again any excess liquid was drained off the blots, that were then wrapped in a fresh piece of Saran Wrap, sealed hermetically, and exposed to autoradiographic films for 3-10 h at RT. Clones that showed a significantly more intense hybridisation signal with cDNA of T4.67 on SM+*Rhizoctonia* in comparison with the hybridisation signal obtained with cDNA of T4.67 on SM+g were selected and submitted to sequencing.

Gene expression studies by Northern blot analysis. Northern blot gel electrophoresis was carried out on 1.2% [w/v] formaldehyde gels. RNA samples were mixed with the denaturing solution (55% [v/v] formaldehyde, 22% [v/v] formamide, 1x MOPS, 40 μg mL^{-1} ethidium bromide) and denatured at 65°C for 30 min. Aliquots of 20 mg of RNA per lane and per sample were loaded on the gel, that was run at 5 V cm^{-1} until 28S and 18S RNA bands were clearly separated. RNA was transferred onto nylon membrane (Hybond-N, Amersham Biosciences, Freiburg, Germany) using the BioRad Vacuum Blotter System following the standard protocol described by the

manufacturer. After controlling the integrity of 28S, 18S and 5.8S RNA bands under UV light (Gel Doc 2000, Biorad, CA, USA) the gel was washed with DEPC-treated H_2O for 10 min. Gel slots were filled and closed with agarose (1% [w/v]). After 15 min of polymerisation RNA was transferred with 10x SSC (150 mM sodium citrate, 1.5 M NaCl) at a pressure of 5 Hg for 90 min, followed by a fixation step at 80°C for 30 min.

Membranes were pre-hybridised with formamide containing hybridisation buffer (6x SSC, 5x Denhardt's reagent, 0.5% [w/v] SDS, 1% [v/v] ssDNA , 50% [v/v] formamide and 6% [w/v] dextrane sulfate) at 42°C for at least 30 min. The labelled probe was added to 10-15 mL of hybridisation buffer and blots were hybridised at 42°C overnight. Blots were washed with 2x SSC, 0.1% [w/v] SDS at RT and then with 2x SSC, 0.1% [w/v] SDS at 42°C for 10 min. Membranes were sealed with Saran wrap and exposed to autoradiographic film at -80°C for 5-48 h.

Probe labelling. ***(i)Labelling of fluorescein probes.*** Reverse Northern blots were carried out using a random labelling system based on fluorescein (F1-dUTP) (Gene Images Random Prime Labelling Module, Amersham Biosciences, Freiburg, Germany). 50 ng of tester and driver cDNA was labelled as described by the manufacturer.

(ii) Labelling of radioactive probes. Plasmid inserts, used as probes in the Northern Blot analysis, were amplified by PCR applying RaSH primers. PCR was set up as described above. PCR products were purified using the Qiaquick PCR Purification kit according to the manufacturer's protocol. Probe labelling was performed with 11 μL of purified DNA, 2 μL priming buffer (10X), 2 μL hexamer random primer (0.05 μg μL^{-1}). The mixture was boiled in a water bath for 3 min and then chilled in ice for 15 min.

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Two µL of nucleotide mix (2 mM dATP, dTTP, dGTP), 2 µl ^{32}P-dCPT and 1 µL (1 U) of Klenow fragment were added and labelling was carried out at 38°C for 60 min, followed by an inactivation step at 75°C for 10 min. Probes were purified by Sephadex50 columns.

3.7 Confrontation assays

Culture conditions. In confirm the results obtained in RaSH experiments, confrontation assays were performed as follows: *T. harzianum* T4.67 or T4.31 were grown on cellophane covered malt extract agar plates in confrontation with *R. solani* in constant darkness at 25°C (Figure 14). Mycelia were harvested from those strains "before contact", at "contact" and "after contact" with *Rhizoctonia* and used for isolation of total RNA.

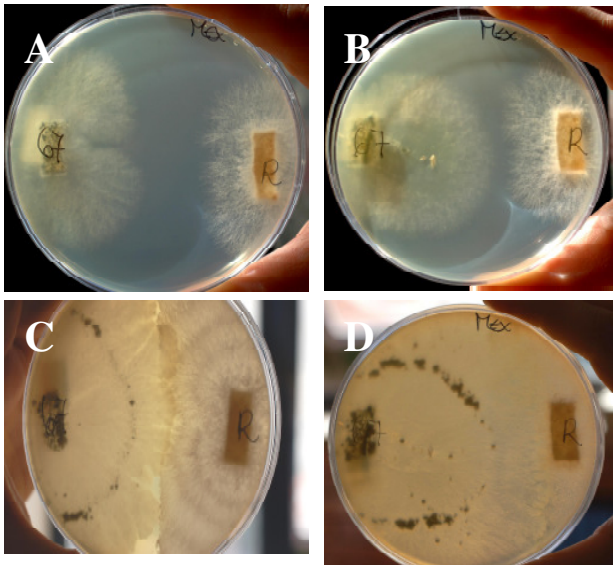

Figure 14:

Confrontation experiment permitting mycoparasitic interaction between *T. harzianum* and *R. solani* mycelia on agar plates, demonstrating stages 1 day after inoculum (A), pre-contact (B), contact (C), and after-contact (D).

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RNA isolation. Fungal mycelia were immediately flash-frozen after sampling and then ground to powder under liquid nitrogen. RNA was extracted as described by Chirgwin *et al.*, (1979). Mycelia were then transferred into a 2 mL reaction tube containing 700 µL Chirgwin reagent (74% [w/v] guanidin thiocyanate, 0.8% [w/v] sodium lauryl sarcosinate, 40 mM sodium citrate, pH 7.0) and 6 µL 2-mercaptoethanol. After addition of 70 µL sodium acetate (2M, pH 4.0), 700 µL phenol and 200 µL chloroform : isoamyl alcohol (49:1) mixture, samples were vortexed and incubated on ice for 15 min. A centrifugation step at 20.000 x g (4°C, 10 min) followed and the supernatant was transferred into a new reaction tube, where RNA was precipitated adding an equal volume of isopropanol. Samples were incubated at –20°C at least for 60 min, and then centrifuged at 20.000 x g (4°C, 30 min). RNA pellet was washed once with 70% [v/v] ethanol, air-dried and then resuspended in 50-100 µL diethyl pyrocarbonate (DEPC) treated water.

RNAs extracted from mycelia of T4.67 and T4.31, respectively, were purified from the time steps "before-", "during-" and "after-contact". RNA isolated from *R. solani*, and from *Trichoderma* T4.67 and *Trichoderma* T4.31 pure cultures served as controls. Northern blots were performed as described above, loading 20 mg of RNA per lane. Inserts from selected clones obtained by the RaSH experiment were applied as probes.

Scanning Electron Microscopy (SEM). Tissue sections (2cm x 2cm x 2cm in size)to be observed by SEM were cut with a sterile surgical blade from PDA substrate plates with interacting *Trichoderma* and *R. solani* ("contact" stage) strains and fixed in 2.5% glutaraldehyde for 90 min, then washed three times for 5 min with Ringer solution, and postfixed in 1% OsO_4 for 2 h. After three 5 min-washes with distilled water, samples

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were acetone-dehydrated with two successive 10 min washes in 25%, 50%, 70%, 80%, 95%, and 100% acetone [v/v], respectively. After critical point drying using liquid CO_2, the samples were sputter-coated with gold-palladium, examined and photographed using a Zeiss DMS 960a SEM.

3.8 Bioinformatic methods

To identify individual clones from their nt-sequence, they were first trimmed from vector sequences, and then subjected to a BLASTN search against the JGI TRIRE database (http://gsphere.lanl.gov/cgi-bin/runBlast?db=trire1). This was done in order to bypass the drawback that the genome sequence of *T. harzianum* is not available, in the assumption that the sequences of its clones would show highest similarity to the gene sequences of another *Trichoderma* species. In case of hits, the annotated full-length sequences of the respective *H. jecorina* hypothetical proteins was then used to perform a BLASTX search of the NCBI database (http://www.ncbi. nlm.nih.gov) to identify the protein on the basis of its closest orthologue. In case a clone did not yield any hits in the JGI TRIRE database, they were nevertheless given a try at NCBI in order to identify genes which might have arisen by horizontal transfer from other more distant organisms. *In silico* analysis of the predicted proteins was done with PROTPARAM (http://www.expasy.org/tools/protparam.html), and signal peptide prediction was performed with SignalP (www.cbs.dtu.dk/services/SignalP/) (version 3.0 Bendtsen *et al.*, 2004), both available at Expasy-Tools (http://www.expasy.org/tools).

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3.9 Substrates, buffers, and common techniques

Trichoderma **selective medium (TSM)**

$MgSO_4$ x 7 H_2O	0.16 g
K_2HPO_4	0.72 g
KCl	0.12 g
NH_4NO_3	0.80 g
Glucose	2.40 g
Chloramphenicol	0.20 g
Rosa Bengala	0.12 g
Agar	10.0 g
H_2O dist.	800 ml

Suspend all components in distilled water. Sterilise by autoclaving at 121°C for 15 min. Add then

Propamocarb ®, 66.5% [w/v]	1.05 ml
Pentachlornitratebenzen	0.16 g

Potato dextrose agar - PDA

Potato glucose agar	39 g
H_2O dist.	1000 ml

Suspend potato glucose agar in distilled water. Sterilise by autoclaving at 121°C for 15 min.

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Potato dextrose broth - PDB

Potato glucose broth	24 g
H_2O dist.	1000 ml

Suspend potato glucose broth in distilled water. Sterilise by autoclaving at 121°C for 15 min.

Synthetic Medium - SM + glucose (Yedidia *et al.*, 1999)

$(NH_4)_2NO_3$	1.0 g
$MgSO_4x7H_2O$	0.2 g
K_2HPO_4	0.9 g
KCl	0.15 g
$FeSO_4x7H_2O$	0.01 g
$ZnSO_4x7H_2O$	0.009 g
$MnSO_4xH_2O$	0.007 g
Glucose	30 g
Technical agar	20 g
H_2O dist.	1000 ml

Suspend all components in distilled water. Sterilise by autoclaving at 121°C for 15 min.

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Malt extract agar - MEX, 3% [w/v]

Malt extract	30 g
Technical agar	20 g
H_2O dist.	1000 ml

Suspend malt extract and technical agar in distilled water. Sterilise by autoclaving at 121°C for 15 min.

Luria-Bertani agar – LB agar

Tryptone	10 g
Yeast extract	5 g
NaCl	5g
Technical agar	20 g
H_2O dist.	1000 ml

Suspend all components in distilled water. Sterilise by autoclaving at 121°C for 15 min.

Luria-Bertani broth - LB

Tryptone	10 g
Yeast extract	5 g
NaCl	5g
H_2O dist.	1000 ml

Suspend all components in distilled water. Sterilise by autoclaving at 121°C for 15 min.

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Glycerol, 50% [v/v]

Glycerol, 100% [v/v]	50 ml
H_2O dist.	50 ml

DNA extraction buffer

NaCl	400 mM
Tris-HCl, pH 8.0	10 mM
EDTA, pH 8.0	2 mM
Proteinase K	400 μg mL^{-1}
Sodium dodecyl sulfate	2%

Tris-EDTA buffer - TE, pH 7.5

Tris-HCl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

Tris-Acetate-EDTA buffer - TAE, 50x

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml
H_2O, dist.	ad 1000 ml

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Agarose gel loading buffer, 10x

Bromophenol bue	0.05 g
Xylene cyanol FF	0.05 g
Glycerol	5 mL
EDTA	0.186 g
TAE, 1x	ad 10 mL

10x SSC

Sodium citrate	8.8 g
NaCl	17.5 g
H_2O dist.	ad 200 ml

10x MOPS

4-morpholinpropansulfonic acid (MOPS)	52 g
EDTA	1.86 g
DEPC-H_2O	500 ml

adjust pH to 7 using conc. NaOH

Chrigwin reagent

Guanidin thiocyanate	250 g

Suspend in 293 ml of distilled water and add

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0.75 M sodium citrate, pH 7.0	17.6 ml
10% [w/v] sodium lauryl sarcosinate	26.4 ml

Dissolve the components under stirring at 65°C. Store in aliquots at –20°C until use.

Hybridisation buffer Southern blot (using the Gene Images Kit, Pharmacia)

10x SSC buffer	25 ml
Liquid block reagent	2.5 ml
H_2O dist.	ad 49 ml
20% [w/v] SDS	0.25 ml
Dextrane sulfate	2.5 g

Dissolve components at 60°C and store at –20°C until use.

Hybridisation buffer Northern blot

20x SSC	15 ml
50x Denhardts reagent	5 ml
10% [w/v] SDS	2.5 ml
ss DNA	0.5 ml
formamide	25 ml
Dextrane sulfate	5 g
H_2O dist.	ad 50 ml

Dissolve components at 42°C..

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DEPC-treated water/solutions and materials

Diethyl pyrocarbonate (DEPC) is a strong, but not absolute inhibitor of Rnases. It is commonly used to inactivate Rnases on glass or plastic ware or to create Rnase-free solutions and water. DEPC inactivates Rnases by covalent modification. Add 0.1 mL DEPC to 100 mL of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. Glass and plastic ware should be filled with 0.1% DEPC water, allowed to stand overnight at 37°C, and then be autoclaved to 100°C for 15 min to eliminate the residual DEPC.

Formaldehyde gels (RNA)

RNA-gels (formaldehyde!) should always be carried out under the chemical hood! Treatment of gel sub-cell: fill the cell with H_2O-DEPC and leave for 1h; then wash the cell, gel tray and combs with autoclaved H_2O-DEPC → now everything is ready for preparing the gel.

Formaldehyde-gel volumes	100 ml	150 ml	50 ml
Agarose (1.2% final conc.)	1.2 g	1.8g	0.6 g
DEPC H_2O	72 ml	108 ml	36 ml
10x MOPS	10 ml	15 ml	5 ml

boil/heat and dissolve in the microwave oven and let allow to reach 50 – 60 °C (keep temperature in water bath if necessary), then add

formaldehyde	18 ml	27 ml	9 ml

mix well and pour the gel carefully. Allow 60-70 min for the gel to solidify at room temperature.

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10x RNA Loading Buffer

DEPC-water	25 ml
Glycerin	25 ml
EDTA	0.016g
Bromophenol blue	0.25g

Preparation of competent cells of *E. coli*

SOB medium

Tryptone	8 g
Yeast extract	2 g
NaCl	
KCl	
H_2O dist.	400 mL

Suspend all components in distilled water. Sterilise by autoclaving at 121°C for 15 min. Add $MgCl_2$ and $MgSO_4$ in final concentrations of 10 mM (starting from 1M sterile filtrated solutions).

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TB buffer

1 M HEPES	2 mL
1 M $MnCl_2$	11 mL
1 M $CaCl_2$	3 mL
H_2O dist.	ad 200 mL

adjust pH to 6.7 using KOH

KCl	3.7 g

Suspend the KCl, sterilise by filtration (0.45 μm) and store at 4°C until use.

Inoculate 250 mL SOB medium with 1 mL of pure a pre-culture of *E. coli* and incubate at 18-20°C, 150 rpm, overnight (the final OD should be 0.6). Transfer the culture flask on ice, incubate for 10 min, and centrifuge at 2500 x g for 10 min at 4°C. Resuspend the pellet into 80 mL of ice cold TB and incubate on ice for 10 min. Centrifuge again at 2500 x g for 10 min at 4°C. Resuspend the pellet in 20 mL TB and add then 1.6 mL of DMSO (final concentration 8%). Incubate again on ice for 10 min and then transfer small volumes into cryotubes, that are immediately shock frozen in liquid nitrogen and then stored at –80C.

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4. Results

4.1 Sampling site characteristics

Sardinia, the second-largest Mediterranean island, is characterised by a semi-arid climate and the majority of its ecosystems have developed under severe water scarcity. Topographically it is described as 67.9% hilly and 13.6% mountainous, areas which are divided by the Campidano plain stretching from west to south (European Commission, 1994) and was forested for the most part with shrubs and forest stands until 1800.

