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The bacterial community of tomato rhizosphere is modified by inoculation with arbuscular mycorrhizal fungi but unaffected by soil enrichment with mycorrhizal root exudates or inoculation with *Phytophthora nicotianae*

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A B S T R A C T

Arbuscular mycorrhizal (AM) fungi have been shown to induce the biocontrol of soilborne diseases, to change the composition of root exudates and to modify the bacterial community structure of the rhizosphere, leading to the formation of the mycorrhizosphere. Tomato plants were grown in a compartmentalized soil system and were either submitted to direct mycorrhizal colonization or to enrichment of the soil with exudates collected from mycorrhizal tomato plants, with the corresponding negative controls. Three weeks after planting, the plants were inoculated or not with the soilborne pathogen *Phytophthora nicotianae* growing through a membrane from an adjacent infected compartment. At harvest, a PCR-Denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments amplified from the total DNA extracted from each plant rhizosphere was performed. Root colonization with the AM fungi *Glomus intraradices* or *Glomus mosseae* induced significant changes in the bacterial community structure of tomato rhizosphere, compared to non-mycorrhizal plants, while enrichment with root exudates collected from mycorrhizal or non-mycorrhizal plants had no effect. Our results support that the effect of AM fungi on rhizosphere bacteria would not be mediated by compounds present in root exudates of mycorrhizal plants but rather by physical or chemical factors associated with the mycelium, volatiles and/or root surface bound substrates. Moreover, infection of mycorrhizal or non-mycorrhizal plants with *P. nicotianae* did not significantly affect the bacterial community structure suggesting that rhizosphere bacteria would be less sensitive to the pathogen invasion than to mycorrhizal colonization. Of 96 unique sequences detected in the tomato rhizosphere, eight were specific to mycorrhizal fungi, including two *Pseudomonas*, a *Bacillus simplex*, an *Herbaspirilium* and an Acidobacterium. One *Verrucomicrobium* was common to rhizospheres of mycorrhizal plants and of plants watered with mycorrhizal root exudates.
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1. Introduction

AM fungi have been shown to reduce disease development in a wide range of plant-pathogen associations (St-Arnaud and Vujanovic, 2007). These ubiquitous fungi are grouped into the phylum *Glomeromycota* (Schüßler et al., 2001) and form a mutualistic symbiosis with most land plants, receiving carbon from their host, and delivering minerals and water back (Smith and Read, 2008). They benefit plant growth in a large part through their ability to colonize a wider soil volume and to exploit resources more efficiently than roots. Recently, Maherali and Klironomos (2007) provided evidences supporting that functional ability to protect from soil pathogens may be conserved within AM fungi evolutionary lineages.

AM fungi impact the other soil microorganisms development leading to the formation of the specific zone of soil called the mycorrhizosphere (Linderman, 1988). Root colonization with AM fungi has most of the time been shown to decrease (Christensen and Jakobsen, 1993; Bansal and Mukerji, 1994; Cavagnaro et al., 2006), but also to increase (Posta et al., 1994; Albertsen et al., 2006) or have no effect (Olsson et al., 1996) on the microbial biomass within not only the rhizosphere but also within the mycosphere, the zone of soil under the influence of the mycorrhizal mycelium only. They were also shown to have species-specific impacts by stimulating or inhibiting the growth of specific microbial taxa (Marschner and Timonen, 2006). As some rhizobacteria are known to inhibit pathogen proliferation through various mechanisms (Bowen and Rovira, 1999; Whipps, 2001), one way AM fungi may reduce disease development is therefore by inducing the formation of a bacterial community unfavourable to pathogens development

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(Li et al., 2007). However, the mechanisms controlling the interaction between bacteria and AM fungi in soil are still poorly understood.

AM fungi have been shown to quantitatively change the amount of root exudates but also to induce some qualitative modifications of root exudate composition (Bansal and Mukerji, 1994; Azaizeh et al., 1995; Marschner et al., 1997; Sood, 2003). Carbohydrates (Hooker et al., 2007; Toljander et al., 2007) and citric acid (Tawaraya et al., 2006) were detected in mycorrhizal hyphae exudates. In this manner, the decrease of soil microbial biomass and the modification of the soil microbial community induced by mycorrhizal colonization were suggested to depend on quantitative and qualitative changes of root exudates (Bansal and Mukerji, 1994; Marschner et al., 1997). Filion et al. (1999) observed that extracts from *Glomus intraradices* mycelium grown *in vitro* in a root free compartment had differential effects on soil microbes, stimulating *Pseudomonas chlororaphis* and *Trichoderma harzianum*, reducing conidial germination of *Fusarium oxysporum* f. sp. *chrysanthemi* and having no effect on the growth of *Clavibacter michiganensis*. In addition, chemotactic responses of the plant-growth-promoting rhizobacteria *Azotobacter chroococcum* and *Pseudomonas fluorescens* to exudates of tomato plants colonized with *G. fasciculatum* were significantly stronger than the response to non-mycorrhizal root exudates (Sood, 2003). Furthermore, exudates produced by AM extraradical mycelia influenced the vitality and the community of bacteria extracted from soil, *in vitro*, but this varied with the time of incubation (Toljander et al., 2007).

Phytophthora nicotianae (an Oomycete) is a soilborne pathogen inducing root rot diseases on a huge host range, infecting more than 72 genera from 42 plant families (Erwin and Ribeiro, 1996). It is responsible for large yield losses in many important crops, including tomato. Reduction of the detrimental effect of *P. nicotianae* on tomato plants colonized by the AM fungus *Glomus mosseae* (Trotta et al., 1996; Vigo et al., 2000; Pozo et al., 2002) but also by *G. intraradices* (Lioussanne et al., 2009) has been described. Cell defense responses and the induction of new isoforms of defense-related enzymes after colonization with *G. mosseae* were described and would contribute to limit pathogen proliferation within host roots (Pozo et al., 2002). Vigo et al. (2000) however reported that *G. mosseae* clearly reduced the number of infection loci formed by *P. nicotianae* on tomato roots which supports the hypothesis that the pathogen's ability to reach and penetrate roots may also be affected before root infection.

It has been estimated that only 0.1–10% of the microorganisms found on typical agricultural soils would be culturable using current culture media formulations while culture independent methods based on 16S rRNA gene amplification permit the detection of over 90% of microorganisms that can be observed microscopically *in situ* (Hill et al., 2000). Denaturing gradient gel electrophoresis (DGGE) is a method by which fragments of DNA of the same length but with different sequences can be resolved electrophoretically and used to infer about the microbial diversity within samples (Muyzer et al., 1993). This approach led to observations that microbial communities within different plant rhizosphere were changed by mycorrhizal colonization (Marschner et al., 2001; Wamberg et al., 2003; Marschner and Timonen, 2005).

The aim of the present study was first to verify that inoculation with *G. intraradices* Schenck and Smith and *G. mosseae* (Nicol. and Gerd.) Gerdemann and Trappe modified the microbial community structure of tomato rhizosphere within our experimental conditions, and to compare the effect of the two AM fungal species. Secondly, we tested if enrichment of the rhizosphere soil with root exudates from plants colonized with the same AM fungi would induce changes similar to direct inoculation. Lastly, we assessed if the bacterial community structure of the AM-inoculated and root exudate-enriched non-mycorrhizal plants was modified by inoculation with *P. nicotianae* Breda de Haan. To this end, tomato plants were grown individually in a compartmentalized soil microcosm and submitted to direct mycorrhizal colonization or supplied with exudates from mycorrhizal plants, with the corresponding negative controls. Plants were then inoculated or not with *P. nicotianae*. At harvest, the bacterial community structure was characterized by PCR-DGGE analysis of 16S rRNA gene fragments amplified from DNA directly extracted from the rhizosphere soil.

