Soil Biology & Biochemistry 42 (2010) 473-483



Contents lists available at ScienceDirect

Soil Biology & Biochemistry



journal homepage: www.elsevier.com/locate/soilbio

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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ARTICLE INFO

Article history: Received 19 March 2009 Received in revised form 23 November 2009 Accepted 29 November 2009 Available online 8 December 2009

Keywords: Mycorrhizosphere Arbuscular mycorrhizal fungi Phytophthora nicotianae Bacterial community Exudates DGGE

ABSTRACT

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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^{0038-0717/\$ -} see front matter \odot 2009 Elsevier Ltd. All rights reserved. doi:10.1016/j.soilbio.2009.11.034

(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: Toliander et al., 2007) and citric acid (Tawarava 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 $5 \times$ 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) CaCO₃, 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, $50 \times 16 \times 2.5$ 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 noncompartimentalized 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



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–).

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. 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^{Gi}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 uL volumes. The soil genomic DNA (for the first round) or the amplification products (for the second round) were diluted to 1:100 and 2 uL were added to 1 uL of primers (10 µM, Alpha DNA, Montreal, QC), 1 µL of dNTPs mix (10 mM), 0.5 μ L of bovine serum albumin (BSA, 100×, New England Biolabs, Ipswich, MA), 5 μ L of 10 \times PCR buffer and 2.5 μ L 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 Tag 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 $1 \times$ 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 μ L 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

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
pН	Entire 16S	5'-AAGGAGGTGATCCAGCCGCA-3'	Edwards et al., 1989
341F + GC	V3	5'CGCCCGCCGCGCGCGGCGGGCG	Muyzer et al., 1993
		GGGCGGGGGGCACGGGGGGCCTAC	
		GGGAGGCAGCAG-3'	
534 R	V3	5'-ATTACCGCGGCTGCTGG-3'	Muyzer et al., 1993

filled with 27 mL of TSB. When OD₆₀₀ 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.

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 μ L 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 presenceabsence 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
EGm	G-	P-	40	35.0%	0
E^{G-}	G-	P+	42	36.6%	0
E ^{Gi}	G-	P+	42	38.3%	0
EGm	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. intra-radices*; 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	100	FJ905910	G. intraradices
		entomophila			G. mosseae
		Pseudomonas putida		FJ897848	
		Pseudomonas		FJ587217	
		plecoglossicida			
		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	99.16	AB257649	G. mosseae
		Acidobacterium sp.			
48	TR06	Uncultured bact.	97.05	EU135494	G. intraradices
		Uncultured	94.13	AJ401111	G. mosseae
		Verrucomicrobium sp.			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.