Fifteen non-cultivated, undisturbed sampling sites all over the Sardinian island, representing different ecosystems, were chosen. The selected sampling sites comprised forest stands (3 sites; F), shrublands – maquis (3 sites; S) and grassland – savannas (9 sites; G). Prevalent grassland vegetation consisted of e. g. *Asphodelus ramosus* L., *Cardus* spp., *Pteridium aquilinum*, and various *Gramineae* spp., whereas as shrubland was mainly composed by *Rubus fruticosus, Myrtus communis* L., *Pistacia lentiscus* L., *Chamaerops humilis* L., *Rosmarinus officialis* L., *Phyllirea* spp., *Cistus* spp., *Genista* spp., and forests displayed mostly the species *Quercus suber* L., *Q. ilex* L., *Taxus baccata* L. and *Olea europaea* L. var. *oleaster* Hoff. et Lk.

Geographic coordinates, meterological conditions, and soil properties of these habitats are given in Table 1. Sampling sites F1, F2, G2 and EG4 are all located at an altitude of higher than 1000 m above sea level, being reflected in their characterising lowest minimal, maximal and annual average temperatures. The soil of site F2 comprises the highest content of organic matter. The two rather lowland sites

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Table 1. Geographical coordinates and climatic conditions and soil properties

Soil code	GPS coordinates		Altitude	Yearly rainfall	*Hypocrea/Trichoderma*		Tmin	Tmax	Tave	Sand	Silt	Clay	pH	C	SOM
	N	E	(m)	(mm)	biodiversity index	species	℃	℃	℃	%	%	%		%	%
EG1	41°00'29''	9°15'23''	246	877	0,08	4	6,2	22,6	13,8	75,2	13,3	11,5	5,2	6,2	10,7
EG2	40°19'24''	8°27'19''	45	657	0,57	1	6,5	23,9	14,6	71,6	12	16,3	6	5,9	10,2
EG3	40°18'29''	9°29'31''	378	663	0,04	4	6,7	24,4	14,8	86,3	6,3	7,3	5,4	3,4	5,9
EG4	40°07'16''	9°19'55''	958	970	0,23	5	8	25,1	15,7	72,8	15	12,2	5	9,3	16,1
EG5	39°36'07''	9°06'06''	314	741	0,14	2	7,1	23,8	14,7	41,7	22,1	36,2	7	1,6	2,7
EG6	39°29'12''	9°14'11''	605	638	0,03	3	8	26,2	16,2	49,7	30,6	19,7	4,8	6,7	11,6
EG7	39°05'29''	8°47'01''	204	874	0,17	5	10	25,5	17,2	74,7	14,2	11	6	2,5	4,3
F1	40°51'05''	9°08'59''	1080	800	0,13	6	5,3	22,3	13,1	83,2	8,6	8,2	4,5	6	10,5
F2	40°21'24''	8°55'13''	1019	738	0,21	5	4,8	22,4	12,8	67,8	20	12,2	5	15,3	26,3
F3	40°11'58''	8°35'09''	467	793	0,13	3	7,8	23,4	15	70,3	29,7	10,1	5,5	9,4	16,2
G1	40°37'14''	8°23'14''	128	622	0,27	5	6,5	22,7	14	55,6	25,2	19,2	5,4	4,1	7
G2	40°02'14''	9°28'30''	1093	978	0,42	3	3,5	21	11,4	69,5	17,3	13,2	4,2	8,3	14,4
S1	41°06'26''	9°05'11''	69	747	0,08	5	8,3	24,4	15,4	67,5	16,5	16	5,5	6,3	10,9
S2	40°35'04''	8°09'02''	193	650	0,23	1	10	24,9	16,9	30,5	43,4	25,6	7	4,2	7,3
S3	40°04'06''	8°32'15''	239	574	0,11	2	9,9	24,5	16,8	43,1	28,9	28,1	5,4	4,7	8,2

S1 and EG2 are located in nearly identical climatic conditions and showed very similar soil properties.

4.2 Diversity of *Trichoderma /Hypocrea*

Setting up the parameters for the isolation of strains, it was tested if any of the species of *Hypocrea/Trichoderma* was not able to grow lively on the *Trichoderma* selective medium. As this was the case - some species strains showed only a very cumbersome growth – isolation of the strains from the soil samples was additionally performed on non-selective PDA medium.

From all 482 isolates obtained from the 15 sampled soils, purified DNA was extracted and then subjected to Random Amplified Polymorphism DNA (RAPD) analysis (Figure 15). This pre-screening test permitted the grouping of the isolates according to their RAPD-PCR patterns. Profiles were considered as identical fingerprints when they showed a minimum of 98% similarity. At least one strain of each group of each

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sampling site and all ambiguous RAPD-PCR results, were subjected to species identification by *its1/its2* gene sequence analysis (Figure 16).

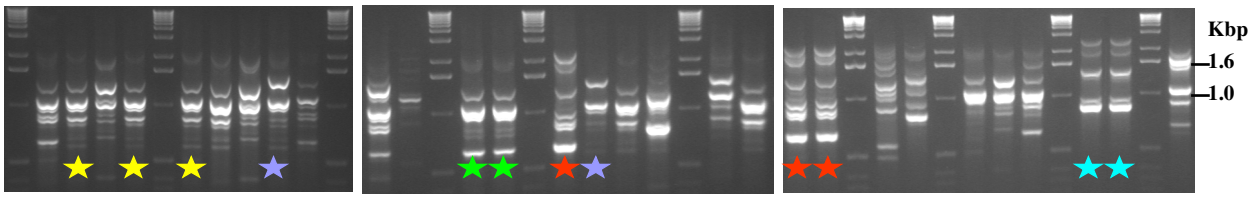

Figure 15:

Pre-screening of *Trichoderma* spp. isolates by RAPD-PCR based on the M13 primer. Profiles exhibiting stars of the same colour were considered as identical fingerprints.

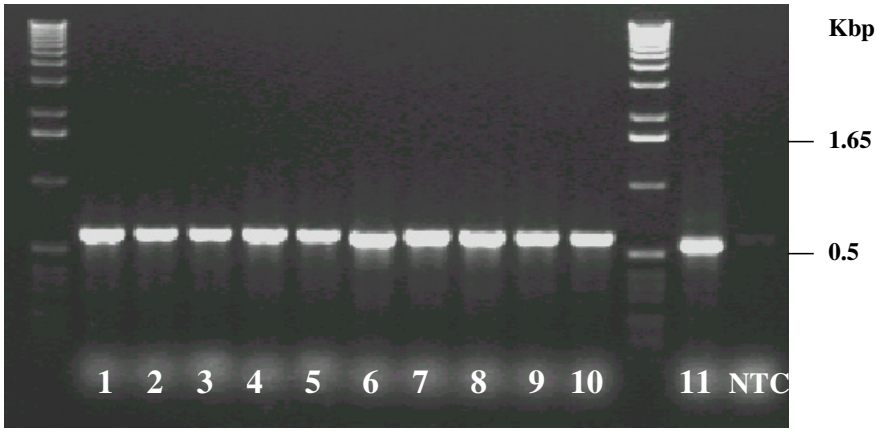

Figure 16:

ITS-PCR for sequence analysis based on the primers ITS1 and ITS4 representing the *its1*-5.8S-*its2* rDNA fragment. (1)-(11): samples; (NTC): no-template control

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Finally the ITS1 and ITS2 region of 231 isolates were sequenced and submitted to *Trich*OKEY (Druzhinina *et al.,* 2005) and *Tricho*BLAST (Kopchinskiy *et al.,* 2005) analysis. Not all *Trichoderma* species can be determined by only these regions (e. g. species from section *Trichoderma*; cf. Druzhinina *et al.,*2005) and hence were subjected to sequence analysis of the long intron of *tef1* (Figure 17). In this manner all except one isolate could be reliably identified (Table 2, Figure 18).

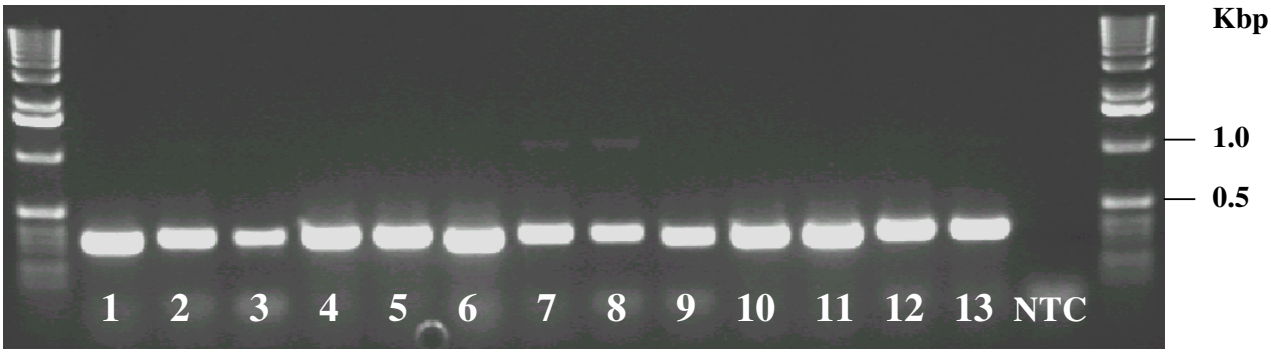

Figure 17:

Amplification products of the long intron of the terminal elongation factor 1-α (*tef 1).* (1)-(13): samples; (NTC): no-template control

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Table2: *Trichoderma* species identified in this study

identified species	**N° of isolates**
T. harzianum	**279**
T. gamsii	**58**
T. spirale	**34**
T. velutinum	**23**
T. hamatum	**21**
T. virens/H. virens	**19**
Trichoderma sp. Vd2	**15**
T. tomentosum	**10**
T. virisdescens/H. virisdescens	**6**
T. koningii/H. koningii	**5**
T. koningiopsis/H. koningiopsis	**4**
H. semiorbis	**4**
T. asperellum	**3**
new isolates	**1**
total	**482**

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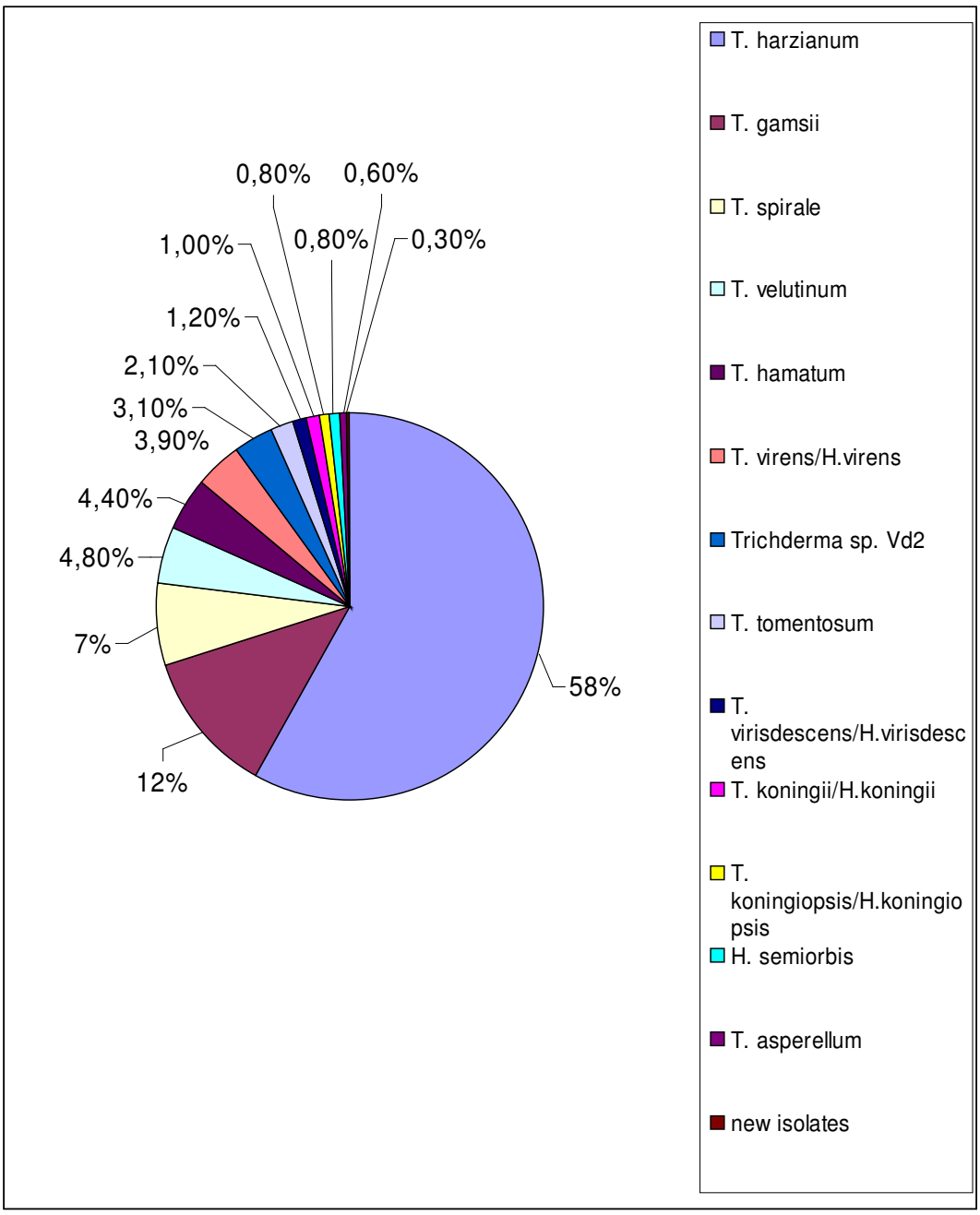

Figure 18:

Frequency of the isolated *Trichoderma* species

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H. lixii/T. harzianum was the most abundant species encountered in this study (57 %), being present in 10 of the 15 sites investigated, exhibiting the highest proportion in 7 of the locations where it was present (G1, S1, S3, F1, F3, EG3 and EG5) and being the only species isolated from site EG2 (Figure 19). These sites are equally distributed over the island, and differ in ecosystem types, soil properties, altitude and climatic conditions. The abundance of *H. lixii/T. harzianum* at these locations is therefore unexplained by the variability of measured abiotic factors.

Two subdominant species for Sardinia were *T. spirale* and *T. gamsii* (7 and 11 % of all isolates, present in 7 and 8 of the 15 samples, respectively). *T. spirale* was most abundant in the forest land of Badde Salighes (F2), where it accounted for > 50 % of the isolates but it did not dominate any other location. *T. gamsii* was the exclusive species found in sample S2 (Torre de la Peña, shrubland), and accounted for the majority of isolates in G2 (Talana; grassland), and GE6 (San Nicolò). In S3 (Narbolia) it was the only found species after *H lixii/T. harzianum* (Figure 19).

Six more species occurred with a frequency of 5 % or less but were detected in several soils (Fig. 1) : *T. hamatum* (5 %, 2 forest and 3 pasture locations on the east side of the island), *T. velutinum* (5 %, 4 locations, mainly grazed grasslands), *H. koningii/T. koningii* (2 %, 5 locations), *T. tomentosum* (2 %, 3 distant locations), *H. viridescens* (1.2 %, 4 locations on the north-eastern side of the island), and *H. semiorbis* (1 %, 2 locations). The latter has so far only been found as its teleomorph in Australia and Tasmania (Chaverri and Samuels, 2003), and this is the first time that its anamorph is found in soil. Five species represent the minority of *Hypocrea/Trichoderma* diversity since they were detected in single soil samples: *H. virens/T. virens* dominated site EG7, *H. koningiopsis/T. koningiopsis* was the subdominant species on another grazing site (EG4),

T. asperellum and *T. atroviride* were detected in the most southern and most northern sites, S1 and EG7 respectively and 2 isolates of *T.* sp. Vd2 *sensu* (Jaklitsch *et al.*, 2006) were detected in soil S1 (Figure 19).

Only a single isolate (C.P.K. 2657, soil F2), while clearly belonging to *Hypocrea/Trichoderma*, could not be identified, but its ITS 1and 2 sequence showed close similarity to Harzianum-Catoptron Clade. Figure 19 shows the phylogenetic position of this new species based on sequences of the long intron of *tef1*, which reveals it as a sister species to *T. aggressivum*. While a phylogeny based on a single gene certainly is not enough to conclude this, it is very likely that this isolate to be an unknown, new species of *Hypocrea/Trichoderma*.

