Evolution de la structure des communautés microbiennes, en particulier celle de Trichoderma sp., en relation avec le développement de foyers infectieux causés par Rhizoctonia solani AG 2-2 dans un champ de betterave

Muhammad Anees

Université de Bourgogne, Dijon, France
Ecole Doctorale Environnements - Santé - STIC - N°490,

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MUHAMMAD ANEES

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Temporal dynamics of components of the microflora, including *Trichoderma* sp. in relation to patch development of the disease incited by *Rhizoctonia solani* AG 2-2 in a sugar-beet field

Jury of Thesis:

Dr. D. Andrivon	INRA, Rennes	Reviewer
Dr. T. Mateille	IRD, Montferrier-sur-Lez	Reviewer
Prof. Y. Moenne Loccoz	Université Claude Bernard-Lyon I, Lyon	Examiner
M. C. Muchembled	ITB, Paris	Examiner
Prof. D. Wipf	Université de Bourgogne, Dijon	Examiner
Dr. C. Steinberg	INRA, Dijon	Supervisor
Dr. V. Edel-Hermann	INRA, Dijon	Co-supervisor

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CONTENTS

LIST OF ABBREVIATIONS

AG	Anastomosis group
ANOVA	Analysis of variance
AUDPC	Area under disease progress curve
BNYVV	*Beet necrotic yellow vein virus*
bp	Base pair
cfu	Colony forming units
CM	Cumulative mortality rate
Ct	Threshold cycle
DA	Disease age
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded ribonucleic acid
dw	Dry weight
Ggt	*Gaeumannomyces graminis* var. *tritici*
HD	High dose
ISR	Induced systemic resistance
ISTH	International Subcommission on *Trichoderma* and *Hypocrea*
ITS	Internal transcribed spacer
LAR	Localized acquired resistance
LD	Low dose
LSD	Least significant difference
MEA	Malt extract agar
MIAE	Microorganismes d'Intéret Agro-Environnemental
NCBI	National Center for Biotechnology Information
NI	Non-inoculated
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PR	Pathogenesis related
rDNA	Ribosomal Deoxyribonucleic acid
TRF	Terminal restriction fragment
TRFLP	Terminal restriction fragment length polymorphism
RNA	Ribonucleic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SNA	Synthetic nutrient agar
TAD	Take all decline

SYNOPSIS

The soil-borne phytopathogenic fungus, *Rhizoctonia solani* AG 2-2 incites damping off and root rot in sugar beet (*Beta vulgaris* L.). The disease occurs in the form of patches of dead or necrotic plants which show temporal and spatial dynamics within and between seasons. The patches never occur at the same place where they were observed in the previous season. The soil from inside the patches was found less conducive towards the disease caused by *R. solani* AG 2-2 as compared to the soil from healthy areas. The objective of the present study was to characterize the mechanisms involved in the build up of disease patches caused by *R. solani* AG 2-2 in a sugar beet field, to evaluate the temporal dynamics of the microflora in relation to the pathogenic activity, and to identify the putative biocontrol agents playing a role in the reduced conduciveness of soil.

Different components of microflora were related to the reduced conduciveness of soil inside the patches. Soil inoculum density was higher inside the disease patches with soil inoculum potential reduced and there seemed to be a progressive decrease in the inoculum density of *R. solani* AG 2-2 measured by real time PCR with the development of patches. The fungal and bacterial community structures were significantly modified inside the disease patches which also showed a progressive deviation with the development of disease. Modification in the community structures of *Trichoderma* spp. and increase in their densities inside the patches were evident in the early and the end of the season respectively. Apparently, the equilibrium established between microflora and the infectious activity of *R. solani* as described above was broken by an exceptional attack of an aerial disease caused by *Cercospora beticola* what revealed possible mechanisms of resilience of all the components of microflora.

The present study marked the role of *Trichoderma* spp. in reduced soil conduciveness along with some unidentified microorganisms. The *Trichoderma* isolates picked from inside the patches were more antagonistic towards the pathogen as compared to the ones from outside. Most of the isolates identified by morphological and molecular tools belonged to *T. velutinum* and *T. gamsii*. The isolates showed different mechanisms to antagonize *R. solani* AG 2-2 including production of volatile and water soluble metabolites, mycoparasitism as well as induced systemic resistance in the host. *T. gamsii* T30 proved to be the best

antagonistic strain based on the different tests used ranging from Petri dishes to microcosms and bioassays and may present a potential biocontrol of *R. solani* AG 2-2.

In conclusion, although mechanisms contributing to the development of patches caused by *R. solani* in a sugar beet field have been established, some others arise and need to be investigated such the vulnerability of the suppressiveness acquired during the patch built up, the role of the intra specific diversity based on the antagonistic activity of some populations of *Trichoderma* and, the mechanism of interactions between these populations and *R. solani*.

GENERAL INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is a dicotyledonous plant belonging to family Chenopodiaceae. It is a biannual plant with the first year to accumulate the sugar in roots and the second year to use the accumulated sugar to produce inflorescence. Sugar beet roots contain 15 – 18 % of sugar which is the principal product of sugar beet cultivation. It is sown from March to mid April and harvested from the start of September up to the end of October in European (septentrional) countries.

Sugar beet is one of the important crops cultivated in France. France is among the largest producers of sugar beet in the world. In 2008, the sugar beet was cultivated on 349,000 hectares by 26,000 farmers. In France, sugar beets are mainly cultivated in heavy soils i.e. silty-loamy soils in the northern part of the country (www.itbfr.org). Various rotation schemes including wheat, rape, barley, and corn can be used but sugar beets are rarely monocropped.

Sugar beet can be affected by aerial bioagressors including insects, some of which like *Pentastiridius* sp. transmitting plant pathogenic stolbur phytoplasma (Sémétey et al., 2007) which are responsible for the low sugar content or SBR disease (Syndrome de Basses Richesses) (Bressan et al., 2008) and fungi such as *Cercospora beticola*, the causal agent of *Cercospora* leaf spot (CLS) (Vereijssen et al., 2004; Khan et al., 2009) or to a lesser extent, at least in France, *Ramularia betae, Alternaria alternata*, *Phoma betae* [*Pleospora betae*] but some fungicides and predictive models may help to control these plant pathogenic fungi more easily than soil-borne pathogens (Chod and Chodova, 2005). Indeed, soilborne plant pathogenic fungi seem more difficult to be controlled probably because their epidemiological statuses and ecological requirements are still to be characterized. Among the soilborne fungi some are directly producing damages such as *Rhizoctonia solani*, *R. violacea*, *Phytophthora drechsleri, Pythium aphanidermatum* and *Rhizopus arrhizus* being responsible at various extents for severe root rotting of the tubers or damping off of seedlings. Others like *Polymyxa betae* have an indirect role by transmitting the beet necrotic yellow vein virus (BNYVV) which is responsible for Rhizomania disease (Liu and Lewellen, 2007).

However, the soil-borne microorganism, *R. solani* is the potential threat to the farmers cultivating sugar beet. *R. solani* is a phytopathogenic fungus which is present in the soil in very low densities but able to cause disease in different plant species because it is a soil saprotrophe and a facultative parasite. This species is complex and includes fourteen

anastomosis groups (AG) which in turn include each many different subgroups. The former are characterized by the ability of their members to anastomose within a group, while the latter are characterized through various biochemical, nutritional, molecular or phenotypic traits. However, apart from AG B which includes fungi which are able to establish a pseudosymbiotic association with orchids, the other AG include plant pathogenic members which all have very broad host spectra. AG 3 appears as having the narrowest host spectrum as it is responsible for diseases mainly on plants of the solanaceous family. The other AG can attack plants from various botanic families and similarly, one plant can be attacked by various AG of *R. solani*. In the case of sugar beet, AG 4 of *R. solani* and at a lesser extent AG 2-2 can cause damping off of seedlings while necroses and root rotting of adult tubers are due to AG 2-2. This subgroup can also cause damages on carrots (Janvier et al., 2006), tomato (de Gurfinkel et al., 1994) or pine (Guillemaut, 2003). Moreover, within this subgroup AG 2-2, three populations of *R. solani* have been identified: the population AG 2-2 IIIB occurs mainly in northern European countries (the Netherlands, Germany) while AG 2-2IV concerns rather southern European countries (Spain, France). The third one, AG 2-2LP concerns mainly bulbs (Guillemaut, 2003). The first one causes more severe and noticeable damages on corn in the Netherlands than the second one in France. However corn in France can harbour and permit the development of *R. solani* causing then a strong primary inoculum towards the forthcoming susceptible crop of sugar beet. The damage caused by this disease is variable and may lead to complete loss of the yield.

The control of this disease is the main problem. The chemical control is not environment friendly. There is only partial genetic resistance available against the disease which is not sufficient for the control. Even long rotation without sugar beet does not guarantee the complete control of the disease because of two main reasons. Firstly, *R. solani* has a broad host spectrum and can survive on the intermediary cultures and weeds; and secondly this fungus has the ability to survive by making sclerotia. The biological control has not been consistently successful against this pathogen but the potential antagonistic activity of soilborne microflora has been recently assessed (Zachow et al., 2008). Therefore, there is a need of an innovative research approach to find new methods to control this devastating pathogen.

R. solani causes disease in the form of patches of dead or necrotic plants with a clear demarcation, surrounded by healthy plants. These patches show temporal and spatial dynamics within and between seasons. A high between-season mobility of patches has been observed when sugar beet was monocropped (Hyakumachi, 1996). The patches never occur at

the same place where they were observed in the last season. In the preliminary studies, higher suppression towards the disease caused by *R. solani* AG 2-2 was observed in the soil from within the disease patches than in the soil from healthy areas in the same laboratory (Guillemaut, 2003). It was hypothesized that the increased suppressiveness inside the diseased patches may be due to the accumulation of the antagonistic microflora against *R. solani* AG 2-2. This accumulation of antagonistic microorganisms and higher suppression may explain the patch mobility between seasons.

The objective of the present work is to characterize the mechanisms involved in the build up of patches caused by *R. solani* AG 2-2 in a sugar beet field and to evaluate the temporal dynamics of the microflora in relation to pathogenic activity. Secondly, it is also to identify the putative biocontrol agents among the emerging groups of microorganisms in the diseased areas by using two complementary approaches to search for putative antagonists: i) a systemic approach by analysing bacterial and fungal densities and the community structures, and ii) a specific approach by collecting isolates from patches of diseased and healthy areas on the basis of the analyses of the structure of microbial communities. These isolates were to be characterized both for their taxonomic position and their antagonistic activity towards *R. solani* AG 2-2.

These isolates were belonging to the *Trichoderma* genus. *Trichoderma* spp. are one of the most antagonistic microorganisms that has been studied or used against various phytopathogenic fungi including *R. solani*. *Trichoderma* spp. are cosmopolitan and abundant fungi in soil in a wide range of ecosystems and climatic zones. They are characterized by rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals. Their economic importance includes their roles as primary decomposers, producers of antibiotics and enzymes as well as biocontrol agents against a wide range of plant pathogens. *Trichoderma* spp. may inhibit the phytopathogenic fungi either by inducing resistance and plant defence reactions or by direct confrontation through mycoparasitism and antibiosis as well as competition.

The experimental field used for this study was developed by F. Montfort (INRA, Agrocampus Ouest, Univ. Rennes-I, France) and V. Faloya who was at that time director of the Experimental Domain of Epoisses, France to study the effect of intercropping and incorporation of mustard on the disease caused by *R. solani* AG 2-2 in sugar beet crop cultivated in rotation with wheat. We contributed to this experimental set up by producing and heavily inoculating, the field with *R. solani* strain G6 (provided by the collection Microorganismes d'Intéret Agro-Environnemental MIAE, Dijon) to artificially initiate the

patchy occurrence of the disease in the sugar beet crop. However, for the present study, only the control areas of the field were investigated where there was no mustard intercropping or mustard incorporation because our objective was to study the microfloral behaviour in response to the infectious activity of *R. solani* AG 2-2 in the sugar beet field without mustard intervention.

The present thesis is composed of five chapters.

- The first chapter is the bibliographic synthesis that covers the state of art of the work done to explain the process of spatial spread of *R. solani* disease in the fields and the factors involved in this process. It equally covers the mechanisms involved in the spatial and temporal dynamics of patches of disease caused by *R. solani*.
- The second chapter is about the spatial distribution of the biotic changes in the soil microflora in relation to decreased conduciveness towards the disease caused by *R. solani* AG 2-2 in the sugar beet field at the end of the season in September 2006. This chapter is based on the work done to confirm the preliminary results about the decreased conduciveness observed inside the disease patches as mentioned above, to investigate the mechanisms involved by evaluating the different microfloral components, and to identify the putative antagonists involved in the decreased conduciveness.
- In a complementary approach the temporal development of the soil inoculum potential, the pathogenic population and the microbial communities, along with the development of the disease is described in the third chapter. This chapter will also show how the behaviour of the soil-borne microbial communities associated to the development of the disease can be obscured by the sudden occurrence of an aerial disease caused by *Cercospora beticola*.

The second and third chapters suggest the probable involvement of *Trichoderma* spp. in the reduced conduciveness and patch dynamics.

- The fourth chapter is about the characterization of the different *Trichoderma* isolates originating from the same field and proposes the potential biological control of the disease. The characterization is based on the morphological, molecular identification as well as the functional diversity in the antagonistic potential against *R. solani* AG2-2.
- Consequently, chapter 5 focuses on the specific interactions between a population of *Trichoderma gamsii* and the population of the pathogenic strain G6 of *R. solani* AG2-2
- A general discussion then comments the relevance of the approach we used, criticises the results we got towards the state of the art at the international level, evaluates

the technical problems we faced, and presents the conclusions we can draw from this discussions as well as the potential for the future studies.

- The brief and concise general conclusions drawn from the work done are given at the end of this dissertation.

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CHAPTER 1: Review Article: Build up of patches caused by *Rhizoctonia solani*

Muhammad Anees, Véronique Edel-Hermann, and Christian Steinberg*

INRA-Université de Bourgogne, UMR 1229 Microbiologie du Sol et de l'Environnement, CMSE, 17 rue Sully, BP 86510, 21065 Dijon, France

Submitted in *Soil Biology & Biochemistry*

* Corresponding author. Tel. : +33 380 693 050 ; fax : +33 380 693 224 ; E-mail: christian.steinberg@dijon.inra.fr

Abstract

R. solani is a complex species that is composed of different anastomosis groups (AG). Although these different AG show differences in their host ranges, generally *R. solani* is a wide host spectrum phytopathogenic species. It has the ability to grow as saprotroph in soil that further complicates the system. The losses caused by *R. solani* are very important and need a formidable control strategy. The patchy appearance of the disease caused by this pathogen is well known. The patches show within and between season dynamics. The factors which affect the disease spread can be grouped into three main categories: host plant, pathogen and environment. However, each of the categories in its detail may depend on or react with others. There are a number of factors that can possibly be involved in dynamics of patches. The mechanisms possibly involved have been discussed in detail in this review which may include biotic and abiotic factors. It is essential to know about the mechanisms involved to develop a formidable control strategy. Although more work is needed to investigate different mechanisms in different AG in different hosts, it seems that many mechanisms are operating at the same time what necessitates an integrative research approach to study and control the diseases caused by *R. solani*.

Keywords: Spatial dynamics, temporal dynamics, primary infection, secondary infection, population dynamics of soil-borne plant pathogens, epidemiology, disease suppression, predictability, control

Introduction

Soilborne root diseases are incited by soilborne microorganisms mainly fungi, such as *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., and the viruses vectored by the soilborne microorganisms. These microbial pathogens live in soil in the presence or absence of their hosts, compete with microflora and have restricted means of spread over space and time, which depends on a number of biological and environmental factors (Foster, 1988). These facts grant an inherent epidemiological variability to the diseases caused by soilborne pathogens and uncertainty to the farmers about the severity of the disease in a given cropping season. Even the losses caused by these pathogens are highly uncertain but the soilborne root diseases reduce the yields considerably and the extent of disease attacks determines the disease losses (Schoeny *et al.*, 2001; Smith *et al.*, 2003). These losses are highly variable but their importance cannot be neglected and they need to be controlled. The efficient economical control of these diseases whether by chemical, biological, genetic or cultural means, depends on the understanding of ecology of these pathogens and epidemiology of diseases (Forster and Gilligan, 2007; Gilligan, 2008).

One of the important epidemiological characteristics of soilborne plant diseases is that they generally occur in patches (Belmar et al., 1987; MacNish, 1996; Pascual and Hyakumachi, 2000; Truscott and Gilligan, 2001). These disease patches comprise killed or stunted plants that vary in sizes. However, the symptoms vary with the type of disease and host species. Patches caused by soilborne pathogens are generally dynamic in the field crops changing their configuration from one season to another (Gilligan *et al.*, 1996; MacNish, 1996; Schneider *et al.*, 2001). Different soil-borne pathogens cause patches of disease with different characteristics and the dynamic nature of soilborne diseases depends on the species of microorganisms causing disease. For instance, take-all disease caused by *G. graminis* var. *tritici* (Ggt) in grasses occurs in patches that increase in intensities for the first few years and then decline in monoculture due to rise of suppressive microorganisms (Cook, 2003). The well-known sugar beet (*Beta vulgaris* L.) soilborne disease especially in U.K. and northern Europe, rhizomania caused by the virus *Beet necrotic yellow vein virus* (BNYVV) and vectored by plasmophorid soil borne fungus *Polymyxa betae* also occurs in patches (Tamada and Abe, 1989; Stacey et al., 2004). However, these patches only expand between the seasons (Tuitert and Hofmeester, 1992). In the present review, we will concentrate on *R. solani* which

is a soilborne phytopathogenic fungus that was originally described by Julius Kühn on potato (*Solanum tuberosum*) in 1858. It is frequently studied because of its ability to cause disease in diverse cultures resulting in considerable losses. *R. solani* is an imperfect, asexual form (anamorph) of *Thanatephorus cucumeris* (Frank) Donk (teleomorph). According to phylogeneticists (Gonzales-Garcia *et al.*, 2006) the name *Thanatephorus cucumeris* should now be used to designate *R. solani* but for a sake of clarity based on what was found in the literature used for that review, the term *R. solani* will be maintained all along the text. *R. solani* is a ubiquitous soil saprotroph and facultative parasite (Ogoshi, 1996). It can grow saprophytically in soil, on soil organic matter or plant debris (Papavizas, 1970). The history of *R. solani* is almost as long as the history of plant pathology. It is a diverse group of fungi and is divided intra-specifically mainly based on its hyphal anastomosis reactions (Ogoshi, 1996; Carling *et al.*, 2002). Fourteen anastomosis groups (AG) have been defined till now. Complementary characteristics including morphology, virulence, host range, nutritional requirements, biochemical characteristics, molecular characteristic and DNA sequences have been used to further subdivide AG 1 to AG 4 and AG 6 to AG 9 (Carling *et al.*, 2002). *R. solani* causes disease all over the world in all kind of crops including food, forage, sugar, oilseed, ornamental crops and has a wide host spectrum (Bolkan and Ribeiro, 1985; MacNish, 1996; Wrather et al., 1997; Demirci, 1998; Ryder et al., 1998; Pascual and Hyakumachi, 2000; Hietala et al., 2005). The width of host range, however, varies with the anastomosis groups. *R. solani* is a fungus that does not produce asexual spores and thus has no big means to disperse (Ogoshi, 1987). Sexual spores called basidiospores can rarely be produced. Despite the absence of spores *R. solani* survives in unfavourable conditions by forming sclerotia containing compact mass of mycelia (Sumner, 1996). Inoculum density of *R. solani* is highly variable and is not correlated to the disease incidence (Kinsbursky and Weinhold, 1988). Most inoculum is present in top soil (MacNish and Dodman, 1987; Paula et al., 2008). The presence of inoculum in the top 10 cm of soil is necessary to cause the disease (Paula *et al.*, 2008).

R. solani has been well documented in literature for production of patches that are dynamic in the field but it is still to be demonstrated why these patches change their configuration from one season to another (Hyakumachi and Ui, 1982; MacNish, 1996; Schneider et al., 2001). For an efficient control of disease, it seems basic to know about mechanisms underlying development and dynamics of these patches without which we may not be able to restrict the pathogen from causing economic losses in all sort of cultures. The main focus should be on understanding the factors that influence the invasion, persistence and

spread of the pathogenic strains, how and why they outcompete the microflora, what controls the variability of epidemics between one location to another as well as from one season to another and makes the disease unpredictable (Gilligan and van den Bosch, 2008). These are the basic questions that need to be addressed for development of a durable control for a given soilborne disease. The biggest obstacle in this direction is the fact that *R. solani* is one of the most complex epidemiological systems as described above. In the present review, we will explore the literature for the mechanisms involved in spread of patches caused by *R. solani*. Various factors involved in the spatial distribution of the pathogen and mechanisms proposed so far to explain the dynamics of disease patches as well as its control strategies will be reviewed.

Spatial distribution and spread of *R. solani*

Soilborne pathogens have limited means of spread in the field and it is usually the host plant that inadvertently seeks out the pathogen by growing near to it (Gilligan, 1983). Therefore, spatial distribution of the inoculum in the field is of crucial importance for soilborne pathogens. This inoculum responsible for the start of the infection is known as primary inoculum and the infection is known as primary infection (Gilligan and Kleczkowski, 1997). Primary infections may contribute to the patterns found in plant pathogen interactions and is partially responsible for the dynamics of the disease observed during the season (Burdon *et al.*, 1989). The primary infection is of vital importance as small changes in initial conditions are later amplified during the course of epidemics (Kleczkowski *et al.*, 1996). Once the infection process is started, *R. solani* may grow from one plant to another and spread the disease as well as the inoculum. This inoculum is known as secondary inoculum and the infection incited by it is denoted as secondary infection (Gilligan, 2002). Secondary infection is responsible for the distribution of the pathogen within the main growing season of the crop.

Thus the disease produced by *R. solani* depends on balance of primary and secondary inoculum which are interdependent (Bailey and Gilligan, 1999). Theoretically the quantity of primary inoculum is the sum of the disease produced and the saprophytic growth till the end of the previous season as well as the saprophytic growth between the two seasons minus the decay of the inoculum during intercrop period (Bailey *et al.*, 2004). This principle is true for any soilborne microorganism that shows primary and secondary infection. Amount of secondary infection also depends on the biological and environmental conditions that can

directly or indirectly influence the rate of growth of the fungus. Because *R. solani* has the ability to grow in the soil, secondary infection is of great importance. This is unlike in rhizomania, where secondary infection is almost negligible and the disease spreads mainly between the seasons. While in the case of Ggt, being a poor saprophyte (Cook, 2003), growth in soil is not that important, however, we cannot neglect the secondary infection caused by root to root contact of the host plants (Colbach *et al.*, 1997).

The main question that is needed to be addressed here is what the factors are that affect spatial distribution as well as growth and spread of *R. solani* in the field. These factors are very important to know in order to understand the disease epidemiology. For instance, in rhizomania, the agricultural machinery plays the most important role in the spread of disease while disease appearance is also dependant on the temperature; hence disinfecting agricultural equipments and altering sowing time has been reported to be beneficial in control of the disease (Blunt *et al.*, 1992; Rush, 2003). On the contrary, *R. solani* is a facultative parasite (Ogoshi, 1996) and the fact that it can grow both parasitically as well as saprotrophically is known since long (Garrett, 1970; Papavizas, 1970). It points towards three main factors responsible for patterns of the spatial distribution and spread of the fungus: the host plant, the intrinsic characteristics of the pathogen and the environment.

Host Plant

The presence of a susceptible host plant is necessary for the disease development and disease spread depends on the degree of susceptibility of the plant population. Here a few questions arise: “Do all the plants in a given population of hosts have the same levels of susceptibility? Does the susceptibility towards *R. solani* of a given plant changes with time?” If the level of susceptibility varies among plants within a population, the ratio of the susceptible and resistant plants will explain the fate of the disease spread. There exists a threshold number of susceptible plants above which the pathogen may invade the field. The presence of enough resistant plants (threshold number) may completely inhibit invasion by the pathogenic fungus (Bailey *et al.*, 2000; Otten *et al.*, 2004a). It is also important to know the factors that can alter the susceptibility of the plants including changes with the age of the plant (Gibson *et al.*, 1999), the genetic make up of the plant (Fu *et al.*, 2005), and the environmental factors (Shehata *et al.*, 1984). Plants become more resistant with age and thus remain healthy even in presence of the fungus. The most fatal is the earliest attack which causes damping off leading

to bare patches. Late attack is in the form of root rot that may not kill the plant but at least cause losses to yield. The genetic resistance/tolerance against the disease due to *R. solani* has been observed in many crops such as tobacco (*Nicotiana tabacum*), peanut (*Arachis hypogaea*), bean (*Phaseolus vulgaris* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolour* L.), and sugar beet (Montoya et al., 1997; Franke et al., 1999; Pan et al., 1999; Pascual et al., 2000; Scholten et al., 2001; Elliott et al., 2008). Recently, genetic resistance/tolerance has also been developed in wheat (*Triticum aestivum* L.) against *R. solani* AG 8 disease by using chemical mutagenesis technique (Okubara *et al.*, 2009). Such techniques could be helpful to create genetic resistance in different hosts against *R. solani* diseases. In the case of sugar beet, although the reduction of disease has been obtained using tolerant varieties coupled with crop rotations (Buhre *et al.*, 2009), complete control of the disease has not been accomplished by using only tolerant varieties so far. Some plant genes are activated by pathogenic invasion resulting into an induced resistance. In this case, the plants produce pathogenesis-related proteins (PR-Proteins) that are involved in control of a wide range of pathogens including *R. solani*. These plant defence reactions have been reported in rice, bent grass (*Agrostis palustris* Huds.) and in wheat (Datta *et al.*, 1999; Fu *et al.*, 2005; Kirubakaran *et al.*, 2008). However, further work is needed to exploit them for the control of the disease. The environmental factors including temperature and moisture have been suggested to change the level of susceptibility of the plant towards the disease (Shehata *et al.*, 1984), however, the mechanisms have not been well demonstrated. From these facts it is apparent that the susceptibility of the plant is variable changing with time and environmental conditions as well as its interaction with different microorganisms. Hence, it can affect the distribution of the disease in the field (Bailey *et al.*, 2000).

Intrinsic characteristics of the pathogen

It is quite basic to know how *R. solani* survives in unfavourable environmental conditions. Unfavourable ecological conditions may comprise the absence of a susceptible host, lack of nutrients, competition with microflora, etc. These conditions are interdependent and may not be separated from each other. For instance, presence or absence of the host may lead to quantitative and qualitative changes in the availability of the nutrient resources for the whole microflora and may in turn be related to its competition with the pathogen and dynamics of antagonistic microorganisms. This is what happens in the case of Ggt where presence of

susceptible host and virulent pathogen is necessary to develop antagonistic microflora (Raaijmakers and Weller, 1998; Walker et al., 2003). In some cases, such as in rhizomania disease, the survival is assured by production of spores that can survive up to 15 years (Sayama *et al.*, 2006). Conversely for Ggt, the survival is the weakest part of the life cycle where it can be controlled (Cook, 2003). However in the case of *R. solani*, the situation is more complex. *R. solani* may survive as sclerotia in the organic matter contents or debris of the previous year crop (Papavizas, 1968) and can cause the disease in the following season. It may also survive on the intermediate crops or the weeds (Paulitz *et al.*, 2002). The broad host spectrum of *R. solani* helps it to survive for longer periods of time. Briefly, some AG (AG1, AG2, and AG4) have a very wide host spectrum (Ogoshi, 1996; Tewoldemedhin *et al.*, 2006). However, some extent of host specificity has been reported for some AG (AG3 and AG8) at least in relation to the important losses they have caused in various cultures (Ogoshi, 1996). *R. solani* AG-8 is known to cause important losses in cereals as well as in legumes (You *et al.*, 2008), but it has been reported that the same AG can infect the broad leaf crops like mustard (*Brassica hirta*) and safflower (*Carthamus tinctorius* L.) (Cook *et al.*, 2002). Concerning wheat, both AG 2-2 and AG 2-1 have also been isolated from disease patches and are highly pathogenic to wheat (Roberts and Sivasithamparam, 1986). Similarly, the bulbous crops can be infected by AG 2-t, AG 2-2, AG 4, AG 5 and AG BI (Schneider *et al.*, 1997). On the other way round, AG 2t is also able to infect sugar beet, crucifers and lettuce seedlings (*Lactuca sativa*) in addition to bulbous crops. On the contrary, AG 3 may be considered as the anastomosis group having the narrowest host range causing disease in potato, tobacco and tomato (*Solanum lycopersicum* L.*)* (Kuninaga *et al.*, 1997), even then, a considerable genetic diversity has been reported within AG 3 along with variability in pathogenicity between the different isolates (Ceresini *et al.*, 2002; Lehtonen *et al.*, 2008).

The second important question regarding the pathogen is the rate of growth to produce secondary infection. Rate of growth of *R. solani* is variable on the surface of soil or through the soil (Otten and Gilligan, 1998). On the soil surface it is about three times higher than through the soil. The reason for decreased growth rate below the surface of soil is the limited supply of air and space. The infectivity of soil borne fungi depends on their ability to grow on or through the soil (MacDonald, 1994) to compete for nutrient sources due to progressive changes in the host plant resistance (Kleczkowski et al., 1996; Gilligan and Kleczkowski, 1997; Gibson et al., 1999; Bailey et al., 2000). Hence, the dynamics of epidemics are partially determined by the growth rate of *R. solani*.

Another important parameter is the competitiveness and virulence of the pathogen in the given environment (Papavizas, 1970). However, although a continuum between virulent and avirulent strains towards various hosts has been described among binucleate *Rhizoctonia* (Herr, 1995; Sharon et al., 2007), less diversity in terms of hypovirulence has been depicted among the *R. solani* species (Cardinale et al., 2006). It has been shown for instance that a double-stranded (ds)RNA was associated with hypovirulence within AG3 (Liu et al., 2003) but also that the occurrence, distribution, and genetic relatedness of dsRNA components were found among 36 isolates of *R. solani* belonging to nine anastomosis groups (AG) without any demonstration of a relationship between dsRNA and hypovirulence (Bharathan et al., 2005). Dark brown pigmentation of *R. solani* hyphae has been associated with melanin biosynthesis that is considered important for its competitiveness and virulence (Hyakumachi *et al.*, 1987; Kawamura *et al.*, 1997; Kim *et al.*, 2001). Melanin is also associated with the protection of microorganisms against environment stresses such as UV light, temperature and solar irradiation (Durrell, 1964). It also provides resistance against microbial attacks (Lockwood, 1960). Further research is expected to better explain the role of melanin in virulence and the role of hypovirulence in the disease epidemiology. More generally, although agents from within the diverse groups of binucleate *Rhizoctonia* spp. and hypovirulent *R. solani* isolates have been demonstrated to be effective in biocontrol of a range of host — *R. solani* disease combinations (Sneh et al., 2004) neither mycoparasitism nor antibiosis was involved in biocontrol of *R. solani* by any of these isolates mentioned in the literature. As well, their occurrence has rarely been associated to disease suppression in soil (Hyakumachi *et al.*, 1990).

Environment

The biotic and abiotic components constitute an ecological complex of environmental factors influencing the growth and spread of *R. solani*. Among biotic components, the whole microflora plays an important role in influencing the growth of *R. solani*. The microorganisms are in fact regulating each other in the microbiological environment. Increase in the pathogenic activity of *R. solani* may lead to increase in activity of antagonistic populations (Anees *et al.*, 2009) that in turn may lead to reduce activity of *R. solani* (Croteau and Zibilske, 1998). For example, the pathogenic colonization may be restricted by competitive or antagonistic microorganisms that constitute the whole microflora (Toyota *et al.*, 1996).

Microorganisms can lead to limit the development and spread of *R. solani* either by the general microbial suppression through exploitation of global resources available (Diab *et al.*, 2003) or by specific suppression (Grosch *et al.*, 2006). The specific microbial suppression results from different antagonistic mechanisms such as production of antibiotics (Arora *et al.*, 2008), mycoparasitism (Rocha-Ramirez *et al.*, 2002; Howell, 2003) or induced resistance (Harman *et al.*, 2004; Shoresh *et al.*, 2005). The most famous antagonists of *R. solani* reported so far are *Trichoderma* spp. and *Pseudomonas* spp. (Scherwinski et al., 2008; Vinale et al., 2008; Scherm et al., 2009). Additionally *Bacillus* spp., *Aspergillus* spp., *Penicillium* spp., binucleate *Rhizoctonia* spp., *Gliocladium* spp. and yeast spp. have also been reported (Mukherjee et al., 1995; El-Tarabily, 2004; Nicoletti et al., 2004; Jabaji-Hare and Neate, 2005; Mojica-Marín et al., 2008; Singh et al., 2008).

