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

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Abstract The relationships 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 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 diseased 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 (T-RFLP) 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 patch mobility in the following season.

Keywords Antagonistic potential · Community structure · Dynamics of patches · Soil inoculum potential · Terminal restriction fragment length polymorphism (T-RFLP) · *Trichoderma* spp.

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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. *Rhizoctonia 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 pose. 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 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 (80×24 m) 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 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 characterised 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). *Rhizoctonia solani* AG 2-2 causes equiv-

alent damages on carrots and sugar beets, and the standardised bioassay proposed by Janvier et al. (2006) was used to assess soil conduciveness towards diseases caused by *R. solani* AG 2-2. This standardised procedure allowed us 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 (8×8×7 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 a 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 sterilised 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 introduced 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 2 weeks and area under the disease progress curve (AUDPC) measured and 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 introduced 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 analysed 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 with 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 programme 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 analysed 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. Ten-fold dilutions were made. Bacteria were quantified on yeast-peptone glucose agar (yeast 5 $g l^{-1}$,

peptone 5 g l^{-1}, glucose 10 g l^{-1}, agar 15 g l^{-1}) (YPGA) supplied with cycloheximide (100 mg l^{-1}). Fungi were quantified on MEA (malt 10 g l^{-1}, agar 15 g l^{-1}) supplied with citric acid (250 mg l^{-1}), chlortetracycline (50 mg l^{-1}) and streptomycin sulphate (100 mg l^{-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 fragment length polymorphism (T-RFLP) of 16S and 18S ribosomal RNA (rRNA) genes for bacteria and fungi, respectively (Edel-Hermann et al. 2004; Pérez-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 (ACGGGCGGTGT GTACA) 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′ (ATTGCAATGCY CTATCCCCA) and digested with the restriction enzyme *Msp*I (Q-BIOgene). The labelled and unlabelled primers were synthesised 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 characterised 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 1,000 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 characterise 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 consisted of 31 different substrates with a negative control while FF plates consisted 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 antagonise 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 $4\times10^{6}\,ml^{-1}$ 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.

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.

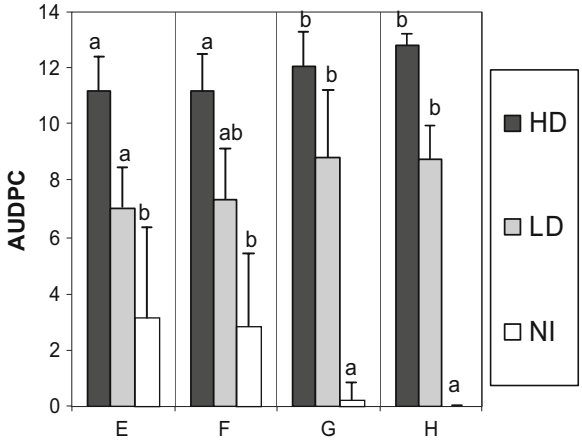

Fig. 1 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 strain G6 on carrots in soils inoculated with 3% (*v*/*v* of soil, high dose, *HD*), 0.3% (*v*/*v* of soil, low dose, *LD*) of sand inoculum, or in non-inoculated soil (*NI*). The different soil samples originated from 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

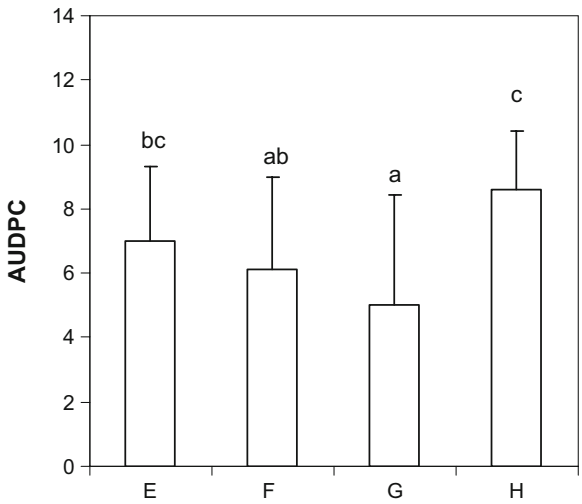

Fig. 2 Area under the disease progress curve (AUDPC) caused by *R. solani* AG 2-2 in carrots in soils amended with buckwheat meal (2% *w*/*v* of soil) and not inoculated. The different soil samples originated from the same patches as in Fig 1. For each characteristic sampling point (*E*, *F*, *G*, *H*), 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