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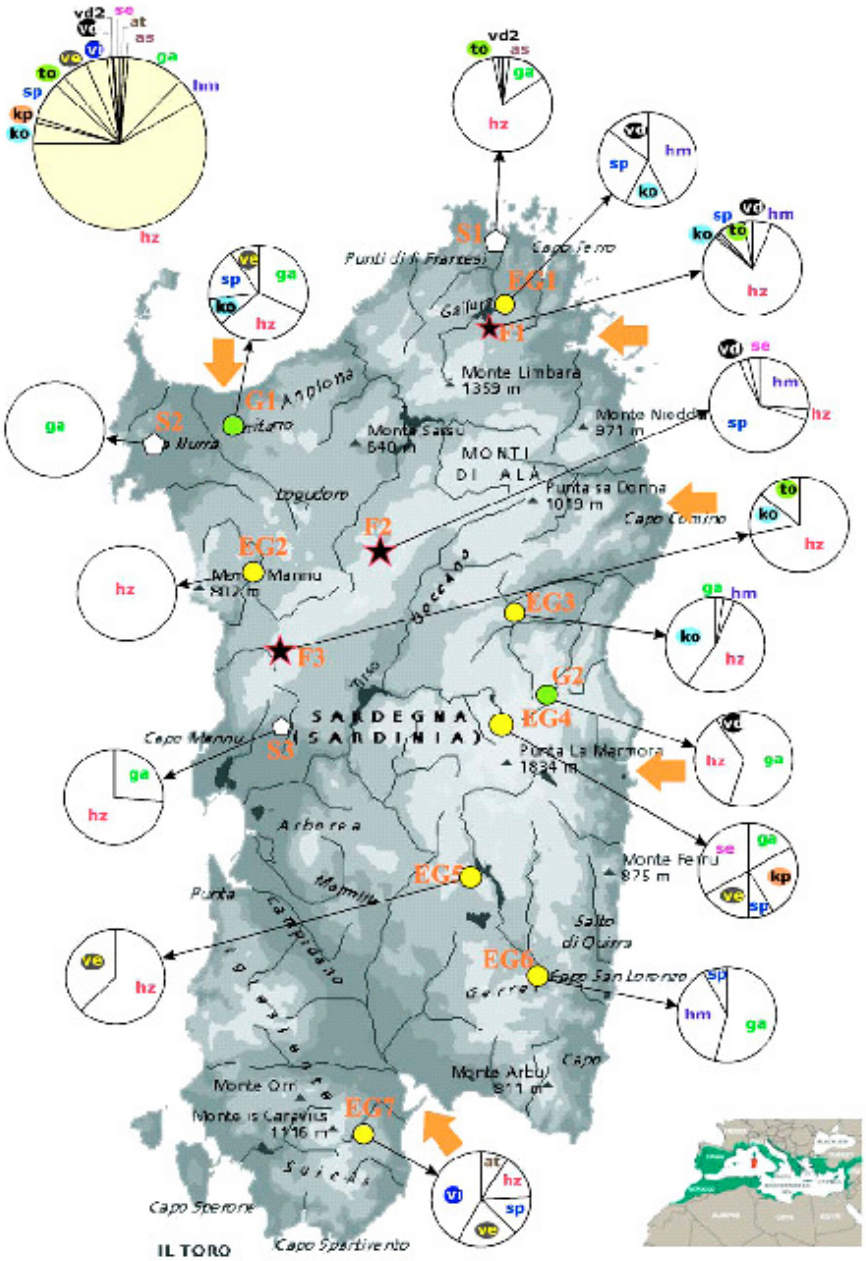

Figure 19:

Distribution and presence of identified *Trichoderma* isolates.

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4.3 Pathogenicity tests

A total of 222 *Trichoderma* strains could be tested for their antagonistic behavior in the greenhouse. Design of a quick and efficient *in vivo* inoculation way by pairing mycelia of *Trichoderma* and *R. solani* on agar support - giving them the possibility of direct contact and interaction – permitted us a selection pushing for elevated biocontrol abilities (Figure 20).

Eighty-two highly antagonistic strains, 29% only moderate antagonistic strains and 54% strains with no ability of mycoparasitism were described in this way. The biocontrol efficient strains consisted of 18 *T. harzianum* (8.1%), 31 *T. gamsii* (14.0%), 11 *T. virens* (4.9%), 1 *T. koningii* (0.5%), 5 *T. hamatum* (2.2%), 3 *T. spirale* (1.3%), 3 *T. cerinum* (1.3%), 6 *T. velutinum* (2.7%), 7 *Trichoderma* sp. Vd2 (3.1%), 2 *T. viridescens* (0.9%), 1 *T. koningiopsis* (0.5%), and of 1 unidentified (0.5%) strains (Table 3, Figure 21). Test plants sown in soil infested by these strains showed significantly diminished disease symptoms in the presence of *R. solani* (Figure 22).

Figure20:

Pathogenicity tests in the greenhouse. Tested isolates and resulting crop protection (left), non-inoculated and *R. solani* inoculated control lines (right).

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Table 3: Antagonistic potential of tested *Trichoderma* species

***Trichoderma* species**	**number of tested isolates**	**number of antagonistic isolates**	**percentage of antagon. Isolates**
T. harzianum	84	18	21.4
T. virens	19	11	57.9
T. koningii	1	1	100
T. hamatum	11	5	45.5
Trichoderma sp. VD2	15	7	46.7
T. gamsii	57	31	54.4
T. spirale	18	3	16.7
T. tomentosum/T. cerinum	3	3	100
T. velutinum	9	6	66.7
T. viridescens	3	2	66.7
T koningiopsis	1	1	100
Yet undescribed	1	1	100
TOTAL	**222**	**89**	**40.1**

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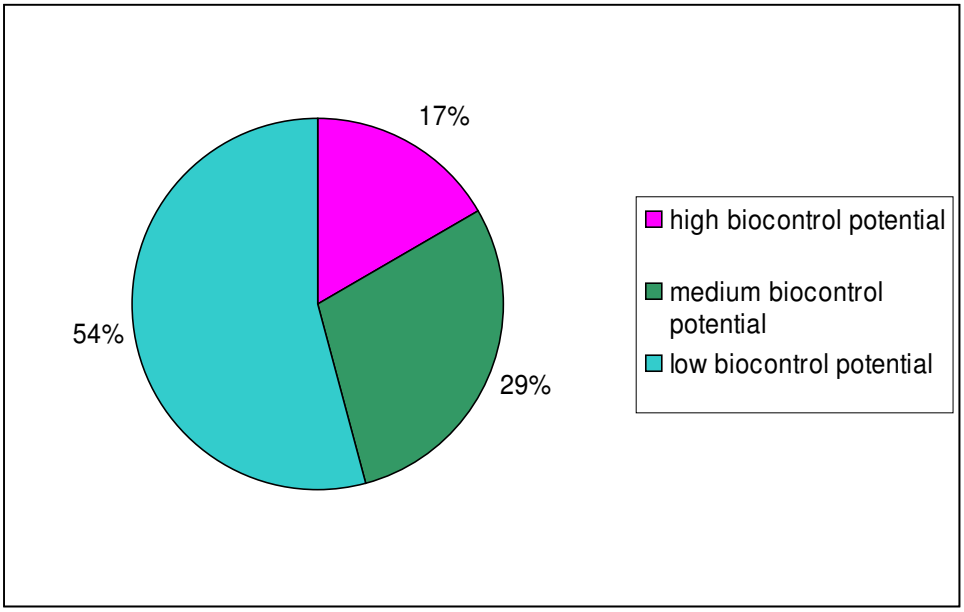

Figure 21:

Frequency of isolates with high, medium and low biocontrol potential.

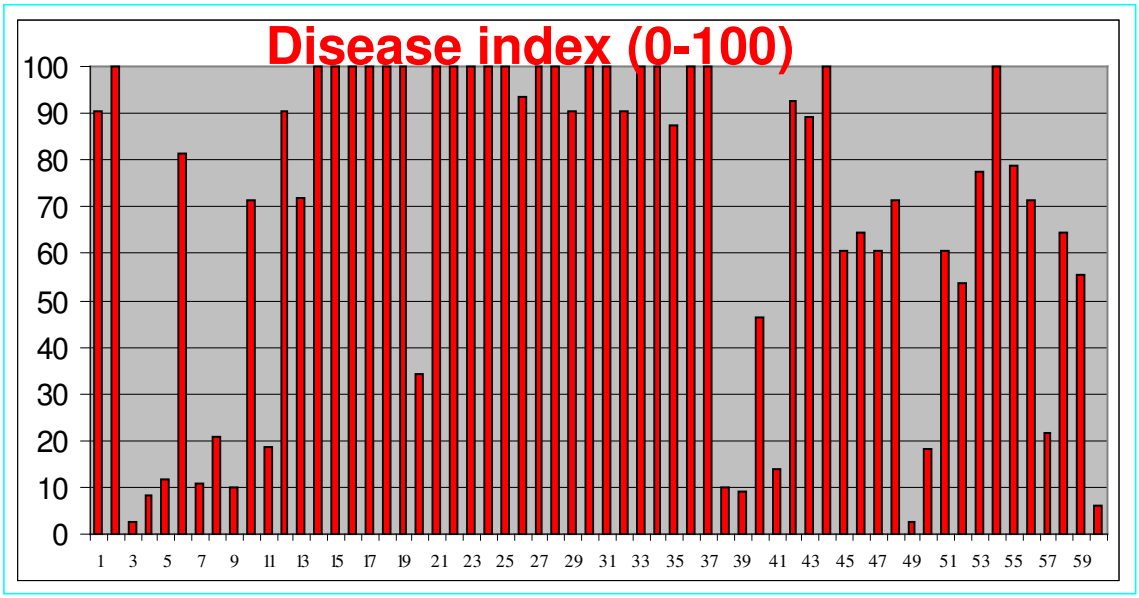

Figure 22:

Disease index based on valutation of crops with no or few disease symptons.

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4.4 Identification of clones describing genes regulated during the interaction of *T. harzianum* T4.67 with *R. solani*

RNA was prepared from driver and tester during a time course of 1- 24 hours and transcribed into cDNA. The RaSH technique (Jiang *et al.*, 2000) allowed rather rapid construction of a subtraction library of differentially expressed tester cDNA. Several hundreds of differentially expressed tester sequence tags were obtained from the hybridisation mix. Two-hundred clones were picked randomly to be characterised by Reverse Northern Blotting (Figure 23).

In the reverse Northern blot analysis inserts of all two-hundred clones were amplified by PCR applying RaSH-F and RaSH-R primers, which bind within pBluescript SK. These gene fragments were then screened with total driver cDNA (T4.67 grown on SM+glucose), as well as with total tester cDNA (T4.67 grown on SM+*Rhizoctonia*) (Figure 23). This analysis strongly reduced the number of positives, resulting in a final fifty clones which showed a significantly more intense hybridisation signal after hybridisation with the cDNA from the latter conditions (i.e. mycoparasitic interaction). These clones were sequenced and used in further analysis.

70

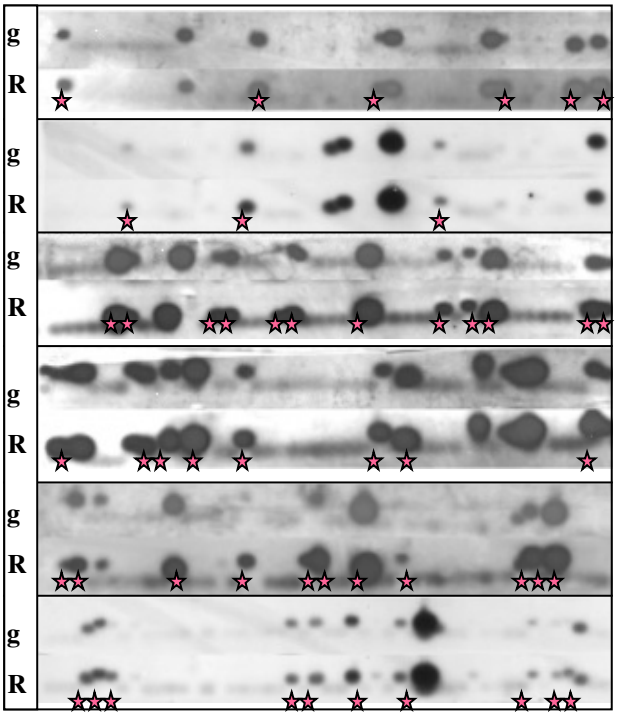

Figure 23:

Reverse Northern Blot analysis of differentially expressed sequence tags identified by RaSH. PCR-amplified products from bacterial clones of the subtracted library were loaded on a 1% TAE-agarose gel and then transferred onto nylon membranes. [α-^{32}P]-labelled cDNAs from *T. harzianum* antagonistic strain T4.67 grown MM substrate complemented with glucose (g) and T4.67 grown in interaction with *R. solani* (R) were applied as probes in the hybridisation reaction. Red stars indicate clones chosen for further sequencing.

4.5 Identification of ESTs

The RaSH-PCR fragments of the 50 selected clones were sequenced in forward and reverse direction and controlled for their (correct) sequence quality.

Thirty-six of the clones were identified as orthologues to sequences of the TRIRE database (Table 4). The remaining 14 clones did not produce any hits upon a BLASTN search against the NCBI database (both within the nr database and the EST-database), and may therefore encode unique *T. harzianum* genes. The 36 clones actually resulted in the long run in 25 different genes, because some of the ESTs

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represented different sequence regions of the same gene, e.g. C91 = D21 = D22 = D29; 10 = 71; 33 = 86 = 127; 88 = 141; 69 = 87 = 95 = 100 = 109 = 110 (Table 4). These findings confirm a notably strong expression of these genes under the test conditions. Four of the putative genes (14, C76, C91, D48) encoded unknown or hypothetical proteins. Search for *T. harzianum* EST orthologues in the NCBI databases didn't retrieve hits, but the sequences showed homologies to *Fusarium graminearum* and *Magnaporthe grisea* proteins, indicating conserved protein structure and function. The main characteristics of the protein encoded by the clone 14 were a signal peptide and four transmembrane helices, indicating its role and location as a membrane-spanning protein. Clone C91 showed similarity to an untranscribed area 5' of the *epl1* gene encoding a protein of the ceratoplatanin-family. These genes seem to be upregulated under mycoparasitic conditions in *H. atroviridis* (Seidl *et al.*, 2006). No further identification of other possible functions could be accounted for the C91 clone, that was isolated four times (C91, D21, D22, D29), indicating a potential significant role during mycoparasitism.

The remaining 21 genes could be clearly identified, representing a wide range of gene functions, however mainly describing proteins involved in transcription and protein synthesis or turnover, and genes encoding extracellular hydrolases (Table 4). Members of the former were: a high-mobility-box protein (C51); ribosomal protein S10 (D90); 40S ribosomal protein 25 (C20); an ARM-repeat protein involved in Puf-RNA folding (C32; Andrade *et al.*, 2001); an U2-associated snRNP A protein (C34); and polyubiquitin (107). The extracellular hydrolases comprised two proteases (a metalloprotease, C63; and an S28 peptidase, 97), a triacylglycerol lipase (69, 87, 95, 100, 109, 110), and an acid phosphatase (88). An orthologue of *H. jecorina* acetyl

xylan esterase I (115; Axe1), and an orthologue of the *H. jecorina* GH family 61 endoglucanase Cel61b (140; Foreman *et al.*, 2003), which corresponds to the previously published EGIV (Saloheimo *et al.*, 1997) but lacks a cellulose-binding domain. Besides these two major groups, most other clones represented enzymes involved in transport, metabolism and energy production: clones 22 and 115a, encoding NADH-dehydrogenase 51 kDa subunit and succinate dehydrogenase, respectively, represented oxidative energy metabolism; a 12-membrane hexose permease (33, 86, 128) and another protein of the major facilitator subfamily (10, 71) representing transport proteins; and tryptophan synthase (74), acetate kinase (75) and saccharopine reductase (81) representing metabolic enzymes. Finally, two clones fell outside of all other categories: a multicopper oxidase (C58) and an adenosyl-methionine-cyclopropan fatty acyl phospholipid synthase (C45).