Because soilborne pathogens live in soil, their activities are highly affected by soil properties and the cultural practices that disturb the soil. Soil physical conditions play an important role in the extent, rate and variability of mycelial growth of *R. solani* (Otten and Gilligan, 1998; Otten et al., 1999). Texture, structure, moisture, nutrition of soil and tillage that define soil physical conditions, have profound effects on the spatial distribution of *R. solani*. Concerning soil texture the disease spreads more in sandy soils than in soils with heavy textures and the patches spread eight times more in sandy soils compared to heavy soils (de Beer, 1965; Gill *et al.*, 2000). Indeed the rate of transmission of disease caused by *R. solani* is higher in sand than in soil and even more in coarse sand compared to fine one (Otten *et al.*, 2004b). Hence, time required for growth and establishment of fungus in soil vary with different soil types (Gill *et al.*, 2000). Of course soil structure having a major influence on water and air movement, has a significant role on root development (Stewart *et al.*, 1999), movement of nutrients (Shipitalo *et al.*, 2000) and in turn on processes involving soil bacteria and fungi (Holden and Firestone, 1997).

The extent and rate of fungal growth may depend on the soil porosity as below the soil surface, there is a confined place to branch and the spread of *R. solani* is restricted. *R. solani* may preferentially follow larger pores to spread over and hyphal densities are higher in gaps than in bulk (Otten and Gilligan, 2006). Another feature of soil pores is known as tortuosity which is the non straight nature of soil pores that depends on soil texture, structure and its degree of wetness. Tortuosity increases with increased degree of wetness. Also the geometry of the air-filled pore volume limits the spread of fungus. Connectivity and tortuosity of air-filled soil pores may play important role in invasion of soil by *R. solani* (Otten *et al.*, 1999). Primary infection is favoured by compacted, high bulk density soils where fungi can't spread

easily and produce locally higher biomasses while large pores make more expansion and saprophytic invasion that favours secondary infection. Thus the air filled pore volume is an important factor affecting the strategies of fungal spread (Glenn and Sivasithamparam, 1990). Another important factor is soil moisture as growth and spread of *R. solani* is favoured by drought (Smiley *et al.*, 1996). High soil moisture affects the development of disease caused by *R. solani* (Gill et al., 2001a) due to its influence on growth and root colonization of *R. solani* which will be discussed later (Shehata et al., 1984; Gill et al., 2001b). *R. solani* can grow and infect the host plant at a wide range of soil temperatures and pH. Temperature ranging from 20-30°C and pH ranging from 4-8 may be considered optimum for growth of *R. solani* in general (Dorrance et al., 2003; Grosch and Kofoet, 2003). However, the disease severity may vary at different temperatures with different AG (Kumar *et al.*, 1999). For instance, AG 8 is favoured by lower temperature ranging from 6 to 20°C while AG 11 by 20 to 25 °C (Smiley and Uddin, 1993; Kumar et al., 1999). Generally higher temperatures (>15°C) may expose the pathogen to microbial competition that may lead to general disease suppression (Smiley and Uddin, 1993; Gill et al., 2001b).

It will be worth to mention the role of soil depth because activity of *R. solani* is mostly confined to the top 10 cm and very small amount of inoculum is present below 5 cm of depth of soil (Papavizas *et al.*, 1975). Fungi are incapable of colonizing at 20-25 cm depth of soil. One of the possible explanations is accumulation of CO_2 that has the capacity to inhibit the growth of *R. solani* in deeper fractions of soil (Papavizas and Davey, 1962; Glenn and Sivasithamparam, 1990).

The distribution, size and quality of substrate present in the soil may affect the activity of soil borne fungal mycelia (Griffith and Bardgett, 2000) and mycelial growth may be proportional to substrate availability. It is known that *R. solani* is able to translocate nutrients and spread at distances away from source (Thornton and Gilligan, 1999; Jacobs et al., 2004). However, sufficient nutrients lead to dense colonies and lack of nutrients makes *R. solani* change its growth strategies to explorative one (unpublished observations).

Cultural practices manipulate the soil structure and its physicochemical properties, and thus have a considerable influence on the fungal growth and spread. Minimum tillage increases the soil borne diseases as the crop residues left on the surface serve as source of nutrition for the soil borne pathogens and provide favourable environment for their growth and nourishment (Rovira, 1986; Bockus and Shroyer, 1998; Cook, 2001) while tillage reduces the disease both by breaking the mycelial network and by burying the trophic bases (Pumphrey et al., 1987). However, tillage reduces the disease but does not remove it

completely and returning back to no tillage, leads to revival of the disease (MacNish, 1985b). Similarly, crop rotation has also been reported to have an effect on the disease. Disease intensity decreases with the increase in length of crop rotation (Gilligan *et al.*, 1996), but again the disease is not completely controlled.

Application of herbicide and their time of application (Smiley et al., 1992), use of fertilizers (Srihuttagum and Sivasithamparam, 1991) and manipulating C/N ratio (Croteau and Zibilske, 1998) have also been reported to reduce the growth and spread of *R. solani*; however, so far the mechanisms behind these effects are not clear. As far as use of fertilizers is concerned, the role of nitrogen was considered important in disease control which seems to be an indirect effect through improvement of plant growth (Papavizas *et al.*, 1975). However, among nitrogen fertilizers, there are contrasting reports of effect of form of nitrogen fertilization (Nitrate or Ammonium) on disease suppression (Elmer, 1997; Rodrigues *et al.*, 2002). Additionally, we can also find an effect of sulphur and phosphorus fertilization (Srihuttagum and Sivasithamparam, 1991; Klikocka et al., 2005). In general, the effect of the fertilizers seems to be indirect through the host plant.

Patchy Nature of Disease

The disease incited by *R. solani* is in the form of patches of dead, chlorotic or stunted plants (Belmar et al., 1987; Gilligan et al., 1996; MacNish, 1996; Hyakumachi et al., 1998; Schneider et al., 2001). Patches usually have distinct edges between the stunted plants and the normal sized plants in the surrounding healthy area of the crops. Plants immediately outside the patch usually show no symptoms of the disease but also have considerable root rot that may be due to late infection (MacNish and Neate, 1996). A biochemical interaction of signals between roots and fungus has been suggested (Kirkegaard *et al.*, 1995). The patch symptoms appear usually when the plants are young and susceptible towards the disease (MacNish, 1996). Patch expression may also depend upon climate, such as for cereal rot caused by *R. solani* AG 8, disease pressure is very high in the Pacific Northwest USA and patches coalesce together (Smiley and Wilkins, 1993) while in Australia there are distinct abrupt edged patches (Sweetingham, 1990). Patch size is highly variable and dynamic (MacNish, 1985a; Gilligan et al., 1996; Schneider et al., 2001; Cook et al., 2002). They are sometimes circular, but more often elongated in the direction of sowing which shows spread of the pathogen by machines (Roberts and Sivasithamparam, 1986). Of course the machines used for soil cultivation also

change the soil structure which in turn affects disease. Certainly, the plant-plant distance in a given row is smaller than the row-row distance in the field, which may affect the patch shape or direction of disease movement. The patches caused by *R. solani* are dynamic in nature (Hyakumachi and Ui, 1982; MacNish, 1985a; Schneider et al., 2001). Thus the distribution, size, shape and number of patches considerably vary between seasons (Cook *et al.*, 2002).

Mechanisms involved in dynamics of patches

Despite several decades of investigation, the mechanisms involved in dynamics of disease patches caused by *R. solani* are still unclear. *R. solani* presents a scenario in which the patch dynamics in various crops infected with various AG have been explained (Table 1). An inherent complexity of this pathogen is that it is a heterogeneous group of many different AG. Additionally, the growth in soil, the broad host spectrum and the ability to survive in unfavourable conditions imparts the system further complications. Thus, a number of hypotheses have been proposed to explain the mechanisms involved in dynamics of patches caused by *R. solani*.

One of the propositions to explain the patchy dynamics of rhizoctonia diseases is based on the density of *R. solani* in the field. De Beer (1965) demonstrated that there was more *R. solani* inside patches than outside. He concluded that pathogen is widely distributed in the field, but with higher concentration inside patches, that may be due to some unknown stimulus. Higher density inside patches being responsible for the disease is quite understandable and it has been recently reported that *R. solani* AG 8 applied in higher densities in barley (*Hordeum vulgare*) caused higher diseases (Schroeder and Paulitz, 2008). However, the basic question here is to know the reason for higher density inside patches that was denoted as 'unknown stimulus' by De Beer. This unknown stimulus may be populations of antagonistic microorganisms involved in the dynamics of patches. In some cases the expansion of patches may be due to receding antagonistic populations instead of advancement of the pathogen (Baker and Cook, 1974). Similarly, the disease suppression taking place in sugar beet field in the part where there was high disease the previous year may indicate the development of a biocontrol in that area (Hyakumachi, 1996). The host monoculture decreased the disease pressure in the part of a wheat field where there was higher disease the previous year and the presence of pathogen and susceptible host were considered important for this kind of decrease (Lucas *et al.*, 1993).

Similarly in flower bulbs, higher isolation of pathogen was reported coupled with reduced disease pressures caused by AG 2t (Schneider *et al.*, 2001). Suppressiveness towards damping off caused by *R. solani* AG 4 in radish (*Raphanus sativus*) was artificially established by successive weekly plantings of the host; the mechanism involved was increased density of *Trichoderma* spp. in suppressive soils as compared to the conducive soils (Henis *et al.*, 1978). When these *Trichoderma* spp. were added to conducive soils in the same densities as observed in suppressive soils, the suppression was achieved (Liu and Baker, 1980). Similar phenomena were observed in a sugar beet field where soil from the patches of disease caused by *R. solani* AG 2-2, had higher density of the pathogen but was more suppressive towards the disease than soil originating from healthy areas (Guillemaut, 2003; Anees *et al.*, 2009). This higher suppressiveness was related to the increased activity of antagonistic populations especially *Trichoderma* spp. The isolates originating from the disease patches showed relatively higher antagonistic potential than isolates from outside in healthy areas (Anees *et al.*, 2009). The influence of the antagonistic fungi, *T. harzianum* was also reported on dynamics of *R. solani* in potatoes in the initial stages; however, the effect could not be seen in the latter stages of plant growth and it was suggested that the pathogenic fungus had overcome the antagonist (Wilson *et al.*, 2008). Apart from *Trichoderma* spp., the microbial diversity of *Bacillus* and *Pseudomonas* communities was related to the suppression of disease caused by *R. solani* AG 3 in potato nevertheless the suppression tests were performed *in vitro* (Garbeva *et al.*, 2006). The microbial diversity in general is also related to the increased suppressiveness and disease control which itself is influenced by the host species (Picard *et al.*, 2004) as well as by the cultivation methods (van Elsas *et al.*, 2002). Thus, cultivation of a specific crop has an impact on the microbial diversity and a specific suppression may be achieved. Hence, it is clear that increased antagonistic activity may be one of the reasons for changing configurations of the patches. As the presence of antagonists has been reported in different crops diseased with different AG, this phenomenon could be considered for diseases caused by all AG of *R. solani*.

Table 1: Factors involved in the dynamics of patches caused by *Rhizoctonia solani*

Factors	Host plant	Anastomosis groups (AG)	References
Stimulation of the growth of the pathogen inside the patch	Wheat, sugar beet	AG 2-2, AG 8	(de Beer, 1965; Anees *et al.*, 2009)
Antagonistic microorganisms	Wheat, sugar beet, potatoes	AG 2-2, AG 3, AG 4, AG 8	(Baker and Cook, 1974; Liu and Baker, 1980; Hyakumachi, 1996; Cardinale et al., 2006; Wilson et al., 2008)
Soil microbial diversity	Potato	AG 3	(van Elsas *et al.*, 2002; Garbeva *et al.*, 2006)
Hypovirulence and accumulation of double stranded RNA	Potato	AG 3	(Hyakumachi *et al.*, 1990; Jian *et al.*, 1997; Sneh *et al.*, 2004; Lakshman *et al.*, 2006)
Tillage	Cereals, Common Bean	AG 8, *AG --	(MacNish, 1985b; de Toledo-Souza et al., 2008; Schroeder and Paulitz, 2008)
Crop rotation	Cereals, sugar beet, potato, common beans, carrot	AG 2-2, AG 3, AG 8, *AG --	(Davis and Nunez, 1999; Larkin and Honeycutt, 2006; Schillinger Paulitz, 2006; Larkin and Griffin, 2007; de Toledo-Souza et al., 2008; Buhre et al., 2009)
Soil moisture	Wheat, potato, lupin, pea, rye grass	AG 1, AG 3, AG 4, AG 8, AG 11,	(Shehata et al., 1984; Lootsma and Scholte, 1997; Gross et al., 1998; Kumar et al., 1999; Gill et al., 2001a)
Soil texture	Cereals, Common beans	AG 8, *AG --	(de Beer, 1965; MacNish and Neate, 1996; Gill et al., 2000; de Toledo-Souza et al., 2008)
Soil structure	Barley	AG 8	(Otten and Gilligan, 2006; Schroeder and Paulitz, 2008)
Host susceptibility		AG 2-1	(Bailey *et al.*, 2000)
Temporal niche differentiation	Bulbous crops	AG 2t	(Schneider *et al.*, 2001)

* Unknown AG

The changes in patch configuration may also be attributed to the tillage effects that may cause changes in the levels of inoculum potential as in case of cereals (MacNish, 1996). Soil tillage may affect the pathogen directly but it could also have an effect on conduciveness or suppressiveness of soil because the compaction increases disease and mixing reduces it (MacNish, 1984; Schroeder and Paulitz, 2008). The effect can be explained by decomposition of the debris and residues of crop that harbour the soilborne pathogens because biomass decay is maximum in conventional tillage (Almeida *et al.*, 2001). With no tillage, the percentage of organic carbon increases in the top 0-5 cm (Schillinger *et al.*, 2007) which is the main part of soil where most of *R. solani* inoculum is found. That is why the soilborne populations of *Rhizoctonia* spp. were more abundant in the no-tillage areas in Brazil (de Toledo-Souza *et al.*, 2008). Apart from this, a number of explanations about the disease reduction by tillage are given in the literature such as breaking the contacts between hyphae and nutrient sources and hence reducing the soil inoculum potential (McDonald and Rovira, 1985), reducing macropores that are usually used by the fungus to grow (Otten *et al.*, 1999) and increasing soil tortuosity (Roseberg and McCoy, 1992). In brief, there is an increased probability of exposure of *R. solani* to microbial competition and antagonistic attacks. Tillage also plays an important role in the dispersal of the inoculum. Indeed, the patches of disease caused by *R. solani* AG 8 in wheat expand along the direction of cultivation or machine movement that suggests the dispersal of the pathogen by machines (MacNish, 1985a).

Nevertheless *R. solani* has a broad host-spectrum; crop rotation has also been reported to decrease intensity in some of the recent reports (Table 1). For instance, the disease intensity reduced when wheat was grown in rotation with barley (Schillinger and Paulitz, 2006) or by green manuring with mustard (*Brassica* sp.) in potato cropping (Larkin and Griffin, 2007) or by growing sugar beet after wheat (Brantner and Windels, 2008). Of course, the length of rotation can also be an important factor (Peters *et al.*, 2004). However, the increased suppressiveness by monoculture as above has been well mentioned in literature. Which mechanisms can be behind the reduction of disease by crop rotation or by monoculture are not well demonstrated. The characteristics of the soil microbial community including microbial activity and diversity, and population of beneficial organisms may be improved by crop management practices which comprehend crop rotation and the choice of host as an important factor (Larkin, 2006).

The soil structure has a major influence on the proliferation of *R. solani* observed in barley infested with *R. solani* AG 8 in which the pathogen grew faster in the soil core containing non tilled soil (Schroeder and Paulitz, 2008). This effect was specific to *R. solani*

as *R. oryzae* had similar growth in both cases i.e. tilled and non tilled soil cores. Similarly the soil texture also affects the patchy nature of disease caused by *R. solani* AG 8 in wheat that reduced in heavy soils as explained earlier (MacNish and Neate, 1996).

Changes in patch configuration in cereals between seasons may also be due to changes in environmental factors such as soil moisture which may allow expression or suppression of the disease caused by *R. solani* present at different levels of inoculum (Gill et al., 2001a). Decrease in disease has been reported about potato diseased with AG 3 and about lupins (*Lupinus angustifolius* L.) diseased with AG 11 (Lootsma and Scholte, 1997; Kumar et al., 1999). An increased microbial activity at higher soil moisture levels or higher tortuosity of soil or both can explain the reduction of disease caused by *R. solani* (Otten et al., 1999; Gill et al., 2001b). An interaction of soil temperature and moisture has also been reported where suppression towards the disease was higher at temperatures higher than 15°C at higher moisture levels coupled with higher microbial activity (Gill et al., 2001b). It means that the microbial activity may be the principle effect of the higher moisture on the disease occurrence followed by soil tortuosity. However, there are reports where the disease intensities increase with higher moisture levels (Shehata *et al.*, 1984; Gross *et al.*, 1998). Hence there are contrasting reports for the effect of soil moisture on disease intensity, for instance in beans (Paula *et al.*, 2007). Although, there is no agreement for the effect of soil moisture in the literature, it is obvious that the soil moisture plays an important role in defining patch dynamics. The mechanisms involved in this direction are to be demonstrated. From the above, it is clear that the environmental and the soil related factors as well as tillage and crop rotation could have an influence on the diseases caused by various AG in different host crops.

The competition and temporal niche differentiation are the mechanisms proposed to explain the dynamics of *Rhizoctonia* bare patches in flower bulbs caused by AG 2t (Schneider *et al.*, 2001). The microbial competition perhaps induces the replacement of AG 2t isolates of *R. solani* with other AG or other microorganisms that are not virulent towards bulbous crops and there are series of niches one after the other at the same position, depending on soil temperature and development of the host plant. However, this hypothesis needs further verification in other AG also.

Accumulation of hypovirulent strains in the diseased areas can lead to disease suppressiveness followed by disease dynamics. For instance, accumulation of hypovirulent strains has been proposed in disease decline caused by AG 2-2 in sugar beet monoculture (Hyakumachi *et al.*, 1990). The virulence of fungi has been shown to be diminished by nuclear or cytoplasmic factors such as viruses, virus-like particles carrying genetic material

known as double stranded RNA (dsRNA) (Elliston, 1982). There are contradictory reports in literature about the role of dsRNA in the decreased virulence in *R. solani*. For some specific strains of AG 3, a direct relation was shown between virulence and the presence or absence of dsRNA (Jian *et al.*, 1997). While others have reported the presence of dsRNA as common characters in *R. solani* in all AG with no direct correlation with virulence in this pathogen (Kousik et al., 1994; Bharathan et al., 2005). Hence, further research is expected to evaluate the development and role of hypovirulent strains in the disease suppression.

It is also possible that evolutionary changes in pathogens may occur along a time scale and selection favours non virulent strains of the pathogen in susceptible populations of host plants and vice versa (Thrall and Burdon, 2003).

While discussing the different probable mechanisms that may be involved in the dynamics of a patch, it is important to know the role of primary and secondary infection in the development of a patch. It is suggested that the patches of disease are predefined and are likely to be determined by saprophytic growth of inoculum from previous infections while primary infections dominate an epidemic development (Gilligan and Kleczkowski, 1997; Gill et al., 2002). The proposition is based on the assumption that contrary conditions may be required for the spreading of *R. solani* in the field and for its growth and increase of biomass which is considered essential for the host infection upon contact with roots (Gilligan and Bailey, 1997; Otten et al., 1999). For instance, high moisture blocks the aeration and increases the tortuosity thus prohibits the spreading of the fungus due to reduced air filled pores and as a result the fungus grows and increases its biomass without spreading too much (Otten *et al.*, 1999). Hence, the patches may result from overwintered inoculum at the onset of favourable conditions and symptoms rapidly develop giving an expression of expanding patches (Aoyagi *et al.*, 1998). But, it has been mentioned earlier that the higher moisture conditions also inhibit the disease infection and secondly, it is also known that the disease occurs more in sandy soils than in heavier soils. Of course, the sandy soils have less water holding capacity and hence decreased tortuosity which encourages the fungal spread. Another argument is the limited time available for the fungus during the season that may not be sufficient to grow, spread and attack the roots that necessitate a predefined structure of mycelial network before the start of the season (Gill *et al.*, 2002). This argument was based on experiments in which artificially designed pots with two compartments were used; one of the two compartments was infested artificially with *R. solani* AG 8 at different times before and after sowing, and the other compartment remained uninfested, although the fungus may approach it by growing across the walls of the compartments. In these experiments, less disease was observed in the non-

infested compartments when the fungus was inoculated at or after the time of sowing. However, this argument needs further verification in the field conditions.

It seems that the patch development of *R. solani* in various crops is the result of both primary and secondary infection. However, it is very difficult to quantify the primary and secondary infection separately. Of course, the higher primary inoculum should result in higher primary and secondary infection. The rapid demarcations of the patches are explained by the fact that the young roots are more sensitive to pathogen attacks and the aggressiveness of *R. solani* towards plants decreased with plant maturity or in other words, plants may become more resistant or rhizosphere may be pre-occupied by some other microorganisms (Gill *et al.*, 2002). There may exist a threshold distance between infected plant and susceptible plant for fungal spread below which it may invade and above which finite patches develop (Bailey *et al.*, 2000). Hence fungus spreads and causes the disease up to the time that the plants are susceptible and this creates a border line between healthy and disease plants, so that finite growth of fungus due to unavailability of next susceptible plants may stop the patch expansion in one side. Although, fungus may continue to grow in soil but it may not contribute to the growth of bare patches during the season. Such growth becomes the primary inoculum for the next season if it survives adequately between the seasons and it may increase the patch area in the following season. Here the question will be again about the presence or absence of *R. solani* as we know that the mere presence of the pathogen may be considered as a powerful indicator of the disease in the given area (Anees *et al.*, 2009). Further research is expected to explain the primary and secondary growth of *R. solani in situ* in natural soil and to accurately identify the mechanisms involved in the spread and dynamics of patches. The problem in this direction is to mark the movement of fungus in soil or to differentiate the primary infection from secondary infection.

Apparently, there is no single mechanism but rather a group of mechanisms in operation defining the patch dynamics within and between seasons. There is a need to explain the spread of patches caused by various AG in various host plants and their dynamics considering multiple mechanisms being operational and it would be better to use an integrative approach to explain the mechanisms. Off course, the knowledge of the mechanisms evolved is of utmost importance to control the disease. In rhizomania disease of sugar beet, it has been reported that the patch expansion during the season is almost negligible because the fungus carrying the virus cannot grow saprophytically in the field (Rush, 2003). The patch dynamics are due to movement of the spores between seasons that can survive up to 15 years in unfavourable conditions (Sayama *et al.*, 2006). The disease appears as soon as the threshold

limit of inoculum density is achieved in the field and hence, it may take many years for infection being visible after invasion of the pathogenic viruliferous fungal vector (Gilligan and van den Bosch, 2008). So during this preparatory period, there is a potential danger of inoculum spread to the surrounding healthy areas by agricultural machinery. Therefore, the control strategy for the rhizomania disease should be designed keeping all these factors in mind. Similarly, in the take-all disease in cereals the disease intensity increases for the first few years in monoculture and then declines due to development of antagonistic microorganisms i.e. 2,4-diacetylphloroglucinol producing *Pseudomonas* spp. that suppress the pathogen and control the disease in the coming seasons if present above a threshold inoculum density (Raaijmakers and Weller, 1998). This case study has been well demonstrated in literature and the disease suppression by host monoculture is known as take-all decline (TAD). However, TAD can only be established by monoculture of the host crop and in case of change of host, the soil suppressive effects are reversed leading to the increased conduciveness. Additionally, the mechanisms for triggering the antagonistic bacterial secondary metabolism in the rhizosphere to produce the antibiotic compounds are still obscure (Haas and Keel, 2003). That is why their use as biocontrol agents against take-all disease has not shown consistent results. Nevertheless, it is hypothesized that *Pseudomonas* spp. may be activated to produce antibiotics by root exudates and the root exudates may be influenced by the pathogenic activities (Walker *et al.*, 2003). The knowledge of such a mechanism in TAD seems important to establish a consistent biocontrol of take-all disease.

Disease control

A variety of measures have been reported in the literature to control the disease. As far as chemical control is concerned, contrasting reports can be found. No available seed treatment fungicides move systemically to roots (Paulitz *et al.*, 2002). Several fungicides that exhibited activity against *R. solani* AG 8 in laboratory were not effective in field trials (Smiley et al., 1990; Cook et al., 2002). No change or rather increase in root rot activity was observed with fungicide seed treatment in wheat (Smiley et al., 1990; MacNish and Neate, 1996). In flowers, *Rhizoctonia* disease is mainly controlled by full field application of fungicides or by the soil disinfection that is not environment-friendly. In conclusion, there is no method of chemical control of epidemics caused by *R. solani* that is effective, economically practical and environmentally safe at the same time. Cultural practices that can be used mainly include

tillage (Roget *et al.*, 1996; Smiley *et al.*, 1996), disruption of soil in the seed zone during seeding and placement of fertilizer below the seed in direct seeding crop (Roget *et al.*, 1996; Cook *et al.*, 2000), paired rows of wheat in field (Cook *et al.*, 2000), starter fertilizer in wheat (Patterson *et al.*, 1998) and application of N to reduce *Rhizoctonia* bare patch in some cases (MacNish and Neate, 1996). Crop rotation has also been reported to decrease the disease in various AG as described earlier, however, it cannot be used alone to control the disease because the pathogen has a wide host spectrum and can escape even the long rotations (Balali *et al.*, 1995). Biological control is an important measure that may be exploited in future as it may control the disease without destroying the environment. There are many reports of biocontrol using various microorganisms, *Trichoderma* spp. and *Pseudomonas* spp. being the most important. Biocontrol mechanisms comprehend antibiosis, siderophore production, induced resistance, and competition (Kloepper et al., 1980; Cardoso and Echandi, 1987; Harris et al., 1997). However, no big successes in fields have been seen in this direction. The earthworms (*Apporrectodea trapezoids)* have also been reported to reduce disease on wheat and in pastures (Stephens and Davoren, 1997). No or partial host resistance is observed but that is not sufficient for complete control of the disease. Finally, monoculture of a susceptible host leads to rhizoctonia disease decline presenting a natural way of disease control involving the accumulation of antagonists (Hyakumachi, 1996; Wiseman *et al.*, 1996) but obviously, it is neither perennial nor reliable as depending on too many uncontrolled parameters. Many mechanisms seem to operate hence we need an integrative approach for this purpose. For instance, recently it has been shown that the combined use of crop rotation, cultivation and resistant cultivars of sugar beet reduced the severity of the disease caused by *R. solani* AG 2-2 (Buhre *et al.*, 2009). Such an integrative approach will be much more fruitful if we can understand the different mechanisms underlying the epidemic.

Conclusions

We have seen above that there are a number of mechanisms that may be involved in the disease expression and dynamics. They both depend on the plant susceptibility and the population dynamics of *R. solani*, the later being regulated by the biotic and abiotic components of the soil, which in turn are tributary of agricultural practices. Modelling is probably the only approach that can integrate these parameters and identify their respective role in given climatic and geographical conditions

Modelling represent a powerful tool to predict population behaviour based on the individual behaviour. However, the predictability of a system depends on the extent to which it has been demonstrated. In the case of *R. solani*, there are numerous factors that need further understanding. Therefore, one finds very limited number of reports about modelling of this pathogen and most of them come from the group of Cambridge who introduced the concept of pathozone (Kleczkowski et al., 1996; Bailey and Gilligan, 1997; Gibson et al., 1999; Bailey et al., 2004). The concept of pathozone has been introduced as a theoretical area around the root where the pathogen must be present to cause the disease (Gilligan and Bailey, 1997). The pathozone is a convenient way to summarize the complicated growth dynamics through soil and soil–plant–pathogen interactions, including the variation that is characteristically associated with these processes (Otten and Gilligan, 2006). This concept was used to model the extent of the biological control of *R. solani* attacking radish (*Raphanus sativus*) seedlings by *T. viride* in microcosms (Bailey and Gilligan, 1997). The authors showed that the antagonistic fungus reduced the pathozone of radish seedlings for *R. solani*. They modelled the interaction of *T. viride* and *R. solani* but did not take into account the microflora, what needs to be done. Although none of these models has been validated in the field so far, an epidemiological study was conducted in a sugar beet field and models were fitted to plant data to describe the positive role of mustard as an intercrop on the disease severity (Motisi, 2009). Unfortunately, the soil microbial components were not explicitly considered in this study, limiting thus the significance of the conclusions drawn concerning the mechanisms involved in the control of the disease. On the other side, the population dynamics of *R. solani*, the structure of soil microbial communities and the evolution of the soil inoculum potential to *R. solani* diseases were measured *in situ* without the measure of the disease severity in the crop above ground in a similar study (Friberg et al., 2009). In the later case, the authors had to conclude in terms of risks about the benefits and putative negative impacts brought by the incorporation of mustard in the wheat-sugar beet rotation.

This situation showed the point of combining field observations to take into account the epidemic behaviour as revealed through the plant health and the epidemic processes as revealed through primary and secondary infections resulting from the population dynamics of the pathogen. It demonstrates even more the need for understanding the mechanisms determining the population dynamics in the soil environment to elaborate the appropriate epidemiological theory and to explain the dynamics of the patches. The knowledge of these mechanisms is vital to develop a control strategy adapted to the ecology of the pathogen of interest as we saw that the epidemiological theory doesn't stand equally to TAD, rhizomania

or *R. solani* diseases. Getting data from both field and meso- or microcosm experiments is required to model the whole process to accurately predict the fate of the disease and to optimize the control practices.

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CHAPTER 2: Biotic changes in relation to local decrease in soil conduciveness to disease caused *by Rhizoctonia solani*

Muhammad Anees[1], Arne Tronsmo[2], Véronique Edel-Hermann[1], Nadine Gautheron[1], Vincent Faloya[3] and Christian Steinberg[1*]

[1]INRA-Université de Bourgogne, UMR 1229 Microbiologie du sol et de l'environnement, CMSE, 17 rue Sully, BP 86510, 21065 Dijon, France; [2] Norvegian University of Life Sciences, Department of chemistry, biotechnology and food science, PO box 5003, NO-1432 Aas, Norway; [3]INRA, Unité Expérimentale d'Epoisses, 21110 Bretenières, France; *Author for correspondence (phone +33 380 693 050 E-mail: christian.steinberg@dijon.inra.fr).

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Abstract

The relations between biotic changes and local decrease in soil conduciveness in disease patches towards the disease incited by *Rhizoctonia solani* AG 2-2 in a sugar beet field in France were investigated. Soil samples from healthy and diseased areas were analysed for bacterial and fungal densities, molecular and physiological microbial community structures, and antagonistic abilities of *Trichoderma* isolates that were collected from diseased and healthy areas. Although the inoculum density was higher inside the disease patches, the respective soil was less conducive towards disease incited by *R. solani* AG 2-2. It was concluded that the pathogen was present in healthy areas but did not incite disease in field conditions. Conversely, the response of the microflora to previous development of *R. solani* in disease areas prevented further pathogenic activity. Indeed, genetic and physiological structures of the fungal communities and physiological structures of the bacterial communities were modified in disease patches compared to healthy areas. The terminal restriction fragment length polymorphism analysis revealed that the peaks corresponding to *R. solani* and *Trichoderma* spp. were higher inside the patches than in the healthy areas. *Trichoderma* isolates from the disease patches were more antagonistic than those from the healthy areas. These results suggest that disease caused by *R. solani* AG 2-2 induced changes in genetic and physiological structure of microbial populations and development of antagonists. The decreased conduciveness inside the patches may help explain the patch mobility in the following season.

Key words: Antagonistic potential. Community structure. Dynamics of patches. Soil inoculum potential. Terminal restriction fragment length polymorphism (T-RFLP), *Trichoderma* spp.