2. Methods

2.1. Experimental design

Using a compartmentalized microcosm (described below), twelve tomato plants were, in a first step, either submitted to direct root colonization with AM fungi or to enrichment with root exudates from mycorrhizal tomato plants, with the corresponding negative controls. Thus, half of the plants was supplied with sterilized pure water (E−) and either colonized with *G. intraradices* (Gi), with *G. mosseae* (Gm) or not colonized (G-). The other half of the plants received 2 mL daily of a standardized tomato root exudate solution collected from plants colonized with *G. intraradices* (E^{Gi}), *G. mosseae* (E^{Gm}) or not colonized (E^{G-}). Three weeks later, in a second step, plants were either inoculated (P+) or not inoculated (P−) with *P. nicotianae*. Therefore, the experiment included the twelve following treatment combinations: E-G-P-, E-GiP-, E-GmP-, E^{G-}G-P-, E^{Gi}G-P-, E^{Gm}G-P-, E-G-P+, E-GiP+, E-GmP+, E^{G-}G-P+, E^{Gi}G-P+, E^{Gm}G-P+. The experimental design was a split-plot with the six mycorrhizal inoculation/exudate application treatments randomized in the main plots, and *P. nicotianae* inoculation treatments randomized in the subplots. There were four blocks, each containing one experimental system with a full complement of treatment combinations, giving therefore four independent replicates per treatment. Two blocks were set out first, and the other two blocks were set out three weeks later.

2.2. Biological material and growth conditions

The growth substrate, a 2:2:1 mix of field sandy loam soil, sand and a commercial potting mix (Tropical Plant Soil, Modugno-Hortibec Inc., St-Laurent, QC, pH 5.3–6.3, 0.06% N, 0.1% P, 0.4% K, conductivity 0.5–2.0 mmhos cm^{-1}), was autoclaved twice for 60 min at 121 °C. To reintroduce a microbial community exempt of mycorrhizal fungi in the growth substrate, a 500 g subsample of the sandy loam soil was mixed in 1.5 L of sterilized Milli-Q water (Milli-Q synthesis, RiOs, Millipore, Mississauga, ON), agitated for 30 min, passed through Whatman No. 1 and 42 filters, and added to 5 kg of growth substrate. The mix was homogenised daily at 26 °C for two weeks before use.

Leek (*Allium porrum* L. cv. Farinto) and tomato (*Solanum lycopersicum* L. cv. Cobra) seeds were surface-sterilized 15 min in 70% ethanol, followed by 20 min in 1.5% sodium hypochlorite plus 1% Triton X100 and rinsed three times in sterilized Milli-Q water. Seeds were germinated 48–96 h on Tryptic Soy Agar (TSA, Quélab, Montreal, QC) and transferred to the experimental units. Seedlings were thinned to one per compartment and grown in a greenhouse with 16 h daylight (22–20 °C). Plants were fertilized with 20 mL of 5× Long Ashton nutrient solution (Hewitt, 1966) per week and watered with deionized water as needed.

Ri T-DNA-transformed *Daucus carota* L. roots colonized with *G. intraradices* Schenck and Smith (DAOM 181602) were grown in minimal medium solidified with 0.4% (w/v) gellan gum (Gel Gro, ICN Biochemical, Cleveland, OH), as described in Fortin et al. (2002), for six months in the dark at 26 °C. The spores were separated from the gel in sodium citrate buffer (Doner and Bécard, 1991) and suspended in sterile water. *G. mosseae* (Nicol. & Gerd.) Gerdemann and Trappe (BEG 12) spore production and disinfection were performed as described in Budi et al. (1999) with modifications. Spores were recovered by wet sieving and decanting, and purification was

carried out by two successive centrifugations at 1600 g for 2 min in a density gradient with a 60% (w/v) sucrose layer at the bottom. Spores were collected from the gradient interface, thoroughly washed with sterile water and disinfected using a Buchner filtration system fitted with a Whatman No. 4 filter paper. The spores were suspended 30 s in 70% ethanol, rinsed three times in sterile water, immersed for 20 min in 2% (w/v) chloramine T plus one drop of Tween 80, rinsed ten times for 1 min in sterile water, and then incubated 24 h in Tryptic Soy Broth with 0.025% (w/v) ampicillin and 0.01% (w/v) streptomycin sulfate, at 24 °C, in darkness. Finally, they were transferred to a new Buchner filtration system, rinsed in sterile water, incubated for 20 min in 2% (w/v) chloramine T and rinsed again ten times for 1 min before being suspended in sterile water. Viability of the spores was estimated by incubation in 0.1% (w/v) MTT for 72 h at 22 °C (Walley and Germida, 1995). They were cold-treated at 4 °C for two weeks in sterile water prior to inoculation (Juge et al., 2002).

P. nicotianae Breda de Haan (isolate 201) was grown on autoclaved V8 agar [V8 juice diluted 1:10 in Milli-Q water, 0.2% (w/v) $CaCO_3$, 1% (w/v) gellan gum, 0.005% (w/v) piramicin, 0.025% (w/v) ampicillin, 0.001% (w/v) rifampicin] at 26 °C, under 16 h of light per day (Tuite, 1969). After two weeks, to inoculate each experimental unit, the mycelium from 10 Petri dishes was scraped, mixed in 100 mL sterile Milli-Q water and blended two times for 4 s before use.

2.3. Experimental set-up, plant inoculation and production of root exudates

The compartimentalized microcosm used in this experiment is described in Lioussanne et al. (2009). Briefly, 50 × 16 × 2.5 cm units divided in six growth compartments were built using polyethylene plates with both sides covered with a Ø 48 μm nylon membrane (A subunits, Fig. 1). Each compartment was supplied with a bottom layer of 20 mL autoclaved quartz gravel to favour drainage, topped with 300 mL of growth substrate. To ensure a fast and homogenous mycorrhizal colonization (treatment Gi or Gm), the A subunits containing tomato plantlets were then placed between two non-compartimentalized B subunits containing leek plants previously colonized with *G. intraradices* on one side and with *G. mosseae* on the other side. Units containing two weeks-old leek plantlets had previously been inoculated with a water suspension of 500 viable spores of *G. intraradices* or *G. mosseae* poured on the roots and grown for seven weeks until use. Control uninoculated plants (G-) were obtained by insertion of a plastic sheet to prevent mycelium growth between compartments. Beginning two weeks after planting until harvest, 2 mL of tomato root exudates (collected from tomato plants colonized with *G. mosseae*, with *G. intraradices* or non-colonized; see below) or sterile Milli-Q water were applied daily on the soil of each compartment. In a second step, three weeks later, B subunits containing leek plants were taken away and replaced on one side by a *P. nicotianae* infested unit (Fig. 2) prepared as follows: 12 tomato seedlings were planted and grown for one week, a 100 mL suspension of *P. nicotianae* mycelium was then evenly spread on the surface of the substrate and the infested plantlets were grown for two weeks before being cut to the soil level. An in-between unit containing only the substrate was added to create a gradient of exudates between plant compartments and the *P. nicotianae*-infested unit. Pathogen uninoculated control (P−) was obtained using a plastic sheet to prevent mycelium growth and zoospore swimming between compartments.