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Table 4: Characteristics and putative functions of clones isolated with RaSH

Clone ID	Frequency	Transcript size (bp)	Identified gene of *T. reesei* or nearest neighbour	Scaffold	Area in scaffold	Putative function
C14	1	725	PalI related protein	14	425000	Unknown
C20	1	694	40S ribosomal protein 25	10	673500	Translation, biogenesis
C32	1	1997	Puf family RNA-binding protein	46	73000	Translation, biogenesis
C34	1	1838	U2-asscociated snRNP A protein, leucin-rich repeat	24	435000	RNA processing and modification
C45	1	2675	SAM-dependent methyltransferase	19	9500	Lipid transport and metabolism
C51	1	300	High mobility group box	8	96000	Regulation of transcription, DNA-dependent
C58	1	393	Multicopper oxidase typeI	7	470000	Oxidation of phenol and diamines
C63	1	1308	Peptidase M20	27	8300	Proteolysis, peptidolysis
C76	1	1187	Hypothetical protein, involved in membrane traffic	32	144900	Intracellular trafficking, secretion and vesicular transport
C91	4 (C91, D21, D22, D29)	-	unknown	47	25800	Unknown
D48	1	1234	Hypothetical protein, conserved	19	131000	Unknown
D90	1	595	Ribosomal protein S10	9	880500	Protein biosynthe-sis, translation, biogenesis
10	2 (10, 71)	1609	Tetracycline resistance protein TetB	7	500700	Tetracycline:hydrogen antiporter activity
22	1	1594	NADH dehydrogenase (domain)	46	21500	NADH dehydro-genase activity, energy conduction and conversion
33	3 (33, 86, 127)	2256	General sugar transporter	7	27000	Carbohydrate transport, sugar porter activity
74	1	2590	Tryptophan synthase	23	500100	Amino acid metabolism and transport
75	1	1734	Acetate and butyrate kinase	38	11000	Organic acid metabolism, phosphorylation
81	1	2699	Saccharopin dehydrogenase/reductase	28	200000	Amino acid transport and metabolism
88	2 (88, 141)	1486	Hypothetical protein	8	153000	Unknown
97	1	1780	Peptidase S28	39	158000	Serin-type peptidase activity, posttranslational modification, protein turnover
107	1	1319	Ubiquitin or ubiquitin like proten	13	510700	posttranslational modification, protein turnover, chaperones
109	6 (69, 87, 95, 100,109, 110)	656	Serine esterase Cutinase, hydrolase	5	542100	Hydrolase activity, extracellular, metabolism
115	1	770	Cutinase, acetyl xylan esterase II precursor	22	92600	Hydrolase activity
115a	1	763	Succinate dehydrogenase,cytochrome b subunit	5	551000	Tricarboxylic acid cycle, carbohydrate transport and metabolism, electron transport
140	1	1218	Endoglucanase candidate, fam. 61 (Cel61b), cellulose growth specific Glycoside hydrolyse	4	764000	Nucleic acid binding

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4.6 Expression analysis of selected genes during the antagonistic interaction with *R. solani*

According to the TRIRE and NCBI database research results, fourteen clones (C51, C63, D90, 10, 22, 33, 74, 75, 81, 97, 109, 115, 115a, 140) were used as probes to examine the expression of these genes during mycoparasitic interaction between *T. harzianum* and *R. solani*. Only four of the 14 clones confirmed the differences in expression patterns as anticipated by the reverse Northern blot experiments (Figure XX). The NADH dehydrogenase (clone 22) and Cel61b endoglucanase (clone 115) genes were not expressed at all during growth on SM+g. Their growth on SM+*Rhizoctonia* is described by a low level expression until 8h after contact, resulting in a dramatically increased expression after 12 to 24h of mycoparasitic interaction. Expression patterns of the putative acetyl xylan esterase (*axe1*; clone 115) showed a basal level of expression from 1h of growth under both target conditions, but increased significantly after 12 to 24h of growth on SM+*Rhizoctonia*. In contrast expression of the hexose transporter (clone 33) increased already after 3 h of growth on SM+*Rhizoctonia*, compared to a more ore less constant low level expression rate on SM+g. The clones C51, C63, D90, 10, 74, 75, 81, 97, 109, and 115a didn't exhibit any differential expression patterns during mycoparasitic interaction and are shown as examples for "false positives" (Fig. 24). Interestingly, all genes but the hexose transporter displayed two mRNA transcripts of different size, that could be due to different lengths of the 5' and 3' untranslated regions or alternative splicing.

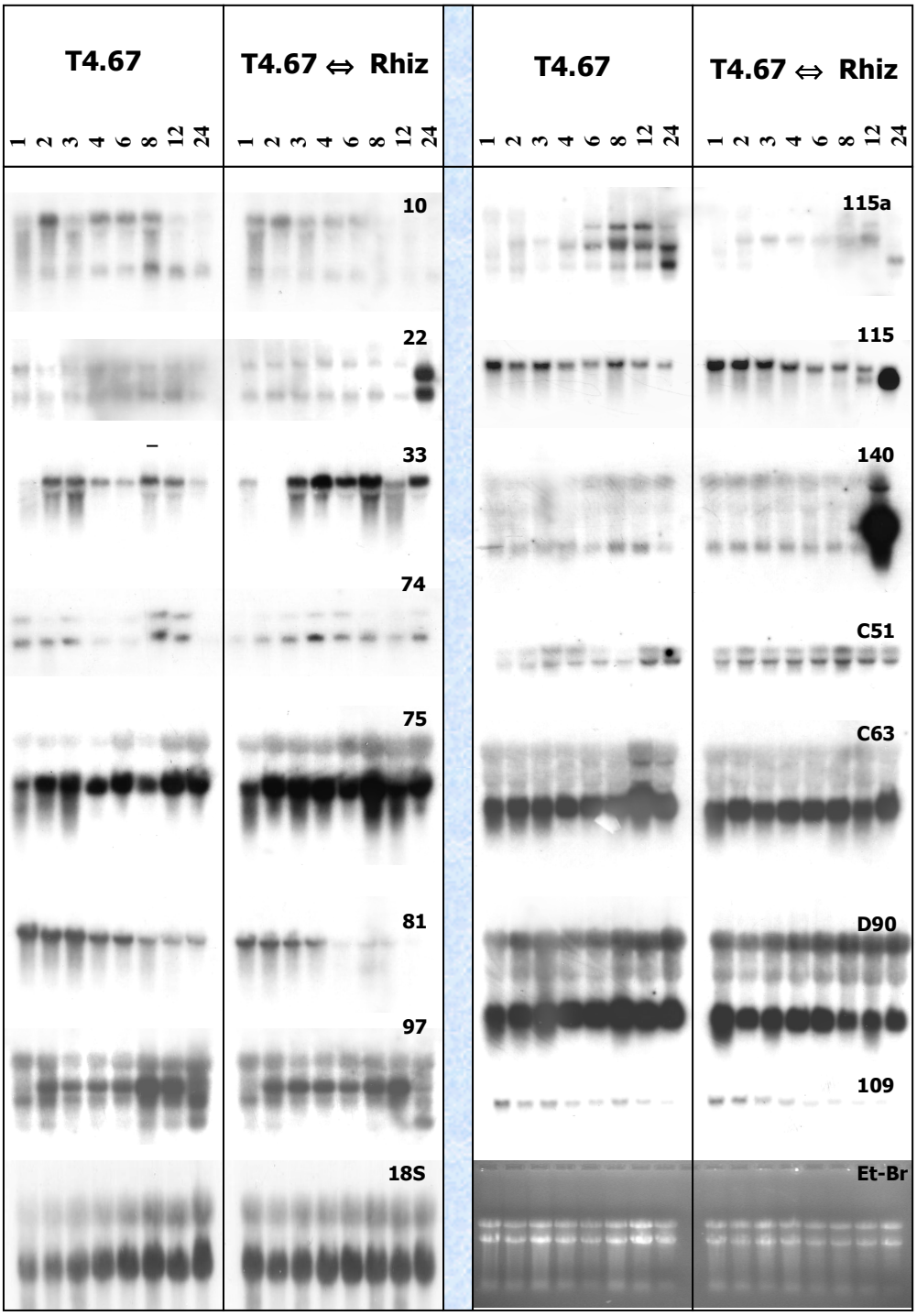

Figure 24:

Expression patterns by Northern blot analysis of selected subtracted library clones. RNA was prepared after 1, 2, 3, 4, 6, 8, 12, and 24h from *T. harzianum* antagonist isolate T4.67 grown on minimal medium containing glucose (driver), as well as from T4.67 during interaction with *R. solani* (tester) and loaded in the given order (20 µg per lane). Hybridisations were performed with [α-^{32}P]-labelled sequence tags as obtained by the RaSH experiment. 18S rRNA was used as hybridisation control.

4.7 Increased expression of the genes up-regulated during antagonistic interaction with a superior biocontrol strain of *T. harzianum*

Differential expression of genes corresponding to clones 22, 33, 115, and 140 was further characterised by carrying out confrontation experiments. Setting up experimental conditions for the confrontation assay, consisting in a direct mycoparasitic interaction of *Trichoderma* and *R. solani* on plates, gave us the possibility to learn about the expression of these genes under *in vivo* conditions. Furthermore, RNA was extracted not only from the potent biocontrol strain *T. harzianum* T4.67, but also from *T. harzianum* T4.31, a weakly antagonising strain not capable of biocontrol of *R. solani*, before – during and after interaction with *R. solani*. Scanning electron microscopy (SEM) of interaction between T 4.67 and T 4.31 with *R. solani* under the described *in vivo* conditions of the confrontation experiment, confirmed an elevated ability of antagonism of T 4.67 to sporulate abundantly in the presence of *R. solani* in comparison with T 4.31 (Figure 25).

The significant differences in gene expression of 22, 33, 115, and 140 of *Trichoderma* T4.67 interacting with *Rhizoctonia* unlike growing on SM+g suggested a possible marker gene character of these ESTs. Four other genes (88, 97, C45, and C14) which were not expected to be upregulated during mycoparasitism were included as a control.

The results (Figure 26) showed that indeed seven genes were expressed under these conditions, and only the transcript of C14 could not be detected. NADH dehydrogenase (22), endoglucanase Cel61b (140) and the S28 peptidase (97, originally not recognized as "mycoparasitic") were most strongly expressed. Only the acid phosphatase (88) and the cyclopropan fatty acyl phospholipid synthase (C45) failed to show a specific difference in the expression pattern, whereas all the other genes were

significantly overexpressed in the good biocontrol strain. The S28 peptidase thereby displayed two major transcripts, of which the larger one was only detected in the better biocontrol strain T4.67. Interestingly, genes represented by clones 22, and 140 do not seem to be expressed at all in the non-antagonistic isolate T4.31.

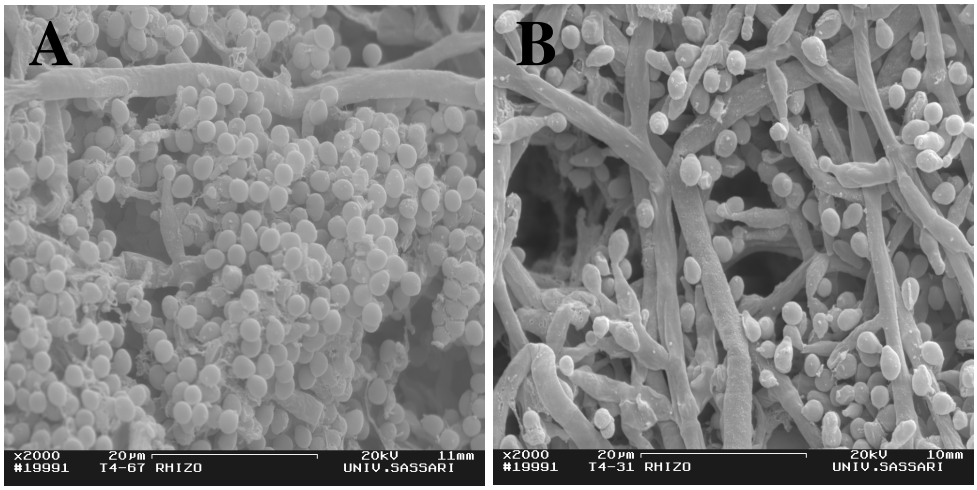

Figure 25:

Scanning electron microscopy (SEM) of interaction between the (A) antagonistic strain T 4.67 and (B) T 4.31, not capable of biocontrol, with *R. solani*

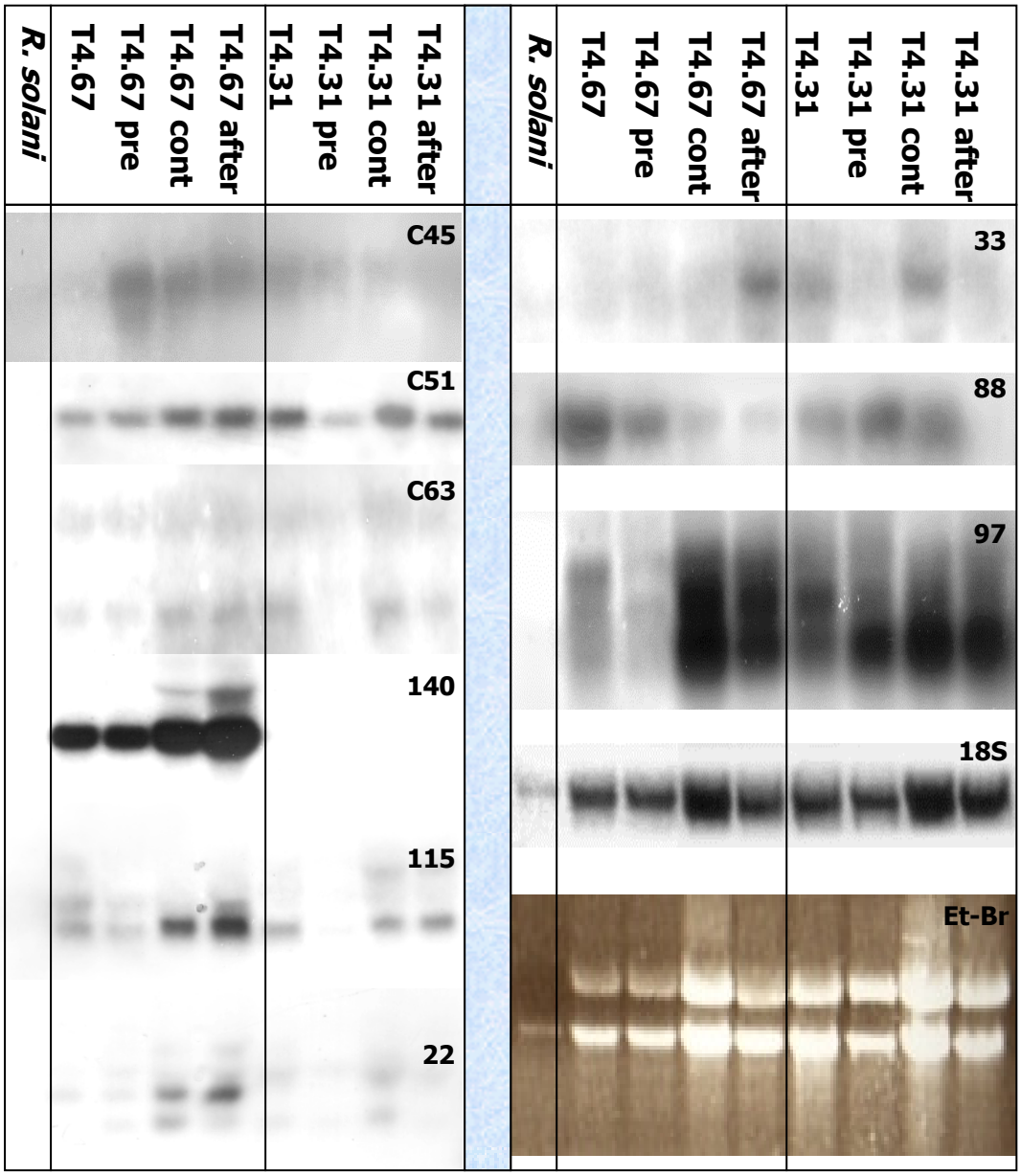

Figure 26:

Analysis of transcript abundance of genes expressed during *in vivo* mycoparasitic interaction with *R. solani* by Northern blotting. 20 µg of total RNA were applied in every lane, obtained from *Trichoderma harzianum* isolates T4.67 (antagonist) and T4.31 (non-antagonist) before – during – and after contact with *R. solani* in constant darkness and from pure cultures of the three fungi under otherwise equal conditions. Hybridisation was performed with the [α-^{32}P]-labelled clones/fragments C45, C51, C63, 140, 115, 22, 33, 88, 97; 18S rRNA was used as hybridisation control.