Introduction

Rhizoctonia solani is worldwide known as a soil-borne fungal plant pathogen with a broad host spectrum able to affect the growth and yield of many plant species. *R. solani* AG 2-2 causes severe damage to sugar beet through root rot and damping off (Hyakumachi and Ui 1982). The disease occurs in the form of patches of damaged or dead plants in various crops (MacNish 1985; Schneider et al. 2001). These patches are highly mobile and never occur in the same place where they were observed the previous year (Hyakumachi and Ui, 1982). MacNish (1985), for instance, showed that there could be dramatic changes in area, size and shapes of patches in cereals caused by *R. solani* AG 8. Similar information was reported concerning *R. solani* AG 2-t in tulips (Schneider et al. 2001). Long distance transmission is observed between seasons and is generally attributed to water movement and mainly to mechanical dispersal of inoculum during harvest and cultivation procedures (Gill et al. 2002; MacNish 1996; Truscott and Gilligan 2001).

The production of patches with changing configurations is a phenomenon related to epidemics of soilborne plant diseases mainly caused by restricted dispersal of inoculum. Patches arise from the presence of natural primary inoculum carried over from previous crops in the field. During the parasitic phase, this primary inoculum infests the host plant, and then two types of strategy may be used by the secondary inoculum of the pathogenic fungus within the root system of the host plants. The first one results in local increase of the inoculum through autoinfection of the host plant, and the second corresponds to the short distance transmission through alloinfection between contiguous plants. In the case of *R. solani*, the structure of the soil in the upper layers including the connectivity and the tortuosity of the air-filled pore volume may determine the spread of the fungus (Harris et al. 2003).

The rates of transmission of infection from *R. solani* inoculum or infected plants to susceptible hosts are critical determinants of epidemics (Otten et al. 2004). Therefore, besides the quantification of the pathogenic fungi, the soil inoculum potential as defined by Bouhot (1979) needs to be assessed to evaluate the risks that new patches arise. Briefly, soil inoculum potential is the pathogenic energy present in the soil and is assessed by growing susceptible host plants in the soil under the environmental conditions favourable for the disease expression. There is a threshold concentration of inoculum below which symptoms are not apparent although the pathogen continues to increase within the host. Cultivation may lead to the disappearance of patches in successive seasons if the density of inoculum within a patch falls below the critical threshold. It may also increase the period for which a field is infectious without the infection being apparent (Truscott and Gilligan 2001). Conversely, there is also a

threshold level with respect to the host population above which the plant pathogenic fungus may be able to invade plant roots (Gubbins et al 2000).

In the case of *R. solani*, the mechanisms that control occurrence, size, shape and persistence of patches in the field have received little attention but models have been formulated to describe and propose hypotheses about the growth and behaviour of this fungus in the soil in controlled conditions (Bailey et al. 2004; Otten et al. 2004). Some of these models took into account the presence of a *Trichoderma* strain interacting with a strain of *R. solani* in the presence of radish host plants in microcosms (Kleczkowski et al. 1997). However, the behaviour of the population of *R. solani* within the whole microflora as well as the behaviour of the microflora towards *R. solani* during the development of primary and secondary inoculum in natural conditions are still obscure.

Besides abiotic factors such as soil structure and moisture, various biotic factors may also affect the behaviour of plant pathogenic fungi. Mycoparasitism exerted by *Trichoderma* has been described as a natural means of control of *R. solani* (Verma et al. 2007) and temporary out-competition of the pathogenic fungus by indigenous micro-organisms was suggested to explain why the disease patches caused by *R. solani* AG2-t in flower bulbs did not reoccur at the same site (Schneider et al. 2001). It could, for example, be that the root exudates or the compounds resulting from the decaying host plant may provide nutrients in a way that might stimulate the microbial communities. Intense microbial activity has already been shown to limit the development of pathogenic populations in various pathosystems such as wilts due to *Fusarium oxysporum* (Steinberg et al. 2007) or damping off due to *Pythium aphanidermatum* (Grunwald et al. 2000) or *R. solani* (Diab et al. 2003). While specific suppression of soilborne diseases is due to the activity of a particular microbial group such as 2,4-diacetylphloroglucinol producers towards *Gaeumannomyces graminis* var. *tritici*, responsible for take all decline of wheat (Raaijmakers and Weller, 2001), general suppression of soilborne disease relies on the activities of the whole microflora (Weller et al. 2002). From the above mentioned studies it appears that every soil has some potential for disease suppression and that soil suppression has to be considered as a continuum from highly conducive to strongly suppressive. Depending on the pathosystem and the environmental conditions (biotic and abiotic), suppressiveness can be definitively established or temporarily acquired (Weller et al. 2002). Therefore, a local combination of specific and general suppression of disease caused by *R. solani* AG 2-2 in artificially infested sugar beet fields from the second year of cultivation on could explain the dynamics of disease patches observed in Japan by Hyakumachi (1996). Disease severity was high in the plots where there was less disease the previous year while it was low in the plots where there was high disease the previous year. Similar observations were reported from a French sugar beet field naturally infested by *R.*

solani where the soil inside the patches at the end of the season was less conducive towards the disease than the soil from the healthy areas (Guillemaut 2003).

The objectives of the present study were to investigate the soil conduciveness in disease patches relative to that in healthy areas towards the disease incited by *R. solani* in a sugar beet field, by analysing different components of the microflora and by comparing the behaviour including saprophytic growth and infectious activity of the pathogenic population of *R. solani* within the biotic soil environment. A second aim was to look for antagonists possibly involved in the modification of soil conduciveness inside the patches. Two complementary approaches were used to search for putative antagonists: i) a systemic approach by analysing bacterial and fungal densities and the community structures, and ii) a specific approach by collecting *Trichoderma* isolates from patches of diseased and healthy areas and testing them for their antagonistic potential.

Materials and methods

Experimental design and soil sampling

Sampling was done at the INRA Experimental Unit of Epoisses, Côte d'Or, France (5°05'56"E; 47°14'20"N) in a sugar beet field (80 X 24 m) that was inoculated in 2005 with barley seeds infested with the pathogenic strain G6 of *R. solani* AG 2-2, isolated from a diseased sugar beet in France. The strain was provided by the collection "Microorganisms of Interest for Agriculture and Environment" (MIAE, INRA Dijon, France). The soil from the experimental field had a silt clayey texture (sand 6.1%, silt 57.7%, clay 36.2%) with pH 7.1, 2.5% organic matter, and C/N ratio of 9.7 and a Cation Exchange Capacity of 20.1 cmol(+) kg^{-1} soil. Sampling was performed in September 2006 during the epidemics. Soil was sampled along three independent transects, each of them crossing a gradient from a disease patch to a healthy area. The transects were about 10 to 12 m long and the disease patches were about 3 to 4 m in diameter. The three transects concerned independent disease patches in the field that were considered as representative of the history of the field. Each transect included four characteristic sampling points (E, F, G, H) where sugar beets were selected: a fully necrotic plant in the centre of the disease patch (E), a weakly necrotic plant at the edge of the disease patch (F), the first apparently healthy plant outside the disease patch (G), and a healthy plant in the healthy area characterized by group of healthy plants with no apparent disease symptoms (H). Soil was sampled from the top layer up to 10 cm depth in a radius of 10 cm around selected sugar beets. Each sample consisted of approximately 2 kg of soil. Soil was

sieved (6 mm) to remove gravel, plant roots and debris. Aliquots of soil (100 g) were passed through a 2 mm sieve and stored at -20°C for molecular analyses. The rest of the soil was air dried at 20°C and stored in paper bags at room temperature. The dried soil was used within 10 months. This procedure mimicked natural summer air drying and is more appropriate than storing at 4°C, which is not recommended for long periods as slow microbial activity has been reported in cold storage (Brohon et al. 1999). The soil was moistened back to the original carrying capacity one week before further microbiological measurements were conducted.

Soil conduciveness and soil inoculum potential

The conduciveness of the different soil samples towards the disease caused by *R. solani* was assessed by performing bioassays in climate chambers (Alabouvette et al. 2006). In the bioassays, carrot (*Daucus carota* cv. Yukon) was used as a susceptible host plant to *R. solani* AG 2-2 (Janvier et al. 2006). *R. solani* AG 2-2 causes equivalent damages on carrots and sugar beets, and the standardized bioassay proposed by Janvier et al. (2006) was used to assess soil conduciveness towards diseases caused by *R. solani* AG 2-2. This standardized procedure allowed to use more seedlings of carrots per replicate than a bioassay based on the use of sugar beet plantlets (Bakker and Schneider 2004). Briefly, seeds of the susceptible variety of carrot were sown in rectangular plastic pots (8 X 8 X 7 cm) on 260 g of sand (20 seeds per pot) and covered by 20 ml of calcined clay to hold the irrigation water. Pots were incubated in climate chamber with controlled temperature of 20°C during the day (16 h of light) and 18°C at night for 12 days. The fungal inoculum consisted of the strain G6 grown on sand prepared as follows. Sand (0.5 to 1.2 mm) was autoclaved for 1 h on three consecutive days at 105°C and stored at room temperature for three days before inoculation. The sand (200 g) was mixed with 26 ml of sterilized malt broth (230 g l^{-1}) and five plugs of 10-day old culture of G6 on malt extract agar (MEA) and incubated for 3 weeks at 25°C. Soil (50 ml) containing sand inoculum 3% (high dose, HD), 0.3% (low dose, LD) or 0% (non inoculated, NI) v/v was brought to the crown of 12 day old carrot seedlings and temperature was increased to 25°C day and 20°C night. All experiments were performed in triplicate such that 60 plants were used per characteristic sampling point per transect. The number of damped off seedlings was counted regularly on alternate days for two weeks and area under the disease progress curve (AUDPC) measured in cumulative mortality rate (CM) was calculated. Results were compared by analysis of variance (ANOVA) and Fisher LSD tests using XLSTAT-Version 2007.5 (Addinsoft).

Bioassays as above, but without addition of *R. solani* strain G6 inoculum were conducted to assess the capacity of the native population of *R. solani* to incite the disease. For each soil

sample, 50 ml of soil amended with buckwheat meal (2% w/v of soil) was brought to the crown of 12 day old seedlings as an additional source of nutrients for the resident *R. solani* inoculum. All experiments were performed in triplicate and the results analyzed as above.

Density of *R. solani* AG 2-2

The quantification of *R. solani* in the different soil samples was performed by real time polymerase chain reaction (PCR). DNA was extracted from samples of 1 g of soil using the method described by Edel-Hermann et al. (2004). Briefly, the DNA extraction was done by the help of chemical extractant (sodium dodecyl sulphate, SDS) as well as physical disruption (bead-beater). Lysis buffer consisted of Tris-HCL 50 mM pH 8, EDTA 20 mM pH 8, NaCl 100 mM and SDS 1%. The DNA extracts were purified twice using a polyvinylpolypyrrolidone spin column and once using a Geneclean® kit (Q-BIOgene, Evry, France). DNA extractions were performed in triplicate for each characteristic sampling point of each transect. DNA extracts were stored at –20°C.

The real time PCR assay was based on primers G6-F2 (5'-AGGTTGTAGCTGGCTCCATTAG-3') and G6-R2 (5'-GTAGGGGTCCCAATCATTCA-3') that specifically target the ribosomal internal transcribed spacer (ITS) region of *R. solani* AG-2-2 (Edel-Hermann et al. 2009). Real-time PCR was performed in a final volume of 25 µl, with 5 µl of soil DNA (20 ng), 0.5 µM of each primer G6-F2 and G6-R2 and 12.5 µl of Absolute Q-PCR SYBR Green Rox Mix (ABgene, Thermo Fisher Scientific, Courtaboeuf, France) containing 3 mM $MgCl_2$. The program included 15 min at 95°C and 35 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C using a ABI Prism 7900 detection system (Applied Biosystems, Foster City, CA). A standard curve was generated using ten-fold dilution series of plasmid DNA containing the cloned ITS region of the strain G6, corresponding to 10^9 to 10^2 copies of target DNA per PCR reaction. The curve was used to quantify the amount of target DNA in the different DNA samples. All samples were analyzed three times in independent real time PCR experiments such that nine analyses per characteristic sampling point of each transect were performed. Results expressed as number of copies g^{-1} soil (dw) were compared by ANOVA as above.

Microbial densities

The densities of cultivable bacteria and fungi were estimated using standard dilution plating known as colony forming units (cfu) procedure as described by Pérez-Piqueres et al. (2006).

Briefly, 5 g of soil was suspended in 45 ml of sterile water and shaken for 20 min. Tenfold dilutions were made. Bacteria were quantified on yeast peptone glucose agar (yeast 5 g liter^{-1}, peptone 5 g liter^{-1}, glucose 10 g liter^{-1}, agar 15 g liter^{-1}) (YPGA) supplied with cycloheximide (100 mg liter^{-1}). Fungi were quantified on MEA (malt 10 g liter^{-1}, agar 15 g liter^{-1}) supplied with citric acid (250 mg liter^{-1}), chlortetracycline (50 mg liter^{-1}) and streptomycin sulphate (100 mg liter^{-1}). Bacterial and fungal densities were evaluated in triplicate for each characteristic sampling point in each transect and were compared by ANOVA as above.

Microbial community structures

The genetic structure of microbial communities in the different soil samples was investigated using terminal restriction length fragment length polymorphism (T-RFLP) of 16S and 18S ribosomal RNA (rRNA) genes for bacteria and fungi, respectively (Edel-Hermann et al. 2004; Perez-Piqueres et al. 2006). Bacterial 16S rDNA was amplified by PCR using the primer 27F (AGAGTTTGATCCTGGCTCAG) labelled with the fluorescent dye D3 (Beckman Coulter, Fullerton, CA, USA) and the primer 1392R (ACGGGCGGTGTGTACA) and digested with the restriction enzyme *Hae*III (Q-BIOgene). Fungal 18S rDNA was amplified by PCR using the primer nu-SSU-0817-5' (TTAGCATGGAATAATRRAATAGGA) labelled with the fluorescent dye D3 (Beckman Coulter) and the primer nu-SSU-1536-3' (ATTGCAATGCYCTATCCCCA) and digested with the restriction enzyme *Msp*I (Q-BIOgene). The labelled and unlabelled primers were synthesized by Proligo (Paris, France) and MWG Biotech (Courtaboeuf, France), respectively.

Fluorescently labelled terminal restriction fragments (TRF) were separated and detected using a capillary electrophoresis sequencer CEQTM 2000XL (Beckman Coulter). The sizes of the TRF were determined by comparison with Size Standard-600 (Beckman Coulter). Fungal and bacterial community structures were characterized by the size and fluorescence intensity of the TRF. For each PCR product, the T-RFLP analysis was performed in triplicate. Mean values for the intensity of peaks found in at least two of the three analyses were considered. Each experiment was performed in triplicate using three independent DNA extracts for each characteristic sampling point in each transect. Community structures were compared by principal component analysis (PCA) using ADE-4 software (Thioulouse et al. 1997). The significance of the resulting structures was checked using Monte-Carlo tests with 1000 random permutations of the data. The number of TRF detected per soil sample was also counted and compared by ANOVA as above. The same procedure of T-RFLP analysis of the 18S rDNA was used to characterize the strain G6. For this purpose fungal DNA was extracted from cultures on MEA using a rapid minipreparation procedure (Edel et al. 2001).

Microbial physiological structure was assessed for the soil samples from the characteristic sampling points E (centre of disease patches) and the characteristic sampling points H (centre of healthy areas) from each transect using Biolog EcoPlates for bacteria and FF plates for fungi. EcoPlates consist of 31 different substrates with a negative control while FF plates consist of 95 different substrates and one negative control. For bacteria, the same protocol was used as described by Pérez-Piqueres et al. (2006). For fungi, a few modifications were made in the protocol by adding soil (4 g) in 60 ml of 0.85% sterile NaCl solution, shaking for 10 min and centrifuging for 10 min at 100 g. For bacteria analysed in EcoPlates, cycloheximide (50 µg ml^{-1}) was added to the soil extract to limit fungal growth. For fungal analysis in FF plates, antibiotics including streptomycin sulphate (50 µg ml^{-1}) and chlortetracycline (50 µg ml^{-1}) were added to the soil extract to limit bacterial growth. Plates were incubated at 25°C. The experiment was performed in triplicate for each characteristic sampling point in each transect. Colour formation was measured at 590 nm. Readings were made at regular intervals to determine when maximum optical density was reached. The microbial physiological structure was determined near the top of the exponential phase, after 136 h for bacteria and 288 h for fungi. Community level physiological profiles of the different treatments were compared by PCA as above.

Isolation and antagonistic potential of *Trichoderma* isolates

Trichoderma isolates were collected from the different sampling points in order to test their antagonistic potential. Among those possessing colony morphology indicative of *Trichoderma*, isolates were randomly taken from MEA used in quantification of the fungi. Sixteen isolates originating from E (T30, T31, T40, T41), F (T32, T42, T43), G (T33, T44, T45) or H (T34, T35, T36, T37, T46, T47) were transferred on fresh MEA and their identification as *Trichoderma* was confirmed by microscopic observations. The characteristic 18S TRF profile of the 16 isolates of *Trichoderma* was determined using the same procedure of T-RFLP analysis as above.

Trichoderma isolates were tested for their potential to antagonize the strain G6 of *R. solani in vitro* on MEA. Discs of culture media (5 mm) from the edge of growing fungal colonies were used to inoculate plates. The strain G6 was grown on the same plates as *Trichoderma* isolates 6 cm apart and incubated at 20°C. Radii of colony of the strain G6 approaching and not approaching the colony of *Trichoderma* isolates were measured twice a day for 3 to 4 days. Experiments were performed in triplicate. Inhibition of growth rate of the strain G6 was assessed as percentage of difference of radius not approaching and approaching *Trichoderma* over not approaching and compared by ANOVA as above.

Bioassays were performed as described above to assess the *in vivo* antagonistic potential of the selected *Trichoderma* isolates from the disease patches and healthy areas. Conidial suspensions were prepared for each isolate from 18 day old cultures on MEA incubated at 25°C. The suspensions were adjusted to 4 X 10^6 ml^{-1} using a haemocytometer (Thoma, Preciss France). Each carrot pot was supplied with 15 ml of conidial suspension at the time of sowing. Control pots were supplied with sterile water. Twelve days after sowing, carrot seedlings were supplied with 50 ml of natural soil from a non-inoculated sugar beet field situated near the experimental area. Millet seed inoculum was produced by adding five pieces of MEA culture of the strain G6 into millet seeds previously autoclaved for 1 h on three consecutive days at 105°C. The inoculated millet seeds were incubated for two weeks at 25°C with periodic shaking. Fourteen days after sowing, pots were inoculated with millet seed inoculum of the pathogen, 4 seeds per pot, one at each corner of the pot. Positive controls were maintained by inoculating with pathogen without antagonist and negative controls without pathogen and antagonists. Temperature was maintained at 25°C day and 20°C night for the whole experiment. Experiments were performed in triplicate such that 60 plants were used per treatment. Number of damped off seedlings were counted and AUDPC was calculated and analysed by ANOVA as above.

Results

Soil conduciveness and soil inoculum potential

In non-inoculated soil (NI), the disease development measured as AUDPC was significantly higher inside disease patches (E and F) than outside in the healthy areas (G and H) (Fig. 1). Similar levels of disease were observed in E and F while almost no disease was noticed in G and H. In high dose (HD) inoculated soil, the soil from the disease patches (E and F) produced less disease than that from healthy areas (G and H). For HD, no significant differences were observed between E and F, and between G and H. For low dose (LD), we observed similar results as for HD (Fig. 1). However, for LD, disease in F was suppressed ($p=0.1$) as compared to G and H, showing a similar trend as observed in HD.

In the soil inoculum potential bioassays amended with buckwheat meal disease was considerably higher (Fig. 2) as compared to the control (Fig. 1). Disease was significantly higher in H than in F and G but not significantly different from E (Fig. 2).

Quantification of *R. solani* AG 2-2

As expected, the number of target copies of DNA of *R. solani* AG 2-2 was significantly higher in the centre of disease patches (E) than in other characteristic sampling points and it declined gradually along the transect from E to H (Fig. 3). The results were similar to the disease results obtained for non inoculated soil in Fig. 1, where higher disease was observed in diseased patches as compared to healthy areas.

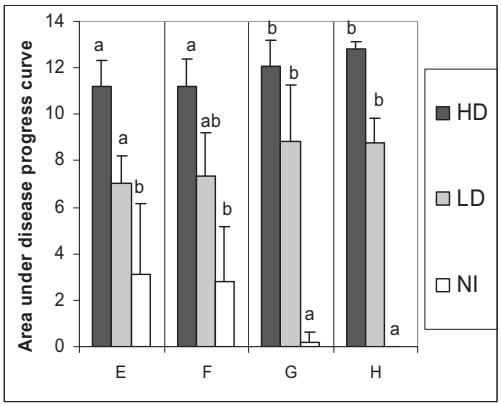

Fig.1 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 strain G6 on carrots in soils inoculated with 3% (v/v of soil, high dose, HD), 0.3% (v/v of soil, low dose, LD) of sand inoculum, or in non inoculated soil (NI). The different soil samples originated from inside disease patches (E), inside disease patches near to healthy areas (F), inside healthy areas near to disease patches (G) and inside healthy areas (H). For each characteristic sampling point (E, F, G, H) and each transect, AUDPC was calculated using 60 plants. Bars represent the mean values obtained for three independent transects. ANOVA and LSD tests were performed separately for each dose of inoculum. Bars designated by a different small letter are significantly different, $P < 0.05$. Error bars represent SD.

Microbial densities

There was no significant difference among the different treatments for bacterial densities with log of cfu g^{-1} of soil in E (6.7 ± 0.14), F (6.8 ± 0.10), G (6.8 ± 0.12) and H (6.8 ± 0.17). The fungal densities expressed in log of cfu g^{-1} of soil were in E (4.9 ± 0.34), F (4.8 ± 0.15), G (4.7 ± 0.19) and H (5.03 ± 0.13). The fungal density was significantly ($p = 0.05$) higher in E than in G while no significant differences were observed between F and G. The fungal density was significantly lower in F and G than in the treatment H. No significant differences were observed between E and H.

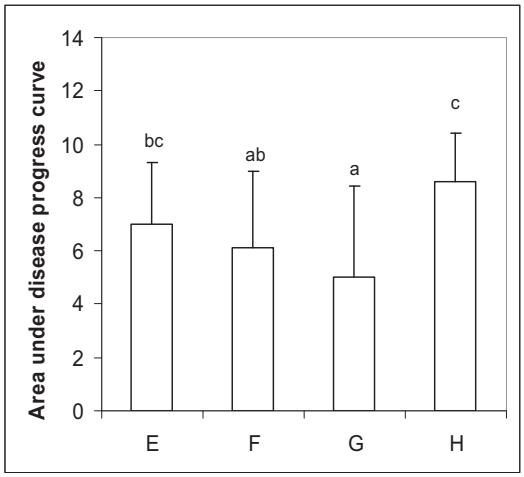

Fig. 2 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 in carrots in soils amended with buckwheat meal (2% w/v of soil) and not inoculated. The different soil samples originated from the same patches as in Fig 1. For each characteristic sampling point (E, F, G, H), AUDPC was calculated using 60 plants. Bars represent the mean values obtained for three independent transects. Bars designated by a different small letter are significantly different, $P < 0.05$. Error bars represent SD.

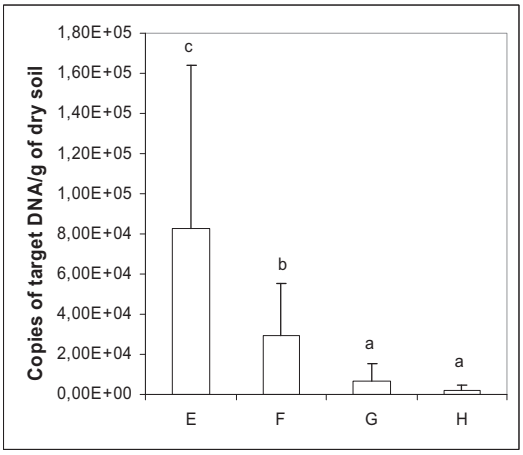

Fig. 3 Number of copies of target DNA of *R. solani* AG 2-2 quantified by real time PCR. The different soil samples originated from the same patches as in Fig 1. For each characteristic sampling point (E, F, G, H), the bar corresponds to the mean value among three independent PCR experiments per three independent soil samples per three independent transects. Bars designated by different letters are significantly different, $P < 0.05$ (LSD). Error bars represent SD.

Bacterial and fungal community structure

The total number of TRF detected was 260 for bacteria and 245 for fungi. The number of TRF detected per soil sample did not differ among characteristic sampling points neither for the bacteria (mean 105 TRF) nor for the fungi (mean 47 TRF). PCA was performed by integrating the relative intensities of the different TRF, calculated as the percentage of the total intensity of TRF for a given sample in a given analysis. Permutation tests revealed no significant discrimination of the bacterial community structures (Fig. 4a). However, the structures of the fungal communities were significantly different ($p = 0.001$) between different characteristic sampling points (Fig. 4b). The most important difference was observed between E and H, with a continuum from E to F, G, and H. An important difference in the relative intensity of some peaks was found between the diseased and healthy areas. The relative intensity of a peak at 565 bp was 8% and 6.1% in E and F respectively, but only 1.6% and 1.3% in G and H, respectively. T-RFLP analysis of the strain G6 of *R. solani* revealed that this peak corresponds to the TRF of *R. solani* (565 bp). A second peak, at 581 bp, was much more abundant in disease areas, with 8.3% and 9.4% in E and F respectively, than in healthy areas, with 4.3% and 2.2% in G and H, respectively. T-RFLP analysis of the 16 isolates of *Trichoderma* collected in our study showed that this peak corresponds to the TRF of *Trichoderma* (581 ± 0.24 bp). Other peaks also highly differed in their relative intensity between the diseased and healthy areas. The relative intensity of a peak at 97 bp was 19% in E but only 2% in H. In contrast, the relative intensity of the peaks at 71 bp and 249 bp was less important in E (2% and 3% respectively) than in H (10% and 9% respectively).

The community structures in the characteristic sampling points E (centre of the patches) and H (centre of the healthy areas) could be clearly discriminated by PCA based on substrate metabolism for both, the bacteria (Biolog EcoPlates) (Fig. 5a), and the fungi (Biolog FF plates) (Fig. 5b).

Antagonistic potential of *Trichoderma* isolates

The *in vitro* tests showed that the inhibition of growth rate of the strain G6 was generally higher by the *Trichoderma* isolates from E and F than the inhibition by the isolates from G and H (Fig. 6). All the isolates from E (T30, T31, T40 and T41) and two from F (T42, T43) inhibited the growth rate of the strain G6 more than did two isolates from G (T33, and T45) and all from H (T34, T35, T36, T37, T46 and T47). The percentage of inhibition of the growth rate of *R. solani* AG 2-2 was the highest by isolates from E (38 ± 6) and lowest by isolates from H (9 ± 8), with intermediate values for isolates from F (29 ± 9) and G (21 ± 10).

In *in vivo* bioassays in natural soil the three isolates originating from the diseased area (T30 and T40 from E, and T43 from F) were able to suppress the disease (Fig. 7). In contrast, the disease was not significantly reduced by the isolates originating from G and H.

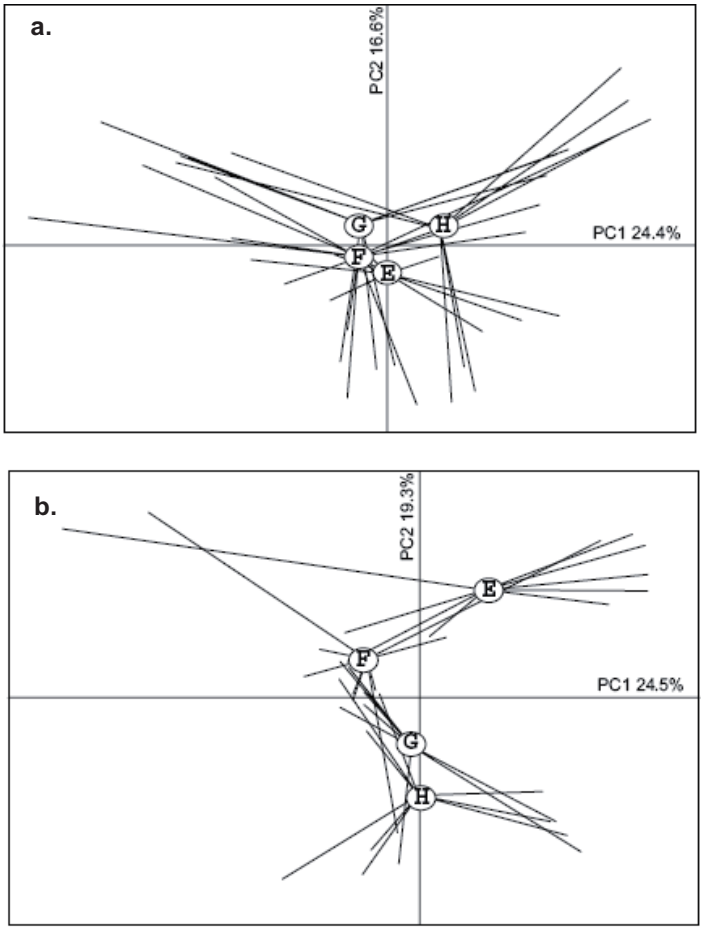

Fig. 4 Principal component analysis of 16S (a) and 18S (b) terminal restriction fragment length polymorphism data sets from the same patches as in Fig 1. For each characteristic sampling point, analysis was performed in three independent transects, each with three independent soil samples. Each characteristic sampling point is represented by its gravity center. The branches show the divergence of the repeats from the respective gravity centers.

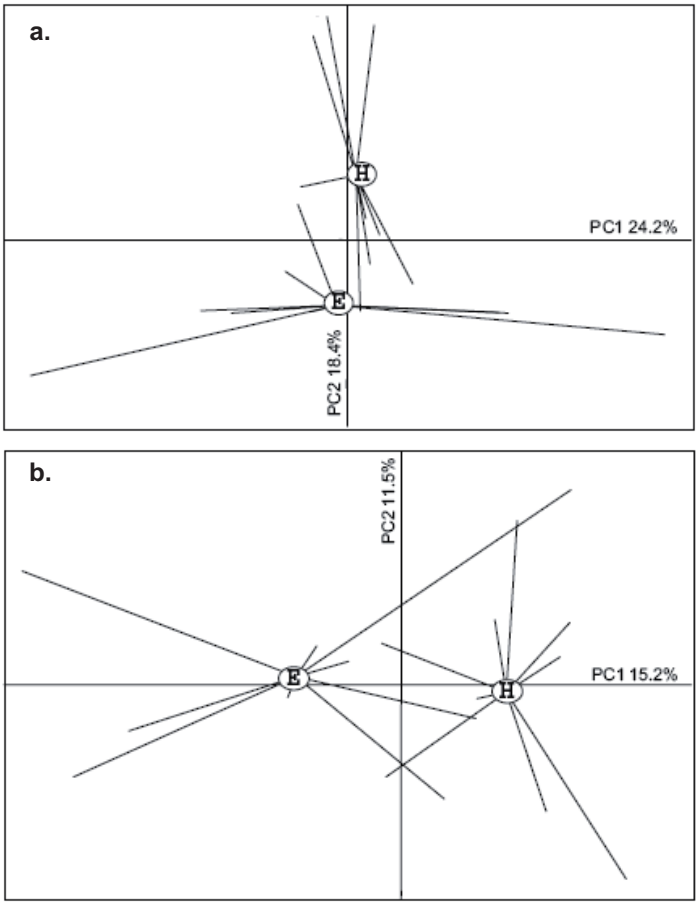

Fig. 5 Principal component analysis of the physiological fingerprints of the bacterial (a) and the fungal (b) communities inside the diseased patch (E), and inside the healthy area (H). For each characteristic sampling point, analysis was performed in three independent transects, each with three independent soil samples. Each characteristic sampling point is represented by its gravity center. The branches show the divergence of the repeats from the respective gravity centers.