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20R54 Uncultured bact.99.61#7921683NoUncultured Pavobacteria bact.99.08FIG1587FIG128323R55Uncultured Pavobacteria bact.99.60FIG128323R200Uncultured Pavobacteria bact.99.50FIG128324R210Uncultured Pavobacteria bact.97.60FIG128325R21Uncultured Pavobacteria bact.99.60R27384Yes26R22Uncultured Carribacters p.98.824EF663074		TR19	Uncultured Acinetobacter sp.	99.02	FJ268994	
Inclutured back.99.08Fj615857TR55Unclutured Flavobacteria back.99.63DQ29797823TR20Unclutured Flavobacteria back.99.63DQ29797823TR20Unclutured Flavobacteria back.97.43EF082914Yes24TR21Exigoboacterian solvicurum99.76Fj79564Yes7820Uncultured Carubacterian solvicurum98.80AB273845Yes7821Uncultured Carubacterian solvicurum98.86EF074067Yes7823Uncultured Carubacterian solvicurum98.86EF074067Yes7835Uncultured Aquifaca bact.98.50EB09100Yes7856Escherichia coli98.50EB09100Yes7857Uncultured Aquifaca bact.98.50EF66430Yes7858Uncultured Aguifaca bact.98.61EF651669No797TR60Uncultured aphiganomas sp.98.52FN94971No798TR55Uncultured abstensis99.61AM043966Yes797TR61Uncultured annonia-soldizing bact.95.26FI918078Yes798Uncultured annonia-soldizing bact.99.24Ad474998No799TR24Uncultured annonia-soldizing bact.99.24Ad474998Yes798Uncultured Marcomicopia bact.97.29AP322067Yes798Uncultured annonia-soldizing sp.99.24Ad474998Yes799TR25Uncultured Marcomicopia bact.99.	20	TR54	Uncultured Bacteroidetes bact.	99.61	AY921683	No
Incluited Flavobacteria bact.99.08FF664331R850Uncluited Soil bact.99.50EF65126323R720Uncluited Flavobacteria bact.99.50EF65126324IR21Uncluited Flavobacteria bact.99.76EJ795554Yes25R721Uncluited Bact.99.76EJ795554Yes7822Uncluitued bact.99.76EJ795554Yes7833Uncluitued bact.99.82EF663074			Uncultured bact.	99.08	FJ615857	
TRS5Uncultured soli bact.99.63DQ29797823TR20Uncultured Flavobacteria bact.97.43EF082914Yes25TR21Exguobacterian solitricum97.74EF082914Yes78.20Uncultured Earobacteria bact.98.50A8273845Yes78.21Uncultured Curvibacter sp.98.86EF074067Yes78.31Uncultured Curvibacter sp.98.86EF074067Yes78.56Escherichia coli99.50EU809100Yes78.57Uncultured Aquiface bact.98.61EF664147Yes36TR59Uncultured philogononas sp.98.41EF665166No37TR60Uncultured obespretobacteria98.18EF664147Yes39TR61Uncultured obespretobacteria98.61EF064147Yes39TR51Uncultured obespretobacteria98.61EF064147Yes39TR61Uncultured obespretobacteria98.61EF064147Yes39TR52Sphiligononas sp.94.7AF1020Yes41TR25Sphiligononas sp.94.62F17887Yes42TR25Sphiligononas sp.99.61AP474998Yes43TR62Uncultured heta proteobacteria99.62AP37208Yes44TR27-TR30Uncultured heta proteobacteria99.62AP374087Yes44TR27-TR30Uncultured Mathoff sp.93.46F172877Yes45Uncul			Uncultured Flavobacteria bact.	99.08	EF664335	