5. Discussion

5.1 Diversity of *Trichoderma/ Hypocrea* isolates in Sardinia

The idea of the first part of this investigation was to study *Hypocrea/Trichoderma* soil diversity of the Sardinian island ecosystem known for its floristic richness in order to detect potentially species capable of elevated mycoparasitic interaction with fungal plant pathogens, representing new biocontrol agents. Although the isolated species belonged nearly exclusively to common soil species already recorded in Eurasia, Africa and other continents, a set of highly efficient biocontrol *Trichoderma* strains were provided.

The study on *Hypocrea/ Trichoderma* species variety in soils of Sardinia showed a rather low diversity, illustrating pan-European and pan-global species, hence not belonging natively to the island, as yet described in various continental locations. On the basis of reliable molecular phylogeny methods (Druzhinina *et al.,* 2006) and the increasing interest in *Trichoderma/Hypocrea* species for research purposes and industrial applications, the number o newly discovered and identified species is growing.

The general soil diversity of *Hypocrea/ Trichodema* in Sardinia is relatively low, recording fifteen species among the 482 isolates obtained from fifteen soil samples, resulting in a diversity index (DI) of 0.03. Similar studies carried out in South-East Asia (Kubicek *et al., 2003*), South America (Druzhinina *et al.*, 2004), and China (Zhang *et al.*, 2005) showed diversity indexes of DI=0.15 (14 different species in a total of 96 isolates), DI=0.2 (10 different species over a total of 53 isolates) and DI=0.096 (13 different species over a total of 135 isolates), respectively. The striking low diversity index of this study could be due to the particular high number of isolates tested, as *T. harzianum, T.*

hamatum, T. spirale, T. virens, T. atroviride, T. asperellum, and T. koningii are all easily detectable, common soil species. The DI most similar to Sardinia geographic scale was applied in the study of Austrian virgin forest soil (Wuczkowski *et al.,* 2003), where 7 species were detected among 46 isolated strains (DI=0.15). The highest diversity of *Hypocrea/Trichoderma* was so far found in the rhizosphere of *Coffea arabica* in Ethiopian highland forests (every third strain is a different species, estimated diversity index 0.38 (T. M. Belayneh and I.S. Druzhinina, ms in preparation).

Within the Sardinian ecosystem highest DI values were observed in soils sampled on the eastern rocky side of the island (EG1 and EG4), in the Limbara and in the Gennargentu mountains, respectively. At the same time, pastures EG2 and EG5 and the shrublands S2 and S3, located on the low western part of Sardinia had the least number of *Hypocrea/Trichoderma* species per sample unit.

The diagnostic tool of ITS1 and ITS2 sequence analysis provided the opportunity of the detection of potentially endemic alleles within the *Trichoderma* isolates of this study (Kubicek *et al.,* 2003; Druzhinina *et al.*,. 2005; Zhang *et al.*., 2005; Hatvani *et al.,* 2007), as the alleles of all species of the genus are known. Strains endemic to the Sardinian island should be indicated by the presence of unique ITS1 and ITS2 alleles. Only one strain (*T. hamatum*) exhibited a single-nucleotide-polymorphism (SNP) in the ITS1 sequence, while all the remaining isolates possessed the well-established ITS1 and ITS2 alleles. This indicates that the *Hypocrea/Trichoderma* diversity in Sardinia is likely dominated by invasive species. In addition no impact on the appearance of most *Hypcrea/ Trichoderma* spp. was found to depend on major environmental properties (such as soil type and climatic conditions). Carriage of fungal spores has been shown to be possible over distances such as Africa and North America (Aylor, 2003). The lower western coast is

mainly exposed to Atlantic mistrals which potentially carry lower number of *Hypocrea/Trichoderma* propagules. The absence of species from section *Longibrachiatum* in this study would be an argument in favour of colonisation via air because these species have much larger conidia than other *Hypocrea/Trichoderma* species and are presumably less efficiently carried by clouds or dust.

Anyway, the possibility of incidence of endemic species of *Hypocrea/ Trichoderma* on the Sardinian island should not be completely excluded. Their existence could be supposed in remaining native forests associated with the rhizosphere of relict plants such as *Quercus ilex*, and *Taxus baccata.*

Regarding the distribution of Hypocrea/Trichoderma, this study contributed with two interesting findings. *T. tomentosum* was isolated repeatedly in different Sardinian soils. The location of *T. tomentosum* in Europe is interesting because of an ongoing speciation study on *T. tomentosum* and *T. cerinum* at the University of Vienna. The two species are believed to be very closely related, due to their very similar phenotype and their identical ITS sequences. The almost exclusively occurrence of *T. tomentosum* in America and of *T. cerinum* in Eurasia was an evidence for their allopatric speciation. The findings of *T. tomentosum* in Sardinia allow the possibility that these two species are only allelic variants of one and the same species. The second relevant finding is the detection of the anamorph of *H. semiorbis* in two Sardinian soils: while the anamorph of *H. semiorbis* has so far never been isolated, the teleomorph is believed to be restricted to Australia and Tasmania (Chaverri and Samuels, 2003). A disparity in the distribution of the teleomorph and anamorph in *Hypocrea/Trichoderma* is not without precedent, and has been observed for *H. schweinitzii* (Turner *et al.,*1997) and *H. pilulifera* (Hagn *et al.,* 2003).

In addition to this, a comparison of the ITS 1and 2 and *tef1* sequences of the only putative new species from this study with sequences present in databases showed that they are identical to those of *H. alni*, which has been isolated from several locations in Central and North Europe (W.M. Jaklitsch, ms in preparation) and whose anamorph is abundant in Austrian soils (M. Friedl and I.S. Druzhinina, unpublished data).

5.2 Biocontrol potential of the isolated strains

The greenhouse studies on the biocontrol abilities of 223 isolated *Trichoderma* strains, revealed a high percentage (37%) of strongly antagonising isolates, followed by xxx% of biocontrol strains of medium quality and xxx% of the isolates not capable of antagonism with the fungal plant pathogen *R. solani*. Test criteria were pushed towards a severe selection of only optimal biocontrol agents, by considering the elevated concentration and pathogenicity of *R. solani*. All the species were already described as successful agents of biological control against various plant pathogenic fungi (Harman *et al.*, 2004; Baek *et al.*, 1999; Turoczi *et al.*, 1996). The most frequently found effective biocontrol strains in this study were *T. harzianum* (21.4%), *T. virens* (57.9%), *Trichoderma sp. VD2* (46.7%), *T. hamatum* (45.5%), and interestingly *T.* gamsii (54.4%). *T. gamsii* which was described only recently (Jaklitsch *et al.,* 2006) was shown to be an effective biocontrol species as it is demonstrated by the high proportion of isolates with high antagonistic efficiency identified in this study. Concering this matter it was remarkable that *T. asperellum* and *H. atroviridis*/*T. atroviridis* were isolated only two times. These two species are well known biocontrol agents which usually dominate infrageneric communites of temperate soil (Druzhinina, Friedl, Komon-Zelazowska, unpublished data). The weak representation of these highly competitive species may be due to their

inability to adapt to semi-arid climatic conditions in Sardinia, or by the fact that they are suppressed by other dominant species. This might be an important issue in view of the widespread use of these two species as biocontrol agents.

5.3 Identification of genes involved in antagonism towards *R. solani*

Identification of genes which play key roles in physiological processes has strongly advanced by the aid of techniques which identified differentially expressed genes. Despite the widespread use of this principle with industrially applied and plant pathogenic fungi, it has suprisingly not been thoroughly applied to the study of mycoparasitism and biocontrol. During this Ph.D. two papers appeared on *T. harzianum* gene expression (Vizcaino *et al.*, 2006, 2007) and one paper concerning a substractive hybridisation in a strain of *T. hamatum* (Carpenter *et al.*, 2005). The papers on *T. harzianum* dealt with EST genomics, unfortunately not distinguishing between houskeeping genes and such genes specifically involved in possible mycoparasitism. Housekeeping genes are strongly expressed under all conditions and are consequently not specific to mycoparasitism. Furthermore the target condition of these analyses was growth of *T. harzianum* on cell walls of plant pathogenic fungi, hence not considering the interaction with the living host. The paper on *T. hamatum* uses a general NCBI BLAST search to identify the clones obtained by the substractive hybridisation. This approach could be problematic when the gene fragment encodes a poorly conserved part of the protein. The search results then usually don't describe actual characteristics and identity of the encoded gene. In this case the best hits can come up with proteins from other kingdoms, e.g. bacteria plants or even mammals. In contrast, in the present study – which first searched for full length orthologues in the closely related fungus *H.*

jecorina/*T. reesei* – all of the clones gave closest neighbours in other fungal species, or rather showed that their similarity was highest with closest neighbours from GenBank (*G. zeae* and *M. grisea*). For the 14 clones which could not be identified, no proteins were found with similarity in non-fungal kingdoms. One should remember that – while cases of apparently horizontal transfer have been found in *Trichoderma* and other fungi (El-Bondkly, 2006) - the annotation of the genome sequences of several filamentous fungi has provided evidence for only a small number of cases of horizontal transfer, in most cases from bacteria (Braun *et al.*, 2000).

The clones/genes identified in this study roughly fell into three groups: proteins involved in protein synthesis and turn-over; proteins involved in metabolism, and proteins involved in the hydrolysis of macromolecules. As a general rule, this reflects the necessity to degrade the cell wall of the mycoparasitic host, to take up and metabolise its constituents, but also to provide the energy needed for an induced synthesis, modification and excretion of the hydrolases and eventually to compete with the metabolic rate of the host. Such an interpretation is also supported by the finding that most of the genes tested were more expressed in the better biocontrol strain, thereby indicating that the major advantage of this strain may consist in a more rapid and efficient reaction to the presence of the host.

Increased expression of a respiratory-chain NADH-dehydrogenase (clone 22) appeared abruptly after 24 h of interaction between *T. harzianum* T4.67 and *R. solani*, resulting in two strong transcripts differing in size, whereas no expression at all was detectable from 1 to 24 h of growth for T4.67 grown alone (driver). Different transcript sizes could depend upon alternative splicing or different lengths of the 5' and 3' untranslated

region. In addition confrontation test analysis demonstrated the solely expression of the NADH-dehydrogenase by the biocontrol strain (T 4.67) compared to T 4.31 not capable of mycoparasitism. The identified gene fragment 22 was homologue to the domain of a respiratory-chain NADH dehydrogenase 51 kDa subunit of *T. reesei*, belonging to an oligomeric enzymatic complex located in the inner mitochondrial membrane. This subunit is described for mammal cells, but shows sequence similarities to different regions of the bacterial hydrogenase alpha subunit, and being part of a quinone-ubiquinone oxydoreductase system should also be present in fungi (Weiss *et al.*, 1991). Whether or not this NADH dehydrogenase plays an important role in mycoparasitism remains to be explained in detail.

The isolated hexose/ general substrate transporter, exhibited with driver as well as tester RNA an almost constant expression signal starting from 2 h of interaction, although expression levels of the hexose transporter gene were always more intense in the tester than in the driver. In the confrontation experiment the transporter was strongly expressed in the biocontrol strain during the "after contact" stage, but these findings were not in a striking contrast to the T 4.31 expression pattern, showing a weak signal during the "contact" phase. The hexose transporter belongs to the major facilitator superfamily (MFS), also called the uniporter-symporter-antiporter family (Pao *et al.*, 1998). Like the ATP-Binding Cassette (ABC) superfamily, the MFS occurs ubiquitously in all classifications of organisms. MFS transporters are single-polypeptide secondary carriers capable only of transporting small solutes in response to chemiosmotic ion gradients. ABC superfamily and MFS account for half of the solute transports encoded within the genomes of microorganisms, and are also prevalent in higher organisms (Pao *et al.*, 1998). The sugar transporters belong to a superfamily of membrane proteins responsible for the binding and transport of various carbohydrates,

organic alcohol, and acids in a wide range of prokaryotic and eukaryotic organisms (Paulsen *et al.*, 1996). These integral membrane proteins are predicted to comprise twelve membrane spanning domains. It is likely that the transporters have evolved from an ancient protein present in living organisms before the divergence into prokaryotes and eukaryotes. In mammals, these proteins are expressed in a number of organs (Henderson and Maiden, 1990).

Expression patterns of a high mobility box gene (C51), a metalloprotease (C63), and a cyclopropane synthase (C45) didn't reveal any differences between the tester and the driver target conditions. Anyway these genes were also included in the confrontation experiment, where all three of them were upregulated during the pre-contact, contact, and after-contact stages of the biocontrol strain T 4.67, in contrast to the weak antagonist T 4.31 where expression signals of these genes were less intense or even absent. The isolation of a cyclopropane synthase clone was intriguing. This enzyme catalyzes the addition of a methylene group from S-adenosylmethionine to the cis-double bond of oleic acid (Bao *et al.*, 2002). In *Mycobacterium tuberculosis*, this enzyme is related to α-mycolic acid synthesis and is likely to affect cell wall structure, and has been found to be a virulence factor. High mobility group (HMG) box domains are involved in binding DNA, and may be involved in protein-protein interactions as well. The HMG proteins represent a class of chromatin-associated proteins discovered more than 30 years ago (Goodwin *et al.*, 1973). HMG-box domains are found in one or more copies in HMG-box proteins, which form a large, diverse family involved in the regulation of DNA-dependent processes such as transcription, replication, and strand repair, all of which require the bending and unwinding of chromatin. Many of these proteins are regulators of gene expression. (Thomas and Travers, 2001). The HMG box is a characteristic L-shaped domain of about 80 amino acid residues, which binds the

DNA minor groove (Thomas and Travers, 2001). By binding to DNA, HMG boxes induce DNA bending, and this "architectural feature" can be considered as their major function. HMG-box proteins are found in a variety of eukaryotic organisms, and can be broadly divided into two groups, based on sequence-dependent and sequence-independent DNA recognition; the former usually contain one HMG-box motif, while the latter can contain multiple HMG-box motifs (Serra and Bianchi, 2007). In fungi the HMG-box domains are found in mating type proteins (MAT) involved in the sexual reproduction of fungi (Barve *et al.*, 2003). Proteases can be divided in four main types, of which again metalloproteases are the most diverse with more than 50 families. Divalent cations, usually zinc, active the water molecule in these enzymes. The majority of zinc-dependent metallopeptidases (with the notable exception of the carboxypeptidases) share a common pattern of primary structure (Jongeneel *et al.*, 1989) in the part of their sequence involved in the binding of zinc, and can be grouped together as a superfamily, known as the metzincins, on the basis of this sequence similarity (Murphy *et al.*, 1991). The metal-binding sites were shown to mostly possess a conserved HEXXH motif, forming the catalytic centre of the proteases (Rawlings and Barret, 1995).