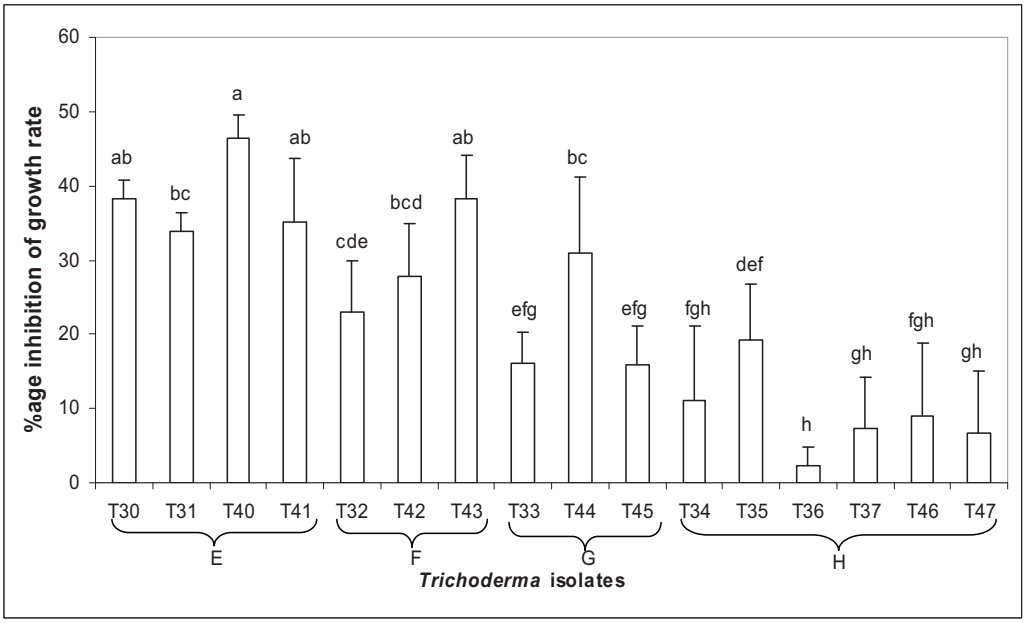

Fig. 6 Percentage of inhibition of growth rate of *R. solani* AG 2-2 strain G6 caused by *Trichoderma* isolates cultured together on malt agar plates. The *Trichoderma* isolates were collected from the the same patches as in Fig 1. Different small letters on top of the bars indicate significant differences ($P < 0.05$). Error bars represent SD.

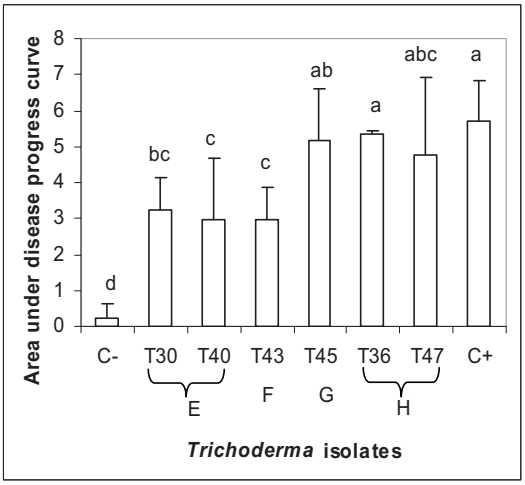

Fig. 7 Area under disease progress curve caused by *R. solani* AG 2-2 strain G6 on carrots in natural soil in the presence of different isolates of *Trichoderma* that were isolated from the different soil samples originating from the same patches as in Fig 1. "C-" denotes the treatment neither with *R. solani* nor *Trichoderma* added while "C+" indicates the treatment with *R. solani* without *Trichoderma*. Different small letters on top of the bars indicate significant differences ($P < 0.05$).

Discussion

The main aim of the present study was to investigate the biotic changes related to the local decrease in soil conduciveness towards the disease incited by *R. solani* AG 2-2 inside disease patches compared to that in healthy areas in a sugar beet field located in the Experimental Unit of Epoisses (France). The approach attempted to take into account the response of the whole indigenous microflora to the parasitic activity of *R. solani* in natural conditions and thus complements the investigations performed by the group of Cambridge in controlled conditions to identify and model the mechanisms involved in growth and development of *R. solani* AG 2-2 in soil (Bailey et al. 2004; Kleczkowski et al. 1997; Otten et al. 2004). By investigating three independent transects, i.e. three unconnected disease patches, the results can be assumed to be representative of the field situation. The present study confirms the local decrease in soil conduciveness towards the disease inside the patches, and suggests that there may be development of specific antagonists inside the disease patches in response to the pathogenic activity of *R. solani* AG 2-2. These results confirm the findings of Guillemaut (2003) who observed the decrease in soil conduciveness towards *R. solani* AG 2-2 within disease patches as compared to that in the healthy areas. These results are also congruent with those of Schneider et al. (2001) and Hyakumachi (1996), who observed higher disease in the part of the field where disease was low the previous year and lower disease in the part of the field where there was high disease the previous year. Rise of natural suppressiveness has been well demonstrated in the case of *G. graminis* var. *tritici* (responsible for take-all disease) where it takes two to four years of wheat monoculture to develop. Weakly aggressive *G. graminis* var. *tritici* genotypes may accumulate during wheat monoculture (Lebreton et al. 2004). However, the decline is mainly due to an increase of specific antagonistic microorganisms during monoculture that inhibit the disease (Weller et al. 2002).

The high disease occurrence inside the patches has generally been attributed to a high density of the primary inoculum in the soil (Truscott and Gilligan 2001). The density of *R. solani* AG 2-2 assessed by real time PCR using specific primers showed that the number of copies of target DNA was highest in the centre of the patches and it decreased along the respective transects. This was further confirmed by the bioassays in controlled environmental conditions that resulted in higher disease inside and negligible disease outside the patches. These findings are also congruent with those of De Beer (1965) who demonstrated that the density of the pathogen was higher inside the patches of disease than outside. However, our study showed that while inoculum density was higher inside the disease patches inoculum potential was higher in the healthy areas. Thus, the amendment with an external nutritional

source (the buckwheat meal) might have broken the environmental as well as biological limits to pathogen growth and disease development. Two important conclusions can be drawn from these results: i) Primary inoculum of the pathogen was also present in the healthy area but at the date of the soil sampling it was restricted from causing the disease and developing any secondary inoculum. ii) The development of the disease depends not only on the pathogen inoculum density but also on other biological and environmental factors that alter the soil inoculum potential as defined by Bouhot (1979). Moreover, comparing results from the bioassays with and without addition of buckwheat meal demonstrated increased rise of disease in the healthy areas of the three transects (G and H). This also reflects the presence of some suppressive agents in the disease patches. These results suggest that the pathogen is widely spread and its ability to cause disease cannot be determined only by its density. Although abiotic factors were not assessed in this study, the interactions among the soil abiotic as well as biotic components including the pathogen and the antagonistic populations also need to be considered for a more complete understanding of the system as it was demonstrated for *G. graminis* var. *tritici* (Weller et al. 2002).

No significant differences in the bacterial densities were observed among different characteristic sampling points. For the fungi, however, significant differences in cfu per g of soil were observed between characteristic sampling points. The highest densities were observed in the sampling points that were most and least affected by the disease. Although statistically significant, it is difficult to attribute the higher number of fungi to the secondary inoculum of *R. solani* in the case of characteristic sampling points E or to a fungal community responsible for general suppression in the case of characteristic sampling points H. As only 0.9 to 22 % of the total microbial communities can be cultivated (Herbert 1990), these results may not be sufficient to draw solid conclusions.

For the bacterial communities, the culture independent T-RFLP analysis did not reveal any differences in the genetic structures of bacterial populations from different sampling points. However, the physiological structures assessed by Biolog technology were significantly different between characteristic sampling points. The root exudates of healthy sugar beets in characteristic sampling points H and of necrotic sugar beet in characteristic sampling points E were certainly different and may have stimulated different enzymatic machineries among the respective bacterial communities to allow them to metabolize the compounds released by the plants. The same explanation stands also for fungal community physiological patterns assessed using FF biolog plates. The fact that the same TRF may correspond to a variety of bacteria (Pérez-Piqueres et al. 2006) explains why the changes in physiological structure did not lead to changes in the genetic structures of the bacterial

communities. Another explanation may be the inter- or intra specific differences at the community level of bacteria that led to the differences in the physiological fingerprints.

In the case of the fungal communities, modifications were also observed in genetic structure as well as in the expression of genes involved in metabolic processes. A significant difference in the balances among the populations of the fungal communities of the characteristic sampling points E and H was revealed by the T-RFLP analysis and the Monte Carlo tests. However, the number of TRF per sample was not significantly different among the characteristic sampling points. Moreover, as expected, the intensity of the peak corresponding to the TRF of the strain G6 was found to be higher in characteristic sampling points E (disease patches) than in characteristic sampling points H (healthy areas). In addition to this, the mean value of the peak obtained by T-RFLP analysis for the *Trichoderma* isolates was also higher in sampling points E. Although the same TRF may correspond to different fungi, our results support the hypothesis of accumulation of antagonistic *Trichoderma* populations inside the patches that may be partially responsible for the observed reduced conduciveness. Accumulation of *Trichoderma* spp. has been previously reported by Mghalu et al. (2007) by repeated inoculation of soil with *R. solani* leading to suppression of radish damping off. Moreover, various species of *Trichoderma* have been shown as efficient antagonists towards *R. solani* both in natural (Verma et al. 2007) and controlled conditions (Bailey et al. 2004; Kleczkowski et al. 1997). Recently, some *Trichoderma* spp. associated with sugar beet roots and showing antagonistic ability to a broad range of pathogens including *R. solani* were isolated (Zachow et al. 2008). Similarly, the relative values of some other peaks revealed by the T-RFLP analysis were found to be highly different between the fungal community structures of the characteristic sampling points E and H. This suggests that other microorganisms could also be involved in the suppression as suggested earlier in the case of *R. solani* AG 2-t in tulip crops (Schneider et al. 2001). These microorganisms could be identified using cloning and sequencing of the discriminant TRF. These differences in microbial communities may also be related to the increased release of nutrients by infected plants inside the patches.

To further investigate the probable role of *Trichoderma* spp., a specific approach was followed by isolating *Trichoderma* spp. from different soil samples and testing them for their potential to antagonize the strain G6 *in vitro* using plate assay. Isolates from characteristic sampling points E were able to reduce the growth rate of the strain G6. This was further confirmed by conducting antagonistic bioassays in controlled environmental conditions. It may be concluded that the development of the secondary inoculum of *R. solani* at the surface of the diseased sugar beet (autoinfection) or during moving to neighbouring sugar beet (alloinfection) stimulated the development of specific populations of *Trichoderma* within the

Trichoderma community of characteristic sampling points E. This resulted in the accumulation of potent antagonistic isolates inside the patches where they play a role in reduced conduciveness of soil. This is congruent with Baker and Cook (1974) who associated the expansion of patches with receding antagonistic populations. They also showed that the sclerotia around the diseased roots did not germinate because of greater abundance of the antagonistic populations. Similarly, Hyakumachi (1996) proposed that antagonistic mechanisms are involved in the development of suppressiveness against *R. solani* affecting its pathogenicity, growth rate and the propagule viability.

The present study suggests that the higher amounts of disease caused by the pathogen led to the modification of the genetic and physiological structure of microbial populations resulting in the development of antagonistic microorganisms that in turn suppressed the disease. The reduced conduciveness inside the patches may contribute to the suppression of the disease in the following season in the same area and causes the mobility of the patches.

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CHAPTER 3: Relative importance of a soilborne and an aerial disease in relation to the evolution of the microflora in a diseased field

Muhammad Anees, Véronique Edel-Hermann, and Christian Steinberg*

INRA-Université de Bourgogne, UMR 1229 Microbiologie du Sol et de l'Environnement, CMSE, 17 rue Sully, BP 86510, 21065 Dijon, France.

*Author for correspondence (phone +33 380 693 050 E-mail: christian.steinberg@dijon.inra.fr).

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Abstract

The aim of the present study was to investigate the relative influence of the soilborne diseases caused by *Rhizoctonia solani* AG 2-2 and the airborne disease caused by *Cercospora beticola* on the evolution of soil microflora in sugar beet field. Soil was sampled from healthy and diseased areas based on the age of the disease incited by *R. solani* AG 2-2 in July (not infested with *C. beticola*) and in August (all the plants were infested by *C. beticola*). Decrease in conduciveness of soil towards the disease was observed in disease areas in July related to a progressive decrease of inoculum density of R. solani along with reduced soil inoculum potential inside the patches with the disease development. A progressive deviation of molecular community structures of bacteria and fungi in July was also evident along with modification of molecular community structures of *Trichoderma* spp. However, this equilibrium between the microfloral components and the infectious activity of R. solani was disturbed in August probably by the infestation of *C. beticola*. There were no significant differences in soil conduciveness and no discrimination in the community structures of bacteria, fungi and *Trichoderma* spp. Overall, the inoculum density of *R. solani* was also reduced in August as compared to that in July. This resilience of microflora also reveals the weakness of the equilibrium stated above towards the probable influence of the aerial disease. Hence *C. beticola* damaging sugar beet foliage above ground may also have an impact on the soil microflora below ground.

Keywords: Terminal restriction fragment length polymorphism; real time PCR; Temporal dynamics; soil suppressiveness

Introduction

Soilborne root diseases are incited by microorganisms that live in soil in the presence or absence of their hosts, compete with microflora and have restricted means of spread over space and time, which depends on a number of biological and environmental factors. Therefore, the inoculum density and the spatial distribution of the primary inoculum is important for these diseases and usually it is the host plant roots which seek out these pathogens and gets infested. *Rhizoctonia solani* is an important soilborne fungal plant pathogen able to affect the growth and yield of many plant species. It is a ubiquitous saprophytic fungus that can grow in the soil using crop residues and spread to the adjacent susceptible host plants (Friberg et al. accepted; Ogoshi 1996). It causes disease in various crops in the form of patches of damaged or dead plants which show spatio-temporal dynamics (Hyakumachi and Ui 1982; MacNish 1985; Schneider et al. 2001). *R. solani* AG 2-2 causes severe damage to sugar beet through root rot and damping off (Hyakumachi and Ui 1982). During the parasitic phase, the host plant is infested by the active primary inoculum of *R. solani* AG 2-2. Thereafter the pathogen either grows inside the plant or incites secondary infection to the adjacent susceptible host plants by saprotrophic growth. The rates of transmission of infection from *R. solani* inoculum or infected plants to susceptible hosts are critical determinants of epidemics (Otten et al. 2004). Therefore, besides the quantification of the pathogenic soilborne fungi, the soil inoculum potential needs to be assessed to evaluate the risks that new patches arise (Bouhot 1979; Friberg et al. accepted).

Besides the abiotic factors, the whole microflora plays an important role in influencing the infectious activity and growth of *R. solani*. Increase in the pathogenic activity of *R. solani* may lead to an increased activity of antagonistic populations that in turn may inhibit the activity of *R. solani* (Anees et al., 2009; Croteau and Zibilske 1998). Microorganisms can limit the development and spread of *R. solani* either by the general microbial suppression through exploitation of global resources available (Diab et al. 2003), or by specific suppression (Grosch et al. 2006). The specific microbial suppression results from different antagonistic mechanisms such as production of antibiotics (Arora et al. 2008), mycoparasitism (Howell, 2003; Rocha-Ramirez et al. 2002) or induced resistance (Harman et al. 2004; Shoresh et al. 2005). *Trichoderma* spp. are among the most famous antagonists of *R. solani* reported so far (Scherm et al. 2009; Scherwinski et al. 2008; Vinale et al. 2008). The importance of *Trichoderma* spp. in the decrease of conduciveness towards the disease caused by *R. solani* has been recently underlined (Anees et al. 2009). Additionally, the infectious activity of *R. solani* AG 2-2 was shown to be related to the alterations in the structures of soil

microbial communities. The study was based on a sugar beet field sampled at the end of the season in September 2006. However, the changes in microfloral components along the development of disease were not considered.

Conversely, aerial diseases incited have more efficient ways than soilborne diseases to infest the entire fields by quick-spread mechanisms causing huge losses. *Cercospora beticola* Sacc. is the most destructive leaf-spot disease of sugar beet causing losses of up to 42% in yield (Holtschulte 2000; Shane and Teng 1992). It quickly spreads through wind, water, insects, rain splashes, infected seeds or even through roots (Khan et al. 2008; Vereijssen et al. 2004; Weiland and Koch 2004). Warm and humid weather favors the disease (Holtschulte 2000). *C. beticola* produces conidia that can survive in the soil on infected leaves in the form of stromata up to two years (Khan et al. 2008). In the favorable season the stromata sprouts and produces conidia that are spread in the surrounding plants by different mechanisms. The ways to control the fungus are either by applying fungicides to plant varieties having moderate to high genetic resistance to the disease or by using long rotations by avoiding cultivation of the host crop (Windels et al. 1998; Wolf and Verreet 2002). A few studies have attempted to develop a biocontrol of the pathogen especially by *Trichoderma* spp. (Galletti et al. 2008). Regardless the fact that the pathogen can survive in the soil on diseased leaves, no data is available concerning its influence on the soil microflora. However, there are studies showing that the inoculum survives better on the surface than below the surface of the soil, suggesting the role of the microflora in the degradation of the plant residues resulting into decreased survival of the pathogen (Khan et al. 2008).

The aim of the present study was to evaluate the relative influence of the soilborne disease caused by *R. solani* AG 2-2 and the airborne disease caused by *C. beticola* on the evolution of the soil microflora in a sugar beet field. For this purpose, different components of the microflora were analyzed being influenced by the saprophytic growth and infectious activity of the pathogenic population of *R. solani* in the biotic soil environment. We investigated both the global bacterial and fungal communities and the specific community of *Trichoderma* spp. i) temporally in course progress of *Rhizoctonia* disease as well as ii) influenced by a severe attack of *C. beticola* in natural field conditions.

Materials and methods

Experimental design and soil sampling

Sampling was done in 2007 at the INRA Experimental Unit of Epoisses, Côte d'Or, France (5°05'E; 47°14'N) in a sugar beet field (80 X 24 m) that was inoculated in 2005 with the pathogenic strain G6 of *R. solani* AG 2-2 (Motisi et al. 2009) adjacent to the field previously sampled (Anees et al. 2009). The progression of the disease caused by *R. solani* was continuously checked thanks to fortnightly crossings of the plots. A coloured plastic stick was set in the soil close to each altered plant to indicate the date of occurrence of the first symptoms. The visible symptoms which were taken into account were necroses observed first at the basis of the weak leaves and on the crown. When there was a doubt, a slight scraping of the soil around the crown (2 to 3 cm depth) allowed ensuring of the presence or not of necroses. This notation was performed by Motisi et al. (2009), in the frame of a programme aiming at introducing mustard (*Brassica juncea*) in the wheat-sugar beet rotation. No fungicide was used. In the present study, we only focussed on the 4 plots in which no mustard was grown.

Sampling was done twice during the epidemics, once in July and once in August in four entirely separated plots corresponding to four independent *Rhizoctonia* disease occurrences in the field considered as representative of the history of the field. At each sampling date (July and August), the biotic components of the soil samples (i.e. characteristic sampling points) have endured the same climatic (water and temperatures) conditions allowing then to compare their respective status. In both cases (July and August), the sampling procedure was the same as previously explained by taking soil around the sugar beet roots (Anees et al. 2009). The sampling was based on the age of the disease (DA) incited by *R. solani* AG 2-2 around the selected sugar beet plants as indicated by the coloured plastic sticks what denoted the characteristic sampling points. DA is defined as the number of days, at a given sampling date, since the first *Rhizoctonia* disease symptoms were observed in the given infested plant. In July, the *Rhizoctonia* patches were well established without any infection by *C. beticola*, while in August, the whole field was severely infested by natural infection of *C. beticola*.

In July, the soil was sampled from the diseased plants as follows: D1 (DA = 79 days), D2 (DA = 33 days), D3 (DA = 5 days) and around the healthy plants H1 (no *Rhizoctonia* disease symptoms). It should be noted there that D1 corresponded to characteristic sampling points where seedlings of sugar beet where attacked very early what leaded to the early death of

these seedlings. This resulted in 4 characteristic sampling points per plot, leading to 16 characteristic sampling points in July. In August, all the plants around which the soil was sampled were affected by the leaf spot disease caused by *C. beticola*. The soil was sampled around the plants based on the values of DA incited by *R. solani* in August: D4 (DA = 75 days), D5 (DA = 47 days) and H2 (no *Rhizoctonia* disease symptoms). This resulted in 3 characteristic sampling points per plot, leading to 12 characteristic sampling points in August. Soil was sampled from the top layer up to 10 cm depth in a radius of 10 cm around selected sugar beets. Each sample consisted of approximately 2 kg of soil. Soil was sieved (6 mm) to remove gravel, plant roots and debris. Aliquots of soil (100 g) were passed through a 2 mm sieve and stored at -20°C for molecular analyses. The rest of the soil was air dried at 20°C and stored in paper bags at room temperature (Anees et al. 2009). The soil was moistened back to the original carrying capacity (i.e. 20%) one week before further microbiological measurements were conducted.

Soil inoculum potential and soil conduciveness

The conduciveness of the different soil samples towards the disease caused by *R. solani* was assessed by performing bioassays in climate chambers as previously described (Alabouvette et al. 2006; Anees et al. 2009). Briefly, seeds of the susceptible variety of carrot (*Daucus carota* cv. Yukon) used as the standard test plant, were sown in plastic pots (8 X 8 X 7 cm) on 260 g of sand (20 seeds per pot) and covered by 20 ml of calcined clay to hold the irrigation water. Pots were incubated in climate chamber with controlled temperature of 20°C during the day (16 h of light) and 18°C at night for 12 days. The fungal inoculum consisted of the strain G6 grown on sand (Anees et al. 2009). Soil (50 ml) containing sand inoculum 3% (high dose, HD), 0.3% (low dose, LD) or 0% (non inoculated, NI) v/v was brought to the crown of 12 day old carrot seedlings and temperature was increased to 25°C day and 20°C night. All experiments were performed in triplicate. The number of damped off seedlings was counted regularly on alternate days for two weeks and area under the disease progress curve (AUDPC) measured in cumulative mortality rate (CM) was calculated. Results were compared by analysis of variance (ANOVA) and Fisher LSD tests using XLSTAT- Version 2007.5 (Addinsoft).

Bioassays as above, but without addition of *R. solani* strain G6 inoculum were conducted to assess the capacity of the native population of *R. solani* to incite the disease (Anees et al. 2009). Briefly, for each soil sample, 50 ml of soil amended with buckwheat meal (2% w/v of soil) was brought to the crown of 12 day old seedlings as an additional source of

nutrients for the resident *R. solani* inoculum. All experiments were performed in triplicate and the results analyzed as above.

Density of *R. solani* and *Trichoderma* spp.

The quantification of *R. solani* and *Trichoderma* spp. in the different soil samples was performed by real time polymerase chain reaction (PCR). DNA was extracted from samples of 1 g of soil using the method described by Edel-Hermann et al. (2004). Briefly, the DNA extraction was done by the help of chemical extractant (sodium dodecyl sulphate, SDS) as well as physical disruption (bead-beater). Lysis buffer consisted of Tris-HCL 50 mM pH 8, EDTA 20 mM pH 8, NaCl 100 mM and SDS 1%. The DNA extracts were purified twice using a polyvinylpolypyrrolidone spin column and once using a Geneclean® kit (Q-BIOgene, Evry, France). DNA extractions were performed in triplicate for each characteristic sampling point of each transect. DNA extracts were stored at –20°C.

The real time PCR assay to quantify *R. solani* was based on primers G6-F2 (5'-AGGTTGTAGCTGGCTCCATTAG-3') and G6-R2 (5'-GTAGGGGTCCCAATCATTCA-3') that specifically target the ribosomal internal transcribed spacer (ITS) region of *R. solani* AG-2-2 (Anees et al., 2009; Edel-Hermann et al., 2009). PCR was performed in a final volume of 25 µl, with 5 µl of soil DNA (20 ng), 0.5 µM of each primer G6-F2 and G6-R2 and 12.5 µl of Absolute Q-PCR SYBR Green Rox Mix (ABgene, Thermo Fisher Scientific, Courtaboeuf, France) containing 3 mM $MgCl_2$. The program included 15 min at 95°C and 35 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C using an ABI Prism 7900 detection system (Applied Biosystems, Foster City, CA). A standard curve was generated using ten-fold dilution series of plasmid DNA containing the cloned ITS region of the strain G6, corresponding to 10^9 to 10^2 copies of target DNA per PCR reaction. The curve was used to quantify the amount of target DNA in the different DNA samples.

The quantification of *Trichoderma* spp. by real time PCR was based on the use of the primers uTf (5′-AACGTTACCAAACTGTTG-3′) and uTr (5′-AAGTTCAGCGGGTATTCCT-3′) which are specific for the ITS region of *Trichoderma* spp (Hagn et al., 2007). PCR was performed in a final volume of 25 µl, with 5 µL of DNA, 5 µL qPCR Rox- and Go Green Mastermix (Q-BIOgene, Evry, France), 0.5 µL BSA, 0.625 µL DMSO and 0.8 µL of each primer. The program included 10 min at 95°C and 45 cycles of 95°C for 30s, 55.5°C for 30s and 72 °C for 30 s using the same detection apparatus as above. A standard curve was generated in the same way as above using ten-fold dilution series of plasmid DNA containing the cloned ITS region of the strain T21 of *Trichoderma* (Hagn et al.

2007), corresponding to 10^6 to 10^2 copies of target DNA per PCR reaction. The curve was used to quantify the amount of target DNA in the different DNA samples.

All samples were analyzed three times in independent real time PCR experiments such that nine analyses per characteristic sampling point (of each plot) were performed. Results expressed as number of copies g^{-1} soil (dw) were compared by ANOVA as above.

Microbial community structures

The genetic structure of microbial communities in the different soil samples was investigated using terminal restriction length fragment length polymorphism (T-RFLP) of 16S, 18S for bacteria, fungi (Edel-Hermann et al. 2004; Perez-Piqueres et al. 2006) and ITS region of ribosomal DNA (rDNA) for *Trichoderma* spp., respectively. Bacterial 16S rDNA was amplified by PCR using the primer 27F (AGAGTTTGATCCTGGCTCAG) labelled with the fluorescent dye D3 (Beckman Coulter, Fullerton, CA, USA) and the primer 1392R (ACGGGCGGTGTGTACA) and digested with the restriction enzyme *Hae*III (Q-BIOgene). Fungal 18S rDNA was amplified by PCR using the primer nu-SSU-0817-5' (TTAGCATGGAATAATRRAATAGGA) labelled with the fluorescent dye D3 (Beckman Coulter) and the primer nu-SSU-1536-3' (ATTGCAATGCYCTATCCCCA) and digested with the restriction enzyme *Msp*I (Q-BIOgene). ITS region of rDNA of *Trichoderma* spp. in the soil was amplified by primers uTf labelled with the fluorescent dye D3 (Beckman Coulter) and uTr. For PCR amplification of ITS region of *Trichoderma* spp. a total of 25 µl reaction mix contained 10 µl of DNA solution, 0.32 µM of each primer, 0.2 mM dNTP, 2.5 mM magnesium chloride, 1× *Taq* polymerase buffer and 2 U *Taq* Polymerase (Q-BIOgene). The PCR programme was the same as used by Hagn et al. (2007) except that 40 PCR cycles were used instead of 30. The restriction enzyme was selected from a T-RFLP simulation by comparing 31 different sequences of ITS region of *Trichoderma* spp. obtained from the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH) database (www.isth.info). The theoretical number of different terminal restriction fragments (TRF) generated was predicted for *Alu*I, *Bst*UI, *Hae*III, *Hha*I, *Mbo*I, *Mse*I, *Msp*I, *Rsa*I, *Sau3*A and *Taq*I restriction enzymes. *Hae*III was predicted to be the most polymorphic one with 13 different theoretical TRF among 31 different sequences. The PCR products obtained with primers uTf-D3 and uTr were checked by electrophoresis, purified, quantified and digested with *Hae*III as above. The labelled and unlabelled primers were synthesized by Proligo (Paris, France) and MWG Biotech (Courtaboeuf, France), respectively. Fluorescently labelled terminal restriction fragments (TRF) were separated and detected using a capillary

electrophoresis sequencer CEQTM 2000XL (Beckman Coulter). The sizes of the TRF were determined by comparison with Size Standard-600 (Beckman Coulter). The microbial community structures were characterized by the size and fluorescence intensity of the TRF. For each PCR product, the T-RFLP analysis was performed in triplicates. Mean values for the intensity of peaks found in at least two of the three analyses were considered. Each experiment was performed in triplicate using three independent DNA extracts for each characteristic sampling point in each transect. Community structures were compared by principal component analysis (PCA) using ADE-4 software (Thioulouse et al. 1997). The significance of the resulting structures was checked using Monte-Carlo tests with 1000 random permutations of the data. The same procedure of T-RFLP analysis of the ITS rDNA was used to characterize the *Trichoderma* strains (Anees et al. submitted). For this purpose fungal DNA was extracted from cultures on MEA using a rapid minipreparation procedure (Edel et al. 2001).

Results

Soil inoculum potential and soil conduciveness

In the bioassays to assess the soil conduciveness of the soil samples collected in July, overall, disease produced in NI soil was very low (Fig. 1a). However, significantly higher disease was produced in D2 than H1; the increase in disease was not significant in other two cases i.e. D1 and D3 (Fig. 1a). In HD, the disease was significantly suppressed in D2 and D3 as compared to H1. However, there was no significant difference between D1 and H1. In LD we observed a similar trend in results as observed in HD except that the disease suppression was not significant in D1 and D3 as compared to H1. Although highest disease was produced in D2 in NI (Fig. 1a), significantly lower disease was produced in D2 than H1 in bioassays to assess the soil inoculum potential (Fig. 2a). Generally, addition of buckwheat meal to natural soil raised the disease considerably in both the diseased and healthy areas (Fig. 2a) as compared to NI (Fig. 1a). However, the decrease in disease in D1 and D3 was not significant as compared to H1 (Fig. 2a).

For the soil samples collected in August, in the bioassays aimed to assess the soil conduciveness towards the disease caused by *R. solani* AG 2-2, higher disease was observed in H2 than in D4 in LD (Fig. 1b). However, no significant difference was observed in NI or HD (Fig. 1b). Addition of buckwheat meal to natural soil raised the disease considerably in

both the diseased and healthy areas (Fig. 2b) as compared to the non-amended soil (NI) (Fig. 1b). Disease produced in H2 was significantly higher than in D4 and D5 (Fig 2b).

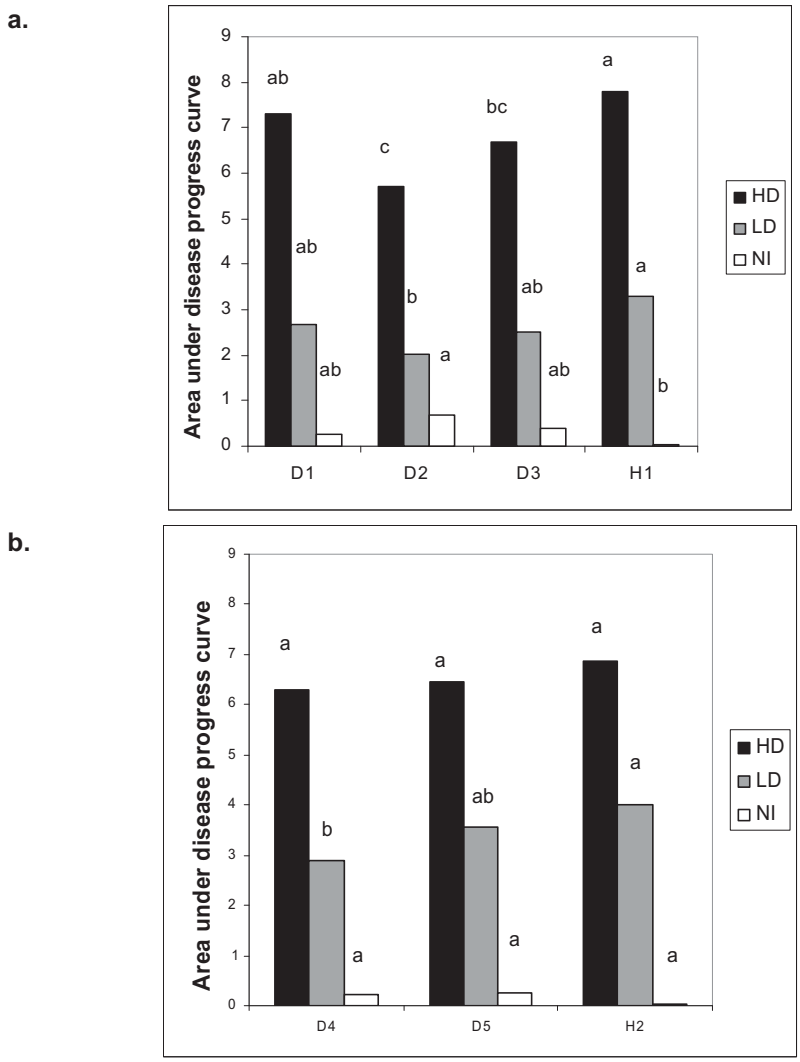

Fig.1 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 strain G6 on carrots in soils inoculated with 3% (v/v of soil, high dose, HD), 0.3% (v/v of soil, low dose, LD) of sand inoculum, or in non inoculated soil (NI) sampled a) in July with disease ages of infested plants caused by the strain G6 (DA) as follows: D1 (DA = 79 days), D2 (DA = 33 days), D3 (DA = 5 days) and H1 (healthy areas); and b) in August D4 (DA = 75 days), D5 (DA = 47 days) and H2 (no *Rhizoctonia* disease symptoms) . In August all the plants around which sampling was done were infested by *Cercospora beticola*. For each characteristic sampling point (D1, D2, D3, H1 and D4, D5, H2) in each plot, AUDPC was calculated using 60 plants. Bars represent the mean values obtained for four independent plots. ANOVA and LSD tests were performed separately for each dose of inoculum. Bars designated by a different small letter are significantly different, $P < 0.05$.