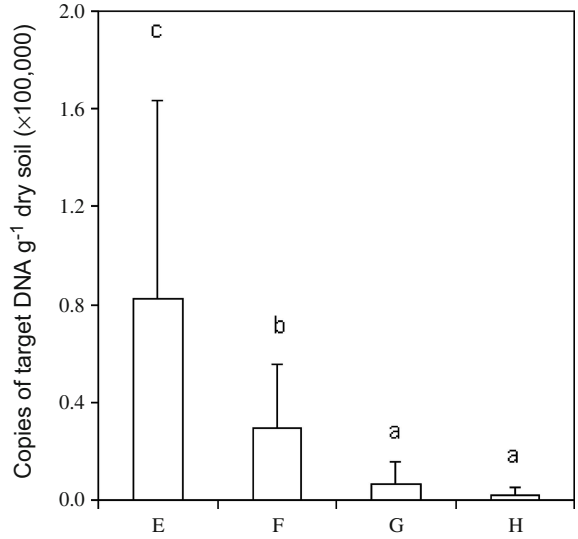

Fig. 3 Number of copies of target DNA of *R. solani* AG 2-2 quantified by real-time PCR. The different soil samples originated from the same patches as in Fig 1. For each characteristic sampling point (*E*, *F*, *G*, *H*), the bar corresponds to the mean value among three independent PCR experiments 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 either for the bacteria (mean 105 TRF) or 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

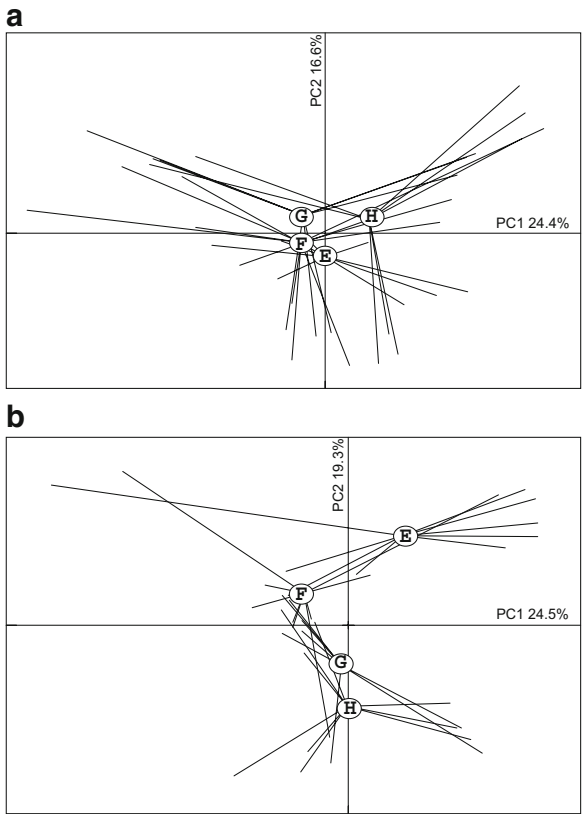

Fig. 4 Principal component analysis of 16S (**a**) and 18S (**b**) terminal restriction fragment length polymorphism data sets from the same patches as in Fig 1. For each characteristic sampling point, analysis was performed in three independent transects, each with three independent soil samples. Each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

much more abundant in diseased 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