Being especially interested in antagonistic, mycoparasitic interactions and the proteins enduing important roles in this situation, we had a closer look at four further clones, a phosphatase with a single trans-membrane domain (clone 88), a S28 peptidase/serine protease (clone 97), an acetyl xylan esterase (*axe1*, clone 115) and the Cel61b endoglucanase (clone 140). Only the acetyl xylan esterase and the Cel61b endoglucanase showed striking expression increases of the tester (after 12 to 24 h) in confrontation to the driver, while phosphatase and peptidase didn't evidence differences in gene expression between tester and driver test conditions. The

phosphatase confirmed in the following confrontation assay these data, displaying no expression differences, neither during interaction of the antagonistic strain with *R. solani* nor during interaction of the non-antagonistic strain with this pathogen. In contrast, all three extracellular enzymes were significantly over-expressed during antagonism. The identified serine protease/ S28 peptidase showed a strong switch-on expression pattern passing over from the pre-contact state to contact with the pathogen. Serine proteases are ubiquitous proteolytic enzymes that exploit serine in their catalytic activity, being found in viruses, bacteria and eukaryotes. They include a wide range of peptidase activity, including exopeptidase, endopeptidase, oligopeptidase and omega-peptidase activity. The esterase clone seems to be directly involved in mycoparasitism, coding for a cutinase, an extracellular enzyme with a lipase active site (Ettinger *et al.*, 1987). Cutinases hydrolyse cutin, facilitating fungal penetration through the cuticle. The biochemical nature of cutinases is that of serine esterases and the TRIRE database search for this clone showed 69% coverage and 77% identity to an acetyl xylan esterase precursor (AXE1 TRIRE) of *T. reesei*, and 90% coverage and 71% identity to AXE of *Penicillium purpurogenum,* and could be a candidate acetyl xylan esterase itself, even being shorter at the N-terminus. The third extracellular enzyme turned out to be a fragment of a glycoside hydrolase-encoding gene belonging to family 61, one out of 85 families known, possessing a fungal cellulase-binding region (Henrissat, 1991). Its homology to Cel61b gene of *H. jecorina* was 100% on the TRIRE database search. Cel61b protein was described by Foreman (2003), but further characterisation is still lacking. Glycosyl hydrolases catalyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Davies and Henrissat, 1995). The only known activity within the family 61 is that of endoglucanase. Cel61b endoglucanase seems to be induced in the same or a similar way like other cellulases,

that is by cellulose and sophorose as described recently (Schmoll *et al.*, 2004, 2005; Zeilinger *et al.*, 1998). The cellulose binding domain (CBD) of Cel61b could be a clue to a significant role during mycoparasitism, being present also in cellobiohydrolases and xylanases that are essential in microbial degradation of cellulose and xylanes. Also the GUN4 TRIRE protein belongs to family 61, showing quite high homologies with similar proteins of other organisms. Homology to Cel61b of clone 140 is much lower as 70% of coverage and 48% identity, probably indicating a *T. harzianum* specific protein. Interestingly, the Cel61b is only expressed by the tester after 12 h, and not at all by the driver. Total absence of expression during interaction of the non-antagonistic *T. harzianum* isolate T 4.31 with *R. solani* is indicative of a specific role of the Cel61b endoglucanase in overcoming the pathogenic fungus, as could be the case for the NADH-dehydrogenase and the acetyl xylan esterase already mentioned above.

The main purpose of this study was to isolate genes which could be used as markers for the identification of biocontrol strains and their monitoring after application in the field. Indeed, several such genes were identified, the most promising ones being gene 22 (NADH dehydrogenase), gene 115 (acetyl xylan esterase) and gene 140 (Cel61b endoglucanase). Although in theory these need not necessarily to be identical with genes which are critical or essential for mycoparasitism and biocontrol, they turned out to be more expressed in the effective biocontrol strain than in the non-antagonistic strain. If the hypothesis of the enhanced metabolic activity of this strain is correct, it would be logical to expect that other good biocontrol strains of *T. harzianum* - or even any good antagonistic *Trichoderma* spp. - are also characterised by faster growth and faster sporulation. The thorough description of soils of the island of Sardinia for their diversity in/of *Trichoderma* spp. and the following screeing for potential biocontrol

strains in the greenhouse, provided us a vast assortment of strains that will serve as a basis to test such hypothesis.

6. References

Andrade M.A., Petosa C., O'Donoqhue S.I., Muller C.W., Bork P. (2001). Comparison of ARM and HEAT protein repeats. J. Mol. Biol. 309, 1-18

Ait-Lahsen H., Soler A., Rey M., De la Cruz J., Monte E., Llobell A. (2001). An antifungal exo-alpha-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. Appl. Environ. Microbiol. 67, 5833–5839

Aljanabi S.M., Martinez I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR based techniques. Nucleic Acids Res 25, 4692-4693

Antal Z., Kredics L, Manczinger L., Ferenczy L. (2001). Extracellular enzyme profiles of mycoparasitic *Trichoderma* strains. IOBC/wprs Bull 23, 337-340

Aylor D.E. (2003). Spread of plant disease on a continental scale: role of aerial dispersal of pathogens. Ecology 84, 1989-1997

Bao X., Katz S., Pollard M., Ohlrogge J. (2002). Carbocyclic fatty acids in plants: biochemical and molecular genetic characterization of cyclopropane fatty acid synthesis of Sterculiafoetida. Proc Natl Acad Sci USA 99, 7172-7177

Baek J.M., Howell C.R., Kenerley C.M. (1999). The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. Curr Genet 35, 41-50

Barve M. P., Santra D. K., Ranjekar P. K., Gupta V. S. (2004). Genetic diversity analysis of a world-wide collection of *Ascochyta rabiei* isolates using sequence tagged microsatellite markers. World Journal of Microbiology & Biotechnology 20, 735–741

Bendtsen J.D., Nielsen H., Heijne von G., Brunak S. (2004). Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol., 340, 783-795

Benitez T., Rincon A. M., Limon M. C., Codon A. C. (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7, 249-260

Bisby G.R. (1939). *Trichoderma viride* Pers. ex Fries, and note on *Hypocrea*. Trans. Br. Mycol. Soc. 23, 149-168

Bissett J. (1992). *Trichoderma atroviride*. Can J Bot 70, 639–40

Bissett J. (1991a). A revision of the genus *Trichoderma*. II. Infrageneric classification. Canad. J. Botany 69, 2357-2372

Bissett J. (1991b). A revision of the genus *Trichoderma*. III. Sect. *Pachybasium*. Canad. J. Botany 69, 2373-2417

Braun E.L., Halpern A.L., Nelson M.A., Natvig D.O. (2000). Large-scale comparison of fungal sequence information : mechanisms of innovation in *Neurospora crassa* and gene loss in *Saccharomyces cerevisiae*. Genome Res. 10, 416-430

Brunner K., Zeilinger S., Ciliento R., Woo S.L., Lorito M., Kubicek C.P., Mach R.L. (2005). Improvement of the fungal biocontrol agent Trichoderma atroviride to enhance both antagonism and induction of plant systemic disease resistance. Appl Environ Microbiol 3959-3965

Buchert J., Oksanen T., Pere J., Siika-aho M., Suurnäkki A., Viikari L. (1998). Application of *Trichoderma reesei* enzymes in pulp and paper industry in *Trichoderma and Gliocladium: Enzymes, biological control and commercial applications* (Harman, G. E. & Kubicek, C. P., eds), Taylor & Francis, Ltd., London

Cabib, E. (1987). The synthesis and degradation of chitin. Adv Enzymol Relat Areas Mol Biol 59, 59-101.

Carpenter M.A., Stewart A., Ridgway H.J. (2005). Identification of novel *Trichoderma hamatum* genes expressed during mycoparasitism using subtractive hybridisation. FEMS Microbiol. Letters 251, 105-112

Carsolio C., Benhamou N., Haran S., Cortes C., Gutierrez A., Chet I., Herrera-Estrella A. (1999). Role of the *Trichoderma harzianum* endochitinase gene, *ech42*, in mycoparasitism. Appl Environ Microbiol 65, 929-935

Chaverri P., Samuels G.C. (2003). In: Studies in Mycology: *Hypocrea/Trichoderma* (*Ascomycota*, *Hypocreales*, *Hypocreaceae*): species with green ascospores.

Chirgwin J.M., Przybyla A.E., MacDonald R.J., Rutter W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299

Cohen-Kupiec R., Broglie K.E., Friesem D., Broglie R.M., Chet I. (1999). Molecular characterization of a novel β-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. Gene 226, 147–154

Cook R. J., Baker K. F. (1983). The nature and practice of biological control of plant pathogens. American Phytopathological Society, St.Paul, MN.

Cortes C., Gutierrez A., Olmedo V., Inbar J., Chet I., Herrera-Estrella A. (1998). The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol Gen Genet. 260, 218-225

Danielson R.M., Davey C.B. (1973). The abundance of *Trichoderma* propagules and the distribution of species in forest soils. Soil Biol Biochem 5,486-494

Davies G., Henrissat B. (1995). Structures and mechanisms of glycosyl hydrolases. Structure 3, 853-859

Day P.R. (1965). Particle fractionation and particle-size analysis. In *Methods of soil analysis*. Black, C.A. (ed). Madison, WI; American Society of Agronomy, 545-567

Deacon J.W. (1997). Modern Mycology. Blackwell Scientific, Oxford.

De La Cruz J., Pintor-Toro J.A., Benitez T., Llobell A., Romero L.C. (1995). A novel endo-beta-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J. Bacteriol 177, 6937-6945

De La Cruz J, Llobell A. (1999). Purification and properties of a basic endo-B-1,6-glucanase (Bgn16.1) from the antagonistic fungus *Trichoderma harzianum*. European Journal of Biochemistry. Vol. 265. 1999. Pag. 145-151

Delgado-Jarana, J., Pintor-Toro J. A., Benítez, T. (2000). Overproduction of ß-1,6-glucanase in *Trichoderma harzianum* is controlled by extracellular acidic proteases and pH. Biochim. Biophys. Acta 1481, 289-296

Delgado-Jarana J., Rincón A.M., Benítez T. (2002) Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization. Microbiology 148, 1305–1315

Donzelli B.G.G., Harman G.E. (2001). Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. Appl Environ Microbiol 67, 5643-5647

Druzhinina I.S., Kopchinskiy A.G., Komon M., Bissett J., Szakacs G., Kubicek C.P. (2005). An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fung. Genet. Biol. 42, 813-828

Druzhinina I., Chaverri P., Fallah P., Kubicek C.P., Samuels G.J. (2004). *Hypocrea fluciconidia*, a new species with yellow conidia from Costa Rica. Studies in Mycology 50, 401-407

Druzhinina I.S., Kopchinskiy A., Kubicek C.P. (2006). The first one hundred *Trichoderma* species characterized by molecular data. Mycoscience 47, 55–64.

Druzhinia I., Kubicek C.P. (2005). Species concept and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters?. J. Zhejiang University Science 6B (2), 100-112

Elad, Y., Kapat, A. (1999). Role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol. 105,177-189

Elad Y., Chet I., Henis Y. (1981). A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica 9, 59-68

El-Bondkly A.M. (2006). Gene transfer between different *Trichoderma* species and *Aspergillus niger* through intergeneric protoplast fusion to convert ground rice straw to citric acid and cellulases. Appl. Biochem. Biotechnol. 135, 117-32

Ettinger W.F., Thukral S.K., Kolattukudy P.E. (1987). Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi. Biochemistry 26, 7883-7892

Flores A., Chet I., Herrera Estrella A. (1997). Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1. Current Genetics 31, 30-37

Foreman P.K. (2003). Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. J. Biol. Chem. 278, 31988-31997

Fuglsang C.C., Berka R.M., Wahleithner J.A., Kauppinen S., Shuster J.R., Rasmussen G., Halkier T., Dalboge H., Henrissat B. (2000). Biochemical analysis of recombinant fungal mutanases. A new family of α-1,3-glucanases with novel carbohydrate-binding domains. J Biol Chem 275, 2009–2018

Gams W. (1971). *Cephalosporium*-artige Schimmelpilze (Hyphomycetes). G. Fischer, Stuttgart

Gams W., Bissett J. (1998). Morphology and identification of *Trichoderma*. In: G.E. Harman, C.P. Kubicek (Eds.), *Trichoderma and Gliocladium*. Vol. 1. Basic Biology, Taxonomy and Genetics, Taylor & Francis, London, UK, 3-34

Geremia R.A., Goldman G.H., Jacobs D., Ardiles W., Villa S.B., Van Montagu M., Herrera-Estrella A. (1993). Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. 8, 603-613

Gielen S., Aerts R., Seels B. (2004). Biocontrol agents of *Botrytis cinerea* tested in climate chambers by making artificial infection on tomato leafs. Commun Agric Appl Biol Sci. 69, 631-9

Gooday G.W. (1990). The ecology of chitin degradation. Adv. Microb. Ecol. 11, 387-430

Goodwin G.H., Sanders C., Johns E.W. (1973). A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. Eur J Biochem 38,14–19

Hagn A., Pritsch L., Schloter M., Munch J.C. (2003). Fungal diversity in agricultural soil under different framing management systems, with special reference to biocontrol strains of *Trichoderma* spp. Bio. Fert. Soils 38, 236-244

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

Hatvani L., Antal S., Manczinger L., Szekeres A., Druzhinina I.S., Kubicek C.P. (2007). Green mold diseases of *Agaricus* and *Pleurotus* are caused by related but phylogenetically different *Trichoderma* species. Phytopathology 97, 532-537

Henderson P.J., Maiden M.C. (1990). Homologous sugar transport proteins in *Escherichia coli* and their relatives in both prokaryotes and eukaryotes. Philos. Trans. R. Soc. Lond. B Biol. Sci., 326, 391–410

Henrissat B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280**,** 309–316

Horsch M., Mayer C., Sennhauser U., Rast D. M. (1997). beta-*N*-acetylhexosaminidase: a target for the design of antifungal agents. Pharmacol Ther 76, 187-218

Hjeljord L., Tronsmo A. (1998). Trichoderma and *Gliocladium* in biological control.: An overview, in: G.E. Harman, C.P. Kubicek (Eds.), *Trichoderma* and *Gliocladium*: vol. 2, Enzymes, Biological Control and Commercial Application, Taylor & Francis, London, UK, 129-151

Inbar J., Chet I. (1995). The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harizanum*. Microbiology 141, 2823-2829

Jacobs H., Gray S.N. (2003). Crump DH Interactions between nematophagous fungi and consequences for their potential as biological agents for the control of potato cyst nematodes. Mycol Res 107, 47-56

Jaklitsch W.M., Samuels G.J., Dodd S.L., Lu B.-S., Druzhinina I.S. (2006). *Hypocrea rufa/Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. Studies in Mycology 56, 135–177

Jiang H., Kang D.C., Alexandre D., Fisher P.B. (2000). RaSH, a rapid subtraction hybridisatin approach for identifying and cloning differentially expressed genes. Proc. Natl. Acad. Sci. USA 97, 12684-12689

Jongeneel C.V., Bouvier J., Bairoch A. (1989). A unique signature identifies a family of zinc-dependent metallo peptidases. FEBS Lett. 242, 211-214

Kim D.J., Baek J.M., Uribe P., Kenerley C.M., Cook D.R. (2002). Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*. Curr Genet 40, 374–384

Klein D., Eveleigh D.E. (1998). Ecology of *Trichoderma*. In: Kubicek C.P., Harman G. E. (Eds.). *Trichoderma* and *Gliocladium*. *Basic Biology, Taxonomy and Genetics*, Vol. 1, Taylor and Francis, London, 57-73

Kapat A., Zimand G., Elad Y. (1998). Effect of two isolates of *Trichoderma harzianum* on the activity of hydrolytic enzymes produced by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 52, 127–137

Kobayashi S., Toshitsugu K., Shin-ichiro S. (1997). A novel method for synthesis of chitobiose via enzymatic glycosylation using a sugar oxazoline as glycosyl donor. Tetrahedron Lett 38, 2111-2112

Kopchinskiy A., Komon M., Kubicek C.P., Druzhinina I.S. (2005). *Tricho*BLAST: a multiloci database for *Trichoderma* and *Hypocrea* identification. Mycological Research 109, 657 –660

Kredics L., Antal Z., Doczi I., Manczinger L., Kevei F., Nagy E. (2003). Clinical importance of the genus *Trichoderma* – a review. Acta Microbio. Immunol. Hungarica 50, 105-117

Kubicek C.P., Bissett J., Druzhinina I., Kullnig-Gradinger C.M., Szakacs G. (2003). Genetic and metabolic diversity of *Trichoderma*: A case study on South-East Asian isolates. Fungal Gent. Biol. 38, 310-319

Kubicek C.P., Penttilä M.E. (1998). Regulation of plant polysaccharide degrading enzymes by *Trichoderma*. In: Kubicek C.P., Harman G. E. (Eds.) *Trichoderma* and *Gliocladium*. Vol. 2. *Enzymes, Biological Control and Commercial Applications*. Taylor and Francis, London, 49-71

Kulkarni N., Shendye A., Rao M. (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev. 23, 411-456