33 R20 Uncultured Flavobacteria bact. 99.50 FF631283 25 R21 Lögubbacterium sibricum 99.76 F795654 Yes 1722 Uncultured Larvibacter sp. 98.50 A273845 Tess 1723 Uncultured Curvibacter sp. 98.86 EF663704 Tess 17657 Uncultured Sp. 99.50 E0809100 Tess 17657 Uncultured partebacterium 93.66 EF664147 Yes 1765 Uncultured partebacterium 93.65 EF664147 Yes 17660 Uncultured partebacterium 93.66 EF664147 Yes 1760 Uncultured Partebacteria 98.11 EF651267 No 1760 Uncultured Statomios sp. 98.11 EF651267 No 1761 Uncultured Automios sp. 98.11 EF651267 No 1761 Uncultured Automios sp. 99.47 AF191022 Tes 1765 Sphingomonas sp. 99.47 AF191022 Tes 1766 Sp		TR55	Uncultured soil bact.	99.63	DQ297978	
23IRQ0Uncultured Involvateria batc.9/43EH08.914Yes782IRQ1Explored action as provided and as provided at a specific as provided at			Uncultured Flavobacteria bact.	99.50	EF651263	
25 IR21 Local uncel auxilianted auxiliantexiliantexilianted auxilianted auxiliantexilianted auxilianted aux	23	TR20	Uncultured Flavobacteria bact.	97.43	EF082914	Yes
Inc.2Uncultured curvibater sp.98.24EF66307430TR56Escherichia coli98.84EF707467TR57Uncultured Auribater sp.98.86EF707467TR57Uncultured Aurificae bact.99.82CP001396No35TR24Uncultured Aurificae bact.98.61EF6645036TR59Uncultured Aurificae bact.93.86EF66447Yes36TR59Uncultured Aurificae bact.98.811EF665166No37TR60Uncultured Aurificae bact.98.811EF665247No39TR61Uncultured bact proteobacteria98.11EF665247No39TR61Uncultured Aurificae bact.96.86EF01808841TR25Sphingomonas sp.AM303496Yes5TR62Uncultured Aurifica bact.97.29A92206743TR62Uncultured Aurificater sp.99.47AF19102244TR27-TR30Uncultured Aurificater sp.99.48EU2917044TR27-TR30Uncultured Aurificater sp.99.62-100A8293358Yes46TR31Uncultured Aurificater sp.99.62A03564247TR36Uncultured Aurificater sp.99.37EF5224797Yes47TR36Uncultured Aurificater sp.95.33EF516243Yes47TR36Uncultured Aurificater sp.95.34M91008847TR36Uncultured Aur	25	IKZI TECO	Exiguodacterium sidiricum	99.76	FJ/95054	Yes
Inclusion Onclusion Processing Processing 30 TK56 Escherichia coli 99.82 CP001396 No 31 TK57 Uncultured bact. 99.50 EU809100		TR22	Uncultured Curvibacter sp	98.50	FF663074	
30 TR56 Escherichia coli 99.82 CP001306 No 7R57 Uncultured bact. 99.50 EU809100 - 315 TR24 Uncultured porteobacterium 93.61 EF66430 - 35 TR24 Uncultured porteobacteria 98.61 EF664167 Yes 36 TR59 Uncultured Splingomonas sp. 98.41 EF651669 No 37 TR60 Uncultured Malsonia sp. 98.61 AM043966 Yes 39 TR51 Uncultured Malsonia sp. 99.61 AM043966 Yes 41 TR25 Splingomonas sp. 99.61 AM043966 Yes 43 TR62 Uncultured Malsonia sp. 99.62–100 AB274998 No 44 TR27-TR30 Uncultured Malsonia sp. 99.62–100 AB292067 Yes 46 TR31 Uncultured Malsonia sp. 98.49 EU299170 Yes 47 TR30 Uncultured Malsonia sp. 98.49 EU299170 Yes <t< td=""><td></td><td>1125</td><td>Uncultured Curvibacter sp.</td><td>98.86</td><td>EF074067</td><td></td></t<>		1125	Uncultured Curvibacter sp.	98.86	EF074067	
FR57 TR58Uncultured Aquificae bact.99.50 98.61EU80910035TR54 TR54Uncultured Aquificae bact.98.61EF664137Yes36TR59Uncultured Sphingomonas sp.98.41EF661477Yes37TR60 	30	TR56	Escherichia coli	99.82	CP001396	No