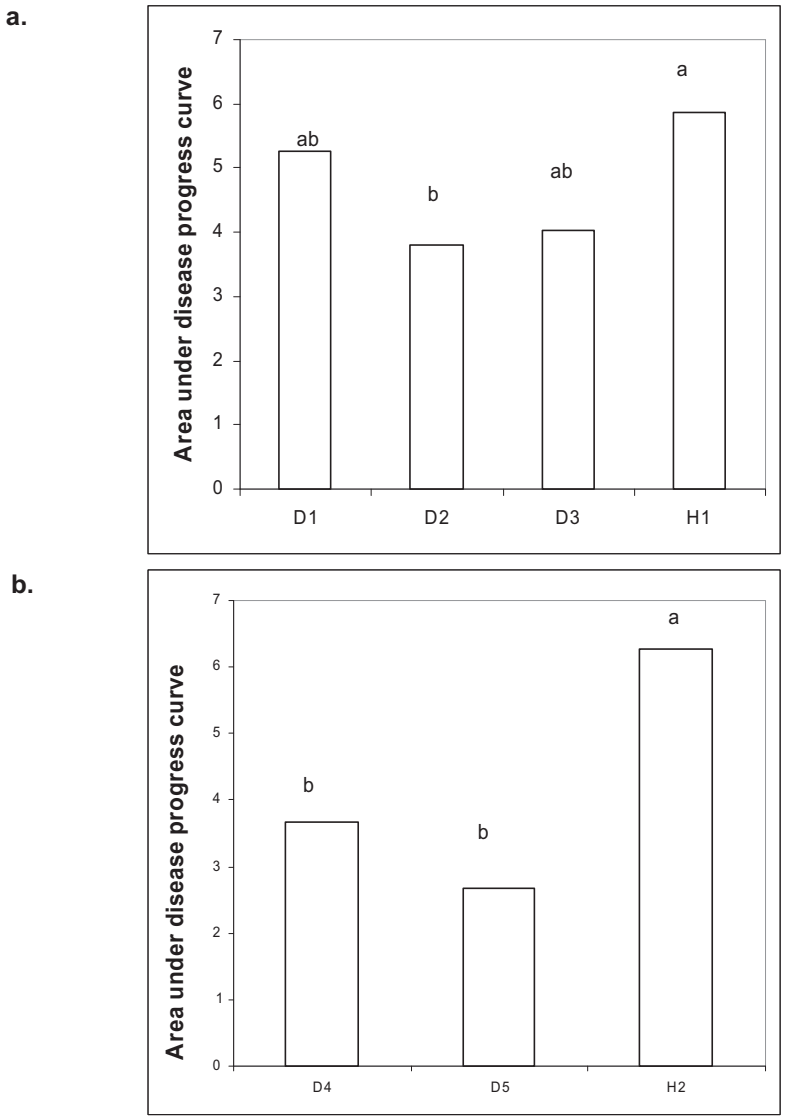

Fig. 2 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 in carrots in soils amended with buckwheat meal (2% w/v of soil) and not inoculated. The different soil samples originated from sampling same as in Fig 1 in a) July and b) August. For each characteristic sampling point AUDPC was calculated using 60 plants as in Fig 1. Bars represent the mean values obtained for four independent plots. Bars designated by a different small letter are significantly different, $P < 0.05$.

Density of *R. solani* and *Trichoderma* spp.

In the samples collected in July, the number of copies of target DNA of *R. solani* per g of dry soil assessed by real time PCR was higher in all the disease areas than in H1 (Fig. 3a). It was the highest in D3, followed by D2 and then D1. For the density of *Trichoderma* spp. in soil,

the log of number of copies of target DNA/g of dry soil was 4.69 ± 0.15, 4.65 ± 0.39, 4.71 ± 0.27, 4.57 ± 0.21 for D1, D2, D3 and H1 respectively with no significant differences.

In the samples collected in August, the number of copies of target DNA of *R. solani* AG 2-2 per g of dry soil was significantly higher in the disease areas (D4 and D5) as compared to healthy areas (H2) (Fig. 3b). The highest number of copies was observed in D5 followed by D4. The log of number of copies of target DNA of *Trichoderma* spp was 5.13 ± 0.18, 4.98 ± 0.44, 4.82 ± 0.17 for D4, D5 and H2 respectively with no significant differences.

Microbial community structures

For the community structures of bacteria, a highly significant discrimination was observed. The most important difference was observed between D1 and H1 on one side, and D2, D4, D5 and H2 on the other side, while D3 lying in between (Fig. 4). For the samples collected in July, D1 and H1 were placed together while a continuum up to D2 through D3 was evident. However, D4, D5 and H2 were placed together close to D2. The relative intensities of peaks at 375 bp were more important in D2, D3, D4, D5 and H2 than D1, H1 (Table 1). For July sampling, the relative intensities of peaks at 215 bp, 260.6 bp, and 294 bp were more important in D2 than H1, D1, and D3 (Table 1). The relative intensities of peaks at 323 bp were more important in all the disease areas (D1, D2, D3) than in H1. For the sampling in August, the differences were less clear between healthy and disease areas as compared to what was observed in sampling in July except that the relative intensities of peaks at 215 bp and 294 bp were slightly more important in D4 than H2 and D5.

For the community structures of fungi, a highly significant discrimination was observed (Fig. 5). A similar situation was observed for fungi as was observed for bacteria. An important difference was observed between D1 and H1 on one side and D2, D3, D4, D5 and H2 on the other side. In July sampling, D2 and D3 showed the most important deviation from D1 and H1; while the difference was not obvious between D1 and H1. A relatively smaller deviation was observed among H2, D4 and D5 as compared to what was observed between D1, H1 and D2, D3. In July, the highest difference was found for the peaks at 309 and 565 bp between H1, D1 and D2, D3 (Table 1); the relative intensity of a peak at 309 bp was more important in D1, H1, while the intensity of the peak at 565 bp was more important in D2, D3; the relative intensities of peaks at 96 bp and 564 bp were more important in H1 and D3 respectively. In August, the most important difference was observed for the peak at 564 bp which was more important in H2 (Table 1); similarly, the relative intensities of peaks at

565 bp and 580 bp were more important in D4 and D5 than those in H2; the relative intensity of a peak at 96 bp was more important in D4.

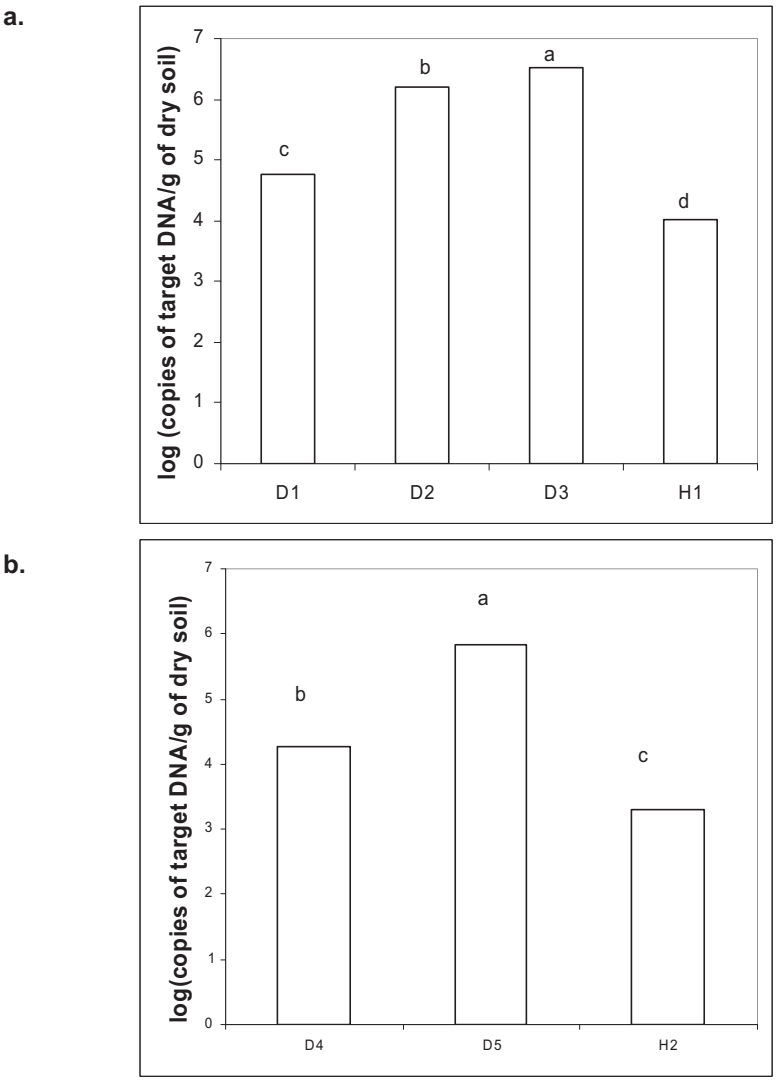

Fig. 3 Number of copies of target DNA of *R. solani* AG 2-2 quantified by real time PCR. The different soil samples originated from sampling same as in Fig 1 in a) July and b) August. For each characteristic sampling point the bar corresponds to the mean value among three independent PCR experiments per three independent soil samples per four independent plots. Bars designated by different letters are significantly different, $P < 0.05$ (LSD).

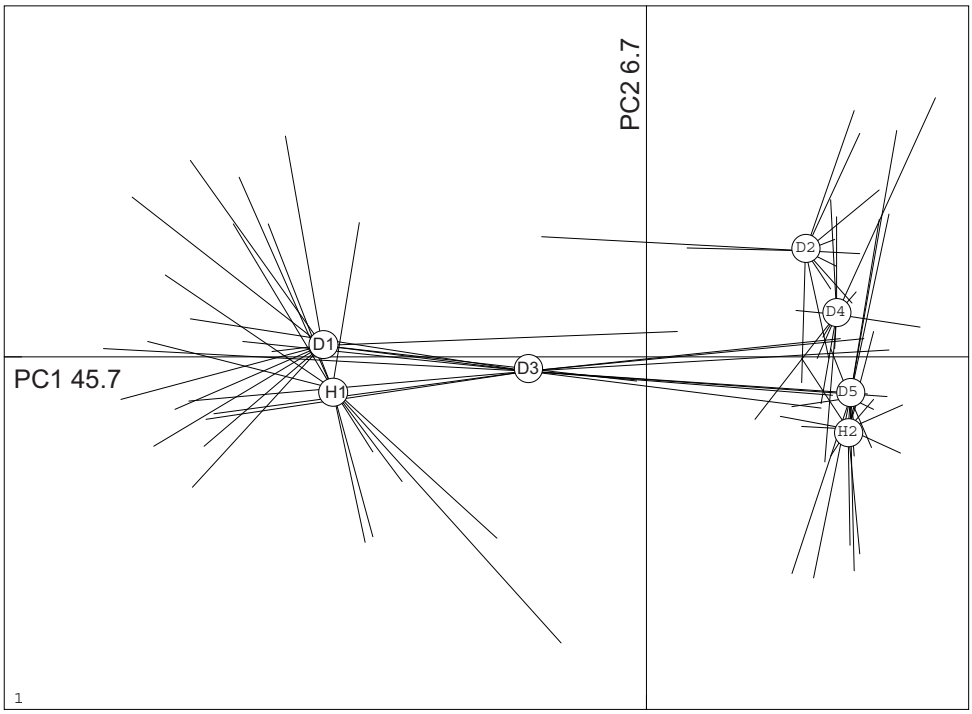

Fig. 4 Principal component analysis of 16S terminal restriction fragment length polymorphism data sets from sampling same as in Fig 1 in a) July and b) August. For each characteristic sampling point, analysis was performed in four independent plots, each with three independent soil samples. Each characteristic sampling point is represented by its gravity center. The branches show the divergence of the repeats from the respective gravity centers.

A significant discrimination in the community structures of *Trichoderma* spp. was observed in such a way that D1, D2 and D3 could be grouped together while the D4, D5, H1 and H2 could make one group (Fig 6). In July, all the disease areas (D1, D2, and D3) were well discriminated as compared to H1. The highest difference was found for the peak at 368 bp which was more important in the disease areas of July (D1, D2, and D3) than in the other areas (Table 1). The relative intensities of the peaks at 366 bp and 367 bp were higher in D2, D3 and H1, H2, D4 respectively. The relative intensity of peaks at 364 bp and 370 bp were more important in D1 and D2 respectively. Concerning the samples collected in August, the community structures of *Trichoderma* spp. were not significantly different.

Table 1: Percentage intensity of the important TRF resulting from the terminal restriction fragment polymorphism

Organisms	TRF	H1	D1	D2	D3	H2	D4	D5
Bacteria	(214,74-) **215,30**±0,28 (-215,82)	1,2	1,4	2,6	1,7	1,9	2,5	1,9
	(260,2-) **260,59**±0,28 (-261,35)	0,4	0,3	1,3	0,8	1,3	1,1	1,3
	(293,9-) **294,28**±0,26 (-294,69)	0,9	0,5	1,4	0,0	0,5	1,5	0,1
	(322,7-) **323,05**±0,15 (-323,38)	1,3	1,6	1,8	1,5	1,8	1,9	1,8
	(375,24-) **375,51**±0,08 (-375,68)	0,0	1,1	3,8	2,1	3,3	3,7	3,8
Fungi	(95,86-) **96,47**±0,08 (-96,73)	14,8	6,5	8,1	7,8	4,8	10,9	4,7
	(308,13-) **308,64**±0,15 (-309,02)	25,2	32,1	10,1	13,9	1,4	2,3	2,1
	(563,69-) **564,09**±0,15 (-564,33)	1,1	0,9	0,9	14,5	27,5	5,2	0,9
	(564,34-) **564,86**±0,35 (-565,59)	6,4	3,5	24,9	20,5	0,0	2,8	11,1
	(579,38-) **579,91**±0,21 (-580,52)	7,8	5,7	4,8	3,5	5,0	8,0	5,4
Trichoderma **spp.**	(363,9-) **364,45**±0,43 (-365,15)	0,9	4,5	1,5	0,5	0,3	1,0	1,6
	(365,2-) **365,91**±0,32 (-366,45)	1,6	3,8	9,3	8,1	3,8	2,7	4,8
	(366,47-) **367,17**±0,34 (-367,71)	7,3	3,3	1,5	1,4	6,5	7,2	4,1
	(367,74-) **368,24**±0,26 (-368,97)	5,2	26,0	17,1	21,8	10,6	6,6	12,8
	(369,06-) **369,67**±0,38 (-370,28)	0,3	0,2	2,1	0,8	0,3	0,2	0,5

T-RFLP analysis of the *Trichoderma* strains originating from a diseased sugar beet field infested with *R. solani* adjacent to the field under study were also investigated (Anees et al. 2009). For all the strains of *T. gamsii*, the TRF size ranged from 369 to 370 bp (370.25 for T30; 370.35 for T31; 369.43 for T35; 369.50 for T40; 369.47 for T43). For all the strains of *T. velutinum*, the size of TRF ranged from 366 to 367 bp (367.37 for T34; 367.45 for T36; 366.95 for T37; 367.12 for T42; 367.04 for T44; 367.32 for T46; 366.95 for T47). TRF size for *T. harzianum* T45 was observed at 366.21 bp. TRF size for strains of *T. tomentosum* was observed at 366.95 for T32 and 364.59 for T33.

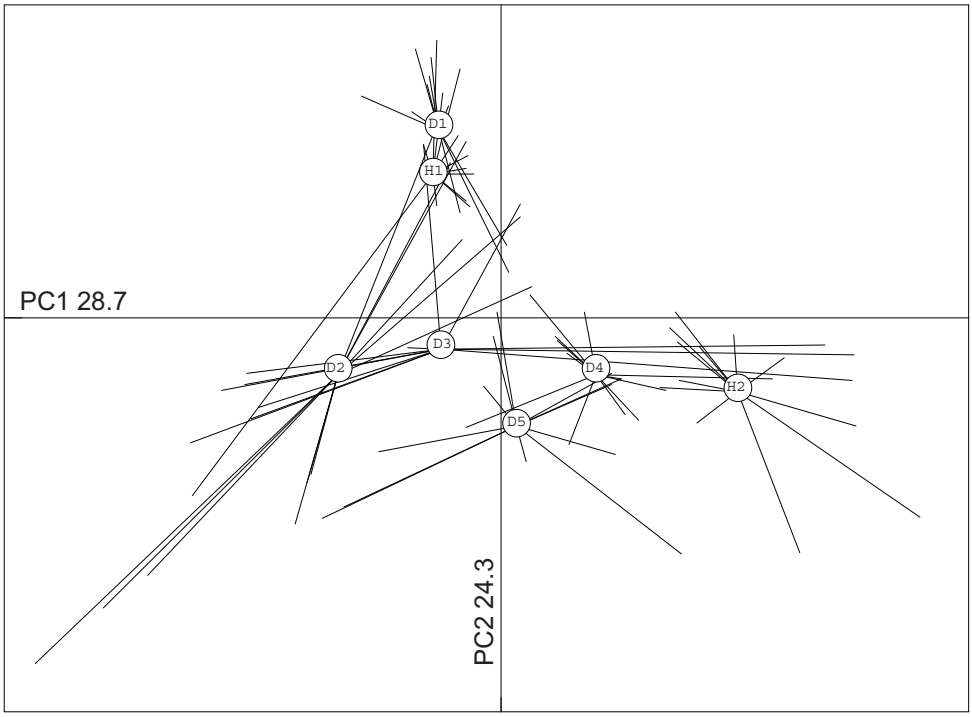

Fig. 5 Principal component analysis of 18S terminal restriction fragment length polymorphism data sets from sampling same as in Fig 1 in a) July and b) August. For each characteristic sampling point, analysis was performed in four independent plots, each with three independent soil samples. Each characteristic sampling point is represented by its gravity center. The branches show the divergence of the repeats from the respective gravity centers.

Discussion

Influence of infectious activity of *R. solani* without *C. beticola* on the evolution of microflora

The first part of the study was to reveal the effect of disease caused by *R. solani* AG 2-2 on the evolution of microflora in a sugar beet field based on sampling in July when there was no attack of the aerial pathogen *C. beticola*. This study confirms the reduced conduciveness towards the disease caused by *R. solani* AG 2-2 inside the patches of disease incited by the same fungal pathogen in relation to the modification of microfloral components in a different field and at a different time as compared to the previous study (Anees et al. 2009). Thus it supports the repeatability of the phenomenon in space and time with the exception of the diseased areas D1 (DA = 79 days). No difference was observed between D1 and the healthy areas (H1) for all the components of microflora except the inoculum density which was

significantly higher in terms of number of copies of target DNA/g of dry soil in D1 than in H1. Similarly, the only deviation in microbial community structures between D1 and H1 was observed for *Trichoderma* spp. D1 represented the primary infection and the earliest attack of the *R. solani* AG 2-2 disease in the field on the young seedlings that died early in the season. After their deaths, the plantlets completely rotted and dried out. It seems that as soon as the infectious activity in these areas started, it was followed by the changes in community structures of *Trichoderma* spp. However, after infection and an early death of the plantlet, the pathogen either moved to the nearby susceptible host to survive as the saprotrophic ability of the pathogen is well known (Ogoshi 1996) or became inactive or ultimately died because of further unavailability of nutrients in D1. Thus D1 may represent the premature death of the epidemic of *R. solani* AG 2-2 with no changes in components of microflora. That may be why the characteristic sampling points of D1 partially resemble those of the healthy areas in terms of microbiological behaviours.

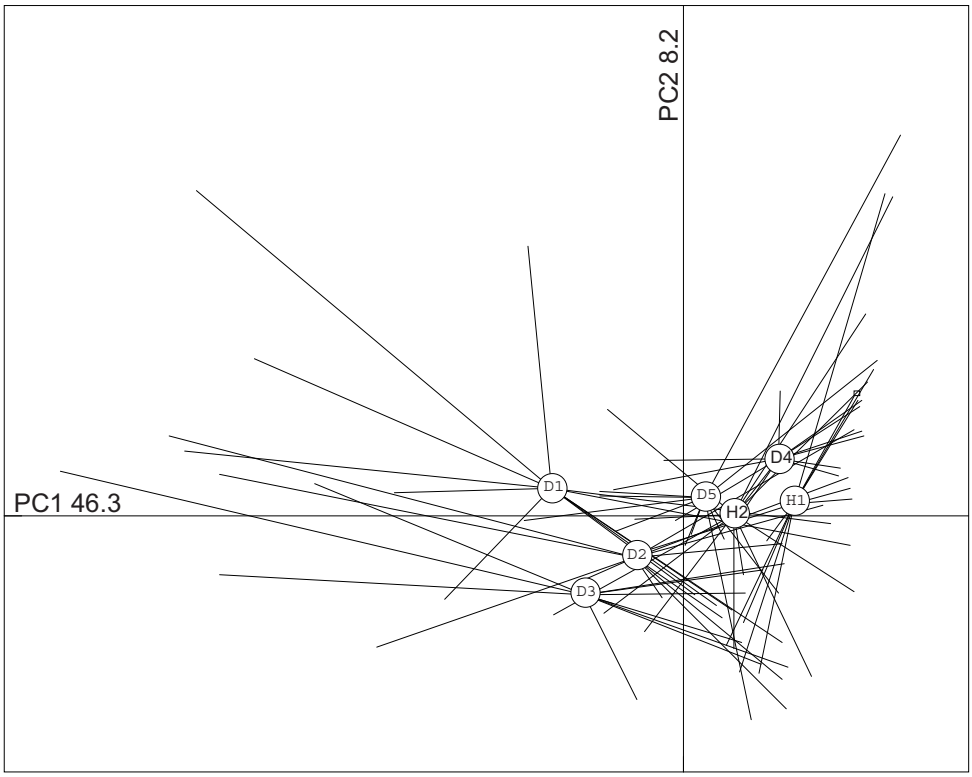

Fig. 6 Principal component analysis of ITS rDNA terminal restriction fragment length polymorphism data sets of *Trichoderma* spp. from sampling same as in Fig 1 in a) July and b) August. For each characteristic sampling point, analysis was performed in four independent plots, each with three independent soil samples. Each characteristic sampling point is represented by its gravity center. The branches show the divergence of the repeats from the respective gravity centers.

Conversely, in the characteristic sampling points of D2 (DA = 33 days) and D3 (DA = 5 days), we found partially rotted living plants of sugar beet. The sampling points D2 were the least conducive towards the disease caused by *R. solani* AG 2-2 coupled with decreased soil inoculum potential and inoculum densities followed by the sampling points D3. These results may suggest a progressive decrease along with time in both the conduciveness towards the disease caused by *R. solani* AG 2-2, the inoculum density revealed by real time PCR and the soil inoculum potential after the commencement of disease attack of *R. solani* AG 2-2. The highest inoculum density at disease age of 5 days (D3) is consistent with the previous findings that the increase in the density of mycelium increases the probability of infection (Gilligan and Bailey 1997). These results also suggest that the primary infection leads to the parasitic amplification through autoinfection at the surface of the tuber or/and to putative alloinfection by spreading to the adjacent susceptible host plants giving an expression of patch development and expansion as already shown in the case of take all epidemics (Bailey and Gilligan 1999).

Additionally, the community structures had also changed as a result of infectious activity of *R. solani* AG 2-2. The most important differences for the global community structures of bacteria and fungi were between H1 and D2, while D3 lying in between. These results also support the hypothesis of temporal deviation, maximum at 33 days of disease age with the exception of D1 explained above. The peaks which were responsible for this deviation could have been identified. For example in bacteria, the TRF with mean at 375 bp was more important in D2 followed by D3, D1 and H1. Similarly, for fungal community structures, the most important differences were obtained for the TRF 565 bp which corresponds to one resulting from the T-RFLP of *R. solani* AG 2-2 obtained previously (Anees et al. 2009) and for an unknown TRF with mean size at 309 bp. The TRF at 565 bp was more important in D2 and D3, whereas the TRF at 309 bp was more important in D1 and H1. However, the peak for *Trichoderma* spp. (580 bp) as determined through the T-RFLP performed on 18S rDNA region, was found everywhere with similar relative intensities which was also confirmed by the specific real time PCR to assess the density of *Trichoderma* spp. in the soil. This is contrary to what was observed in September 2006 where an increased intensity in the peak of *Trichoderma* was observed (Anees et al. 2009). The two studies were based on the sampling at two different epidemiological periods of the disease with the difference of about 2 months that may explain this difference in results.

However, there was a significant discrimination observed for the community structures of *Trichoderma* spp. based on T-RFLP of their ITS region, which confirms that the relative abundance of the various species of *Trichoderma* may be changed in response to the infectious activity of *R. solani* towards the sugar beet. This also confirms the role of

Trichoderma spp. in reduced conduciveness previously suggested. This change in community structures of *Trichoderma* spp. may be principally attributed to the TRF with mean value at 368 bp that was more abundant in all the disease areas (D1, D2 and D3) as compared to the healthy areas (H1). The community structure of *Trichoderma* isolates based on T-RFLP analysis of their ITS region revealed that this peak may be attributed to either *T. gamsii* or *T. velutinum*. The majority of *Trichoderma* strains isolated in the previous study belonged to these two species (Anees et al. 2009). Similarly, some strains of *T. gamsii* proved to be good antagonists towards *R. solani* AG 2-2 (Anees et al. submitted). *Trichoderma* have already been shown as efficient antagonists towards *R. solani* both in natural (Verma et al. 2007) and controlled conditions (Bailey et al. 2004; Kleczkowski 1997). Accumulation of *Trichoderma* spp. has been previously reported by Mghalu et al. (2007) by repeated inoculation of soil with *R. solani* leading to suppression of radish damping off. Moreover, some *Trichoderma* spp. associated with sugar beet roots and showing antagonistic ability to a broad range of pathogens including *R. solani* were isolated (Zachow et al., 2008).

Relative influence of infectious activity of *R. solani* and *C. beticola* on the evolution of microflora

The relative importance of the soilborne disease in comparison with an airborne disease on microflora was evaluated in the present study. The airborne disease is more efficient by quickly spreading and causing huge losses in the field. The same is true for the leaf spot disease caused by *C. beticola* which spreads very quickly in the field (Weiland and Koch, 2004). In our case, during the sampling performed in August, the whole field was under a severe attack of this disease and no fungicide was used to allow thus the epidemics to propagate within and across the plots. Besides its ability to cause more losses in lesser time as compared to the soilborne disease *R. solani* which have only a limited ability to spread in the field, it is interesting to investigate whether *C. beticola* is also more efficient in influencing the microflora as compared to *R. solani*. Therefore, soil was sampled around the diseased plants affected by *R. solani* (D4 and D5) and the plants showing no disease symptoms of *R. solani* (H2) but the foliage of all these plants was affected by *C. beticola*.

Surprisingly, there were no significant differences or only minor differences among H2, D4 and D5 for the soil conduciveness and the soil inoculum potential towards the disease caused by *R. solani*. Additionally, no considerable discrimination was observed in the microbial community structures among the three characteristic points within the four plots. Comparing the second sampling with the first sampling, it is obvious that overall the

inoculum density of *R. solani* has been reduced considerably. The maximum deviation was revealing when comparing H2, which had the symptoms of leaf spot disease but no symptoms of *R. solani* and H1, the healthy plants. Going in depth of these modifications and identifying the TRF which are principally responsible for this deviation led us to a few candidates. For bacteria, the TRF at the mean size of 375 bp was more important in H2 but absent in H1. For fungi, two TRF were mainly responsible for the differences. The first one was at mean size of 309 bp which was more important in H1 and the second was at mean size of 564 bp being more important in H2. Although, we are not able to link these TRF to any candidate species, there is no information about the 18S rDNA allowing to identify the putative TRF of interest in *C. beticola* in the databases available on the web so far, all these results lead to the conclusion that *C. beticola* had partially influenced the microfloral evolution below the soil surface along with the probable effect of seasoning.

Secondly, the infestation by *C. beticola* suppressed most of the expected influences of *R. solani* on the microfloral components leading the temporal dynamics to a static point with no differences between diseased areas by *R. solani* and healthy areas with no *R. solani* disease as was evident from the previous sampling. The changes in structures of *Trichoderma* spp. observed earlier in the microflora were also reverted. Thus the present results suggest an effect of this external factor of aerial disease (*C. beticola*) on the microflora. *C. beticola* is an aerial disease for which recently a soilborne epidemiological phase has been suggested (Schneider and Vereijssen, 2006; Vereijssen et al., 2006). Moreover, it has been shown that this pathogen can penetrate through roots to the upper parts of the plant (Vereijssen et al., 2005). However, only the foliage above ground is damaged in the form of leaf spots indicating that the infection was from an aerial origin. This is the first report of the comparative influence of the two diseases (*C. beticola* and *R. solani*) on the microflora, although the seasonal effect could not be circumvented from the present set of experimental observations.

There is actually a lack of data about the impact of the disease caused by *C. beticola* on the evolution of soil microflora but the present study demonstrates that this impact is not neutral. The epidemic aerial invasion of *C. beticola* revealed the weakness of the equilibrium which was established by the soil microflora in response to *R. solani* infectious activity. This equilibrium involved changes in the structure of microbial communities and more specifically, in the *Trichoderma* community, leading to the modification of both soil conduciveness, soil inoculum potential and promotion of soil microbial populations which are antagonistic towards *R. solani*. One may have wonder why the so locally acquired soil suppressiveness to *R. solani* disease was not persistent and did not result in a disappearance of the disease. The aerial invasion, as probably mediated by the sugar beet, can be seen from the

soil microbiologist point of view, as an external factor that activates the resilience mechanisms within the soil biotic components and may contribute to explain the non preservation of the reduced conduciveness achieved within the diseased patches.

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CHAPTER 4: Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*

Muhammad Anees [a], Arne Tronsmo [b], Véronique Edel-Hermann [a], Linda Gordon Hjeljord [b], Cécile Héraud [a], Christian Steinberg[a,*]

[a]INRA, Université de Bourgogne, UMR 1229 Microbiologie du sol et de l'environnement, CMSE, 17 rue Sully, BP 86510, 21065 Dijon, France; [b]Department of chemistry, biotechnology and food science, PO box 5003, NO-1432 Aas, Norway.

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[*] Corresponding author. Email address: christian.steinberg@dijon.inra.fr; Telephone: +33 (0) 380 69 30 50; Fax: +33 (0) 380 69 32 24

Abstract

The aim of the present study was to characterize sixteen isolates of *Trichoderma* originating from a field of sugar beet where disease patches caused by *Rhizoctonia solani* were observed. The isolates were identified using both molecular and morphological characters and both approaches were found complementary. Production of water-soluble and volatile inhibitors, mycoparasitism and induced systemic resistance in plant host were investigated using *in vitro* and *in vivo* tests in disinfested and non-disinfested soils. This functional approach revealed the intraspecific diversity as well as biocontrol potential of the different isolates. Different antagonistic mechanisms were evident for different strains. The most antagonistic strain, T30 was identified as *T. gamsii*. This is the first report of an efficient antagonistic strain of *T. gamsii* being able to reduce the disease in different conditions. The ability to produce water-soluble inhibitors or coil around the hyphae of the pathogen *in vitro* was not related to the disease reduction *in vivo*. Additionally, the strains collected from the high disease areas in the field were better antagonists. The antagonistic activity was not characteristic of a species but that of a population of strains.