Kullnig C., Mach R.L., Lorito M., Kubicek C.P. (2000). Enzyme diffusion from *Trichoderma viride* (= *T. harzianum*), P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma ech42* gene expression before mycoparasitic contact. Appl. Environml. Microbiol. 66, 2232-2234

Kullnig-Gradinger C.M., Szakacs G., Kubicek C.P. (2002). Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. Mycol Res 106, 757-767

Li A., Antizar Ladislao B., Khraisheh M. (2007). Bioconversion of municipal solid waste to glucose for bio-ethanol production. Bioprocess Biosyst. Eng 30, 189-196

Lisboa De Marco J., Valadares-Inglis M.C.l, Felix C.R. (2004). Purification and characterization of an N-acetylglucosaminidase produced by a *Trichoderma harzianum* strain which controls *Crinipellis perniciosa*. Appl Microbiol Biotechnol 64, 70-75

Lorito M., Farkas V., Rebuffet S., Bodo B., Kubicek C.P. (1996). Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J. Bacteriol. 178, 6382-6385

Mahadevan P.R., Tatum E.L. (1967**).** Localization of structural polymers in the cell wall of *Neurospora crassa*. The Journal of Cell Biology, 35, 295-302

Maniatis T., Fritsch E.F., & Sambrook, J. (1982). *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Mauch, F., Mauch-Mani B., Boller T. (1988). Antifungal hydrolases in pea tissues. II. Inhibition of fungal growth by combinations of chitinases and b-1,3-glucanases. Plant Phisiol. 88, 936-942

Mazzola M. (2002). Mechanisms of natural soil suppressiveness to soilborne diseases Antonie Van Leeuwenhoek 81, 557-64

Migheli Q., González-Candelas L., Dealessi L., Camponogara A., Ramón-Vidal D. (1998). Transformants of *Trichoderma longibrachiatum* overexpressing the beta-1,4-endoglucanas gene *egl1* show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology 88, 673-677

Montero M., Sanz L., Rey M., Monte E., Llobell A. (2005). BGN16.3, a novel acidic β-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. FEBS Journal 272, 3441-3448

Murphy G.J., Murphy G., Reynolds J.J. (1991). The origin of matrix metalloproteinases and their familial relationships. FEBS Lett 289, 4-7

Nei M., Li W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76, 5269-5273

Nelson D.W., Sommers L.E. (1996). Total carbon, organic carbon and organic matter. *In* D.L. Sparks et al. (ed.) Methods of soil analysis, Part 3. 3rd ed. SSSA, Book Ser. 5. SSSA, Madison, WI., 961–1010

Olmedo-Monfil V., Mendoza-Mendoza A., Gómez I., Cortés C., Herrera-Estrella A. (2002). Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene *prb1* in *Trichoderma atroviride*. Mol. Genet. Genomics 267, 703–712

Paulitz T.C., Bélanger R.R. (2001). Biological control in greenhouse systems. Ann. Rev. Phytopathol. 39, 103-133

Pao S.S., Paulsen I.T., Saier Jr M.H. (1998). Major facilitator superfamily. Microbiol Mol Biol Rev 62, 1-34

Paulsen I.T., Brown M.H., Skurray R.A. (1996). Proton-dependent multidrug efflux systems. Microbiol Rev 60, 575-608

Persoon C.H. (1794). Disposita methodical fungorum. Roemer's Neues Mag. Bot. 1, 81-128

Pozo M.J., Baek J.M., Garcia J.M., Kenerley C.M. (2004). Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet Biol 41, 336-348

Prabavathy V.R., Mathivanan N., Sagadevan E., Murugesan K., Lalithakumari D. (2006). Self-fusion of protoplasts enhances chitinase production and biocontrol activity in *Trichoderma harizanum*. Bioresource Technol 97, 2330-2334

Ramot O., Cohen-Kupiec R., Chet I. (2000). Regulation of β-1,3-glucanase by carbon starvation in the mycoparasite *Trichoderma harzianum*. Mycol Res 104, 415-420

Rawlings N.D., Barrett A.J. (1995). Families of aspartic peptidases, and those of unknown catalytic mechanism. Methods Enzymol. 248, 105-120

Rawlings N.D., Barrett A.J. (1995). Evolutionary families of metallopeptidases. Methods Enzymol. 248, 183-228

Rifai M.A. (1969). A revision of the genus *Trichoderma*. Mycol. Papers 116, 1-116

Rocha-Ramírez et al. (2002). *Trichoderma atroviride* G-protein alpha-subunit gene tga1 involved in mycoparasitic coiling and conidiation. Eukaryotic Cell, 1, 594-605

Roiger D.J., Jeffers S.N., Caldwell R.W. (1991). Occurance of *Trichoderma* species in apple orchard and woodland soils. Soil Biol. Biochem. 23, 353-359

Rossman A.Y. (1996). Morphological and molecular perspectives on systematics of the *Hypocreales*. Mycologia 88, 1-19

Sahai A.S., Manocha M.S. (1993). Chitinases of fungi and plants: involvement in morphogenesis and host parasite interaction. FEMS Microbiology Review 11, 317-338

Saloheimo M., Nakari-Setaelae T., Tenkanen M., Penttilae M. (1997). Eur. J. Biochem. 249, 584-591

Sambrook, J., Fritsch E.F., Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuels G.J. (1996). *Trichoderma*: a review of biology and systematics of the genus. Mycological Research 100, 923-935

Samuels, G.J. (2005). Changes in taxonomy, occurrence of the sexual stage and ecology of *Trichoderma* spp. Phytopathology 96, 195-206

Samuels G.J., Dodd S., Lu B., Petrini O., Schroers H., Druzhinina I. S. (2006). The *Trichoderma koningii* morphological species. Studies in Mycology. 56, 67-133

Samuels G.J. (2006). *Trichoderma*: Systematics, the Sexual State, and Ecology. Phytopathology 96, 195-206

Samuels G.J., Dodd S.L., Lu B.-S., Petrini O., Schroers H.-J., Druzhinina I.S. (2006). The *Trichoderma koningii* aggregate species. Studies in Mycology 56, 67-133

Sanz L., Montero M., Redondo J., Llobell A., Monte E. (2005). Expression of an a-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J 272, 493–499

Schirmbock M., Lorito M., Wang Y. L., Arisan-Atac I., Scala F., Kubicek C. P. (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytophathogenic fungi. Applied Environmental Microbiology. 60, 9-16

Schmoll M., Kubicek C.P. (2003). Regulation of *Trichderma* cellulase regulation: lessons in molecular biology from an industrial fungus. A review. Acta Microbiol. Immunol. Hung. 50 (2-3), 125-145

Schmoll M., Zeilinger S., Mach R.L., Kubicek C.P. (2004). Cloning of genes expressed early during cellulase induction in *Hypocrea jecorina* by a rapid subtraction hybridization approach. Fung. Gent. Biol. 42, 877-887

Schmoll M., Franchi L., Kubicek C.P: (2005). Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (Anamorph *Trichoderma reesei*), modulates cellulase gene transcription in response to light. Eukaryot Cell 4, 1998-2007

Schrot M.N., Hancock J.G. (1982). Disease-suppressive soil and root-colonizing bacteria. 216, 1376-1381

Seaby D. (1998). *Trichoderma* as a weed mould or pathogen in mushroom cultivation. In: Harman G.E., Kubicek C.P., eds. *Trichoderma* and *Gliocladium*. Vol. 2. Enzymes, biological control and commercial applications. London: Taylor & Francis, 267–287

Seidl V., Marchetti M., Schandl R., Allmaier G., Kubicek C.P. (2006). Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitiors. FEBS J 273, 4346-4359

Sessa L., Bianchi M.E. (2007). The evolution of High Mobility Group Box (HMGB) chromatin proteins in multicellular animals. Gene 387, 133-140

Steyaert J., Stewart A., Jaspers M.V., Carpenter M., Ridgway H. J. (2004). Co-expression of two genes, a chitinase (*chit42*) and proteinase (*prb1*) implicated in mycoparasitism by *Trichoderma hamatum*. Mycologia 96, 1245-1252

St. Leger R.J., Charnley A.K., Cooper R.M. (1987) Characterization of cuticle degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch. Biochem. Biophys. 253, 221-232

Suarez B., Rey M., Castillo P., Monte E., Llobell A. (2004). Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl Microbiol Biotechnol. 65, 46-55

Suarez M.B., Sanz L., Chamorro M.I., Rey M., Gonzalez F. J., Llobell A., Monte E. (2005). Proteomic analysis of secreted proteins from *Trichoderma harzianum*. Identification of a fungal cell wall-induced aspartic protease. Fungal Genet Biol 42, 924-934

Summerell B.A., Rugg C.A., Burgess L.W. (1993). Mycogeography of *Fusarium* : survey of *Fusarium* species associated with forest and woodland communities in north Queensland, Australia. Mycol Res 97, 1015-1019

Szekeres A., Leitgeb B., Kredics L., Antal Z., Hatvani L., Manczinger L., Vagvolgyi C. (2005). Peptaibols and related peptaibiotics of *Trichoderma*. A review. Acta Microbiol Immunol Hung. 52, 137-168

Thomas, J.O., Travers A.A. (2001). HMG1 and 2 and related "architectural" DNA-binding proteins. Trends Biochem. Sci. 26, 167-174

Turoczi G., Fekete C., Kerenyi Z., Nagy R., Pomazi A., Hornok L. (1996). Biological and molecular characterisation of potential biocontrol strains of *Trichoderma*. J Basic Microbiol 36, 63-72

Turner D., Kovacs W., Kuhls K., Lieckfeldt E., Peter B., Arisan-Atac I., Strauss J., Samuels G.J., Boerner T., Kubicek C.P. (1997). Biogeographic and phenotypic variation in *Trichoderma* sect. *Longibrachiatum* and associated *Hypocrea* species. Mycological Research 101, 449-459

Velusamy P., Immanuel J.E., Gnanamanickam S.S., Thomashow L. (2006). Biological control of rice bacterial blight by plant-associated bacteria producing 2,4-diacetylphloroglucinol. Can J Microbiol 52, 56-65

Vinale F., Marra R., Scala F., Ghisalberti EL, Lorito M., Sivasithamparam K. (2006). Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. Lett Appl Microbiol 43, 143-148

Viterbo A., Harel M., Chet I. (2004). Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol Lett. 238, 151-158

Vizcaino J.A., Gonzalez F.J., Suarez M.B., Redondo J., Heinrich J., Delgado-Jarana J., hermosa R., Gutierrez S., Monte E., Llobell A., Rey M. (2006). Generation, annotation and analysis of ESTs from *Trichoderma harzianum* CECT 2413. BMC Genomics 7, 193

Vizcaino J.A., Redondo J., Suarez M.B., Cardoza R.E., Hermosa R., Gonzalez F.J., Rey M., Monte E. (2007). Generation, annotation, and analysis of ESTs from four different *Trichoderma* strains grown under conditions related to biocontrol. Appl Microbiol Biotechnol 75, 853-862

Wardle D.A., Parkinson D., Waller J.E. (1993). Interspecific competitive interactions between pairs of fungal species in natural substrates. Oecologia 94, 165-172

Weinding R. (1934). Studies on a lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. Phytopathology 24, 1153-1179

Weiss H. , Friedrich T. , Hofhaus G. , Preis D. (1991). The respiratory-chain NADH dehydrogenase (complex I) of mitochondria. Eur J Biochem 197, 563-576

Widden P., Abitbol J.-J. (1980). Seasonally of *Trichoderma* species in a spruce forest soil. Mycologia 72, 775-784

Widden P. (1979). Fungal populations from forest soils in Southern Quebec. Can. J. Bot. 57, 1324-1331

Williams J.G.K., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18, 6531-6535

Williams J., Clarkson J. M., Mills P. R., Cooper R. M. (2003). Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus.* Appl Environ Microbiol 69, 4192-4199

Wuczkowski M., Druzhinia I., Gherbawy Y., Klug B., Prillinger H. Kubicek C.P. (2003). Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. Mircobiol. Res. 158, 1-9

Yedidia I., Benhamou N., Chet I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microbiol. 65, 1061-1070

Zeilinger S., Mach R.L., Kubicek C.P. (1998). Two adjacent protein binding motifs in the cbh2 (cellobiohydrolase II-encoding) promotor of the fungus *Hypocrea jecorina* (*Trichoderma reesei*) cooperate in in the induction by cellulose. J. Biol. Chem. 273 (51), 34463-34471

Zeilinger S., Galhaup C., Payer K., Woo S.I., Mach R.I., Fekete C., Lorito M., Kubicek C.P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal. Genet. Biol. 26, 131-140

Zhang C., Druzhinina I.S., Kubicek C.P., Xu T. (2005). *Trichoderma* biodiversity in China: evidence for a North to South distribution of species in East Asia. FEMS Microbiol Lett 251, 251-257

Appendix

FASTA files of sequenced RaSH clones

>c13 sequence exported from chromatogram file X
ANTGGGGTTNATCGAAGGGTCGNACGGGGTAATCGNATAAGCTTTGGATATCNGAATTTC
CTGCAGCCCGGGGGGNATCCCACTTAGNTTCTAGAGCGGCCCGCCACCGCGGTGGAGCTC
CAGCTTTTGTTCCCTTAANGTTTGGAGGGGGTTAATTTAANNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNN

>c14 sequence exported from chromatogram file
CGATGGGTNTCGAGACAGGGTCCTCACCCGAGTTCTGATTCTGCACCCCATTGCTGCTGG
CCTTACCTTCTTGGCCTTCTTGCTCTGCTTGGGCACCAGCTTCCTCGGCTCCTTTGTCGC
CTCCCTCTTCTCCTTCCTGGCCAGGTCTCGAG

>c18 sequence exported from chromatogram file X
CGATGGGTNTCGAGGTCGAGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGG
ATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTANGT
TGAGGGTTAATTANNNNNNNNNNNNNNNNNNNNNNNNN

>c19 sequence exported from chromatogram file
CNATGGGTNTCGAGNCAGGTAAAGGCCATTTCTGCCTTAGCAACGTTCAAAGCATGTGCA
ATGGATCCAATTTCGGTGAAGATGGTATCTCGCCCTCGGTTGCCAGGCCAGGTCTCGAG

>c20 sequence exported from chromatogram file
CGATGGGTNTCGAGNACAGGCGCCAAATCTCAATGCTTTAACTTTCAAACATGGTGGTAA
ATTTACTCGCCGATGGCACGGGTGTAGATCTTCATCTTGCTATGAGTGACGACCGGCTTG
ATCATGCCCTTCTCCTCGAG

>c32 sequence exported from chromatogram file
CGATGGGTNTCGAGNCAGGAGCTCCAGGGAACTTATACGCAGCTTGCTGAAGGACGATAC
GCCAAGTTTCTCATTGGAAAGCTCCTTACCCACAATGATGATGAAATCCGCGACATCATC
ATCCCCAGCTTCTACGGCAAAGTTCGAAAGCTCATAAACCACTCTGAGGCCTCGTGGATC
CTGGCCAGGTCTCGAG

>c34 sequence exported from chromatogram file
CATGGGTNTCGAGACAGGACCGGAAGACGGACACCGCCAAATGACCCAATAGCGATAATG
CTCCTTCTTGGTAAGAGGGTTATCCGCCAACACCAAGTGCGTCAGCCGCGAAAACGTCCC
CAGCGCATCCACGTCCGCCAGCTCCACCAGGTGATTCGACGCCAGCACCAGGTTCCGCAA
GTTGGGCACCGCGGCCGCCACCGTGGGCTGGATGCTCGTGACGCGGTTGCGCGCCAGCAG
CAGCGTCGTTATGCGCGGCGAGAGCGGGAAGTTGCCCAGCACCTGGCCAGGTCTCGAG

>c44 sequence exported from chromatogram file
CNATGGGTNTCGAGACAGGAAAAATATACCCGTCATATCGTCCTCATCTACCGCGCTTCA
TTCGAATAAATAGCACGATTGCGGTTGGTAGTTGTTACGTAGAGTGTCAGGTCATCAGGC
CAGGCCAGGTCTCGAG

>c45 sequence exported from chromatogram file
CGATGGGTNTCGAGNCTGGCCAGGGNAAGACATATTTGTTCATGAAGAGACCCCAGATGA
GATCCTCATACCGCCACGCCCTTCGGAGACCGGCGATCTGGAGGAAGAAGACACCATCGT
CATCAAGCATGTTGTAAACCTGGTCTCGAG