Root exudates of tomato plants colonized with *G. intraradices*, *G. mosseae* or without mycorrhizal colonization were collected according to Pinior et al. (1999). Tomato plants inoculated as described previously were grown for five weeks, the root system of each tomato plant was washed under tap water and incubated in Erlenmeyer flasks filled with 100 mL sterilized Milli-Q water for 22 h. Solutions were sterilized by filtration through Whatman No. 4 and No. 42 filter papers and then Ø 0.22 μm nitrocellulose filters (Millipore) and lyophilized. Concentrations were adjusted to a ratio of 1 g of root fresh weight equivalent to 20 mL of exudate solution with sterilized Milli-Q water. The pH was then adjusted to 6.0 before being sterilized again by filtration through Ø 0.22 μm nitrocellulose filters and solutions were kept at −20 °C until use.

2.4. Plant and soil harvesting

After six weeks of growth, plants inoculated with *G. intraradices* or *G. mosseae* showed 23.5% and 44.9% of root length bearing mycorrhizal colonization, respectively, while plants not inoculated were not colonized (Lioussanne et al., 2009). The growth substrate was withdrawn from each compartment containing tomato plants, and the whole root system was separated from the soil using sterilized forceps and gloves. The whole soil from each experimental unit was then immediately placed in individual sterilized plastic bags and frozen at −20 °C.

2.5. DNA extraction and PCR amplification

Each soil sample was homogenized before the total DNA was extracted from a 0.6 g subsample using the UltraClean Soil DNA

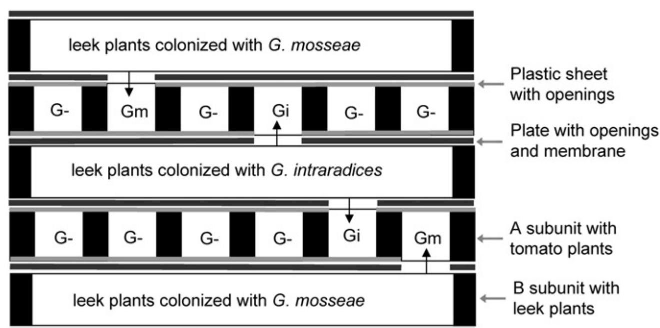

Fig. 1. Mycorrhizal inoculation of tomato plants: A subunits divided in six compartments containing tomato plants were attached to non-compartimentalized B subunits containing leeks previously colonized with *G. intraradices* or *G. mosseae*. Plastic sheets were attached to both sides of each A subunit and openings were cut in front of designated compartments to allow mycorrhizal mycelium growth from leeks. Subunits were separated with a Ø 48 μm nylon membrane sticked on a plate with openings. Tomato plant colonized with *G. intraradices* (Gi), *G. mosseae* (Gm) or non-inoculated (G−).

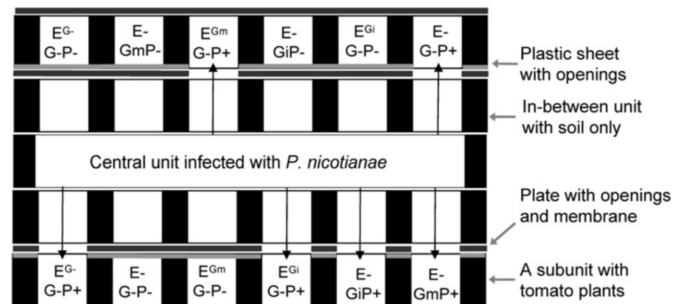

Fig. 2. Subunits reassembled to allow pathogen inoculation: new plastic sheets attached to A subunits containing tomato plants were cut in front of designated compartments to allow *P. nicotianae* growth from the infected central unit through a Ø 48 μm nylon membrane. Tomato plants directly inoculated with AM fungi or submitted to enrichment with mycorrhizal roots exudates with the negative controls (E−G−, E−Gi, E−Gm, E^{G-}−G−, E^{Gi}G−, E^{Gm}G−) were inoculated (P+) or not with *P. nicotianae* (P−).

Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA). PCR amplification of a 16S rRNA gene fragment was performed using a nested protocol with the universal primers pA and pH (Edwards et al., 1989) in the first round and the primers 341F + GC and 534R in the second round, to amplify the V3 region (Muyzer et al., 1993) (Table 1). All PCRs were conducted in 50 μL volumes. The soil genomic DNA (for the first round) or the amplification products (for the second round) were diluted to 1:100 and 2 μL were added to 1 μL of primers (10 μM, Alpha DNA, Montreal, QC), 1 μL of dNTPs mix (10 mM), 0.5 μL of bovine serum albumin (BSA, 100×, New England Biolabs, Ipswich, MA), 5 μL of 10 × PCR buffer and 2.5 μL of dimethyl sulfoxid (DMSO, Sigma–Aldrich, Oakville, ON, for the second round only). The PCR amplifications were performed in a TC-514 thermal cycler (Techne Inc., Princeton, NJ) and consisted in an initial denaturation at 95 °C for 5 min, after which the temperature was adjusted to 80 °C and 1.25 U of Taq DNA polymerase (TAQ PCR core kit, Qiagen, Mississauga, ON) were added. Then, for the first round, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min were performed. The second round consisted in a Touch down protocol with decreasing annealing temperatures from 65 °C to 55 °C for 35 cycles (Muyzer et al., 1993).

2.6. DGGE profiling of the bacterial community

DGGE analysis was performed using the Dcode Universal Mutation Detection System (BioRad, Missauga, ON) according to the manufacturer instructions except that the Model 485 Gradient Former was used for the gel preparations instead of the Model 475. Twenty μl of PCR product were loaded in each well and the electrophoresis was run at 60 °C for 16 h at 60 V in an 8% acrylamide/bisacrylamide (37.5:1) gel with a 40–70% denaturant gradient, where 100% denaturant corresponded to 7 M of urea and 40% (v/v) formamide. A molecular marker (described below) was loaded in the first and the last well of the DGGE gels to facilitate gel-to-gel comparisons. Gels were stained 15 min in 1× SYBR Gold (Invitrogen, Carlsbad, CA), digitized using a Chemi-Doc apparatus (BioRad) and analyzed using the Quantity One 4.0 software (BioRad). Because each migration position usually corresponds to a different sequence variant, the matrix obtained was considered as a taxa presence–absence matrix for statistical analyses. Bands were numbered in order of appearance from top to bottom of gels.

In order to prepare the molecular marker, two distinct bands from a DGGE gel performed using samples from the present experiment were collected with a sterilized scalpel and deposited in 50 μL TE 10 mM buffer, pH 7.6. The DNA was extracted by incubation at 50 °C for 10 min, crushing with a microcentrifuge tube pestle, centrifuged at 10 000 g for 1 min and the V3 region was reamplified as described above. Pure cultures of *Escherichia coli* (XL1-blue Mrf, Stratagene, Cedar Creek, TX) and of bacterial isolates 10D, 10G and 60A (Lioussanne, 2007) were also used. Each isolate was incubated 72 h in Tryptic Soy Broth (TSB, Quélab, Montréal, QC), and 3 mL were transferred and incubated in new Erlenmeyers filled with 27 mL of TSB. When OD_{600} was between 0.65 and 0.9, the DNA was extracted from 3 mL of culture broth by two successive treatments in 1.5 mL 1 M NaCl each followed by a centrifugation at 10 000 g for 4 min and removal of the supernatant. The extracted DNA was then suspended in TE 10 mM buffer, pH 7.6 and stored at −20 °C (Versalovic et al., 1994). The V3 region of the 16S rRNA gene was then directly amplified as described above, except that no BSA and DMSO were incorporated in the PCR mix. Equal volumes of each PCR product were then mixed and 10 μL were charged as molecular markers in DGGE gels.