Key words: *Trichoderma gamsii*, *Trichoderma velutinum*, water-soluble inhibitors, volatile inhibitors, biocontrol

Introduction

Trichoderma spp. are cosmopolitan and abundant fungi in soil in a wide range of ecosystems and climatic zones. They are characterized by rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals (Klein & Eveleigh 1998). Their economic importance includes their roles as primary decomposers, producers of antibiotics and enzymes as well as biocontrol agents against a wide range of plant pathogens (Hjeljord & Tronsmo 1998; Kubicek & Penttila 1998; Rossman 1996; Sivasithamparam & Gisalberti 1998). *Trichoderma* spp. may inhibit the phytopathogenic fungi either by inducing resistance and plant defence reactions or by direct confrontation through mycoparasitism and antibiosis as well as competition (Howell 1998, 2003; Papavizas 1985; Verma et al. 2007).

A wide range of defence mechanisms against invasion is triggered in plants following contact with pathogenic and non-pathogenic microorganisms. This kind of resistance may include the localized acquired resistance (LAR), systemic acquired resistance (SAR) or the induced systemic resistance (ISR) (Vallad & Goodman 2004; van Loon et al. 1998). In LAR, only the tissues exposed to the primary infection become more resistant while in the other two cases, a signal propagates the enhanced defensive capacity throughout the plant. SAR is commonly triggered by local infection and is correlated with activation of pathogenesis-related (PR) genes that generally requires the involvement of the signal molecule salicylic acid (SA). However, ISR is generally triggered by non-pathogenic rhizobacteria and followed by the expression of the genes involved in the jasmonate/ethylene signalling pathways, which does not involve the accumulation of PR proteins. The types of resistance induced by *Trichoderma* spp. are mainly considered as LAR or ISR similar to the resistance induced by rhizobacteria (Harman et al. 2004; Shoresh et al. 2005). To assess ISR induced by *Trichoderma* spp., the biocontrol fungus is inoculated at different times or sites than the pathogenic attack.

In the direct interactions between *Trichoderma* spp. and the phytopathogenic fungi, mycoparasitism is one of the mechanisms observed in which the antagonist coils around the hyphae of the pathogen, develops hook like structures known as appressoria coupled with production of lytic enzymes and then penetrates the pathogen hyphae (Chet 1987; Kubicek et al. 2001). Coiling of the phytopathogenic fungal hyphae by *Trichoderma* spp. is one of the parameters used to characterize the mycoparasitism (Howell 2003; Rocha-Ramirez et al. 2002). *Trichoderma* spp. have also been reported to produce a plethora of secondary metabolites showing anti microbial activity (Vinale et al. 2008). The chemical composition of these compounds depends on the strains and they may be classified as volatile, water-soluble

or water-insoluble compounds (Ghisalberti & Sivasithamparam 1991). The competition for space, infestation sites and nutrients has also been shown to be possible mechanisms involved in the biocontrol activities of *Trichoderma* spp. (Chet 1987; Dennis & Webster 1971a; Dennis & Webster 1971b; Tronsmo & Hjeljord 1998) The knowledge of mechanisms of interaction of *Trichoderma* spp. with phytopathogenic fungi and plant host is of utmost importance to enhance the practical application of these beneficial microorganisms.

Trichoderma spp. are among the microorganisms most frequently used as antagonists against *Rhizoctonia solani* (Hjeljord & Tronsmo 1998; Hyakumachi 1996). *R. solani* is a soil-borne phytopathogenic fungus known worldwide for causing root diseases in diverse cultures (Ogoshi 1996). *Rhizoctonia*-diseased areas have been previously shown to become more suppressive towards coming invasion by the same pathogen (Anees et al. 2009; Guillemaut 2003; Hyakumachi 1996) and hence, may present a potential reservoir of beneficial microorganisms; however, such areas have been little evaluated. Comparing the structure of fungal communities in healthy and diseased areas in fields affected by *R. solani* revealed that *Trichoderma* spp. accumulated in the latter and were proposed to be responsible for the increased suppressiveness (Anees et al. in press; Mghalu et al. 2007). Similarly sclerotia of *Rhizoctonia* on potatoes have been recently shown to be a source of beneficial antagonistic microorganisms e.g. *Trichoderma* spp. (Grosch et al. 2006). These findings suggest that putative actively antagonistic populations of *Trichoderma* are more likely to be found within diseased areas than in healthy areas. It would be worthwhile then to check for naturally occurring *Trichoderma* in soils where *R. solani* is active to determine their putative intrinsic abilities to control this pathogenic fungus.

Phenotypic characterization presents a potential method to taxonomically identify *Trichoderma* spp. by using a morphological key (Samuels GJ, Chaverri P, Farr DF, McCray EB, http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm). However, because of the increasing number of species recorded within the genus, the few morphological characters of anamorph and teleomorph have reached their limit for identifying species. Thus they need to be combined with molecular data resulting from DNA sequencing (Samuels 2006b). As a single gene is generally not considered sufficient, a multigene approach using at least two unlinked loci is advised. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is one of the most reliable targets to identify a strain at the species level (Kullnig-Gradinger et al. 2002). However, some closely related species share the sequences of their ITS regions. For example this is the case for some species in *Trichoderma* sect. *Trichoderma*, (Samuels 2006b). Being more variable, the gene *tef1* encoding the translation elongation factor 1-α can be used to reflect differences within and among groups of closely related species. In this way, combination of the ITS region and *tef1* allow most identifications at the

species level (Samuels 2006b). For identification of *Trichoderma* strains, *Trich*OKEY and *Tricho*BLAST (www.isth.info) are convenient tools available online that are based on the sequence comparisons of ITS or *tef*1 regions (Druzhinina et al. 2005; Kopchinskiy et al. 2005).

The primary aim of the present study is to characterize *Trichoderma* spp. originating from a sugar beet field containing patches infected by *R. solani,* using a set of complementary approaches. These field isolates of *Trichoderma* were tested for their antagonistic abilities towards *R. solani* AG 2-2 both *in vitro* and *in vivo*. *In vitro* tests were performed to characterize their mycoparasitism and ability to produce water-soluble metabolites or volatile inhibitors. *In vivo* tests were performed to characterize the isolates for induction of resistance in the host or direct antagonism of the pathogen. The isolates were identified using morphological and molecular characters. Morphological characterization was based on microscopic measurements of mycelial fragments as well as growth rates of different isolates on different media at different temperatures, while molecular identification was based on sequence comparisons of the ITS and *tef1* regions.

Materials and Methods

Fungal isolates

The study was conducted with sixteen isolates of *Trichoderma* (T30 to T37 and T40 to T47) collected in 2006 from soil samples originating from a sugar beet field at the INRA Experimental Unit of Epoisses, Côte d'Or, France (5°05'E; 47°14'N) (Table 1). The field was chosen because patches of root rotting caused by *R. solani* were observed (Anees et al. 2009). The isolation was performed using malt extract agar (MEA) as previously described by Anees et al. (in press). The *Trichoderma* isolates were stored in the collection "Microorganisms of Interest for Agriculture and Environment" (MIAE, INRA Dijon, France). Additionally, two external isolates of *Trichoderma* were also included in this study: one isolate (T38) originating from another suppressive soil in Norway (*Pythium* suppressive soils) and one isolate (P1, ATCC74059) known for its antagonistic activity against *R. solani* (Tronsmo 1989).

Table 1- Morphological and molecular identification of the strains of *Trichoderma* used in this study.

Strain	MIAE accession No.*	Morphological identification**	Genbank accession No.		Molecular identification	Definitive identification
			ITS sequence	*tef1* sequence		
T30	MIAE29	*T. gamsii*	Tobe deposited		*T. gamsii*	*T. gamsii*
T31	MIAE30	*T. gamsii*			*T. gamsii*	*T. gamsii*
T32	MIAE31	NI			*T. tomentosum*	*T. tomentosum*
T33	MIAE32	NI			*T. tomentosum*	*T. tomentosum*
T34	MIAE33	*T. velutinum*			*T. velutinum*	*T. velutinum*
T35	MIAE34	*T. gamsii*			*T. gamsii*	*T. gamsii*
T36	MIAE35	*T. velutinum*			*T. velutinum*	*T. velutinum*
T37	MIAE36	*T. velutinum*			*T. velutinum*	*T. velutinum*
T40	MIAE37	*T. gamsii*			*T. gamsii*	*T. gamsii*
T41	MIAE38	*T. velutinum*			*T. velutinum*	*T. velutinum*
T42	MIAE39	*T. velutinum*			*T. velutinum*	*T. velutinum*
T43	MIAE40	*T. gamsii*			*T. gamsii*	*T. gamsii*
T44	MIAE41	*T. velutinum*			*T. velutinum*	*T. velutinum*
T45	MIAE42	*T. harzianum*			*T. harzianum*	*T. harzianum*
T46	MIAE43	*T. velutinum*			*T. velutinum*	*T. velutinum*
T47	MIAE44	*T. velutinum*			*T. velutinum*	*T. velutinum*
T38		*T. hamatum*			*T. hamatum*	*T. hamatum*
P1		*T. atroviride*			*T. atroviride*	*T. atroviride*

* Collection MIAE, Microorganisms of Interest for Agriculture and Environment (INRA, Dijon, France)
** NI: not identified.

Morphological identification

The identification was performed using an interactive key for strain identification (Samuels GJ, Chaverri P, Farr DF, McCray EB, http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) based on morphology and differences in growth rates on potato dextrose agar (PDA) and synthetic nutrient agar (SNA, a defined low-sugar medium containing 1 g of KH_2PO_4, 1 g of KNO_3, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose, and 20 g of agar in the final volume of 1 litre in distilled water) (Nirenberg 1976) along with microscopic measurements of mycelial parts. All

measurements of morphological characters (shape and size of conidia, size of conidiophores, presence or absence of sterile hairs) were taken from slide mounts prepared by the tape touch method (Harris 2000) in a drop of lactofuchsin. Growth rates were measured every 24 h for 4 days on 9 cm PDA and SNA plates inoculated with a plug (5 mm) from the actively growing edge of colonies (Samuels et al. 2002) incubated at 20, 25, 30, 35, and 40°C in dark (Chaverri et al. 2003). The experiments were performed in triplicate.

Molecular identification

The molecular identification of *Trichoderma* isolates was based on DNA sequencing of two unlinked loci, the ribosomal ITS region and the *tef1* gene. Fungal DNA was extracted from cultures on MEA using a rapid minipreparation procedure (Edel et al. 2001). The ITS region was amplified by PCR using primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) in a final volume of 50 µl by mixing 2 µl of DNA with 0.5 µM of each of the primers, 150 µM of dNTP, 6 U of Taq DNA polymerase (Q-Biogen, Evry, France) and PCR reaction buffer. Amplifications were conducted in a mastercycler (Eppendorf, Hamburg, Germany) with an initial denaturation of 3 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50°C, 1 min extension at 72°C and a final extension of 10 min at 72°C. Aliquots of 2 µl of PCR products were checked by electrophoresis in a 2 % agarose gel and stained with ethidium bromide for visualization under UV light. The PCR products were cloned with pGEM@-T Easy Vector System (Promega, Charbonnières, France) and sequenced using primers SP6 and T7 by Cogenics (Meylan, France).

The *tef1* fragment was amplified by PCR using the primers ef1 (5'-ATG GGT AAG GA(A/G) GAC AAG AC) and ef2 (5'-GGA (G/A)GT ACC AGT (G/C)AT CAT GTT) (Geiser et al. 2004) and the same PCR conditions as above with the exception of the following parameters that differed: the concentration of primers (0.4 µM) and dNTP (250 µM), and the temperature of denaturation (95°C) and annealing (57°C). The PCR products were checked by electrophoresis as above and directly sequenced using primers ef1 and ef2 by Cogenics (Meylan - France).

For each PCR product, sequences from both strands were assembled using SeqMan 6.0 (DNASTAR Inc., 2004). Sequence identities were determined using both a specific database for *Trichoderma* and the Genbank general database. We used successively the different tools available online from the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info): *Trich*OKEY v. 2.0 based on an oligonucleotide barcode within the ITS1 and ITS2 sequences, *Tricho*MARK to analyse both ITS and *tef1*sequences, and *Tricho*BLAST to detect sequence similarity in the ITS region and the largest 4th intron sequence of *tef1* gene

(Druzhinina et al. 2005; Kopchinskiy et al. 2005). In some cases, BLAST analyses were also performed from the National Center for Biotechnology Information (NCBI) available online. Moreover, as per requirement, the alignments of sequences were performed with the help of the programme ClustalX v 2.0 (Larkin et al. 2007) and then sequences were manually edited by visual adjustments by the help of the computer program Seaview (Galtier et al. 1996).

Assessment of antagonistic activity using *in vitro* tests

Trichoderma isolates were evaluated for their potential to antagonize *in vitro* the phytopathogenic fungus *R. solani* using three different tests. The strain G6 of *R. solani* AG 2-2 (MIAE collection, INRA, France) was used. For all the *in vitro* tests, discs (5 mm) from the edge of growing fungal colonies were used to inoculate MEA in sterile plastic round 9 cm Petri dishes. The plates were incubated in the dark at 20°C.

In the first test, each isolate of *Trichoderma* was grown in dual culture with the strain G6 of *R. solani*. The strain G6 and the *Trichoderma* isolate were inoculated 6 cm apart on the same plate and incubated at 20°C. Radii of colony of *R. solani* approaching and not approaching the colony of *Trichoderma* isolate were measured twice a day for 3 to 4 days. Experiments were performed in triplicate. Inhibition of growth rate of *R. solani* was assessed as percentage of difference of radius not approaching and radius approaching *Trichoderma,* over radius not approaching and compared by analysis of variance (ANOVA) and Fisher LSD tests using XLSTAT- Version 2007.5 (Addinsoft). Part of the results from this test were presented in a previous publication (Anees et al. in press).

The second test was designed to measure the ability of *Trichoderma* isolates to produce water-soluble inhibitors against *R. solani* strain G6. *Trichoderma* inoculum discs were placed in the centre of MEA plates covered with a 50 µm thick cellophane membrane and incubated for 5 days at 20°C. The cellophane membranes along with the mycelia of *Trichoderma* isolates were then removed and *R. solani* was cultured in the same plates (Dennis & Webster 1971a). Growth of *R. solani* was recorded after 24, 48 and 72 h. For the control plates, *R. solani* was cultured instead of *Trichoderma* sp. on MEA covered with cellophane membranes and followed by the same procedure as above.

The third test was designed to measure the ability of *Trichoderma* isolates to produce volatile inhibitors. The MEA plates were inoculated centrally with agar disks of *Trichoderma* isolates and the lid of each dish was replaced by a bottom dish containing MEA newly inoculated with *R. solani*. The two dishes were taped together with adhesive tape (Dennis & Webster 1971b). The growth of *R. solani* was recorded after 24, 48 and 72 h. In the control, *R. solani* was cultured in the same way but without *Trichoderma* isolates. For both volatile and

water-soluble inhibitors tests, the percentage of inhibition was measured by dividing the difference between the radial growth of control and antagonised culture of *R. solani* by the radial growth of the control and multiplied by 100. The experiments were performed in triplicate and results were compared by ANOVA as above.

Finally, direct inspection by light microscopy was carried out before and after contact of the hypha of the *Trichoderma* isolates with *R. solani* (Tronsmo & Dennis 1978). In addition to this, interactions between hyphae of *Trichoderma* isolates and *R. solani* were recorded as inhibition zones before contact between colonies, or as inhibition of the pathogen after contact with the antagonist, or no visible inhibition after contact between colonies.

Assessment of antagonistic activity using *in vivo* tests

Bioassays were performed in climatic chambers in order to test the antagonistic activity of the selected *Trichoderma* isolates against *R. solani* strain G6 causing disease on a susceptible host plant. Conidial suspensions of *Trichoderma* were prepared from 18-days -old cultures on MEA incubated at 25°C and adjusted to 4 X 10^6 ml^{-1} conidia using a haemocytometer (Thoma, Preciss France). Millet seed inoculum of *R. solani* was produced by adding five pieces of MEA culture to millet seeds previously autoclaved for 1 h on three consecutive days at 105°C. The inoculated millet seeds were incubated for two weeks at 25°C with periodic shaking. Carrot (*Daucus carota* cv. Yukon) was used as a host plant susceptible to *R. solani* (Anees et al. in press). Twenty seeds of carrot were sown in each square plastic pot (8 X 8 X 7 cm) containing 260 g of sand covered by 20 ml of calcined clay to hold the irrigation water. Two different tests were conducted. To characterize resistance induced in the plant host by *Trichoderma* isolates, 15 ml of the conidial suspensions of *Trichoderma* isolates were added directly to the sand without soil at the time of sowing. Twelve days after sowing, 50 ml of disinfested or non-disinfested soil was brought to the crown of carrot seedlings. Two days later, this soil was inoculated with millet seed inoculum of *R. solani* using 4 seeds per pot (one at each corner of the pot). To characterize direct interaction of *Trichoderma* isolates with *R. solani in vivo*, the carrot seeds were sown in the sand without *Trichoderma*. After 12 days, 15 ml of conidial suspensions were mixed with 50 ml of disinfested or non-disinfested soil and brought to the crown of the seedlings. Two days later, this soil was inoculated with millet seed inoculum of *R. solani* as above.

In all experiments, the temperature was maintained at 25°C day and 20°C night (16 h day length). Experiments were performed in triplicate such that 60 plants were used per treatment. Number of damped off seedlings were counted on alternate days for two weeks and the area under disease progress curve (AUDPC) measured as cumulative mortality rate was calculated.

The percentage of inhibition of the disease by the *Trichoderma* isolates was calculated by subtracting AUDPC values of the treatments from those of the respective controls, dividing that by AUDPC values of the controls and multiplying by 100. The results were analysed by ANOVA as above.

Results

Identification of *Trichoderma* isolates

The isolates were identified using both morphological and molecular characters. Eight out of the 18 isolates could be successfully identified using the morphological key. These isolates corresponded to one of the following species: *T. gamsii* (5 isolates), *T. harzianum* (1 isolate), *T. hamatum* (1 isolate) and *T. atroviride* (1 isolate) (Table 2). The key failed to identify T34, T36, T37, T41, T42, T44, T46 and T47. However, the characteristics of producing small ellipsoidal smooth conidia and the presence of flexuous or undulate conidiophores extensions made it possible to identify these eight isolates as *T. velutinum* (Bissett et al. 2003), a species not included in the key. Finally, the identification of T32 and T33 using the morphological key remained ambiguous. The presence of sinuous or spiral sterile hair, small subglobose to oblong conidia and very slow growth at 35°C on PDA and SNA after 72 hours in the dark were characteristics of the species *T. tomentosum*, whereas presence of long phialides and high growth at 25°C and 30°C on PDA were not.

The morphological identification was completed by a molecular identification based on ITS and *tef1* sequences. The identification of the strains T34, T36, T37, T41, T42, T44, T46 and T47 as *T. velutinum*, T45 as *T. harzianum*, T38 as *T. hamatum*, and P1 as *T. atroviride* was confirmed using *Trich*OKEY and was further confirmed by similarity search of *tef1* intron-4 by *tricho*BLAST. For both the strains T32 and T33 the best match using *tricho*BLAST was *T. tomentosum* with 99 % and 94 % of similarity for ITS and the largest 4th intron regions, respectively. We compared these percentages of similarity with the intraspecific variability found within the species *T. tomentosum*. Concerning the ITS region, 1 % of mismatches is commonly found among sequences of *T. tomentosum* (for example between accession numbers AY605737 and AF149869). Concerning *tef1* intron-4, 6 % of mismatches are commonly found between sequences of this species (for example between accession numbers AY605818 and AY605759). Thus, the variability observed between sequences of the isolates T32 and T33, and the sequences present in ISTH database may be considered as intra-specific variability. Therefore, T32 and T33 may be identified as

T. tomentosum. The strains T30, T31, T35, T40, and T43, which were identified as *T. gamsii* by morphological key, could not be identified by *Trich*OKEY or *Tricho*BLAST because the sequences for this species were not included in the ISTH database at the time of the investigation. The identification of these strains was confirmed as *T. gamsii* by downloading the sequences of the ITS regions of the known *T. gamsii* strains (DQ315459, DQ315448, DQ841730, DQ845432) from the Genbank and aligned them with the sequences of the isolates used in the present study that were identified as *T. gamsii* morphologically. The percentages of mismatches between the probable *T. gamsii* strains and the reference strains were 0 to 0.3 %. Similarly, alignment of the *tef1* intron-4 of the same strains (T30, T31, T35, T40 and T43) with the reference sequences (DQ790653, DQ790649, DQ790655, EF488117, EF488132) was performed as above. The intra-specific variability found in the *tef1* intron-4 was up to 2.8 % among the reference strains while it was 0.3 to 2.2 % between the isolates under query and the reference strains. All these findings, from both morphological and molecular identification confirmed that T30, T31, T35, T40 and T43 belong to *T. gamsii*.

Antagonistic activity measured *in vitro*

Trichoderma strains T30, T40, T43 and P1 showed the highest inhibition (>38 %) of *R. solani* growth in dual cultures (Table 3). On the other hand, T36, T37, T46 and T47 showed the lowest inhibition (<10 %). The remaining strains showed intermediate values of percentage of inhibition of *R. solani* growth: 10 to 20 % (4 strains), 20 to 30 % (3 strains) and 30 to 35 % (3 strains). Additionally, antagonism and hyphal interactions were observed with the naked eye and under the microscope in the dual culture experiments (Table 3, Fig. 1). The strains T30, T31, T40, T43, and P1 were able to inhibit *R. solani* before contact. These strains did not coil over *R. solani* hyphae. On the other hand, T32, T33, T34, T35, T38, T41, T42, T44 and T45 were able to inhibit *R. solani* after contact. Additionally, these strains were able to coil around *R. solani* hyphae, except T42 and T38, for which coiling was not evident. Finally, the strainsT36, T37, T46 and T47 did not inhibit *R. solani*, although they coiled around *R. solani* hyphae.

Table 2- Characterization and identification of the *Trichoderma* isolates using morphological characters. The measurements of cultures on potato dextrose agar (PDA) and synthetic nutrient agar (SNA) media represent the mean of three independent values measured after 72 h in the dark. All the isolates showed green pigmentation of spores, no growth at 40°C on both media, and pustules formation on PDA or SNA.

Strain	Conidia				Sterile hair	Phialides (µm)			Culture on PDA (mm)				Culture on SNA (mm)				Coconut odour	Species*	References
	Shape	Length (L, µm)	Width (W, µm)	L/W ratio		Length	Width at middle	Width at base	20°C	25°C	30°C	35°C	20°C	25°C	30°C	35°C			
T30	Ellipsoidal to oblong	4,2-4,8	2,7-3,2	1,5-1,8	Absent	8,5-10,2	2-4	<2	40,3	54	52	2,3	36	38,3	45	3	Present	*T. gamsii*	Jaklitsch et al. 2006
T31	Ellipsoidal to oblong	4,2-4,8	2,7-3,2	1,5-1,8	Absent	8,5-10,2	2,4-3,2	<2	47	57	54	6	23	40,3	43	7	Present	*T. gamsii*	Jaklitsch et al. 2006
T32	Subglobose to ovoid	3-4	2-2,5	1,2-1,5	Flexuous	14	2,4-3,2	2-2,6	41,3	51	51	2	39	47	46	0,8	Absent	NI	
T33	Subglobose to ovoid	2,8-3,4	2,2-2,7	1,2-1,5	Flexuous	15	2,4-3,2	2-2,6	42,7	51	51	1,3	37	47,7	47	1	Absent	NI	
T34	Ellipsoidal	3,5-4,1	2,2-2,7	1,2-1,5	Flexuous	4-7,5	>3,5	2-2,6	39,7	49	54	9,7	41	50	57	9	Absent	*T. velutinum*	Bissett et al. 2003
T35	Ellipsoidal to oblong	4,2-4,8	2,7-3,2	1,5-1,8	Absent	10	3	2,5	33,3	45	54	5	31	40,7	42	6,5	Present	*T. gamsii*	Jaklitsch et al. 2006
T36	Ellipsoidal	2,8-3,4	2,2-2,7	1,2-1,5	Flexuous	7-12	3	2-2,6	40,67	53	55	10	40	49,7	56	9,2	Absent	*T. velutinum*	Bissett et al. 2003
T37	Ellipsoidal	3-4	2,2-2,7	1,2-1,5	Flexuous	7-12	2,5-3	2,5	42	53	56	11	41	49	56	11	Absent	*T. velutinum*	Bissett et al. 2003
T40	Ellipsoidal	4.2-4.8	2,7-3,2	1.2-1.5	Absent	7-10	3-3,6	1,2-1,7	45,33	53	50	3,3	27	36,7	38	8,3	Present	*T. gamsii*	Jaklitsch et al. 2006
T41	Ellipsoidal	3,5-4,1	2,2-2,7	1,2-1,5	Flexuous	7-10	2,4-3,2	2,5	38,66	47	50	11	35	46,3	54	7,7	Absent	*T. velutinum*	Bissett et al. 2003
T42	Ellipsoidal	2,8-3,4	2,2-2,7	1,2-1,5	Flexuous	7-10	2,4-3,2	2-2,6	39,67	49	54	14	35	46,7	55	9,3	Absent	*T. velutinum*	Bissett et al. 2003
T43	Ellipsoidal	4.2-4.8	2,7-3,2	1,5-1,8	Absent	8,5-10,2	2,4-3,2	2-2,6	44,67	53	52	4	23	36	38	3,2	Present	*T. gamsii*	Jaklitsch et al. 2006
T44	Ellipsoidal	3,5-4,1	2,2-2,7	1,2-1,5	Flexuous	5-10	2.4-3.2	<2	40	48	54	15	41	44,3	59	11	Absent	*T. velutinum*	Bissett et al. 2003
T45	Globose to subglobose	<2.7	2,2-2,7	<1.2	Absent	8,5-10	3.2-3.5	2-2.6	44,67	59	68	40	30	45,3	51	27	Absent	*T. harzianum*	
T46	Ellipsoidal	2,8-3,4	2,2-2,7	1,2-1,5	Flexuous	7-10	2,4-3,2	2-2,6	36	46	49	15	36	47,7	53	11	Absent	*T. velutinum*	Bissett et al. 2003
T47	Ellipsoidal	2,8-3,4	2,7-3,2	1,2-1,5	Flexuous	7-14	1,8-2,4	1,8	37,67	47	52	7	31	42	51	5,3	Absent	*T. velutinum*	Bissett et al. 2003
T38	Ellipsoidal	3,5-4,1	2,7-3,2	1,5-1,8	Straight	ND	ND	ND	41,7	53	35	2,3	27	35,7	29	1,7	Absent	*T. hamatum*	
P1	Globose to subglobose	2,8-3,4	2,7-3,2	<1,2	Absent	8,5-10	2,4-3,0	<2	45	55	40	0,7	30	35,7	32	nil	Present	*T. atroviride*	

* All the species were identified using the morphological key (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm) with the exception of the species *T. velutinum* which was not included in the key; NI = Not identified.

The second part of the *in vitro* characterization of mechanisms of antagonism consisted of testing the strains for their ability to produce water-soluble metabolites that may inhibit the growth of *R. solani* (Table 3). T30, T40 and T43 inhibited *R. solani* growth by more than 50 %, with the highest inhibition produced by T43 (75.6 %). T35, T38, T41, and T45 showed a moderate inhibition (25 to 40 %) while all other strains exhibited less than 25 % inhibition. T34, T46 and P1 were the poorest producers of water-soluble inhibitors with an inhibition of *R. solani* growth of less than 10 %. The reduction of *R. solani* growth by volatile inhibitors was not very remarkable (Table 3). The highest inhibition was produced by T39 (32.1 %) followed by T43 (14.4 %) and T31 (10.3 %). All other strains exhibited poor production of volatile inhibitors with an inhibition of less than 10 %.

Antagonistic activity measured *in vivo*

The *in vivo* tests for characterization of mechanisms included assessment of the induced resistance in plant host by the selected *Trichoderma* isolates and direct interaction of the isolates with *R. solani* in both the non-disinfested and disinfested soil (Fig 2). Selection of strains for the *in vivo* tests was based on the *in vitro* results in dual cultures as explained above, such that T30, T40, T43 were the best inhibitors of *R. solani*; T36 and T47 showed the least inhibition while T45 was intermediate, along with the reference strain P1 which also performed well in dual culture.

Table 3- In vitro antagonistic activity of *Trichoderma* strains against *Rhizoctonia solani* AG 2-2 strain G6 measured as dual cultures (percentage of inhibition of G6 growth when grown together with *Trichoderma* strains); water-soluble metabolites (percentage of inhibition of G6 growth when grown on metabolites of *Trichoderma* strains); volatile inhibitors (percentage of inhibition of G6 growth when grown in the presence of separate cultures of *Trichoderma* strains); interactions between hyphae of the strains and G6 on agar media (interactions were recorded as inhibition zones before contact between colonies, or as inhibition of *R. solani* growth after contact with *Trichoderma*, or as visible inhibition after contact between colonies); microscopic interactions of colonies of the strains and G6. Values in the same column followed by different letters are significantly different (Fisher's LSD, $p<0.05$).

Strain	Percentage of inhibition of *Rhizoctonia solani* growth			Visual interaction visible with naked eye	Microscopic interaction
	Dual culture test	Soluble inhibitors	Volatile inhibitors		
T30	$38,17^{abc}$	$60,69^{ab}$	$8,84^{bcd}$	Inhibition before contact	No coiling
T31	$33,93^{bc}$	$20,23^{de}$	$10,32^{bc}$	Inhibition before contact	No coiling
T32	$22,92^{def}$	$15,61^{de}$	$-5,92^{de}$	Inhibition after contact	Coiling
T33	16^{fghi}	$16,38^{de}$	$-10,35^{e}$	Inhibition after contact	Coiling
T34	$11,17^{ghij}$	$4,05^{e}$	$1,83^{bcde}$	Inhibition after contact	Coiling
T35	$19,29^{efgh}$	$27,36^{de}$	$-2,23^{cde}$	Inhibition after contact	Coiling
T36	$2,35^{j}$	$22,25^{de}$	$2,57^{bcde}$	No inhibition	Coiling
T37	$7,3^{ij}$	$21,00^{de}$	$2,94^{bcde}$	No inhibition	Coiling
T40	$46,31^{a}$	$58,12^{abc}$	$6,24^{bcd}$	Inhibition before contact	No coiling
T41	$35,16^{bc}$	$30,62^{cde}$	$-3,13^{cde}$	Inhibition after contact	Coiling
T42	$27,74^{cde}$	$18,74^{de}$	$-3,13^{cde}$	Inhibition after contact	No coiling
T43	$38,28^{abc}$	$75,62^{a}$	$14,37^{b}$	Inhibition before contact	No coiling
T44	$30,99^{bcd}$	$20,31^{de}$	$7,49^{bcd}$	Inhibition after contact	Coiling
T45	$15,93^{fghi}$	$39,37^{bcd}$	$-4,38^{cde}$	Inhibition after contact	Coiling
T46	$8,95^{hij}$	$6,24^{e}$	$-0,01^{bcde}$	No inhibition	Coiling
T47	$6,7^{ij}$	$11,87^{de}$	$-9,38^{e}$	No inhibition	Coiling
T38	$21,56^{defg}$	$29,87^{cde}$	$10,69^{bc}$	Inhibition after contact	No coiling
P1	$40,32^{ab}$	$9,25^{de}$	$32,09^{a}$	Inhibition before contact	No coiling

In bioassays characterizing direct interaction of *Trichoderma* isolates with *R. solani* in non-disinfested soil, highest antagonistic activities were observed for T30 (67.9 %) and T47 (75.2 %) (Fig 2). The strains T43 and P1 were moderately antagonistic with disease inhibition of 24 % and 14.5 % respectively. T36, T40 and T45 were not antagonistic in this case. The disease intensity was even increased to 19.7 % when T40 was present together with the pathogen in the non-disinfested soil. On the other hand, in the bioassays to assess the direct interaction in disinfested soil, T30, T45, and T47 proved to be highly antagonistic towards disease produced by *R. solani* (63.7 %, 92.2 % and 94.6 % of disease inhibition, respectively), while P1 was moderately aggressive with inhibition of 26.9 % (Table 3). However, T36, T40 and T43 were not antagonistic under these conditions.