F88 35Uncultured Aquificae bact.98.10EF6643035TR24Uncultured proteobacterium93.36EF664147Yes36TR59Uncultured Aghingomona sp.98.41EF651669No37TR60Uncultured Kalstonia sp.98.11EF65247No39TR61Uncultured Chalobacteraceae bact.98.61FR65247No41TR25Sphingomona sp.99.61M403496Yes41TR26Sphingomonas sp.99.61AM03496Yes43TR63Uncultured Manonia-oxidizing bact.99.24AB474998No44TR63Uncultured Verrucomicrobia bact.99.62AP22067Yes44TR37Uncultured Manonia-oxidizing bact.99.62R023358Yes45TR31Uncultured Ramibiacter sp.98.42F1712877Yes46TR32Bacillus sp.99.25EU939500Yes47TR34Bacillus sp.99.25EU939500Yes48TR33Bacillus sp.99.25EU939500Yes49TR34Uncultured Akizonian sp.99.26N035642Yes49TR35Uncultured Akizonian sp.99.26N035642Yes49TR34Uncultured Akizonian sp.99.53HistorianYes49TR35Uncultured Akizonian sp.99.54N034973Yes50TR36Uncultured Akizonian sp.95.6CQ13142Yes <td< td=""><td></td><td>TR57</td><td>Uncultured bact.</td><td>99.50</td><td>EU809100</td><td></td></td<>		TR57	Uncultured bact.	99.50	EU809100	
35TR24Uncultured proteobacterium93.36EF664147Yes36TR59Uncultured falstonia sp.98.41EF651669No37TR60Uncultured kalstonia sp.96.25FN394971No39TR61Uncultured beta proteobacteria98.11EF651269No39TR61Uncultured beta proteobacteria96.86EF018088T41TR25Sphingomonas sp.96.86FT018088T43TR62Uncultured ammonia-oxidizing bact99.47AF191022T43TR63Uncultured beta proteobacterium99.47AF191022T44TR27-TR30Uncultured beta proteobacterium99.47AF29338Yes44TR31Uncultured falizabium sp.98.49R329338Yes46TR32Bacillus sp.98.42EF122377T47TR34Uncultured falizabium sp.99.37EF522797T47TR35Uncultured actificang symbiot bact.95.33EF516243Yes49TR37Uncultured bact.95.33EF516243Yes50TR83Adriba proteobacterium98.39AM395042T52TR64Uncultured alpha proteobacterium95.33EF5162431Yes53TR84Uncultured alpha proteobacterium97.52EF073324Yes54TR83Adribaptores pr.93.46EF074179Yes55TR40Uncultured alpha proteobacter		TR58	Uncultured Aquificae bact.	98.61	EF664530	
36TR59Uncultured Sphingomonas sp.98.41EF651669No37TR60Uncultured Stationia sp.96.25FN349471No39TR61Uncultured beta proteobacteria96.86EF01808841TR25Sphingomonas sp.A4803496Yes43TR62Uncultured ansonia-oxidizing bact99.61A8474998No43TR63Uncultured ansonia-oxidizing bact99.47AF19102243TR62Uncultured errucomicrobia bact.97.29A792206744TR27-TR30Uncultured Ramibacter sp.98.49-98.86EU29917045TR33Bacillus sp.99.47A8293358Yes46TR32Bacillus sp.99.45EU29917047TR33Bacillus sp.99.45EU29917048TR33Bacillus sp.99.45EU29917047TR34Bacillus sp.99.45EU29917048TR32Bacillus sp.99.45AM91008849TR34Uncultured Antibacter sp.98.44PS52279749TR34Uncultured sphapoteobacterium98.79ED16343Yes49TR37Uncultured adobacteriacea bact.98.79D16708049TR37Uncultured Antibacteria97.32EF014334Yes50TR34Uncultured Antibacteria97.32EF073324Yes51TR40Uncultured	35	TR24	Uncultured proteobacterium	93.36	EF664147	Yes
37TR60Uncultured Ralstonia sp.96.25FN34971No39TR61Uncultured Ralstonia sp.96.15FF63247No41TR25Sphingomonas dlaskensis96.66EF01808841TR26Sphingomonas sp.99.61AM403496Yes43TR26Sphingomonas sp.99.47AF19102243TR62Uncultured vernacomicrobia bact.97.29A9206744TR63Uncultured vernacomicrobia bact.97.29A92206744TR32Bacillus sp.98.49EU29917045TR31Uncultured kernacomicrobia bact.97.29A93258Yes46TR32Bacillus sp.98.42FJ71287747TR32Bacillus sp.99.37EF52279748Uncultured Ralizobium sp.98.39AM91008847TR36Uncultured Actidobacteriacea bact.98.79D016708047TR36Uncultured Ralstonia sp.94.53Kr394973Yes49TR37Uncultured Ralstonia sp.95.33Ses38No47TR36Uncultured Ralstonia sp.95.33Kr394973Yes50TR39Uncultured Ralstonia sp.95.33Kr394973Yes51TR44Uncultured Ralstonia sp.97.52EF673324Yes52TR36Uncultured Ralstonia sp.97.33AJ536858No52TR64Uncultured Ralstonia sp.97.73AJ536858No55 </td <td>36</td> <td>TR59</td> <td>Uncultured Sphingomonas sp.