Table 1
Sequences of the primers used for the nested-PCR amplifications of the bacterial 16S rRNA gene.

Primer	Region amplified	Sequence	Reference
pA	Entire 16S	5′-AGAGTTTGATCCTGGCTCAG-3′	Edwards et al., 1989
pH	Entire 16S	5′-AAGGAGGTGATCCAGCCGCA-3′	Edwards et al., 1989
341F + GC	V3	5′CGCCCGCCGCGCGCGGCGGGCG GGCGGGGGCACGGGGGGCCTAC GGGAGGCAGCAG-3′	Muyzer et al., 1993
534 R	V3	5′-ATTACCGCGGCTGCTGG-3′	Muyzer et al., 1993

2.7. Identification of bacterial taxa

Bacterial taxa were identified using a cloning-DGGE strategy, as described in Gonzalez et al. (2003). All PCR products amplified with primers pA and pH, from each mycorrhizal fungi/*Phytophthora* inoculation treatment combination (E-GiP-, E-GmP-, E-GiP+ and E-GmP+), were pooled together and a 2 μL sample was used for cloning. Ligation reaction was performed using the pDrive Cloning Vector DNA of the QIAGEN PCR Cloning Kit and transformation with QIAGEN PCR Cloning*plus* Kit (QIAGEN Inc., Mississauga, ON), following suppliers instructions except that heat shock was performed for 45 s, at 42 °C. Plates were sent to Genome Quebec Innovation Center (Montréal, Canada) for plasmid extraction and sequencing of 144 clones using the pA primer. Sequences were aligned and compared using BioEdit (version 7.0.0). All unique sequences were submitted to BLASTn searches in GenBank (http://www.ncbi.nlm.nih.gov/) to identify clones. A clone subset was chosen in order to include one copy of each single sequence, and the 16S rRNA gene V3 region of each clone was amplified as described before, and 6 μL of PCR products from each reaction were loaded on a DGGE gel and run with the same conditions and molecular markers previously described. The electrophoretic mobility of products from each clone was then compared with the DGGE banding pattern of amplicons from the tomato rhizosphere to identify bands showing a similar migration position.

2.8. Statistical analyses

Correspondence analysis was performed on the taxa presence-absence matrix of DGGE banding patterns using the CORRESP procedure of SAS (release 8.02, SAS Institute Inc.) to describe bacterial taxa associations with mycorrhizal inoculation, exudate application and *Phytophthora* inoculation treatments. Discriminant analysis was used to test the significance of differences between the twelve treatments on bacterial communities with a Fisher test of the Mahalanobis distances between treatment clusters, using the CANDISC procedure of SAS. Rare and common band types were excluded in both analyses because of the distortion they provoke in these types of ordination.

3. Results

A typical DGGE gel profile obtained with the V3 region of 16S rRNA gene fragments amplified from DNA extracted from the rhizosphere of tomato plants after six weeks of growth is shown in Fig. 3. A total of 60 different migrating positions were detected, with a number varying from 26 to 38 per sample. Of these, 26.6% occurred in all samples, while 42% were treatment-dependent (with detection frequencies varying with treatments) (Table 2). Direct inoculation of the mycorrhizal fungi had the largest influence on the bacterial taxa assemblage within the tomato rhizosphere (Fig. 4). The highest number of different bands were observed from samples harvested from rhizosphere soil where *G. mosseae* was inoculated, with 44–49 bands, respectively, in the presence or absence of *P. nicotianae*. The highest number of highly specific bands was also found from the

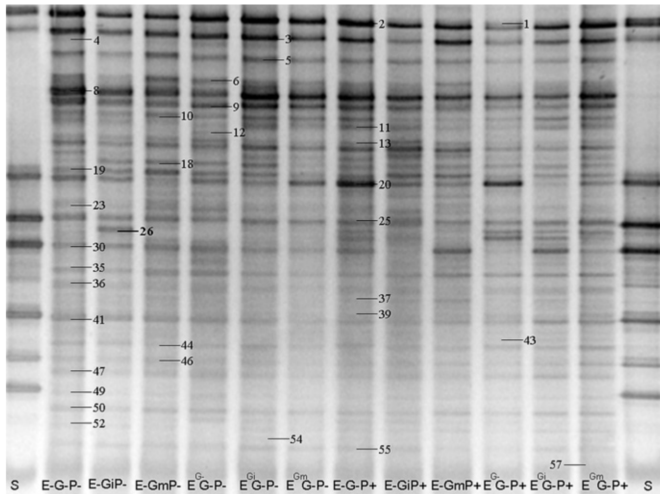

Fig. 3. Typical DGGE gel of bacterial 16S gene sequence variants detected from tomato rhizosphere, after six weeks of growth. Lanes S—molecular markers composed of (from top to bottom) two unidentified bacteria, *Escherichia coli* XL1-blue Mrf, isolate 60A, *E. coli* XL1-blue Mrf, isolates 10G, 10D and 10D; Description of treatments is given in Table 2 while band numbers correspond to those used in Tables 3–5.

rhizosphere of plants inoculated with *G. mosseae* when *P. nicotianae* was co-inoculated. Conversely, the smallest number of bands was observed from control treatments without mycorrhizal inoculation nor exudate application, with only 38 bands in the presence of *P. nicotianae* and 39 in its absence (Table 2). Moreover, band 48 was found to be absent in control treatments without AM inoculation or without exudate enrichment from mycorrhizal plants. Inoculation with *G. intraradices* or *G. mosseae*, as well as the application of exudates from mycorrhizal or non-mycorrhizal plants had no effect on shoot dry weight and root fresh weight of tomato plants compared to those of non-inoculated control plants which received pure water (data not shown).

Table 2
Effect of mycorrhizal inoculation, application of exudates from mycorrhizal roots and *Phytophthora* inoculation on the 16S rRNA gene profile of rhizosphere bacteria of six week old tomato plants.

Exudates supplied[a]	AM fungi inoculation[b]	Phytophthora inoculation[c]	Total number of bands[d]	Common bands[e]	Specific bands[f]
E−	G−	P−	39	43.3%	1
E−	Gi	P−	42	36.6%	3
E−	Gm	P−	44	35.0%	0
E−	G−	P+	38	36.6%	0
E−	Gi	P+	41	30.0%	0
E−	Gm	P+	49	31.6%	4
E^{G-}	G−	P−	43	41.6%	0
E^{Gi}	G−	P−	40	35.0%	1
E^{Gm}	G−	P−	40	35.0%	0
E^{G-}	G−	P+	42	36.6%	0
E^{Gi}	G−	P+	42	38.3%	0
E^{Gm}	G−	P+	42	43.3%	1
Total			60		10

[a] E−: Milli-Q water; E^{G-}: root exudates collected from non-mycorrhizal plants; E^{Gi}: from plants colonized with *G. intraradices*; E^{Gm}: from plants colonized with *G. mosseae*.
[b] G−: plants not inoculated with an AM fungus; Gi: inoculated with *G. intraradices*; Gm: inoculated with *G. mosseae*.
[c] P−: plants not inoculated with *Phytophthora nicotianae*; P+: inoculated with *P. nicotianae*.
[d] Total number of distinct band migration positions detected on DGGE gels.
[e] Percentage of bands detected in all repetitions of each treatment combination.
[f] Number of bands specific to each treatment.