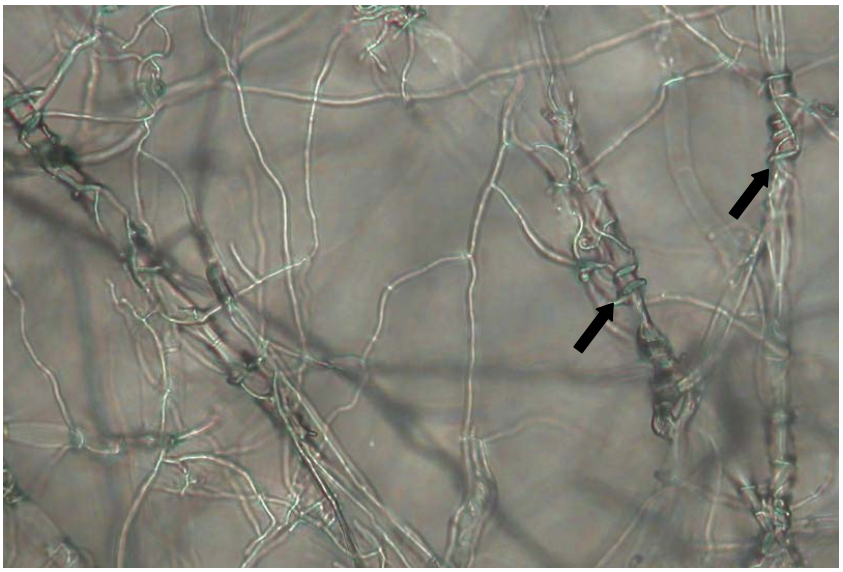

Fig 1- Coiling of hyphae of *Rhizoctonia solani* by *Trichoderma* spp. The arrows indicate the hyphae of *T. tomentosum* strain T33 coiling around the hyphae of *R. solani* AG 2-2 strain G6.

In bioassays aimed at characterization of induced resistance in the plant host in non-disinfested soil, T30, T40 and T43 performed better in terms of percentage of inhibition of the disease caused by *R. solani* in the controlled environmental conditions (Fig 2).

T36 produced 20.2 % of inhibition while all the other strains exhibited inhibition of less than 10 %. In the bioassays for characterizing induced resistance in the plant host in disinfested soil, trends were similar to those noticed in non-disinfested soil for the different strains, except for T45 (Fig 2). T45 reduced the disease by 24.2 % in disinfested soil but only by 5.5 % in non-disinfested soil. T30, T40 and T43 were able to reduce the disease by more than 30 %.

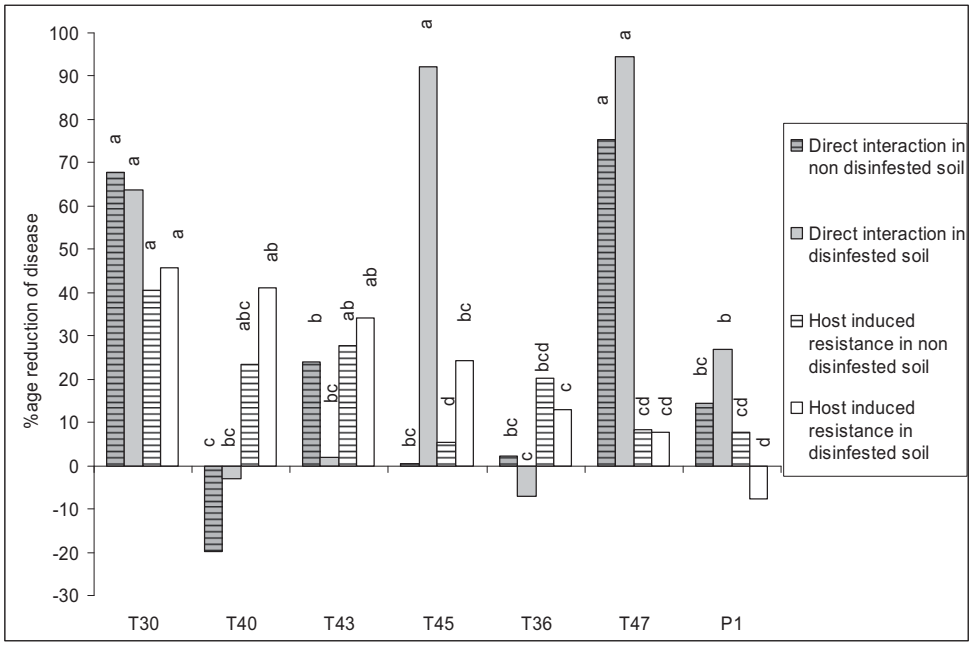

Fig 2- *In vivo* antagonistic tests of *Trichoderma* strains against infection of carrot seedlings by *Rhizoctonia solani* AG 2-2 strain G6. Direct interactions were measured as disease reduction when the strains were present in the crown and root zone at the time of pathogen introduction. Induced resistance was measured as disease reduction when root zones were inoculated with *Trichoderma* strains two weeks before the pathogen was introduced in the stem zone soil. The data are presented as percentage of reduction of disease as compared to controls without *Trichoderma*. ANOVA and LSD tests were performed separately for each of the four series. Bars designated by different small letters are significantly different (Fisher's LSD, $p<0.05$).

Discussion

Based on the limited number of isolates taken from a sugar beet field in which infectious patches occurred, we found an unexpected diversity by using three different approaches. Of course, these *Trichoderma* isolates needed to be characterized and identified. The classical way to identify *Trichoderma* isolates relies on morphological and molecular approaches. Adding the functional approach, including various mechanisms of antagonistic activity towards *R. solani*, the plant pathogenic fungus responsible for the disease observed in the sugar beet field, reveals the intra-specific diversity among the antagonists and brings some new information about the interactions as well as the biocontrol potential that is harboured by the *Trichoderma* group. Thus the functional approach is inevitable for isolating better antagonistic strains, in contrast to mere identification which renders information about inter-specific diversity, as recently done by Migheli et al. (2009).

Morphological identification is one of the key techniques used for identification. However, the few morphological characters with limited variation may lead to an overlap and misidentification of the strains (Kullnig et al. 2001). This shows the necessity of DNA based characters to complete identification, which is evident from the present study. Moreover, the morphological key used in this study is an online interactive key and represents a way to make the morphological identification more convenient. However, it could be even more efficient if the newly identified species were regularly integrated in the key. For instance, in our case, the key could not identify *T. velutinum* because it was not yet included. Concerning molecular techniques, generally, the Genbank database represents the largest reservoir of the sequences; however, it may not be safely used for identification as it contains many erroneous entries that have been submitted with a false identity (Druzhinina & Kubicek 2005). This fact emphasizes the need of a specific database for *Trichoderma* containing only vouchered sequences, such as the ISTH database used in this study. This database has recently been used successfully for identification *Trichoderma* strains (Migheli et al. 2009; Zhang et al. 2005). However, we were not able to identify the strains belonging to *T. gamsii* because it was not present in this database at the time of the investigation.

Similarly, use of two unlinked loci (ITS and *tef1*) further helped in molecular identification in some cases where it was difficult to conclude with the ITS region alone. It can be concluded that both morphological and molecular techniques are complementary for identification of *Trichoderma* strains and should be used in parallel for correct identification. This is consistent with previous experiences (Samuels 2006a). Moreover, the interactive morphological key and a specific molecular database coupled with tools for identification of *Trichoderma* strains has really simplified identification. Although these tools represent an ideal way to identify the *Trichoderma* spp. they need regular updates to include the rapidly increasing number of species of this genus.

The best strains in this study regarding antagonism against *R. solani* AG 2-2 belonged to *T. gamsii*. *T. gamsii* is a species that was recently identified and is a close relative morphologically and phylogenetically of *T. viride* to which many antagonistic strains belong (Brown et al. 1999; Jaklitsch et al. 2006). However, to our knowledge, this is the first report of an efficient antagonistic strain of *T. gamsii* towards *R. solani* AG 2-2. The reason may be because before the reassessment of *T. viride* and definition of *T. gamsii* (Jaklitsch et al. 2006), this species might have been misidentified as *T. viride* or some other closely related species. This is probably why we don't find any report of any strain of this species as a good antagonist in the literature. In the present study, the most efficient strain overall was *T. gamsii* T30, which was able to reduce the growth of the pathogen and inhibit the disease both *in vitro* and *in vivo*. The results indicate that this strain is a good producer of water-soluble metabolites that can inhibit the growth of *R. solani* strain G6 in Petri dishes at a distance before contact between the colonies; however, T30 could not coil around the hyphae of the strain G6. Hence, the principal mechanism of antagonism for this strain may be antibiosis. That may be why the strain was able to reduce the disease in the bioassays conducted to assess the direct interactions of the isolate with the strain G6 in the disinfested as well as non-disinfested soils. Additionally, the *Trichoderma* strain appeared to be able to induce resistance in the host against the infectious activity of by G6, as application of the antagonist to the root zone resulted in reduced disease following subsequent exposure of the plant crown and roots to the pathogen. Of course, the present set of bioassays allowed us to assess the disease resistance induced in the plant host by *Trichoderma* spp. by separating the site as well as time of application of antagonist and the pathogen to avoid direct interaction. Induced resistance is a phenomenon often suggested to be related to

biocontrol by *Trichoderma* spp. and it has been hypothesized that the mechanism may be production of molecules such as enzymes or antibiotics that are sensed by the plant which leads to ISR (Harman et al. 2004; Woo et al. 2006). In our case, the other two strains of *T. gamsii*, T40 and T43 also inhibited the growth of the strain G6 by producing water-soluble inhibitors *in vitro* and inhibited the disease *in vivo* principally through induced resistance, as less disease expression resulted from application of the antagonist to soil around stem. Hence, strains that were able to produce water-soluble metabolites were also able to induce the disease resistance in the plant. Strains T36 (*T. velutinum*) and T45 (*T. harzianum*), which produced from moderate to low extent of water soluble inhibitors *in vitro*, also produced moderate to low disease suppression through induced resistance. Antibiosis *in vitro* was not related to the disease reduction *in vivo* by direct interactions. For instance, T40 and T43 did not consistently reduce the disease in the direct interaction bioassays. *T. harzianum* T45 was unable to reduce disease in non-disinfested soils. The same strain could coil around the pathogen hyphae *in vitro* and reduced the disease *in vivo* significantly by direct interaction, but only in disinfested soil. These results support the hypothesis that T45 is a poor competitor under natural conditions.

The interaction between different isolates and the strain G6 of *R. solani* was observed under the microscope and coiling was noticed. Two important conclusions may be drawn from the present results. Firstly, not all the strains of *Trichoderma* coiled around the strain G6, even of a given species of *Trichoderma*. For instance, the only strain of *T. gamsii* that could coil was T35. Similarly, coiling was evident for all strains of *T. velutinum* except T42. Secondly, coiling action of hyphae of *Trichoderma* was not related to their higher antagonistic abilities. T37 and T46 (*T. velutinum*) could not inhibit the growth of the strain G6 *in vitro*; nevertheless these strains were able to coil around the hyphae of the pathogen. Similarly, the strain T36 of *T. velutinum* was able to coil around *R. solani* but did not reduce disease through direct interaction. However, strain T47 of *T. velutinum,* which could coil around the hyphae of the strain G6 but was considered to be a poor antagonist in dual culture, inhibited the disease by direct interaction with the strain G6 *in vivo* both in disinfested or non-disinfested soils. These results are in accordance with previous reports where no correlation between coiling frequencies and cell wall degrading enzymes were found (Almeida et al. 2007).

T. atroviride strain P1 is a known mycoparasite of different fungal phytopathogens (Hjeljord & Tronsmo 1998). It has been reported that this strain produces diffusible hydrolytic enzymes and inhibits *R. solani* when the fungi are grown together (Kullnig et al. 2000). In the present study, P1 showed pre-contact inhibition of the strain G6 when the fungi were grown together in the confrontation assays and produced volatile inhibitors against the strain G6. In the absence of the pathogen, P1 did not produce strongly inhibitory water-soluble metabolites. Coiling of hyphae around strain G6 by P1 was not evident. In the bioassays, P1 did not strongly reduce disease by direct antagonism in disinfested or non-disinfested soils. These results indicate that *T. atroviride* P1 is a poor mycoparasite of the strain G6.

From the present results, it is clear that antagonism is not a property of a species, as different strains of the same species can exhibit varying potentials of biocontrol. The strains which can express rapidly and efficiently their genes involved in antagonistic activities in the presence of host are in fact better antagonists (Scherm et al. 2009). For instance, although the most efficient strain in our case belonged to *T. gamsii* (T30), we also isolated a strain of *T. gamsii* (T35) that was a poor antagonist of the pathogen used in this study. This fact is also evident from the literature where strains of different species have been used for biocontrol with varying mechanisms (Benítez et al. 2004) which ultimately necessitates the use of a functional approach to characterize the mechanisms and to identify the good biocontrol strains from the field. In general, even a single strain of *Trichoderma* spp. may employ multiple mechanisms simultaneously to antagonise *R. solani* in soil. Therefore, recently Scherm et al. (2009) characterized a few strains based on subtraction hybridization approach and identified the genes which could be used as markers for rapid screening and pre-identification of *T. harzianum* strains for their biocontrol potential.

The main objective of the present study was to characterize the strains of *Trichoderma* originating from a field and the isolation was performed randomly. Among the 16 strains isolated from the same field, the most abundant species were *T. velutinum* and *T. gamsii*, represented by 50 % and 31 % of the strains, respectively. Until now, few data are available concerning the biodiversity of *Trichoderma* in soil. Nevertheless, previous studies have rather reported *T. harzianum* as the dominant species among soil isolates (Migheli et al. 2009; Zhang et al. 2005). In our study, the number of isolates is too small to draw a definite conclusion in terms of diversity.

However, only one out of the 16 isolates belongs to the species *T. harzianum.* Migheli et al. (2009) found very little correlation between species distribution and soil abiotic parameters, with the exception of *T. velutinum* and soil pH. Indeed, they only found *T. velutinum* in soils with pH 5 or less. But this explanation does not fit with our results since the soil pH was 7.1 (Anees et al. in press). Another factor that could affect the distribution of fungal species in soil is the cultivated plant. But until now, no data are available to conclude whether the crop and especially sugar beat influences the diversity of *Trichoderma* species in soil.

Our results indicate that antagonistic *Trichoderma* strains are stimulated by the presence of *R. solani* and may increase in numbers during disease outbreaks in the field. In general the isolates picked from the patches of disease (T30, T40 and T43) induced host resistance more than isolates from healthy areas (T36, T45 and T47), which supports the hypothesis that *Trichoderma* is partly responsible for the increased disease suppression inside patches (Anees et al. in press). Additionally, the strains of *T. gamsii* found in the diseased areas were better antagonists than the strain of the same species found in the healthy area. Hence the increased suppressiveness may not be related to a particular species, i.e., the distribution of different species in the field seems to be random regardless the disease condition. Our results therefore support the theory that soils from diseased sugar beet areas are potential sources of antagonists that may be further exploited in the future.

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CHAPTER *5:* Population dynamics of *Rhizoctonia solani* AG 2-2 strain G6 in relation to the antagonistic fungus *Trichoderma gamsii* strain T30

Abstract

The aim of the present study was to investigate the population dynamics of *Rhizoctonia solani* AG 2-2 strain G6 interacting with *Trichoderma gamsii* strain T30 in controlled environmental conditions in microcosms. The experiments were designed to follow the dynamics in disinfected and non-disinfected natural soils up to 25 days after the inoculation of *R. solani* and *T. gamsii* in three different ratios (1 : 1; 1 : 0.1; 1 : 0.01 of *R. solani* : *T. gamsii*) along with their respective controls. Initially the samples were analysed only from 2nd, 11th and 25th day after inoculation. The strain T30 clearly inhibited the growth of *R. solani* and this inhibition was related positively to the density or dose of the strain T30. Similarly, these experiments also depicted the general suppressive effect of the microflora including the indigenous *Trichoderma* spp. present in the natural soil. Unfortunately, the dynamics of the strain T30 could not be followed due to an unknown problem specific to the primers used to measure the density of *Trichoderma* spp. in these assays. However, the presence of the viable strain T30 was confirmed by conducting some complementary tests. The potential perspectives emerging from the present study have been discussed.

Introduction

Evaluating the mechanisms involved in the spread of disease patches incited by *Rhizoctonia solani* AG 2-2 in sugar beet field; the literature indicates a number of factors ranging from abiotic to biotic in nature. Investigating the biotic factors in two different fields of sugar beet infested with *R. solani* in 2006 and 2007 revealed the acquisition of soil suppressiveness within patches (Anees et al., 2009, chapter 3). Two complementary approaches were used to search for the putative antagonists: i) a systemic approach by analysing bacterial and fungal densities and the community structures, and ii) a specific approach by isolating *Trichoderma* spp. from patches of diseased and healthy areas and testing them for their antagonistic potential. Both approaches suggested the role of *Trichoderma* spp. in the dynamics of disease observed in the field. Changes in community structures of *Trichoderma* spp. were also evident due to infectious activity of *R. solani* AG 2-2. It is hypothesized that increased activity of *R. solani* is followed by the specific development of some populations of *Trichoderma*. However, the mechanisms which are determining the so called specific interactions are not clear. A predator- (or parasite-) prey relationship has been frequently mentioned to describe the way some *Trichoderma* could control plant pathogenic fungi including *R. solani* (Carroll, 1979; Harman et al., 2004). In that case, the incitement of mycoparasitic populations of *Trichoderma* as well as their development can be explained through trophic relationships, although the specificity of the *Trichoderma-Rhizoctonia* populations still needs to be elucidated. The situation is even trickier when considering the development of some dormant populations of *Trichoderma* within a given species of this fungus, at the expenses of *R. solani*, through water-soluble metabolites (Anees et al., 2009, chapter 4).

Considering the processes observed during the built up of patches as described in the previous chapters, two types of nonexclusive hypotheses could be proposed to explain the development of some *Trichoderma* populations at the expense of *R. solani* apart from the mycoparasitism. The first one is based on an indirect interaction mediated by the accumulation of nutrients in the rhizosphere as a consequence of root rotting caused by the infectious activity of *R. solani* as what happens in the interaction of *Gaeumannomyces graminis* var. *tritici* (Ggt), *Pseudomonas* spp. and the wheat host

plants. In that case, it is hypothesized that *Pseudomonas* spp. may be activated by root exudates to produce antibiotics and the root exudates may be influenced by the pathogenic activities (Park et al., 1988; Cook, 2003; Walker et al., 2003). The interaction of a *Trichoderma* strain with a strain of *R. solani* in presence of radish host plants in microcosms in sand has already been modelled (Bailey and Gilligan, 1997; Bailey et al., 2004). It was concluded that the antagonist decreases the pathozone i.e. a theoretical area around the root where the pathogen must be present to cause the disease (Gilligan and Bailey, 1997). Hence the pathogen should be closer to the susceptible roots to incite disease in the presence of *Trichoderma* antagonist than the pathogen should be in its absence. All these studies were, however, performed in controlled environments in sand so that the effect of the microflora on the interaction was not considered.

The second hypothesis is based on secondary metabolites (to be considered as signal molecules) produced by *R. solani* that could directly stimulate susceptible populations of *Trichoderma* which in turn would produced these water-soluble metabolites to destroy *R. solani.* The purpose of such production could be out competition or more likely, the use of the so produced *R. solani* necromass for trophic development, although this has not been shown so far. Once again, anyway, the specificity of the beneficial use of this trophic resource remains questionable.

The *Trichoderma-Rhizoctonia* relationship could be cancelled by violent external factors such as aerial epidemics (chapter 3) but also by external factors such as crop rotation (Vargas Gil et al., 2008) which are not considered as violent ones from an agronomist point of view. However, a self regulation of this interaction is also likely to occur, leading to alternate population densities of *Trichoderma* and *Rhizoctonia* populations as expected in a predator-prey relationship (Lotka, 1934) but in that case, it seems that the density threshold of *R. solani* required to stimulate the predatory activity of *Trichoderma* populations is reached during the secondary infection process as described roughly through the following algorithm;

- Step 1: Increase in the infectious activity of *R. solani*
- Step 2: Changes in structure with increase in the density of some *Trichoderma* spp. and acquisition of soil suppressiveness
- Step 3: Inhibition of disease and further activity of *R. solani* decreases in that area leading to patch mobility

- Step 4: *Trichoderma* decreases
- Step 5: Go to step 1

In our study, higher density of *Trichoderma* spp. inside the patches followed by isolation of *Trichoderma* from the field resulted in a small collection with an unexpected functional diversity (Chapter 4). The antagonistic potential was observed to be variable along with a variety of control mechanisms being active at the same time when confronted with *R. solani* including mycoparasitism, production of water soluble and volatile inhibitors, and induced systemic resistance in the plant host. The best antagonistic strain against *R. solani* AG 2-2 in these tests was *T. gamsii* strain T30. It was able to inhibit growth of the pathogen by producing water-soluble metabolites as well as by inducing systemic resistance (Chapter 4).

The objective of present study was to assess the population dynamics of *R. solani* strain G6 interacting directly with *T. gamsii* strain T30 in controlled conditions in microcosms in absence of the plant. The fungal population dynamics were performed in both disinfected and non-disinfected soil using a real time polymerase chain reaction (PCR) assay specific for each fungus.

Materials and methods

Preparation of fungal inocula

The fungal inocula for plant pathogenic strain G6 of *R. solani* AG 2-2 and *T. gamsii* strain T30 were prepared in sand as follows. Sand (0.5 to 1.2 mm) was autoclaved for 1 h on three consecutive days at 105°C and stored at room temperature for three days before inoculation. The sand (700 g) was mixed with 91 ml of sterilized malt broth (230 g liter $^{-1}$) and five plugs of 4-day old culture of G6 or T30 on malt extract agar (MEA) and incubated for 2 weeks at 25°C. The bottles containing the cultures were shaken every day on regular basis to avoid the formation of the pellets. After 14 days, each culture was sampled in three replicates to measure the density of culture by DNA extraction and real time PCR as described below, and the remaining cultures were preserved at 4 C°.

Soil and treatments

Soil sampling was done from the INRA experimental Unit of Epoisses, Côte d'Or, France, in February 2009 from a field adjacent to the previously used experimental plots (Anees et al., 2009) but this field has not been formerly inoculated with *R. solani* G6 so it has no recent historical infectious activity of the pathogenic population. The soil had a silt clayey texture (sand 6.1%, silt 57.7%, clay 36.2%). The soil was sieved at 4 mm and air dried for four days at 20°C and 354 soil microcosms were prepared in boxes of 100 ml (previously sterilized) containing 50 g of soil. In order to prepare microcosms of disinfected soil, one set of 177 boxes containing 50 g of soil were autoclaved three times on three consecutive days for 1 h at 105°C and stored at room temperature for one week. The second set of 177 boxes was remained non-disinfected. The moisture in the soil microcosms was adjusted to 19% (i.e. 80% of the water holding capacity of the soil on a dry weight basis) with sterile water 3 days before the inoculation.

The study was conducted with eight treatments corresponding to eight different ratios between the inoculum densities of *R. solani* G6 and *T. gamsii* T30 (Table 1). For each treatment, 30 independent microcosms for both sets of soil were prepared in order to evaluate the fungal densities at 10 different sampling times in triplicates. The 10 sampling times were 2, 4, 7, 9, 11, 15, 18, 21, 23 and 25 days after the inoculation of the microcosms. The exceptions were the treatments with the ratios R0/T0, R0/T0.01 and R0/T0.1 for which only 9 microcosms were prepared for both sets of microcosms in order to evaluate the fungal densities at 3 different sampling times in triplicates (2, 11 and 25 days after the inoculation of the microcosms). In this table ratios indicate the different concentrations where "1" means $1x10^5$ copies of target DNA per g soil evaluated by real time PCR.

Microcosms were inoculated with sand inoculum of *R. solani* AG 2-2 G6 and *T. gamsii* T30 by sprinkling both inoculums evenly onto the soil surface in different ratios (Table 1). All the microcosms were inoculated on the same day in the ratio described in the table for both sets of microcosms and then incubated at 20 C°.

From each microcosm, aliquots of soil were stored at -20°C for molecular analysis. Initially, three sampling times were selected to analyze the trend of population dynamics: the beginning (2nd day), the middle (11th day) and the end (25th day) of the incubation. For each of the three sampling times, three independent microcosms were

analyzed. The densities within each of the 2 sets of microcosms (disinfected soil and non disinfected soil) at each sampling time were then compared by analysis of variance (ANOVA) and Fisher LSD tests (p=0.05) using XLSTAT- Version 2007.5 (Addinsoft).

Table 1. Ratios of fungal densities analyzed in this study and number of sampling times and microcosm for each ratio. R1/T0 represents *Rhizoctonia solani* G6 / *Trichoderma gamsii* in the ratios 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1= 1/0.1 ratio, R1/T1= 1/1 ratio and R0/T0 =0/0 ratio; with 1 = 10^5 copies of target DNA per g of soil. For each ratio, 30 independent microcosms were prepared in order to consider 10 sampling with 3 replicates, with the exception of the 3 last ratios in the table: 9 independent microcosms were prepared in order to consider 3 sampling with 3 replicates.

Ratio of fungal densities *Rhizoctonia solani* G6/ *Trichoderma gamsii* T30	Number of sampling times	Number of replicates per sampling time
R1/ T0	10	3
R1/ T0.01	10	3
R1/ T0.1	10	3
R1/ T1	10	3
R0/ T1	10	3
R0/ T0.1	3	3
R0/ T0.01	3	3
R0/ T0	3	3

Quantification of fungi by real time PCR

DNA was extracted from samples of 1g of sand using the method described in chapter 2 for soil DNA extraction. The fungi were quantified by real time PCR using the procedures described in Chapter 4. The same procedures were used to quantify the target DNA copies of *R. solani* and *Trichoderma* in the inocula.

Complementary tests

Following the absence of detection of *Trichoderma* by real time PCR in some DNA samples, additional tests were performed to confirm the presence of *Trichoderma* in the soil microcosms.

Plating of soil: To check the presence of *T. gamsii* in the different microcosms inoculated with different doses of *T. gamsii*, soil aliquots were spread in Petri dishes containing potato dextrose agar (PDA, 39 g/l). Plates were incubated at 25 C° for one and two days and the identification of the *T. gamsii* or other *Trichoderma* spp was done using cultural and morphological characters.

Nested PCR: A nested PCR assay was used to check for the presence of ITS DNA of *Trichoderma* in different soil samples. For this, the soil DNA were used as a template in a first PCR round using universal fungal PCR primers targeting the ITS region, and then the PCR products were used as a template in a second PCR reaction using primers uTf and uTr, specific for *Trichoderma* .

The ITS region was amplified by PCR using the fungal primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) in a final volume of 50 µl by mixing 2 µl of DNA with 0.5 µM of each of the primers, 150 µM of dNTP, 6 U of Taq DNA polymerase (Q-Biogen, Evry, France) and PCR reaction buffer. Amplifications were conducted in a mastercycler (Eppendorf) with an initial denaturation of 3 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50°C, 1 min extension at 72°C and a final extension of 10 min at 72°C. Aliquots of 1 µl of PCR products were used as template in real time PCR with primers uTf and uTr as described above.

Results

Population dynamics of *Rhizoctonia solani*

The quantities of target DNA in the fungal inocula were $3.8 \times 10^7 \pm 2.2 \times 10^7$ copies/g and $2.8 \times 10^7 \pm 7.7 \times 10^6$ copies/g of sand for *R. solani* G6 and *T. gamsii* strain T30. The quantity of sand inoculum needed to inoculate 10^5 copies of target DNA per g of soil was calculated to be 130 mg and 180 mg per microcosm for *R. solani* G6 and *T. gamsii* strain T30, respectively

In the disinfected soil, the density of *R. solani* G6 introduced at 10^5 copies per g of soil without *T. gamsii* significantly increased with time (Figure 1). Twenty-five days

after the inoculation, the fungal density corresponded to a level of 6.5 x 10^6 target copies per g of soil and the slope of the curve indicated that the maximum density was not reached. The influence of different doses of *T. gamsii* on the population dynamics of *R. solani* was checked (Figure 1).

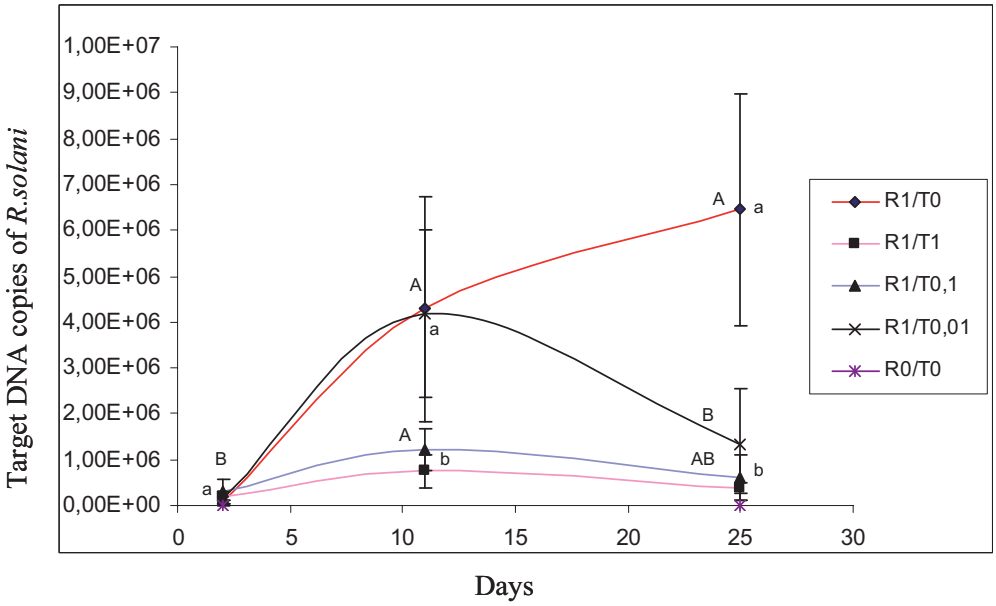


Figure 1. The influence of different doses of *Trichoderma gamsii* T30 on *Rhizoctonia solani* G6 growth in the disinfected soil (R1/T0 represents the *R. solani* G6 / *T. gamsii* T30 in the ratios 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1= 1/0.1 ratio, R1/T1= 1/1 ratio and R0/T0 =0/0 ratio; with 1 = 10^5 copies of target DNA per g of soil). Different small letters indicate significant differences in number of DNA copies at a given time. Different capital letters indicate significant differences in copy numbers among sampling time. For the sake of clarity, similar letters are only represented once at a given sampling date

When *T. gamsii* T30 was introduced in the microcosms at a dose corresponding to 100 times less target DNA copies than that of *R. solani* (R1/T0.01), *R. solani* G6 grew similarly than in the absence of *T. gamsii* until 11 days. But then its population density decreased significantly to reach a value 4.8 times lower than in the control without *T. gamsii* (R1/T0) after 25 days of incubation. The introduction of higher doses of *T. gamsii* T30 (T0.1 and T1) in the microcosms has considerably slow down the growth of *R. solani*, which reached a maximum value of only 2.1× 10^6 (for T0.1) and 7.55 × 10^5 of target DNA per g of soil (for T1) after 11 days of incubation. Although this growth was

significant, the density reached was significantly lower than in the microcosms T0 and T0.1. Finally after 25 days of incubation, whatever the dose of *T. gamsii* introduced in the microcosms, the population density of *R. solani* G6 decreased to a similar value that was significantly lower than in the absence of *T. gamsii.* When the two fungi were introduced at a similar dose in the microcosms (R1/T1), *R. solani* G6 was only able to reach a density 17.2 times lower than in the absence of *T. gamsii.*

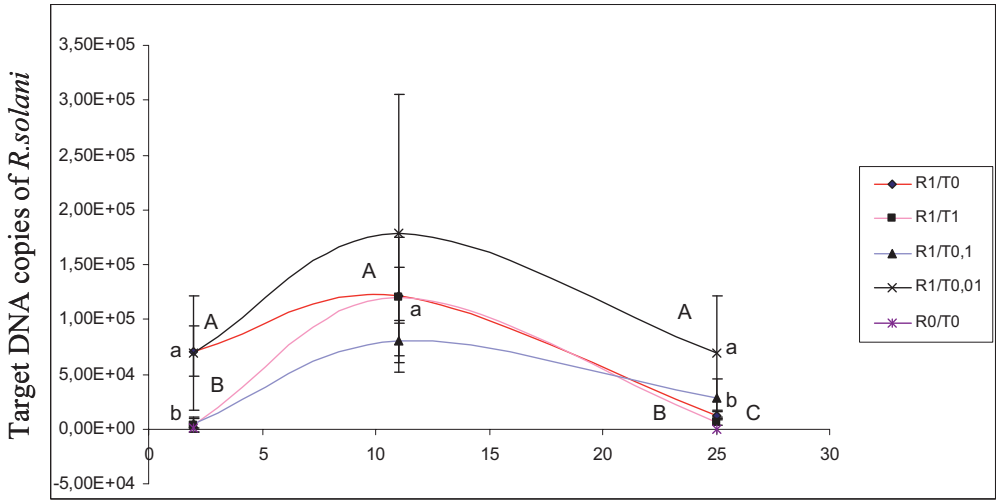
The population dynamics of *R. solani* and the influence of different doses of *T. gamsii* were also assessed in the non-disinfected soil (Figure 2). When *R. solani* was introduced alone in the soil microcosms, its population density increased to reach a maximum level of 1.2×10^5 DNA copies per g of soil after 11 days but this density was not significantly different from the initial density. Then it decreased significantly contrary to what was observed in the disinfected soil. A similar curve was obtained for the population dynamics of *R. solani* G6 when the lowest dose of *T. gamsii* (T0.01) was introduced in the soil microcosms. On the opposite, for the two highest doses of *T. gamsii*, T0.1 (10 times less than *R. solani*) and T1 (similar density than for *R. solani*), the population density of *R. solani* was significantly lower than in the control (R1/T0) as early as 2 days after the inoculation. Then *R. solani* was also able to grow until 11 days of incubation, but significantly decreased after. In all the cases, the densities of copies of target DNA were lower than those observed in disinfected soil.