</td> <td>98.41</td> <td>EF651669</td> <td>No</td>	36	TR59	Uncultured Sphingomonas sp.	98.41	EF651669	No
39TK61Uncultured beta proteobacteria98.11EF663247No41TR25Sphingomonas alaskensis98.11EF613247No41TR25Sphingomonas alaskensis99.61AM403496Yes5phingomonas spAF191022-43TR62Uncultured ammonia-oxidizing bact99.24AB474998No43TR63Uncultured Verrucomicrobia bact.97.29AY922067-44TR27-TR30Uncultured Ramilbacter sp.98.49-98.86EU299170-46TR31Uncultured Ramilbacter sp.99.25EU939660Yes47TR33Bacillus sp.99.25EU939600Yes48TR33Bacillus sp.99.37EF522797-47TR36Uncultured bact.98.79DQ167080-47TR36Uncultured Alastonia sp.96.48FN304973Yes49TR37Uncultured Alastonia sp.96.48FN304973Yes49TR37Uncultured bact.95.33EF516243Yes50TR39Arthrobacter sp.100EU787019Yes51TR64Uncultured Rhobacter inm97.75GQ1142-52TR64Uncultured Rhodoplanes sp.97.56GQ13142-53TR40Uncultured Rhodoplanes sp.99.46EF07179No55TR64Uncultured Rhodoplanes sp.99.46EF07179Yes54TR40Un	37	TR60	Uncultured Ralstonia sp.	96.25	FN394971	No
41TR20 Sphingomonas claskensis96.86FU1808841TR26Sphingomonas claskensis96.86AM403496Yes43TR62Uncultured ammonia-oxidizing bact99.47AF191022Yes43TR63Uncultured ammonia-oxidizing bact97.29AV922067Yes44TR27-TR30Uncultured Verrucomicrobia bact.97.29AV922067Yes44TR27-TR30Uncultured Ramilbacter sp.98.49-98.86EU299170Yes46TR31Uncultured Ramilbacter sp.98.42FJ712877Yes47TR36Bacillus sp.99.37EF522797Yes48TR33Bacillus sp.99.37EF522797Yes49TR34Uncultured duffu-oxidizing symbiot bact.98.78AM910088Yes49TR35Uncultured suffur-oxidizing symbiot bact.98.79DQ167080Yes47TR36Uncultured dactonia sp.96.48FN394973Yes49TR37Uncultured lapha proteobacterium98.33AF922001Yes50TR39Arthrobacter sp.100EU787019Yes51TR64Uncultured lapha proteobacterium97.75GQ13142Yes52TR65Devosia sp.97.56GQ13142Yes54TR65Devosia sp.99.62EV76543Yes55TR40Uncultured Rhodoplanes sp.99.62EV68771No55TR40Uncultured Rhodoplanes sp.<	39	TR61	Uncultured beta proteobacteria	98.11	EF663247	No
A1IR23Splingonious disseries95.01AVAPO3490TesTR36Splingonious sp.99.47AF19102243TR62Uncultured ammonia-oxidizing bact99.24AB474998No44TR27-TR30Uncultured verucomicrobia bact97.29AY92206744TR27-TR30Uncultured beta proteobacterium99.62-100AE293358Yes46TR31Uncultured Rhibbacter sp.98.4998.86EU299170466TR32Bacillus sp.99.25EU393690Yes7R31Uncultured Rhibbacter sp.99.37EF522797F5227977R34Uncultured bact.98.98AM910088Uncultured suffur-oxidizing symbiot bact.98.98AM91008847TR36Uncultured bact.98.98AM910088Yes47TR36Uncultured lastornia sp.96.48FN394973Yes48TR37Uncultured bact.95.33EF516243Yes49TR37Uncultured alpha proteobacterium98.99AY922001Uncultured lapha proteobacterium50TR39Arthrobacter sp.100EU787019Yes51TR65Devosia sp.99.44FJ687971No52TR64Uncultured Rhodoplares sp.99.44EJ687971No55TR40Uncultured Rhodoplares sp.99.44EJ687971No55TR40Uncultured Rhodoplares sp.99.44EJ687971No55TR40Uncultured Rhodop	41	TDOF	Uncultured Uxalobacteraceae bact.	96.86	EFU18088	Vac