A correspondence analysis of the effect of the experimental treatments on the sequence variant frequencies revealed that the two first factors that mostly changed the bacterial community structure within the tomato rhizosphere were root colonization with *G. mosseae* and with *G. intraradices* (data not shown). The two principal components of the discriminant analysis of the bacterial 16S rRNA gene fragment profiles (Fig. 4) described 67% of the variation in the data set. This analysis procedure reduced the different band location variables to a smaller set of hypothetical variables, or principal components (PC), that are ranked for their importance in describing variation in the data set. The first principal component described 41% of the variation between the bacterial community profiles, while PC2 described an additional 26% of the variation. The ordination diagram plotted with respect to these two vectors revealed an excellent homogeneity of the bacterial community structure within replicates of each treatment. Moreover, it showed a close similarity between rhizosphere bacterial communities of control plants (not inoculated and without exudate enrichment) and of non-mycorrhizal plants supplied with exudates from non-mycorrhizal plants, with or without inoculation of *P. nicotianae*. A similar close similarity was noticed between rhizosphere bacterial communities of plants inoculated with *P. nicotianae* only and plants supplied with exudates from plants colonized with *G. intraradices* and inoculated with the pathogen, or with exudates from plants colonized with *G. mosseae* and inoculated or not with the pathogen. On the contrary, points representing the rhizosphere bacterial communities of plants directly inoculated with *G. mosseae* or *G. intraradices*, either inoculated or non-inoculated with *P. nicotianae*, formed clusters that were clearly separated from the other treatments and from each other. The statistical significance of differences between the bacterial communities described by PC1 and PC2 were examined by analysis of Mahalanobis distances between clusters. By this analysis, the rhizosphere bacterial community of the control plants

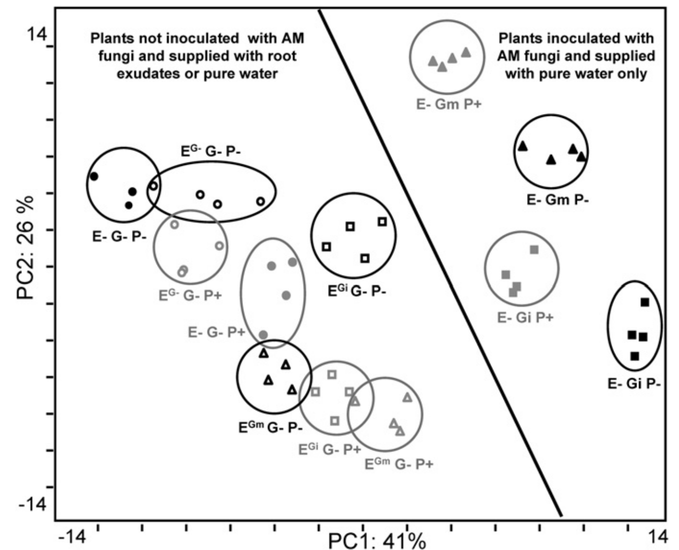

Fig. 4. Discriminant analysis showing bacterial 16S rRNA gene variants associations with regards to direct inoculation with AM fungi or enrichment with root exudate treatments, within the rhizosphere of six week old tomato plants. Points representing the rhizosphere bacterial community of plants inoculated with *G. intraradices* or *G. mosseae* clustered on the right side, while points corresponding to other treatments are scattered on the left part of the graph. Half of the plants was supplied with sterilized pure water (E−) and either colonized with *G. intraradices* (Gi), with *G. mosseae* (Gm) or not colonized (G−). The other half of the plants received daily a tomato root exudate solution collected from plants colonized with *G. intraradices* (E^{Gi}), *G. mosseae* (E^{Gm}) or not colonized (E^{G-}). Three weeks later, plants were either inoculated (P+) or not inoculated (P-) with *P. nicotianae*.

(non-inoculated with AM fungi and *Phytophthora*, and without exudate enrichment) were significantly different from rhizosphere communities of plants only inoculated with *G. intraradices* ($P<0.05$) or *G. mosseae* ($P<0.1$). Meanwhile, no significant difference was measured between these three treatments when *P. nicotianae* was inoculated. The rhizosphere bacterial communities of plants inoculated with the two mycorrhizal species presented no significant difference between each other, with or without *P. nicotianae* inoculation. No significant difference was neither observed between rhizosphere bacterial communities of non-AM plants supplied with exudates from non-mycorrhizal plants or from plants colonized either with *G. intraradices* or *G. mosseae*, with or without inoculation with *P. nicotianae*. The application of exudates from non-mycorrhizal plants on control plants (non-inoculated with AM fungi and *Phytophthora*) did not induce a significant modification of the bacterial community. Mahalanobis distances between clusters were not significant between the direct inoculation of *G. intraradices* and the application of exudates from plants colonized with this mycorrhizal species, but was significant between the direct inoculation with *G. mosseae* and the supply of exudates from plants colonized with the same mycorrhizal species ($P<0.1$), with and without *P. nicotianae* inoculation. The application of *P. nicotianae* did not significantly change the community of control plants, nor the community of plants colonized with either *G. intraradices* or with *G. mosseae*, or supplied with exudates (collected from non-AM inoculated plants or from plants colonized with *G. intraradices* or with *G. mosseae*). In summary, the results (Table 2 and Fig. 4) clearly indicate that PCR-DGGE profiles of 16S rRNA gene fragments from tomato rhizosphere were significantly affected by direct root colonization with either *G. mosseae* or *G. intraradices* but were poorly influenced by *Phytophthora* inoculation and exudate application.

After cloning, ninety-eight unique sequences were obtained from the pooled 16S rRNA gene fragments from the rhizosphere of mycorrhizal tomato plants (Tables 3–5). All sequences showed a percentage of similarity higher than 94% with sequences registered in GenBank, except clones TR77, TR80 and TR81 that showed an identity of 88–90% with sequences of delta proteobacteria. Sequencing the 16S rRNA gene was efficient to identify to the level of species for 7.1%, genus for 43.9%, family or order for 16.3% and phylum or class for 29.6% of the clones.

Table 3
Bacterial taxa specifically detected by DGGE in the rhizosphere of tomato plants inoculated with AM fungi and/or watered with exudates from mycorrhizal tomato plants, after six weeks of growth.

Band[a]	Clone	Most closely related taxa	Similarity %[b]	Accession number[b]	Treatment applied[c]
14	TR01	*Pseudomonas* sp.	99.88	FJ225208	*G. mosseae*
17	TR02	*Pseudomonas entomophila*	100	FJ905910	*G. intraradices* *G. mosseae*
		Pseudomonas putida		FJ897848	
		Pseudomonas plecoglossicida		FJ587217	
		Pseudomonas sp.		EU747694	
26	TR03	*Bacillus* sp.	99.87	AY690698	*G. intraradices*
		Bacillus simplex	99.75	GQ199716	
38	TR04	Uncultured bact.	98.34	DQ537535	*G. mosseae*
		Herbaspirillum sp.	98.21	EU599848	
56	TR05	Uncultured *Acidobacterium* sp.	99.16	AB257649	*G. mosseae*
48	TR06	Uncultured bact.	97.05	EU135494	*G. intraradices*
		Uncultured *Verrucomicrobium* sp.	94.13	AJ401111	*G. mosseae* *Exudates from AM roots*

[a] Bands were numbered in order of appearance from top to bottom on the DGGE gels.
[b] Percent similarity and accession number of sequences with first closest match and then closest match with named sequences with a percent similarity limit of 90% from the GenBank database.
[c] Inoculation with *G. intraradices* or *G. mosseae* or application of exudates from roots colonized with *G. intraradices* or *G. mosseae* on non-mycorrhizal plants.