Quantification of *Trichoderma gamsii*

To quantify *T. gamsii* in the same microcosms of disinfected and non-disinfected soils inoculated with both fungi, the real time PCR assay specific for *Trichoderma* spp (i) showed a linear standard curve, (ii) allowed the quantification of the inoculum of *T. gamsii* T30 and (iii) successfully detected the genomic DNA of the strain T30 used as a positive control, with a Ct value (threshold cycle) of 24.15 in mean. But unfortunately no PCR product was obtained for the soil DNA samples, so that we did not succeed to quantify *T. gamsii* in the soil microcosms. Different complementary tests were performed to find an explanation and resolve the problem:

(1) Plating of different soil samples on PDA medium confirmed the presence of *Trichoderma* both in non-disinfected and disinfected soil samples. The morphology and white color of the colonies obtained together with the spores present were in agreement with the typical cultural characters of the species *T. gamsii*. On the opposite, plating of non-inoculated disinfected soils resulted in no microbial growth, which confirmed the absence of any contamination of the microcosms.

Figure 2. The influence of different doses of *Trichoderma gamsii* T30 on *Rhizoctonia solani* G6 growth in the non-disinfected soil (R1/T0 represents *R. solani* G6 / *T. gamsii*



T30 in the ratio 1/0 and similarly R1/T0.01 = 1/0.01 ratio, R1/T0.1= 1/0.1 ratio, R1/T1= 1/1 ratio and R0/T0 =0/0 ratio; with 1 = 10^5 copies of target DNA per g of soil). Different small letters indicate significant differences in number of DNA copies at a given time. Different capital letters indicate significant differences in copy numbers among sampling time. For the sake of clarity, similar letters are only represented once at a given sampling date

(2) The measurement of the optical density at 260 nm using a Biophotometer (Eppendorf, Hamburg, Germany) confirmed the presence of DNA in all samples (22ng/µl in mean) originating from the different microcosms including the disinfected soils inoculated with *T. gamsii* alone.

(3) The quantification of *T. gamsii* was previously optimized using the absolute Q-PCR SybrGreen Rox Mix from ABgene according to Hagn *et al*. (2007). Following our

negative results with soil samples, we have tried to modify the real time PCR assay using the qPCR Rox- and Go Green Master mix (Q- Biogene) (the same as in the *R. solani* real time PCR assay); but we still did not detect *T. gamsii* in soil samples. In these assays the Ct values obtained for the controls were even higher, showing a lower detection.

(4) Results of nested PCR (ITS1F/ITS4 followed by uTf/uTr) showed an amplification product corresponding to the targeted ITS region of *Trichoderma* for all DNA samples tested originating from the following microcosms: disinfected soil or non-disinfected soil inoculated with *T. gamsii* T30, and non-disinfected soil non-inoculated with *T. gamsii* T30.

(5) The same procedure of DNA extraction and quantification of *Trichoderma* spp by real time PCR was applied to fresh soil of Epoisses that was not stored in boxes like the one originating from microcosms at different incubation times. In that case, the same results were obtained as with the soil DNA extracted from microcosms, which is no PCR amplification using direct real time PCR with primers uTf and uTr.

Discussion

As a preliminary test, only three sampling points were investigated for the densities of *R. solani* and *T. gamsii*. Although it is not possible to know the type of curve that could be fitted or the phases of growth of fungi at different sampling points because of small number of sampling points analyzed so far, this study successfully revealed the perspectives and the potential for future studies. Despite it was not possible to monitor the population of *T. gamsii* T30 for technical reasons, the various complementary tests revealed that the inoculated fungus survived both in disinfected and non-disinfected soil and was viable. Therefore, the population dynamics of the strain G6 of *R. solani* could be discussed in relation to the presence and putative activity of the strain T30 of *T. gamsii*.

The saprophytic ability of the *R. solani* strain when it is alone in the disinfected soil seems to be quite important as the number of copies of the target DNA still increased at 25th day of incubation as compared to 11th day. This is consistent with the already known fact of higher saprophytic ability of the fungus (Ogoshi, 1996). However, the

growth of the strain G6 of *R. solani* was significantly altered when this fungus faced the whole indigenous microflora, added or not with the strain T30. The highest density measured in non-disinfected soil is almost 100 times less than the highest density measured after 25 days in disinfected soil, given that this density could be an underestimation of the carrying capacity of the soil for this strain. This clearly indicates that in natural soil, the development of the primary inoculum is limited despite the saprophytic ability of *R. solani* observed in disinfected soil. *R. solani* survives in soil as sclerotia whom density is generally less than one sclerotium /10 g soil (Neate and Schneider, 1996) what is low compared to the one of spore forming fungi like *Fusarium* spp whom density is from 100 to 1000 propagules / g of soil (Rodríguez-Molina et al., 2000). It is likely that the sclerotia of the primary inoculum might germinate but that their development is reduced or stopped by the activity of the whole microbiota, including or not some antagonistic *Trichoderma* populations and given the abiotic conditions. This may explain why the disease first occurs as sparse spots of sugar beets presenting necroses in the field in early spring (end of May) in France. This mechanism refers to the general microbial suppression (Weller et al., 2002). This general microbial suppression may be through competition for nutrients (Steinberg et al 2007). That is why a robust increase in the disease was observed by addition of buckwheat meal to the natural soil to measure the soil inoculum potential (Anees et al., 2009). This also suggests that the stage of finding and competing for a nutrient source is a critical point in the fungal life history and that may be why the efficiency of a *Trichoderma* strain to control *R. solani* in sand was principally related to inhibition of primary infection (Bailey et al., 2004). As the fungus is able to translocate the nutrients from the trophic basis to the hyphal tips, therefore, once *R. solani* colonized a host plant, it can take advantage of this trophic and spatial resource to develop and spread until the source is exhausted before finding another susceptible host (Jacobs et al., 2004). Therefore threshold number of susceptible host plants has been suggested to be important for the fungus to invade a field (Gubbins et al., 2000). In the soil this might explain the development of the secondary infection leading to patches caused by this secondary inoculum and also leading to the late development of antagonistic population of *Trichoderma*. The process might last as long as the host plants are susceptible or/and no specific antagonistic microorganisms can so far stop the development of the pathogenic population. This phenomenon may explain the abrupt edging of the patches in the fields.

With the development of disease in the patches, the soil becomes less conducive towards the disease with a probable role of *Trichoderma* species inside the patches as explained earlier. Changes in community structures were evident inside the patches and at the end of season higher density of *Trichoderma* spp. was also evident (Anees et al., 2009). The *Trichoderma* spp. inside the patches showed higher antagonistic abilities with the help of a variety of antagonistic mechanisms (Chapter 4). For instance, in case of the strain T30, although no coiling was observed when the two strains were paired in *in vitro* conditions, the production by T30 of water soluble metabolites inhibitory towards the growth of G6 was revealed *in vitro*. As it is very difficult to strictly control the environmental or abiotic variables while we are in the field, therefore, to precisely study the growth dynamics of the two fungi, this microcosmic study was designed with both disinfected soil in the absence of microflora and with non-disinfected soil containing the whole microflora. In these preliminary tests, a direct interaction between T30 and G6 was revealed in the disinfected soil as the presence of the *T. gamsii* strain limited the growth of the *R. solani* strain. This may explain the inhibition of disease by *T. gamsii* T30 in the bioassays using carrots (chapter 2 and 4). Moreover, this limitation of the growth of the *R. solani* strain was positively related to the inoculum density of *T. gamsii* T30 in the microcosms. It is likely that the production of water-soluble inhibitory metabolites was involved in the limitation of G6 in the microcosm experiments, although the production of these metabolites in soil was not yet demonstrated. It can also be considered that the more the inoculum of T30, the more the metabolites are produced, hence the limitation of G6 which is positively related to the initial inoculum density. Moreover, a threshold might be required as the control of G6 by T30 in disinfected soil was efficient depending on the dose of T30 introduced. Although further work is needed to explain the dynamics of G6 and T30, the results from these preliminary experiments are quite encouraging. An initial increase in intensity of *R. solani* followed by a considerable decrease is clear from these results.

A combination of general and specific mechanisms seemed to control the development of the pathogenic population in non-disinfected soil. However, from the present set of experiments, it is difficult to discriminate between the relative contribution of the inoculated microorganisms and the indigenous microorganisms, including some indigenous *Trichoderma* spp. General microbial suppression seems to be quite important from these results as explained above. Although this study focused,

as a first step, on the behaviour of the two populations in soil without any plant residues, adding pieces of sugar beet root in the microcosms may help *R. solani* to break this general suppressive effect and hence help to assess the specific antagonism by the indigenous microflora and the inoculated *Trichoderma* spp. The addition of root residues to the microcosms will also help us to further understand the interaction of the two fungi in the soil and to know whether *Trichoderma* spp. grow at the expense of the mycelium of *R. solani* or the increase of their density is the secondary effect of sugar beet root infestation by the pathogen resulting in rotting and increased nutrient availability for *Trichoderma* spp to grow.

Concerning the monitoring of *T. gamsii* in the soil microcosms, the real time PCR assay we used has already been successfully employed to quantify *Trichoderma* spp in different soils (Hagn *et al*., 2007; chapter 3). But in our study, we could not detect *T. gamsii* T30 in the soil microcosms although (i) the different controls included in our PCR assay were positive and (ii) the presence of *Trichoderma* was confirmed by soil plating. The PCR primers used were designed to be specific for the whole genus *Trichoderma*, so that they should allow the detection of any *Trichoderma* species. Thus the absence of amplification was not specifically related to the strain T30 or to the species *T. gamsii*, since we similarly did not detect the indigenous communities of *Trichoderma* in the non-disinfected soil. On the opposite, *R. solani* could be successfully quantified in the same microcosms using another molecular tool. Thus it seems that the PCR assay targeting *Trichoderma* spp was specifically inhibited in our experiments. Changing the composition of the real time PCR mixture could not circumvent this inhibition. On the other hand, using a nested PCR approach including a first step of amplification with other fungal primers allowed both the detection of *T. gamsii* T30 inoculated in disinfected or non-disinfected soils and the detection of autochthonous *Trichoderma* communities in non-disinfected soil. These results indicate that the inhibition of the PCR was related to a specific non-functioning of the amplification reaction using primers uTf and uTr rather than to a general incapacity of detection of *Trichoderma.* Our results also confirm the presence of *Trichoderma* DNA in the different DNA samples originating from soil extraction, with the exception of the DNA originating from non-inoculated disinfected soils. In addition, the same absence of PCR signal was obtained with the soil of Epoisses sampled directly from the field than with the same soil incubated in aluminium boxes for several days. This result shows that

the absence of molecular detection was not due to the production of specific compounds in the soil incubated in microcosms that could inhibit the PCR reaction, but rather to the presence of such inhibitors directly in the field soil. Whether specific treatments have been applied to this field has been investigated but no explanation to this inhibition has been found until now. Since all the soil samples originating from the different microcosms at the different sampling times have been preserved in the freezer, it will still be possible to monitor *T. gamsii* T30 in the microcosms. For this, one possibility could be to design other PCR primers that target *Trichoderma* spp or *T. gamsii*, since the inhibition was only observed for one primers pair but not the two others used in our study.

The antagonistic efficiency of T30 has already been studied (chapter 4) and is supported by these preliminary experiments. We know that this strain originated from diseased area in the sugar beet field and the strains picked from inside the patches showed better antagonistic ability than the strains outside in the healthy areas (chapter 2). We also know that the different strains showed different antagonistic mechanisms against the strain *R. solani* G6. Therefore, it will be worth to perform similar kinds of experiments with the *Trichoderma* strains isolated from inside and outside the patches of the disease to model their growth dynamics in interaction with the dynamics of *R. solani* AG 2-2. Testing this set of 16 different strains of *Trichoderma* including different populations of the same species should also help to identify criteria for invasion and persistence of both the pathogenic and the antagonistic population. In other words, this should help to understand why the antagonistic population of *T. gamsii* does not maintain at a high density and activity level in healthy areas, preventing thus the development of the primary inoculum of *R. solani.* It should also provide data to identify the costs and benefits for the populations of *Trichoderma* able to control *R. solani* through water soluble metabolites, in relation to the intra specific diversity of *Trichoderma* species (mainly *T. gamsii*) through their antagonistic activity.

Additionally, the strain T30 presents a potential biocontrol of *R. solani* AG 2-2 as a long-term perspective.

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GENERAL DISCUSSION

Development of patches

Rhizoctonia solani is a soilborne phytopathogenic fungus able to cause disease all over the world in all kind of crops (Chapter 1). It is a facultative parasite having the ability to grow saprotrophically (Ogoshi, 1996). It is a complex species divided into fourteen anastomosis groups (AG) which are further subdivided based on complementary characteristics such as morphology, virulence, host range, nutritional requrirements, biochemical and molecular characteristics (Carling et al., 2002). Generally it has a wide host spectrum and is able to produce long surviving sclerotia (Sumner, 1996). Apart from all these characteristics, its inoculum densities are low and variable in the natural soil in the fields (Kinsbursky and Weinhold, 1988; Neate and Schneider, 1996). However, the inoculum densities in the soil are not correlated with the losses it produces; nevertheless they can be very important (Kinsbursky and Weinhold, 1988). Hence, further research is expected to explain the ecology of this complex phytopathogen and to device new innovative ways to control it.

The root rot diseases, and to a lesser extent the damping off caused by *R. solani* AG 2-2 in sugar beet field occur in the form of patches which are highly dynamic in time and space. This was reported from Japan where it was observed that the disease patches caused by *R. solani* AG 2-2 never occurred in the place where they were observed the previous year in sugar beet field which suggested the involvement of microflora in the high mobility of disease patches (Hyakumachi and Ui, 1982). Apart from this, a few studies from the literature has already suggested the role of abiotic factors such as soil moisture and temperature in the development of patches in wheat field by *R. solani* AG 8 (Gill *et al.*, 2001). Similarly, the effect of soil structure and cultivation on the temporal and spatial dynamics of the disease that has been suggested in cereal rot disease caused by *R. solani* AG 8 in Australia (MacNish, 1996). The high mobility of patches was also observed in bulbous crops in Netherlands and the probable reason suggested was temporal niche differentiation (Schneider *et al.*, 2001). The role of soil structure and texture has also been studied in controlled environmental conditions (Otten and Gilligan, 2006). Besides all these abiotic factors, *Rhizoctonia* disease decline

phenomenon has been well described similar to well established take-all decline caused by *Gaeumannomyces graminis* var. *tritici* (Ggt) in wheat (Lucas *et al.*, 1993; Wiseman *et al.*, 1996; Cook, 2003). However, the influence of infectious activity of *R. solani* on the whole microflora during the development of disease patches and the response of components of the soil biota have never been studied. The knowledge of mechanisms involved in development and dynamics of disease patches caused by this pathogen is vital to develop a control strategy adapted to the interest of farmers. A number of mechanisms have been reported previously and it seems that the different mechanisms may be in operation at the same time in the field which necessitates the use of integrative research approaches (Chapter 1). For instance, there is a need to combine the epidemic behaviour reflected by the plant health above ground with the epidemic processes carrying on under surface of the soil through primary and secondary infection.

The main aim of the present thesis project was to uncover the mechanisms involved in the development of the disease patches incited by *R. solani* AG 2-2 in a French sugar beet field mainly concentrating on the behaviour of microflora. The preliminary tests depicted the increased suppressiveness towards the further pathogenic activity of *R. solani* AG 2-2 in bioassays (Guillemaut, 2003). For this purpose soil was sampled from two different infested sugar beet fields once in 2006 and twice in 2007 to uncover the behaviour of microflora towards the infectious activity of *R. solani* AG 2-2. This experimental design allowed us monitoring the microfloral components in the field conditions in relation to the inoculum density and inoculum potential in the soil during the built up of a patch. The major target was to identify and isolate the putative antagonists that may be involved in the increased suppressiveness observed.

In both 2006 and 2007 based on sampling from two different infested sugar beet fields with *R. solani* AG 2-2 described in chapters 2 and 3 of this thesis, a general response of microflora towards the infectious activity of *R. solani* including primary and secondary infections of sugar beet plants during the epidemic was evident. In September (2006, chapter 2), sampling was based on transects across the patches to take a snapshot of the epidemic while in July (2007, chapter 3), we had an approach to assess the temporal influence of the disease caused by *R. solani* AG 2-2 on microflora. In both cases, the results were supporting each other. The pathogen, *R. solani* AG 2-2 incited root diseases in sugar beet that led to a set of changes in microfloral components (Chapters 2 and 3). The soil conduciveness decreased inside the disease patches towards

the future invasion of the same pathogen. This decreased conduciveness was related to the changes in community structures of bacteria and fungi (Chapters 2 and 3). Changes in the community structure of *Trichoderma* spp. early in the season (Chapter 3) and their densities at the end of season were higher inside the disease patches in a different field (Chapter 2). These results suggested the role of *Trichoderma* spp. in the reduced conduciveness along with other unidentified putative antagonists suggested by the community structures of bacteria and fungi (Chapters 2 and 3). This is in accordance with what we observed in our preliminary experiments in the microcosms to study the population dynamics of *R. solani* AG 2-2 strain G6 and *T. gamsii* T30 where we were unable to separate the general suppression in natural non-disinfected soils from the specific microbial suppression by the inoculated antagonist. This was either because of activity of other antagonists than the strain T30 or as a result of general microbial suppression due to an intense microbial activity that has already been shown to limit the development of pathogenic populations in various pathosystems such as wilts due to *Fusarium oxysporum* (Steinberg *et al.*, 2007) or damping off due to *Pythium aphanidermatum* (Grunwald et al., 2000) or *R. solani* (Diab *et al.*, 2003) while specific suppression of soilborne diseases is due to the activity of a particular microbial group such as 2,4-diacetylphloroglucinol producers towards *Gaeumannomyces graminis* var. *tritici*, responsible for take all decline of wheat (Raaijmakers and Weller, 2001). In our case we concentrated mainly on *Trichoderma* spp. because they are known for their antagonistic activities against a wide range of microbial pathogens (Hyakumachi, 1996; Hjeljord and Tronsmo, 1998). *Trichoderma* spp. were isolated from the experimental field from inside and outside the disease patches and tested for their antagonistic potential (Chapter 2). The results further supported the hypothesis because the isolates originating from the disease patches were better antagonists as compared to the isolates from outside the disease patches. The identification of the isolates based on morphology and DNA depicted that the most of the isolates were belonging to *T. gamsii* or *T. velutinum* (Chapter 4) which is contrasting to what has been observed in the diversity studies where generally *T. harzianum* is the most abundant species (Zhang *et al.*, 2005; Migheli *et al.*, 2009). *T. gamsii* is a relatively newly identified species and a close relative of *T. viride* for which the different antagonistic strains are known (Jaklitsch *et al.*, 2006). An important question can be how *Trichoderma* spp. could affect the growth of *R. solani* and its infectious activity in the soil. Therefore, the functional diversity of

different *Trichoderma* strains was checked which revealed a variety of mechanisms that may be involved in the control of disease caused by *R. solani* (Chapter 4). It was noteworthy that the functional diversity allowed to discriminate populations within species, despite a small number of strains was isolated and characterized. The most antagonistic strain was identified as *T. gamsii* that was isolated from the disease patches although some non antagonistic populations belonged to the same species. This clearly shows that the antagonism was not the property of a species and the strains that can rapidly and efficiently express their genes involved in the antagonistic activities in the presence of *R. solani* are better antagonists (Scherm *et al.*, 2009). Such result suggesting that the taxonomic position is not a selective criterion might be cautiously considered when screening for biocontrol agents. From a microbial ecologist point of view, it is therefore surprising that some populations were more or less dormant as long as the density or activity of *R. solani* was below a threshold which is still to be determined and that only these populations and neither all the species nor the communities of *Trichoderma* were stimulated by the pathogenic fungus. The specificity of this interaction needs to be further investigated to depict some putative signal molecules that can be produced by both microorganisms. Indeed, this knowledge may provide tools allowing promoting the *Trichoderma* populations of interest to set up an early control of the primary inoculum of *R. solani* or to lower the threshold required to stimulate the antagonistic populations.

Moreover, the ecological cost and benefits for the *Trichoderma* populations to produce water soluble metabolites that can inhibit the development of *R. solani* or even kill it need to be established to understand these specific interactions and thus to allow us to use it to better control the pathogen. Selected appropriate agriculture practices such as the use of intermediate crops may contribute to the promotion of these benefic populations. Moreover, the selection of an efficient antagonistic population of *Trichoderma gamsii* allows thinking about producing and inoculating a biocontrol agent to reinforce the natural process.

Involvement of *Trichoderma* spp. in the reduced conduciveness in turn may partially explain the high mobility of disease patches from one season to another. On the other way, the suppressiveness acquired during the crop and leading to the mobility of the patches appears to be unfortunately quite temporary. The equilibrium among the various components of the biota within the patch is fragile and can be broken by

external factors such as agricultural practices or aerial diseases. Indeed, the natural infestation by *Cercospora beticola* probably suppressed most of the expected influences of *R. solani* on the microfloral components leading the temporal dynamics to a static point with no differences between diseased areas by *R. solani* and healthy areas with no *R. solani* disease as was evident from the other samplings. *C. beticola* causes the most damaging aerial disease in sugar beet worldwide (Holtschulte, 2000). It is the first report of its probable influence on microflora to our knowledge. It also describes the importance of the external factor although in this case the seasonal effects could not be circumvented (Chapter 3).

Apart from the general response of microflora as explained above, we also had an exceptional case related to the samples from 2007 (chapter 3) where some early attacks had occurred looking like damping off of young plants (when the plants were having more than 11 leaves). In that case, we observed no sugar beet plants at the time of sampling and these characteristic sampling points were called as D1. Surprisingly, D1 partially resembled those of the healthy areas in terms of microbiological behaviours. We may imagine a very early attack by the indigenous pseudo-sclerotia of *R. solani* that germinated and infected the young plantlets but did not produce viable secondary inoculum because the trophic bases provided by the young arising tubers were too small to sustain the invasion through secondary infection. This poor infectious activity that resulted in the death of the plantlets was not strong enough to endure the attack and could stimulate neither the *Trichoderma* populations of interest nor any other component of the microflora. That may be the reason why these areas looked like healthy areas. This further suggests the hypothesis that a threshold development of *R. solani* is required to invade a field because in case of D1, *R. solani* might not have the development beyond a putative threshold that is probably required to incite a response of the other populations. Additionally, this early attack looked like damping off and it is known that AG 4 and AG 2-2 preferentially cause the damping off and root rot diseases respectively. Therefore, *R. solani* AG 4 might have caused this early attack in D1 leading to probable damping off disease and ultimately death of the young sugar beet plantlets. As we have shown that the *Trichoderma-Rhizoctonia* interactions were quite specific, it is possible that the activity of *R. solani* AG 4, if any, either was quite weak or was perceived neither by *Trichoderma* populations nor by the microbial communities. Unfortunately, the presence of AG 4 was not checked in our study.

Apart from the results discussed, some technical limitations and problems were faced ranging from the field to the level of microcosms. *C.beticola* in the experimental field in August 2007 completely infested the field (Chapter 3). Normally, we had to follow the temporal dynamics of the microfloral components further in relation to the infectious activity of *R. solani* AG 2-2 at least up to September but due to this external factor we had to stop. However, the results were helpful in understanding how an aerial disease may affect the microfloral situation in the field (Chapter 3).

Terminal restriction fragment length polymorphism (T-RFLP) was used to assess the community structures of bacteria and fungi (Edel-Hermann *et al.*, 2004). As such a technique includes the entire microflora and is culture independent; it is preferable over the culture dependent methods. However, it is a semi-quantitative method because the same terminal restriction fragments (TRF) may be produced by more than one species or genera and therefore a great care should be taken to exploit the results from such an analysis which is a limitation of this technique (Edel-Hermann *et al.*, 2004) . Although T-RFLP gives an idea of the importance of some specific TRF, it does not allow to isolate and to sequence the TRF of interest directly from the acrylamide gel used for a correct identification. Therefore, it is advised to run T-RFLP analysis of the isolates originating from the experimental area to better interpret the community structure data. Apart from all this, it gives a rapid picture of fingerprints of the communities of the target microorganisms and helps to understand a biological phenomenon due to which this technique was used in our experiments. However, TRFLP yields enormous data that should be carefully handled and manipulated to avoid any possibility of errors.

We also faced problems in quantification of *Trichoderma* spp. in soil. Although enormous energy was invested in setting up a technique to quantify *Trichoderma* spp. in the field soil, the assay installed did not produce the results with the same efficiency as previously reported; the efficiency was lesser by a factor of 100 (Hagn *et al.*, 2007). Worst was the case with quantification of *Trichoderma* spp. in experiments set to assess the population dynamics of *R. solani* and *T. gamsii* conducted in microcosms, where viable and alive *Trichoderma* spp. could not be detected by real time PCR and the different complementary tests revealed that the problem was specific to the PCR reaction involving the PCR primers uTf and uTr (Chapter 5). Therefore, new primers may be designed for the future studies to avoid a similar situation again.

We faced problems in identification of the isolates based on morphology and DNA based characters (Chapter 4). For molecular identification of the isolates, the direct sequencing of ITS region of isolates by using the ITS1F and ITS4 primers pair (Chapter 4) was tried which did not yield the complete ITS sequence and was insufficient for the identification by ISTH database (Druzhinina *et al.*, 2005). That was why we used the PCR cloning technique to get complete sequences of ITS regions of the *Trichoderma* strains. During identification of *Trichoderma* isolates by *Trich*OKey, *T. gamsii* was absent in the database at the time of investigation (Druzhinina *et al.*, 2005). In the morphological key, we faced a similar situation for *T. velutinum*, which was not yet integrated in the key (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm). However use of both tools together made it possible the correct identification of the isolates revealing their complementarities. Therefore, the two techniques should be used together for correct identification.

Potential for future studies

The present study marked the activity of *Trichoderma* spp. that may follow the infectious activity of *R. solani* envisaging the potential for future studies and perspectives. One of the main perspectives of the present study is to characterize the interactions between *R. solani* and the different populations of *Trichoderma* spp. isolated from healthy and diseased areas within sugar beet field while some of them belong to the same species (*T. gamsii*, *T. velutinum* for instance) and exhibited different antagonistic abilities. It will be interesting to know whether *Trichoderma* spp. grow at the expense of mycelium of *R. solani* or it is an indirect effect of root infestation of plant host by *R. solani* followed by accumulation of nutrients in the rhizosphere as what happens in the case of Ggt, *Pseudomonas* spp. and the wheat host plants (Cook, 2003). Additionally, it will also be worth to study the interactions at the metabolic level. This also implies to identify the genes involved in the process as done recently by Scherm et al., (2009). Indeed, these genes are likely to be activated during the interaction of a few strains of *T. harzianum* with a strain of *R. solani*.

From an ecological point of view, the knowledge of the mechanisms that determine and may regulate the interactions of *R. solani* with the different populations of *Trichoderma* spp. is really needed. Indeed, the specificity of these interactions is probably depending on recognition factors among these fungal populations that have not been investigated so far, at least among saprophytic fungi, while examples in literature can be found between fungi and their host plants for instance *R. solani* and rice (Paranidharan et al., 2005), *Alternaria solani* and its host plants (Langsdorf et al., 1990) and in case of mycorrhizal fungi and their associated host plants (Olroyd et al., 2005).

From a practical point of view, this knowledge will take part in the selection of a biocontrol agent to ensure it will fit to the soil environment into which it will be introduced if needed, but also to ensure it will recognize and control the targeted deleterious population, *R. solani* AG 2-2 in our case. Although, the development of the biological control of the pathogen can be a long term perspective, in the present study, we have been able to isolate a strain of T30 belonging to the species *T. gamsii* that has shown the best antagonistic potential in the different assays conducted so far. These two populations may constitute an excellent investigation model to identify the recognition mechanisms among the antagonistic and the deleterious fungi. It can also be used to determine the threshold level of invasion of *R. solani* AG 2-2 according to the biotic and abiotic conditions provided both by the soil environment and the agricultural practices.

At least, from a pathologist and /or an agronomist point of view, the issue of the interactions between G6 and T30 can be observed through the occurrence of symptoms on the sugar beet cropped in that field.

Many scenarii can be considered while many factors (including external factors such as those we faced during our PhD work, for instance) could remain uncontrolled. This is why, it seems necessary to use a mathematical approach that could, thanks to the use of data acquired from field and microcosms experiments, i) determine the parameters of the most important factors, if not all of them, ii) produce simulations and iii) predict the fate of patches caused by the infectious activity of *R. solani*, in presence of introduced biocontrol agents, or in presence of naturally occurring beneficial microorganisms which would have been promoted by appropriate agricultural practices.

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GENERAL CONCLUSION

The general background prevailing to set up the program investigated hereby was relying on field observations, literature survey and preliminary experimentations performed by the team working on the "Impact of agriculture practices on the ecology of soil borne plant pathogenic fungi and the soil quality" at the INRA of Dijon.

In sugar beet fields, patches du to *Rhizoctonia solani* infectious activity appeared as very mobile and their occurrence was unpredictable. They never occurred consecutively at the same place even when the host plant was mono-cropped in the plots. Preliminary experiments carried out by this team suggested that the genetic structures of the soil microbial (both fungal and bacterial) communities within a patch was modified compared to the ones of microbial communities sampled outside of the patches. Moreover, the suppressiveness of the soil towards *R. solani* diseases was increased within the patches while it remained low outside of patches. These observations suggested the stimulation of microbial groups within the microbial communities facing the infectious activity of *R. solani* towards the host plant (sugar beet). It was thus hypothesized that specific antagonistic micro-organisms could emerge from the situation among which some putative biocontrol agents could be isolated, identified, characterized and eventually selected for further practical biocontrol development.

It was therefore decided to investigate the temporal dynamics of components of the microflora, including *Trichoderma* sp. in relation to patch development of the disease incited by *R. solani* AG2.2 in a sugar-beet field, hence my PhD work.

Thanks to the combination of approaches we used (field observations and samplings, Pasteur or classic microbiological methods, molecular methods and bioassays in greenhouses) we have been able to describe partly the contribution of components of the soil microflora to the built up of a patch and in which way, the *Trichoderma* community was responding to the development and/or activity of *R. solani* AG 2-2. We also showed that the equilibrium reached by the biotic components at the end of the season was rather weak, being broken by violent external factors such as the aerial disease, what could explain the unpredictable fate (both disappearance and occurrence) of disease patches caused by *R. solani* AG 2-2.

At last we selected a strain of *T. gamsii* which appears as a good candidate for further biocontrol use.

Although it could seem that we fulfill more or less the program initially set up, it is obvious that the results we got aroused many questions which really need to be investigated to provide even more mechanistic explanations concerning i) the *Rhizoctonia-Trichoderma* interactions and the possible role of signal molecules to identify, ii) but also the above-below ground interactions, the former considering the disease progression as seen at the field scale but also in the frame of an agricultural system (agricultural practices including the uses of inputs such as fertilizers, pesticides, or green manure), the later considering the whole interacting soil biota. Such an integrated approach requires the use of mathematical models to take into account all the factors and their multiple afferent parameters which play a significant role in the epidemiology of rhizoctonia disease, including occurrence, development, activity, persistence or disappearance of a diseased patch.