TR36Sphingomonas sp.99.47AF19102243TR62Uncultured ammonia-oxidizing bact99.24AB474998No44TR63Uncultured Verucomicrobia bact.97.29AY92206744TR77-TR30Uncultured beta proteobacterium99.62-100AB293358Yes46TR31Uncultured Rhizobium sp.98.42FJ71287746TR32Bacillus sp.99.25EU939690Yes7833Bacillus sp.99.37EF5227977844Uncultured Rhizobium sp.94.56AM356427873Uncultured bact.94.56AM356427874Uncultured cAdidobacteriaceae bact.98.79DQ16708047TR36Uncultured Acidobacteriaceae bact.95.33EF516243Yes49TR37Uncultured bact.95.33EF516243Yes49TR37Uncultured bact.97.52EF073324Uncultured50TR49Arhrobacter sp.100EU787019Yes51TR64Uncultured Rhodoplane sp.97.56GQ1314254TR65Devsia sp.99.461EF62431Yes55TR40Uncultured Bhadoplane sp.99.61EF662431100EU78574YesYesYes57TR64Chelatovorus multirophus99.22EU76554Yes57TR40Uncultured Bhadoplane sp.99.24EU76554Yes57TR64Chelatovorus multirophus50.88MM	41	IKZJ	Sphingomonas sp	99.01	AM403496 AF367204	Tes
43TR62Uncultured amnonia-oxidizing bact99.24AB474998No44TR63Uncultured amnonia-oxidizing bact.97.29AY92206744923358Yes44TR27-TR30Uncultured beta proteobacterium99.62–100AB293358Yes46TR31Uncultured Ramilbacter sp.98.49–98.86EU29917046047TR33Bacillus sp.99.25EU393690Yes46TR32Bacillus sp.99.37EF52279747047TR34Uncultured Atizobium sp.98.79DQ16708047TR36Uncultured Acidobacteriaceae bact.98.79DQ16708049TR37Uncultured bact.95.33EF516243Yes49TR37Uncultured alpha proteobacterium98.39AY92200150TR39Arthrobacter sp.100EU787019Yes52TR64Uncultured alpha proteobacterium97.56GQ1314254TR65Devosia sp.99.44FJ687971No55TR40Uncultured Rodoplanes sp.99.46EF074179Yes55TR41Uncultured Rodoplanes sp.99.46EF074179Yes57TR66Chelatvorus multitrophus96.83AM935240-57TR66Chelatvorus multitrophus50EF457243No		TR26	Sphingomonas sp.	99 47	AF191022	
TR63 Uncultured Verrucomicrobia bact. 97.29 AY922067 44 TR27-TR30 Uncultured bag proteobacterium 99.62-100 AR293358 Yes 44 TR31 Uncultured Ramibacter sp. 98.49–98.86 EU299170	43	TR62	Uncultured ammonia-oxidizing bact	99.24	AB474998	No
44TR27-TR30Uncultured beta proteobacterium Uncultured Rniibacter sp.99.62-100AB293358Yes46TR31Uncultured Rniibacter sp.98.49-98.86EU2917046TR32Bacillus sp.99.25EU939690Yes7R33Bacillus sp.99.37EF5227977R34Uncultured bact.98.98AM9100887R35Uncultured sulfur-oxidizing symbiot bact.98.79DQ16708047TR36Uncultured Ralstonia sp.95.33EF51624349TR37Uncultured Ralstonia sp.95.33EF51624349TR37Uncultured alpha proteobacterium98.39AY92200150TR39Arthrobacter sp.100EU787019Yes52TR64Uncultured alpha proteobacterium97.73AJ536858No54TR55Devosia sp.99.46EF074179Yes55TR40Uncultured Rodoplanes sp.99.61EF06243155TR41Uncultured Rodoplanes sp.99.61EF074179Yes57NoTR42Uncultured Methylococales bact.99.22EU27655457TR40Uncultured Rodoplanes bact.99.24EU27655457TR40Uncultured Rodoplanes sp.99.61EF074179Yes57NoEU276554Uncultured Methylococcales bact.99.22EU27655457TR40Uncultured heta proteobacterium95.84EU4359757NoEV457243NoMeso		TR63	Uncultured Verrucomicrobia bact.	97.29	AY922067	