Only clones TR37, TR57 and TR77 were not identified at least to the phylum level. Out of 60 different migrating positions observed on DGGE analyses of rhizosphere samples, 47 had a similar electrophoretic mobility with products from clones and were identified. However, 22 clone PCR products (10 Proteobacteria, 5 Gemmatimonadetes and 4 Acidobacteria) had migration positions not matching with any band from rhizosphere samples (data not shown). Some clone products also showed common migration positions. For example, clones TR46, TR47 and TR48 (identified as *Pseudomonas*) all presented a common migration position with band 8 from tomato rhizosphere. Of them, sequences TR46 and TR47 had only 2 bases of difference while TR46 and TR48 showed 6 differences. On the contrary, PCR products of clones TR51 and TR52 co-migrated with band number 12 but their sequences showed only a weak 78.9% of homology. Fifteen out of 16 bands occurring in all samples were identified as Acidobacteria (7), *Bacillus* (2), *Exiguobacterium* (1) and other Firmicutes (2), *Pseudomonas* (1), *Acinetobacter* (2), *Curvibacter* (2), *Sphingomonas* (2), *Ramlibacter* (4), *Rhizobium* (1) and other Proteobacteria (8), Bacteroides (2) and Actinobacteria (1) (Table 4). Other *Pseudomonas* (1), *E. coli* (1), other *Sphingomonas* (1) and other Proteobacteria (6), other Bacteroides (6), Aquificae (1) and Verrucomicrobia (2) were also highly represented in tomato rhizosphere, being detected in all treatments but not in all samples. Out of eight migrating positions detected in the tomato rhizosphere and specific to mycorrhizal inoculation, five were unambiguously identified, including *Pseudomonas* (2), *Bacillus simplex* (1), *Herbaspirilium* (1) and *Acidobacterium* (1) (Table 3). One additional band, identified as a *Verrucomicrobium*, was common to rhizospheres of mycorrhizal plants and of plants watered with mycorrhizal root exudates, but was never detected in control plant rhizospheres. Nine other bands were found to be present in some treatments but absent in others and were all identified as Proteobacteria, except one Planctomycetale (Table 5).

4. Discussion

The PCR-DGGE analysis of 16S rRNA gene fragments clearly showed that direct root colonization with either *G. mosseae* or *G. intraradices* significantly modified the bacterial community structure of tomato rhizosphere, six weeks after sowing. Meanwhile, under our experimental conditions, the enrichment of tomato rhizosphere with exudates produced by tomato plants colonized with either AM fungi species did not lead to significant changes of the bacterial community structure. The bacterial community was not significantly different between the *G. mosseae* and *G. intraradices* mycorrhizosphere after six weeks of growth. Cloning permitted to successfully identify 62.5% of the bacterial taxa associated with the rhizosphere of tomato plants inoculated with AM fungi to various taxonomic levels. Three specific bands associated with *G. mosseae* were identified as *Pseudomonas*, *Herbaspirilium* and *Acidobacterium* while a *B. simplex* (clone TR03) was found to be associated only with *G. intraradices*. One clone (TR2) associated with both *G. intraradices* and *G. mosseae* was ambiguously identified as *Pseudomonas entomophila*, *Pseudomonas plecoglossicida* or *Pseudomonas putida*. Bands specific to inoculation with *G. intraradices* or *G. mosseae* were removed from the discriminant analyses because of the distortion provoked by rare individuals in this type of analysis. The significant impact of AM fungi inoculation on the bacterial community structure showed by this analysis was therefore determined by significant changes in detection frequencies of a large number of bacterial taxa rather than only by a reduced number of taxa specific to AM inoculation.

Using a similar PCR-DGGE approach, Marschner et al. (2001) previously brought to light differences among rhizosphere bacterial communities between maize plants colonized with *G. mosseae* or with *G. intraradices*, and non-mycorrhizal plants. The two AM fungi species had similar bacterial communities after four weeks while

Table 4
Bacterial taxa detected by DGGE in all treatments in the rhizosphere of tomato plants with treatments-dependent detection frequencies, after six weeks of growth.

Band[a]	Clone	Most closely related taxa	Similarity %[b]	Accession number[b]	Present in all samples
2	TR07−TR12	Uncultured soil bact.	99.19−99.45	DQ123729	Yes
		Uncultured Acidobacteria bact.	98.50−98.77	AY921847	
3	TR13	Uncultured Firmicutes bact.	99.73−100	EF072958	Yes
	TR14				
4	TR43	Uncultured Flexibacteraceae bact.	99.61	EF072248	No
5	TR44	Uncultured Bacteroidetes bact.	99.36	EF663410	No
		Uncultured Saprospiraceae bact.	96.85	EU177690	
6	TR45	Uncultured bact.	99.50	AB686245	No
		Uncultured Flavobacteria bact.	98.90	EF072693	
8	TR46	*Pseudomonas* sp.	99.73−100	EU681010	No
	TR47	*Pseudomonas putida*		EU111737	
		Pseudomonas umsongensis		FJ592169	
	TR48	Uncultured *Pseudomonas* sp.	99.10	AM936017	
		P. putida		EF143407	
9	TR15	*Pseudomonas* sp.	99.63	FJ889622	Yes
		Pseudomonas frederiksbergensis		AY785733	
10	TR49	Uncultured Verrucomicrobia bact.	96.75	EF651216	No
11	TR50	*Pseudomonas fluorescens*	99.75	DQ095891	No
12	TR51	*Pseudomonas* sp.	99.76	AM911656	No
		P. putida		EF615007	
	TR52	Uncultured bact.	95.75	EU262312	
		Niastella sp.	95.50	EU877263	
13	TR53	Uncultured soil bact.	98.16	EU365209	No
		Uncultured *Adhaeribacter* sp.	96.07	EU362135	
18	TR16	*Flavisolibacter ginsengiterrae*	99.03	AB267476	Yes
19	TR17	*Solimonas* sp.	94.60	EU903271	Yes
	TR18	*Acinetobacter* sp.	99.88	AJ551148	
	TR19	Uncultured *Acinetobacter* sp.	99.02	FJ268994	
20	TR54	Uncultured Bacteroidetes bact.	99.61	AY921683	No
		Uncultured bact.	99.08	FJ615857	
		Uncultured Flavobacteria bact.	99.08	EF664335	
	TR55	Uncultured soil bact.	99.63	DQ297978	
		Uncultured Flavobacteria bact.	99.50	EF651263	
23	TR20	Uncultured Flavobacteria bact.	97.43	EF082914	Yes
25	TR21	*Exiguobacterium sibiricum*	99.76	FJ795654	Yes
	TR22	Uncultured bact.	98.50	AB273845	
	TR23	Uncultured *Curvibacter* sp.	98.24	EF663074	
		Uncultured *Curvibacter* sp.	98.86	EF074067	
30	TR56	*Escherichia coli*	99.82	CP001396	No
	TR57	Uncultured bact.	99.50	EU809100	
	TR58	Uncultured Aquificae bact.	98.61	EF664530	
35	TR24	Uncultured proteobacterium	93.36	EF664147	Yes
36	TR59	Uncultured *Sphingomonas* sp.	98.41	EF651669	No
37	TR60	Uncultured *Ralstonia* sp.	96.25	FN394971	No
39	TR61	Uncultured beta proteobacteria	98.11	EF663247	No
		Uncultured Oxalobacteraceae bact.	96.86	EF018088	
41	TR25	*Sphingomonas alaskensis*	99.61	AM403496	Yes
		Sphingomonas sp.		AF367204	
	TR26	*Sphingomonas* sp.	99.47	AF191022	
43	TR62	Uncultured ammonia-oxidizing bact	99.24	AB474998	No
	TR63	Uncultured Verrucomicrobia bact.	97.29	AY922067	
44	TR27−TR30	Uncultured beta proteobacterium	99.62−100	AB293358	Yes
		Uncultured *Ramlibacter* sp.	98.49−98.86	EU299170	
	TR31	Uncultured *Rhizobium* sp.	98.42	FJ712877	
46	TR32	*Bacillus* sp.	99.25	EU939690	Yes
	TR33	*Bacillus* sp.	99.37	EF522797	
	TR34	Uncultured bact.	98.98	AM910088	
		Uncultured sulfur-oxidizing symbiot bact.	94.56	AM35642	
	TR35	Uncultured Acidobacteriaceae bact.	98.79	DQ167080	
47	TR36	Uncultured *Ralstonia* sp.	96.48	FN394973	Yes
49	TR37	Uncultured bact.	95.33	EF516243	Yes
	TR38	Uncultured alpha proteobacterium	98.39	AY922001	
		Uncultured Hyphomicrobiaceae bact.	97.52	EF073324	
50	TR39	*Arthrobacter* sp.	100	EU787019	Yes
52	TR64	Uncultured alpha proteobacterium	97.73	AJ536858	No
		Hyphomicrobium sp.	97.56	GQ13142	
54	TR65	*Devosia* sp.	99.44	FJ687971	No
55	TR40	Uncultured *Rhodoplanes* sp.	99.46	EF074179	Yes
	TR41	Uncultured beta proteobacterium	99.61	EF662431	
		Uncultured Methylococcales bact.	99.22	EU276554	
	TR42	Uncultured beta proteobacterium	96.83	AM935240	
		Uncultured Rhodocyclales bact.	95.84	EU043597	
57	TR66	*Chelatovorus multitrophus*	100	EF457243	No
		Mesorhizobium sp.		CP000390	