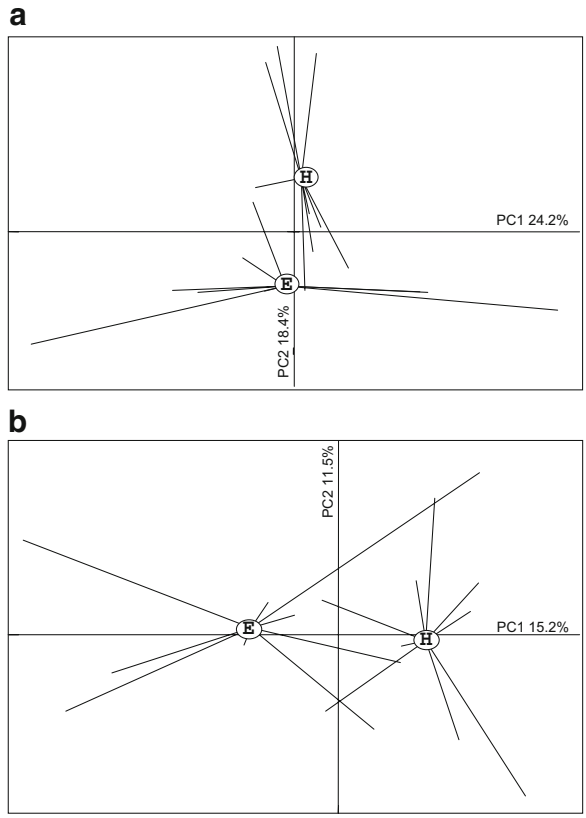

Fig. 5 Principal component analysis of the physiological fingerprints of the bacterial (**a**) and the fungal (**b**) communities inside the diseased patch (*E*), and inside the healthy area (*H*). For each characteristic sampling point, analysis was performed in three independent transects, each with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

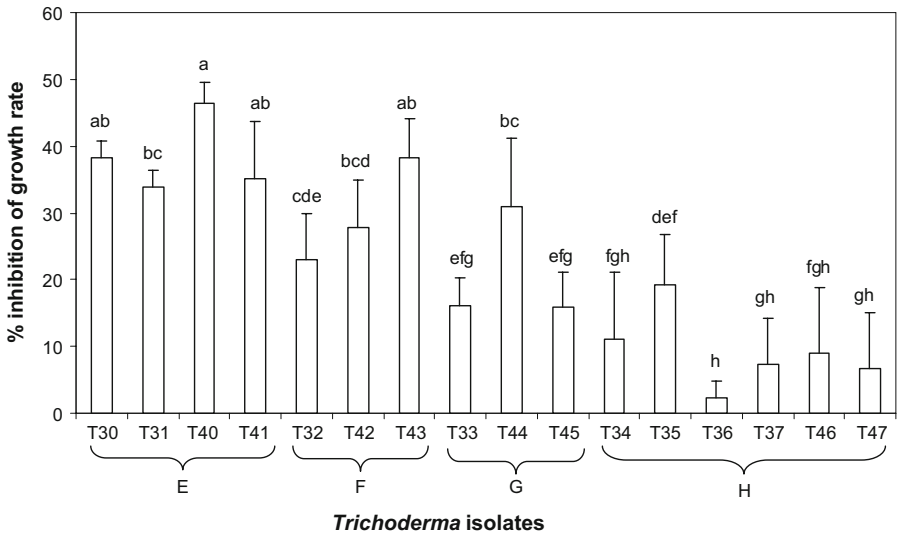

Fig. 6 Percentage of inhibition of growth rate of *R. solani* AG 2-2 strain G6 caused by *Trichoderma* isolates cultured together on malt agar plates. The *Trichoderma* isolates were collected from the same patches as in Fig 1. Different *small letters* on top of the bars indicate significant differences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

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.

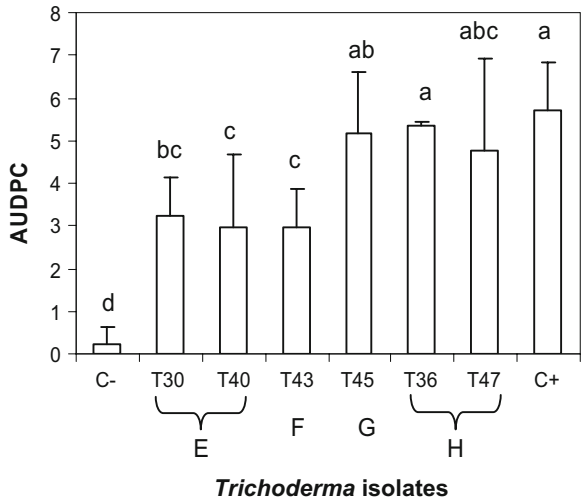

Fig. 7 Area under disease progress curve (AUDPC) caused by *R. solani* AG 2-2 strain G6 on carrots in natural soil in the presence of different isolates of *Trichoderma* isolated from the different soil samples originating from the same patches as in Fig 1. C− denotes the treatment neither with *R. solani* 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 Cambridge group 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 also agree 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 g^{-1} of soil were observed between characteristic sampling points. The highest densities were observed in the sampling points 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 metabolise 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 antagonise 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 in agreement 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 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 cause the mobility of the patches.

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