46TR31Uncultured Ramlibacter sp.98.49–98.86EU29917046TR32Bacillus sp.99.42FJ71287746TR32Bacillus sp.99.37EF522797TR33Bacillus sp.99.37EF522797TR34Uncultured bact.98.98AM910088Uncultured sulfur-oxidizing symbiot bact.94.56AM35642TR35Uncultured Acidobacteriaceae bact.98.79DQ16708047TR36Uncultured Acidobacteriaceae bact.95.33EF51624349TR37Uncultured bact.95.33EF51624349TR37Uncultured alpha proteobacterium98.39AY92200150TR39Arthrobacter sp.100EU787019Yes52TR64Uncultured alpha proteobacterium97.73AJ536858No54TR65Devosia sp.99.44FJ687971No55TR40Uncultured Rhodoplanes sp.99.44FJ687971No55TR40Uncultured Rhodoplanes sp.99.44EI662431Yes57TR42Uncultured Rhodoplanes sp.99.46EF073179Yes57TR66Chelatovorus multirophus95.84EU043597Yes57TR66Chelatovorus multirophus95.84EU043597Yes57TR66Chelatovorus multirophus50.81EV043597Yes57TR66Chelatovorus multirophus50.81EV043597Yes57TR66Chelatovorus mu	44	TR27-TR30	Uncultured beta proteobacterium	99.62-100	AB293358	Yes
TR31Uncultured Rhizobium sp.98.42FJ71287746TR32Bacillus sp.99.25EU939690YesTR33Bacillus sp.99.37EF522797TR34Uncultured bact.98.98AM910088Uncultured Acidobacteriaceae bact.98.79DQ16708047TR36Uncultured Acidobacteriaceae bact.98.79DQ16708049TR37Uncultured Ralstonia sp.96.48FN394973Yes49TR37Uncultured alpha proteobacterium98.39AY92200170TR38Uncultured alpha proteobacterium97.52EF07332450TR39Arthrobacter sp.100EU787019Yes52TR64Uncultured Rhodoplanes sp.97.73AJ536858No54TR65Devosia sp.99.44FJ687971No55TR40Uncultured Rhodoplanes sp.99.46EF074179Yes55TR41Uncultured Rhodoplanes sp.99.46EF074179Yes57TR62Uncultured Rhodoplanes sp.99.46EF074179Yes57TR64Uncultured Rhodoplanes sp.99.25EU27655457TR66Chelatovorus multitrophus95.84EU04359757TR66Chelatovorus multitrophus95.84EU04359757TR66Chelatovorus multitrophus95.84EU04359757TR66Chelatovorus multitrophus95.84 <td></td> <td></td> <td>Uncultured Ramlibacter sp.</td> <td>98.49-98.86</td> <td>EU299170</td> <td></td>			Uncultured Ramlibacter sp.	98.49-98.86	EU299170	
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 ^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	97.09	EF020183
		Sphingomonadaceae bact.		
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 surfacedisinfected 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 soilborne 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 symbioticallymediated 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.

Acknowledgements

We are grateful to Franck Panabières (INRA Antibes) for providing the *P. nicotianae* isolate 201 and to Stéphane Daigle (Institut de recherche en biologie végétale) for statistical analysis. This work was supported by an NSERC grant allocated to M. St-Arnaud and an FQRNT team grant to M. Jolicoeur, M. St-Arnaud and coll.

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