[a] Bands were numbered in order of appearance from top to bottom on the DGGE gels.
[b] Percent similarity and accession number of sequences with first closest match and then closest match with named sequences with a percent similarity limit of 90% from the GenBank database.

Table 5
Bacterial taxa not detected in all treatments by DGGE in the rhizosphere of tomato plants with treatments-dependent detection frequencies, after six weeks of growth.

Band[a]	Clone	Most closely related taxa	Similarity %[b]	Accession number[b]
15	TR67	Uncultured Oxalobacteraceae bact.	99.62	EU641157
24	TR68	Uncultured Sphingomonadaceae bact.	97.09	EF020183
27	TR69	Uncultured soil bact.	98.93	AY989339
		Oxalobacteraceae bact.	98.38	DQ113445
28	TR70	*Hydrogenophaga pseudoflava*	99.87	AF078770
29	TR71	Uncultured soil bact.	97.30	AY493942
		Uncultured *Microbulbifer* sp.	94.62	DQ167111
34	TR72	*Hydrogenophaga* sp.	99.87	AB271047
42	TR73	Uncultured *Afipia* sp.	100	EF650896
45	TR74	Uncultured *Ralstonia* sp.	96.48	FN394973
58	TR75	Uncultured Burkholderiaceae bact.	98.55	AM935631
	TR76	Uncultured bact.	95.11	DQ129385
		Uncultured Planctomycetales	94.99	AF445727

[a] Bands were numbered in order of appearance from top to bottom on the DGGE gels.
[b] Percent similarity and accession number of sequences with first closest match and then closest match with named sequences with a percent similarity limit of 90% from the GenBank database.

these differed after seven weeks. The differences were root-zone specific and were also more important after seven than after four weeks. Similar trends were observed between *G. intraradices* and *G. deserticola* inoculated on canola (a non-mycorrhizal species), clover and two different tomato genotypes (Marschner and Timonen, 2005). After plant genotype, mycorrhizal colonization was the factor that mostly influenced soil-microbial community, and a plant genotype-fungus interaction suggests that the mycorrhizal effect depends on the associated plant species. In the present study, even if the *G. mosseae* spores were surface-disinfected, a proportion of the spores likely still harboured bacteria that probably proliferated as the AM fungus was invading the rhizosphere, while this was not the case for *G. intraradices*, which was inoculated from *in vitro*-produced spores. This may explain the slightly higher bacterial diversity associated with *G. mosseae* inoculation, but was not sufficient enough to induce significant differences between community structures associated with these two AM fungi.

Analysis of the phospholipid fatty acid profiles associated with various AM fungi inocula showed that bacterial communities clearly diverged (Rillig et al., 2005). Furthermore, clustering analysis of 16S rDNA PCR-DGGE data revealed that bacterial community on the surface of *Gigaspora margarita* and *Gi. rosea* spores were different (Long et al., 2008). The bacterial community associated with *Glomus geosporum* and *Glomus constrictum* spores was also shown to be strongly influenced by the mycorrhizal species but weakly by the host plant species (*Hieracium pilosella* and *Plantago lanceolata*) (Roesti et al., 2005). In this work, scanning electron microscopy of *G. geosporum* spores showed erosion of the spore's outer layer and production of mucilaginous material suggesting a bacterial saprophytic activity. Bacterial adherence to spores and hyphae of several AM species, in aseptic conditions, was reported to depend on the bacterial strain and on the fungal species and vitality (Levy et al., 2003; Toljander et al., 2006). Recently, Bharadwaj et al. (2008a) found that species assemblages of cultivable bacteria from surface-disinfected spores of *G. mosseae* and *G. intraradices* were influenced both by fungal and plant species, with spore-type being the most prominent factor. Ten species including *Bacillus brevis* and *P. putida* were associated with both AM fungi species. The authors hypothesized that this effect depends on spore size and surface roughness. The capacity to adhere to *G. intraradices* structures by different bacterial species was previously shown to depend on the capacity to form biofilms (Bianciotto et al., 2001a, 2001b). Thus, AM fungi may specifically favour the proliferation of some bacteria on mycorrhizal structures, serving as substrate or interacting with them. Therefore, nutritional and habitat requirements of the bacteria associated with AM fungi as well as their mechanisms of attachment to mycorrhizal structures will need to be studied to understand their specific interactions with AM fungi.

Pseudomonas species can show various biological effects and were commonly found in association with mycorrhizosphere or AM spores (Andrade et al., 1997; Artursson and Jansson, 2003; Xavier and Germida, 2003; Roesti et al., 2005; Bharadwaj et al., 2008b). The biocontrol of *P. nicotianae* by a *P. putida* was previously shown (Steddon et al., 2002). A *P. putida* and a *P. fluorescens* collected from spores of *G. intraradices* strongly inhibited the growth of *Rhizoctonia solani*, *in vitro* (Bharadwaj et al., 2008b). The bacteria identified in this study in the presence of AM fungi could similarly be antagonistic to soil-borne pathogens and be involved in the AM mediated biocontrol of *P. nicotianae* previously shown (Lioussanne et al., 2009). An *Acidobacterium* has been identified from spores of *G. geosporum* (Roesti et al., 2005) while no *B. simplex* and *Herbaspirillum* were identified before in association with AM structures. Three additional bacteria were specifically found in the presence of either *G. mosseae* or *G. intraradices* but not identified and may also contribute to this effect.