>c49 sequence exported from chromatogram file
CGATGGGTNTCGAGACAGGAAATAACCCCGGAAGAATGCAGGACCACCGAAGATAAAGAG
ATCAGGGTCAGCATCGGCCAGACTGGACTTCTTCACCATGCCAATGGTGAGTCCATTTGA
TAGGTAGGGACCGCGGCGGTGTTTCCACTCTTCAAGGCACGGGTCTCCAGGCCAGGTCTC
GAG

>c51 sequence exported from chromatogram file
CTCGNANNCAGGAGAGGACTCATCGTAAGCGGGGGATTTGCCATGGTGGAAAATTCAGAA
TCATGGTTTAGGTGATTCTTTCTGCGATGATTCACGAAGCATTGGGCTGAGGATTAATGG
CAATGGGCCGCTCGCCTTGCTTAGCCAAGCATGACAGACCCGGGCCAGGTCTCGAG

>c58 sequence exported from chromatogram file
CGATGGGTNTCGAGNCTGGCCAGGCAAGGCTGTGTTTCGCTGTGTCTCGGGAATGGTCAT
CGCGGGATGGGGATTGATTGGGTTGACGGAAAGTGGCTTCTAGAGTGGCAAACGGGTATC
TGTGAAGCTTCCGATGCCCCTGTCTAAAGGTTATCTGACACCTGGTCTCGAG

>c63 sequence exported from chromatogram file
CGATGGGTNTCGAGNCTGGCCAGGACGTTCAGCTTGGAGAGAGCATGGGCGGCTCCGAGA
AATGCAGCTATAGAAGATGTGGCGATCAGGTTGTGGCCACAGCCATGACCGATGTCCGGC
AGAGCATCGTACTCGCAGCACACGACAACCTGGTCTCGAG

>c74 sequence exported from chromatogram file
TGGGTNTNNGACAGGAAACAACCCCGGAAGAATGCAGGACCACCGAAGATAAAGAGATCA
GGGTCAGCATCGGCCAGACTGGACTTCTTCACCATGCCAATGGTGAGTCCATTTGATAGG
TAGGGACCGCGGCGGTGTTTCCACTCTTCAAGGTACGGGTCTCCAGGCCAGGTCTCGAG

>c76 sequence exported from chromatogram file
CGATGGGTNTCGAGNCAGGCAAAGGCTCAGGATTACCTGAGCGTCATTGATAAGGAGCTC
TCCAAATACCCCGCCCTTAACAATCTCGAG

>c91 sequence exported from chromatogram file
TGNGTNTNNANNCTGGCCAGGCATTGACGCAGCCAGACGAGGGAGATGCGCCGCTCAACG
CAACGAAGCTGTCCGAGCCCGCCATGGCGGGACAAACCGGACGCCGCCATTCAGCGATGG
ACAAGATAAAGGAGACACTGCATCTCCAGAAGAAATAAAGGAGAGATAAAATTTGAAGTC
AAAAATACTTACAAAAAAACGTCAAACTGATGATACCTGGTCTCGAG

>c92 sequence exported from chromatogram file
ATGGGTNTCGAGACAGGCACTTTTATCGACTTTATCAAGCAGCTAAATATGGCAACAGCA
CCAAACCCATGGATCAATGCTATCAACGTGGCAGCTATTGCTGTGCAGGGTGTTCCTGAG
CCCATCACCATCGCCGTTTCAATTGCTGCCCAGGCCAGGTCTCGAG

>d17 sequence exported from chromatogram file
CNATGGGTNTCGAGNCAGGATNCGAGGGAAAACGGAAGCATCAACAACGCGCAGGTTGCT
CACCCCTCGGACACGGAAGCGAGAATCAAGAACAGCTTTGGGGTCTCCATCCGCTCCAAT
AGGGCAGCTGCAGGAAGCATGATGGCCCCANGCCTCATTCCTAATGAACTCTTTCACCTG
CTCTGTAGAAGCCAAGTGACGGCCAGGCCAGGTCTCGAG

>d21 (d22) sequence exported from chromatogram file
CGATGGGTNTCGAGANCAGGCATTGACGCAGCCAGACGAGGGAGATGCGCCGCTCAACGC
AACGAAGCTGTCCGAGCCCGCCATGGCGGGACAAACCGGACGCCGCCATTCAGCGATGGA
CAAGATAAAGGAGACACTGCATCTCCAGAAGAAATAAAGGAGAGATAAAATTTGAAGTCA
AAAATACTTACAAAAAAACGTCAAACTGATGATACCTGGCCAGGTCTCGAG

>d29 sequence exported from chromatogram file
CGATGGGTNTCGAGNNCTGGCCAGGTATCATCAGTTTGACGTTTTTTTGTAAGTATTTTT
GACTTCAAATTTTATCTCTCCTTTATTTCTTCTGGAGATGCAGTGTCTCCTTTATCTTGT
CCATCGCTGAATGGCGGCGTCCGGTTTGTCCCGCCATGGCGGGCTCGGACAGCTTCGTTG
CGTTGAGCGGCGCATCTCCCTCGTCTGGCTGCGTCAATGCCTGGTCTCGAG

>d30 sequence exported from chromatogram file X
ATGGGTNTCGAGGTCGAGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGAT
CCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTAAGNTG
AGGGTTAATTANNNNNNNNNNNNNNNNNN

>d39 sequence exported from chromatogram file
CGATGGGTNTCGCAGCNCAGGCGTATGTGGCATGTCTATAGCCCNCAATATTAAGGCCAT
GATGACTTGTCTTTGTCCCCTTTTTCTTTCCAAATTCCAGGTCTCGAG

>d48 sequence exported from chromatogram file
CGATGGGTNTCGAGNCTGGCCAGGCACTCTTCATCTTCNAGGCCATGATCATCCATGGCA
TCGAGGTTGCTCATCGCCCTAAGACTAGAGTACGGAATTAACAAGTTGGTGTGCACCCTC
TTCAGCCTCCGCACTGTTACCACTCCTCATATTCTGGGAGAGCCTGCGCTCGAG

>d54 sequence exported from chromatogram file
TCTCGNANNCANGNAAATAACCCCCGGNAAGAATGCAGGACCCACCCGAAGAATAAAGAG
AATCAGGGTCCAGCATCCGGCCCAGAACTGGACTTCCTTCACCCATGCCCAATGGTGAGT
CCCATTTGATAGGTAGGGACCCGCGGCGGTGTTTCCACTCCTTCAAGGCACGGGTCTCCA
GGCCAGGTCTCGAG

>d75 sequence exported from chromatogram file
CGATGGGTNTCGAGNNCTGGCCTGGAGNCCGTGCCTTGAAGAGTGGAAACACCGCCGCGG
TCCCTACCTATCAAATGGACTCACCATTGGCATGGTGAAGAAGTCCAGTCTGGCCGATGC
TGACCCTGATCTCTTTAACTTCGGTGGTCCTGCATTCTTCCGGGGTTATTTTCCTGGTCT
CGAG

>d83 sequence exported from chromatogram file
CGATGGGTNTCGAGNCAGGANATACCCCGGCAAGAATGCAGGACCACCGAAGATAAAGAG
ATCAGGGTCAGCATCGGCCAGACTGGACTTCTTCACCATGCCAATGGTGAGTCCATTTGA
TAGGTAGGGACCGCGGCGGTGTTTCAACTCTTCAAGGCACGGGTCTCCAGGCCAGGTCTC
GAG

>d90 sequence exported from chromatogram file
CNATGGGTNTCGAGAAGGTCTGCACCGAGATCATCGACCGTGCCAAGAACAAGGACCTCC
GCGCCAAGGGCCCTGTCCGTCTGCCTACCAAGACCCTGACCGTCACTCCCCGCAAGACCC
CTTGCGGTGAGGGTTCCAAGACCTGGCCAGGTCTCGAG

>d91 sequence exported from chromatogram file
CGATGGGTNTCGAGACTGGCCAGGTGTCGCAAGTCGACACGTTCATGGACAATTTTGCAG
CGGAACTGCAAGCTTTGAAAGGAGCCAACGACCAAGTGTTCGGGGTGCTTTTCCCAACCA
CTGGCAAGCTGCTCTGGCAGTGCCGCAAGTTTTTAGATACAGCAATATCGAGGCCGCGTT
CCCCAACGTCCTGGTCTCGAG

>2 10 sequence exported from chromatogram file 255
CAACTTTAAAGGACCTGGGCGATGGTGTTTCTACATCAATGTGCCCATTGGCGTTGCCTCTTTCGTGATT
CTATTTATTTTCCTCCGCGTCAAGTACGAGCGTGACCTGAGCTGGTCGGCTCGCCTCAAGCGCATCGATA
TCTCCGGTAATGCCATCTTCGTGGCCGCAATCGTCTCCGCCCTGATTGCACTCACTTGGGGCGGCACCAT
CTACAGTTGGAGCACCTATCACATCATTGTGCCCCTTGTCCTGGT

>2 22 sequence exported from chromatogram file 538
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CGGTAGAAGTGGCTCAAGCGGCTGATTGCACGCACAACATCCGCACTCTTGTCCATGACAATGACTGCAG
CTGTGCCTAGACCTGATTGGCTATCCTTCAGGGCATCGAAATCCATCAATTGGTCGTCACAGACATTCTT
GGGCAAAATCGGAGTCGAAGAACCACCAGGAATAATGGCTAGCAGATTATCCCAGCCGCCTCGGACTCCA
CCGCAGTGTTTGTCAATAAGCTCGCGAAGGGGAATGGACATTTCTTCTTCAACGGTGCAAGGGTTGTTCA
CATGGCCGGAAATGCAGAACAGCTTAGTTCCCTGGTTGCGCTCGCGACCAAACCCAGCAAACCAACTTCC
TCCACGGCGACAGATTGTCGGCGCAACAGCAACAGTTTCCACGTTGGCAACAGTTGAGGGGCACCCGAAA
ACACCGACCGCAGCAGGGAAAGGAGGCTTGAGTCGAGGCTTTCCAGGT

>2 33 sequence exported from chromatogram file 262
NGGTTTNACGGCCGGTAACAAATCGTCCAATCATGAGCTGATACCATGNCCGTTATAGATGCAATCTGGA
TGACGAAACCGACGGATACAACCAGAGCCCACAGCGAAATGGAAGGCTTACGACCAACACGATCAGCGAT
GGGTGCAGCTACCAAGGCGCCAATAAGAGTACCGATGGACAGAAGAGCGACGATAAGACCAGAGCGAACA
TTGCTAAAGTATTTTGTACCGTCAGAGTGAGTGAGACCGAACCGGTCCAGGT

>2 69 sequence exported from chromatogram file
GCCTTTTTGGTTTTTTTGGTCGGGTTCCTGTACTGGCAATGTTGTCTCCGTCATGGCAGACAACGAGAAC
CTTTGAAGCGTCGTAATTGGCAACAGGTCTGCCATTGTATGGGTCGCCAAAGAGTACCACGGCACTGACT
TTGGCCATTGTCGCAGCATCTAGGTTGCTTGCTGCGTTGTGTACGACCATACTACCTTGTGAGTAGCCAC
CGAGCACGAGCTTGGTGTTGGGGCAGCTGCTGATGACGGATTTGATCTGGTTGGCCATGTCAATTCCTGG
T

>2 74 sequence exported from chromatogram file 203
AATTTTCAAGTTTCGGGACAGCAGTCCTCAAACTCTCGGGGACATTCTGGCCACCAAACT
CTCCAAATCGCTCGGGGATTTTGCCGTGGAGAGCTGCCAACTGAGCTACCAAAGCGCTGT
CTTGCTCTGCCGTGATGCTGTCATCGTCAGTGACAACTGCATTGACAGAGACGGAATCTG
AGCTGGCACCCTGGTTGCCAGGT

>2 75 sequence exported from chromatogram file 227
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AGCAGGCAACGTTGGTGGCGTCGGGGAGTTGCTTCATGCACGAGTCGACAATCGTCAGCGCAGCGCCGTT
ATGTAGGGGAGCCAGGT

>2 81 sequence exported from chromatogram file 324
GTTTCAAGACCTGGGGGTAGAGGTTCGGGTCTCGCGGCTACCGTCCTTGTTCTCGATTTCGAACTTGTGC
TGGAGCATGACAAAGTCACGCTCGTCCTCCTCGTACTGCATCTTCTCCTCAAGAGTGGCGCAGAGGGTGT
CGAGGGGGTTTCCGCGAGGGGTGATGTTGGCATCGGAGAAGATGCCGATCCACTTGAGACCAGAGATCAA
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GATGAGGGGGCGTTGAGGAGCTTCTGGGTAGCCTCCTTCCAGGT

>2 88 sequence exported from chromatogram file 271
TGGCTTCGGGCAGCAGCGGCTGCAGCGATGCCGTTTGTCAGCTCGAACAGCTACTTCTTGACTCCCGAGT
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CTCTACTACGGCCACCTTTAAGAACGGCTACTCAATTTTTGACTACATTAACGTCGCTACAATCCACAAC
TCTTCTATCCCCTCGGACAACCTCCTCACCAACTCTACCCTCACCAACCTCTACGACCTGG

>2 97 sequence exported from chromatogram file 403
CATTGGCTTTAAAAAACTGCGCCCGCGGGACCTGCCCCCACTTGAAGGGCTCGTTGCAGAGGAACCAGTT
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TAGCAAGAGGTGTCGTTGGGGTTGGACCAGTATCCATAACCGGCGCAAGATCCGGGCAAGCTCTGAGTGG
TGAACCACTTGGAGTATCCAGCCAAAGCCTTGGTCAGACCAACTCCCCTGGCTCCAGGAACAGGGCTAGT
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ACGCTCTGCCAAGTATACAATCCGTTGTTCAATGCAGCACCAAAGTCTGCGTC

>2 107 sequence exported from chromatogram file 120
TNNNGNTGAGGTCTTTCAGTGAGGGTCTTGACGAAGATCTGCATACCACCACGTAAGGCGGAGGACCAGG
TGGAGGGTGGACTCCTTCTGGATGTTGTAGTCGGACAGAGTTCGGCCATC

>2-109 sequence exported from chromatogram file
ACCAGGAATTGACATGGCCAACCAGATCAAATCCGTCATCAGCAGCTGCCCCAACACCAAGCTCGTGCTC
GGTGGCTACTCACAAGGTAGTATGGTCGTACACAACGCAGCAAGCAACCTAGGTGCTGCGACAATGGCCA
AAGTCAGTGCCGTGGTACTCTTTGGCGACCCATACAATGGCAGACCTGTTGCCAATTACGACGCTTCGAA
GGTTCTCGTTGTCTGCCATGACGGAGACAACATTTGCCAGGTCTCGA

>2 115 sequence exported from chromatogram file
GGGCCCCCCTCGAGACCAGGCTCTTCTCGCCACGGCCAGCCCAGTGGAGTTGGAGAAGCG
TCAATGCCCCGGAATCCACGTTTTTGGCGCCCGTGAAACTACAGCACCGCCAGGATATGG
CTCTTCTGGTACTGTTGTGAACCTCATTGTCAACTCTCACCCTGGCACGACGGCTGAGGC
CATCAACTACCCGGCTTGCGGTGGTCASAGTCAATGCGGTGGTATTAGCTATACTAACTC
TGTGGTGGCTGGTATCAATGCTGTTGTCCAGGCAGTAAACAATTATCACAACCAATGCCC
CAACACAAAGCTTGTGTTAGTTGGATACTCGCAGGGCGGCCAAATCATGGATGACGCGCT
ATGTGGAGGAGGCGATCCTGGAAATGGCTACCCAAACACT

>2 140 sequence exported from chromatogram file
AANAGGGNNTGTGTAGTTGGTGATTGTCGTGTAAGGGTTGAACAAGATACCAGGGGTCAGTGGGTTTATA
GAGCGAAGTTGCTGGGGTTCCGGCAGGAAGCGACCTGGTGCCCGATCCAGTGACGACAATGTTAACGCAC
TGCGGGTAGTTCTGCATGCCATTCTGG