A *Verrucomicrobium* sp. was absent in control treatments and common to the rhizospheres of mycorrhizal plants and of plants having received mycorrhizal root exudates, and would thus be stimulated by mycorrhizal root exudates. To our knowledge, this is the first time a *Verrucomicrobia* is identified associated with AM fungi. Here, this is the only taxa associated to both direct inoculation and application of mycorrhizal root exudates, therefore not supporting that modification of root exudates mediated by mycorrhizal colonization would be a significant factor in AM fungi impact on rhizosphere bacterial communities. Exudates collected from AM fungi mycelia or from mycorrhizal roots were previously shown to affect microbial activity or chemotaxy *in vitro* (Filion et al., 1999; Sood, 2003; Lioussanne et al., 2008). The effect was either positive or negative depending on the microbial species. Recently, a bacterial community extracted from soil was shown to be significantly affected after 48 h when inoculated with exudates produced by AM mycelia in comparison to a control composed of culture medium (Toljander et al., 2007). Nonetheless, in soil, reduction in exudation through defoliation of pea plants did not modify the DGGE profile of rhizosphere bacteria, whereas missing and additional bands where observed from the rhizosphere of plants precolonized with *G. intraradices* (Vestergård et al., 2008). These findings along with the present results suggest that the rhizosphere microbial community might be much more sensitive to the direct presence of AM fungi mycelium than to a symbiotically-mediated modification in root exudation. Moreover, because of its strong sensitivity to various factors such as plant species, root zone, local soil structure, organic nutrients, pH, temperature (Garbeva et al., 2004; Ehrenfeld et al., 2005), the impact of exudates on the microbial community might be more important *in vitro* than in soil.

The high microbial activity measured in the rhizosphere in comparison to the bulk-soil was often believed to rely on the supply of nutrients liberated by roots through exudation. Soil or rhizosphere enrichment with artificial exudates shifted the microbial community structure more and more consistently as substrate concentration load was increased (Baudoin et al., 2003; Pennanen et al., 2004). However, crude exudates collected from tomato roots may have different effects compared to artificial exudates containing only low molecular weight molecules. We nonetheless cannot rule out the possibility that, in this study, the amount of exudates supplied to the tomato rhizosphere was not sufficient to significantly modify the bacterial community structure in comparison to the water control. Significant differences between the application of mycorrhizal and non-mycorrhizal plant exudates on the bacterial community may have not developed for the same reason. It is also possible that molecules

responsible for the effect of AM fungi mycelium on bacterial community were not hydrophilic and then not recovered by the protocol used to prepare the exudates. Nonetheless, with similarly prepared and concentrated root exudates, Pinior et al. (1999) noted that *G. mosseae*-colonized cucumber exudates inhibited further mycorrhizal colonization, showing that bioactive molecules were present in the exudates. The effect was later shown to be systemic and independent on the presence of the AM fungus mycelium since the exudates collected from the non-mycorrhizal roots of mycorrhizal plants in a split root system induced similar results (Vierheilig et al., 2003). If similar molecules were involved in the inhibition of *P. nicotianae* zoospore chemotaxy observed *in vitro* (Lioussanne et al., 2008), the absence of effects noticed here would less likely be due to an inadequate exudate preparation protocol or to the adsorption of molecules on soil clay particles. Lynch and Whipps (1990) calculated that exudates of barley and maize contained only 9–10% of the amount of substrate required to support the microbial biomass quantified in their rhizosphere. Moreover, Lugtenberg et al. (1999) reported that the ability of *P. fluorescens* WCS365 to use sugars does not play a major role in tomato root colonization. They showed that the mutant PCL1083 from WCS365 impaired in the ability to grow on simple sugars reached the same population levels at the root tip as the wild-type strain. It was also pointed out that bioavailability of some amino acids detected in tomato exudates was too low to support root tip colonization by auxotrophic mutants of *P. fluorescens* strain WCS365. The genes involved in amino acid synthesis were therefore required for root colonization (Simons et al., 1997). Taken together, these facts indicate that microbial establishment in the mycorrhizal rhizosphere would not be strongly related to the supply of exudates but rather would mainly depend on other mechanisms.

Nonetheless, in a case, *G. intraradices* had a negative effect on the population of *P. fluorescens* DF57 both in the rhizosphere and the mycosphere of cucumber plants (Ravnskov et al., 1999). The bacteria did not attach to AM fungus hyphae and were not able to use the hyphae as carbon substrate. The authors postulated that competition for inorganic nutrients other than P could explain their results. An overall decrease of microbial activity after root colonization by AM fungi has also been previously suggested to be due to competition for substrates (Christensen and Jakobsen, 1993; Raiesi and Ghollarata, 2006). Such competition might have however been nullified in the present experiment when exudates from mycorrhizal plants were applied to the rhizosphere of non-mycorrhizal plants to mimic mycorrhizal colonization effects.

In our study, contrarily to the two AM fungi species, inoculation with *P. nicotianae* did not modify the bacterial community structure. The effect of AM fungi on the bacterial community was also not affected by inoculation with the pathogen. Two other Oomycetes taxa, *P. cryptogea* and *Pythium aphanidermatum* were shown not to induce significant changes in the bacterial community found in tomato hydroponic systems (Calvo-Bado et al., 2006). The authors proposed that the microbial communities that established early in the soilless system they used became dominant and resistant to perturbations such as the introduction of a pathogen. Contrarily, Yang et al. (2001) reported different microbial communities in the rhizosphere of healthy and diseased avocado trees infected with *P. cinnamomi*, but when the plants were repeatedly inoculated with a biocontrol *P. fluorescens* strain, the bacterial community was similar to that of healthy plants. At onset of the present experiment, the growth substrate received a standardized soil microflora exempt of AM fungi, which was later significantly affected by AM fungi inoculation but not by *P. nicotianae*. Since AM fungi are carbon sink for plants and constitute a significant part of the soil microbial biomass (Hamel, 2007), they may have a larger impact on the rhizosphere microbes than opportunistic organisms such as *P. nicotianae*. Consequently, AM fungi may favour the establishment in the mycorrhizosphere of stable and resilient microbial communities contrarily to root pathogens, hampering that way their proliferation in the vicinity of roots.

Most bands of the PCR-DGGE were identified to the species or genus level. However, some bands corresponded to several bacterial taxa suggesting that this technique underestimated the bacterial diversity associated with tomato rhizosphere. A low number of studies previously identified bacterial taxa from tomato rhizosphere including mainly Cytophaga-Flavobacterium-Bacteroids (particularly *Flavobacterium aquatile* and *Flexibacter aggregans*) and Proteobacteria (notably *P. putida*) phyla (Kim et al., 2006) but also a *Sphingomonas* sp., three Acidobacteria, a Verrucomicrobia and a *Bradyrhizobium* (Felici et al., 2008). Here, the bacterial taxa identified in most samples were similar but more diverse compared with those previously reported since they were in majority represented by Proteobacteria (including two *P. putida*, five other *Pseudomonas*, four *Ramlibacter*, three *Sphingomonas*, two *Curvibacter*, two *Acinetobacter* and one *E. coli*), seven Acidobacteria, nine Bacteroides (including *Flavobacteria*) and two Verrucomicrobia phyla, even if some Firmicutes (especially three Bacillus) were also encountered. However, the abundance of other Proteobacteria and a Planctomycetale was significantly modified with mycorrhizal inoculation while sequence variants of *Acidobacterium*, *B. simplex*, *Herbaspirilium* and *Pseudomonas* were also specifically associated with the presence of AM fungi.

In conclusion, the results presented here suggest that AM fungi significantly impact the rhizosphere microbial community through mechanisms not related with root exudation of the host plant. The AM fungi may compete for space and nutrients with some microorganisms, or serve of nutritional resource or habitat for others. The direct presence of AM fungi mycelia might also be essential for competence of specific bacteria within the mycorrhizosphere.

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