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Combining potential oomycete and bacterial biocontrol agents as a tool to fight tomato Rhizoctonia root rot

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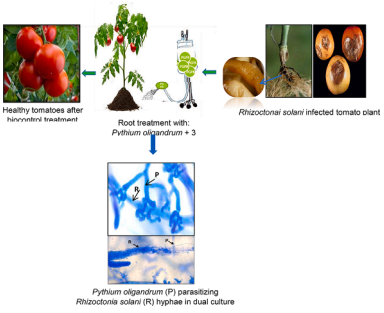
HIGHLIGHTS

- *P. oligandrum* associated with 3 bacteria suppressed tomato Rhizoctonia root rot.
- The microbial association was more effective than the chemical fungicide tested.
- The microbial association enhanced the growth of tomato plants.
- Native rhizospheric bacterial communities depend on the tomato cultivar (SSCP).

GRAPHICAL ABSTRACT



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ABSTRACT

Biological control of Rhizoctonia root rot and growth-promoting potential on two tomato cultivars was performed using *Pythium oligandrum* in combination, or not, with three tomato-associated rhizobacterial strains of *Bacillus subtilis*, *B. thuringiensis* and *Enterobacter cloacae*. The three bacterial strains displayed antifungal activity against *Rhizoctonia solani*, with pathogen growth inhibition up to 60%. *P. oligandrum* also destroyed *R. solani* cells by antibiosis and mycoparasitism processes. In a three-month greenhouse assays, for all trials and the two tomato cultivars used, disease suppression potential, as compared to the controls, was up to 80% using the microorganisms mixtures and 74% by using *P. oligandrum* alone. Increased height in disease-free plants was obtained with the microorganism mixtures (59%) compared to *P. oligandrum* (49%). Plants grown in *R. solani* inoculated peat and challenged with microorganism mixtures were higher than controls, whereas those amended with *P. oligandrum* showed 46 to 87% height increase. All treatments with the microorganisms were more effective in suppressing the disease than chemical fungicide. Increment of aerial parts and root fresh weights on disease-free plants were until 51% for those treated with microorganism mixtures compared to 46% recorded using *P. oligandrum*. On inoculated plants, these parameters were enhanced using the mixed treatment. No differences in the rhizosphere-bacterial populations were observed by Single Strand Conformational Polymorphism (SSCP)

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when, either the rhizobacteria, with or without *P. oligandrum*, or the pathogen, were inoculated. This suggests that the native bacterial communities were not significantly impacted by the various microbial-inoculations. Only differences were obtained for the two tomato cultivars that the native bacterial microflora colonize.

1. Introduction

Plant protection method, *i.e.* biological control, is used as a strategy for reducing disease incidence or severity by applying plant-beneficial microorganisms having direct or indirect effect on the plant pathogens. This work was focused on the rhizosphere because it is a key zone at the frontiers of soil and roots that is widely colonized by microorganisms, including many root pathogens. Here, infection and control of *Rhizoctonia solani* Kühn, one of the most economically important worldwide soil-borne pathogenic fungi able to infect various crops including vegetables (Kurzawinska and Mazur, 2008), were studied.

Microbes associated with plant roots are highly diverse and they are cited as the second genome of the plant due to its important role in plant health and development. For instance, the rhizosphere microbiome has received considerable attention because it has a significant role on the reprogramming of the defense responses of plants (Spence et al., 2014). Plants also shape their rhizospheric microbiome by secreting root exudates (Berendsen et al., 2012). A comprehensive understanding on the mechanisms that govern recruitment and activity of the associated microbes may open up opportunities to utilize the phenomenon in increasing crop productivity (Sarma et al., 2015) and protection. This is a very important item because some microorganisms colonizing the rhizosphere act as potential biocontrol agents.

In previous experiments, traditional methods for controlling it such as cultural practices, solarization and, very frequently chemical control, have been used (Prashar et al., 2013). However, these methods displayed some limitations as they are not effective enough in controlling this disease (Huang et al., 2012). For instance, fungicides can be both expensive and hazardous (Fernando et al., 2007) and, they are losing effectiveness due to the development of resistance in populations of *R. solani.* Therefore, alternative disease control strategy has been developed based on biological control microorganisms (Velusamy and Kim, 2011; Goudjal et al., 2014) and (Binyamin et al., 2019). Rhizobacteria such as *Bacillus subtilis* (Lugtenberg and Kamilova, 2009; Ouhaibi-Ben et al., 2016a), *B. thuringiensis* (Van der Ent et al., 2009; Ouhaibi-Ben et al., 2016a), and *Enterobacter cloacae* (Ouhaibi-Ben et al., 2016a) were shown to be efficient in controlling *R. solani*.

The soil-inhabiting biocontrol oomycete, *Pythium oligandrum*, colonizes the rhizosphere of many crop species and is responsible for reduction of diseases caused by a number of soil-borne fungal pathogens (Benhamou et al., 1999; Vallance et al., 2009; Gerbore et al., 2014); this reduction varies from 15 to 100% (Gerbore et al., 2014). *P. oligandrum* has a mycoparasitic activity against many fungal plant pathogens, including *R. solani* (Benhamou et al., 1999). Additionally, *P. oligandrum* has been reported to promote plant growth (Wulff et al., 1998; Pisarčik et al., 2020) via the production of tryptamine, an auxin-like compound (Le Floch et al., 2003) and (Binyamin et al., 2019), and to induce systemic resistance in many plants (Rey et al., 2008) and (Gerbore et al., 2014).

Since microbes in natural habitats live in communities, it is believed that each microbial component provides specific benefit to plants. Therefore, it has become necessary to test the microbial components of a consortium so that desired benefits can be provided to plants especially under the pathogen challenged conditions (Rudresh et al., 2005). Additionally, biological control has proved to be efficient in preventing some damping off diseases (Velusamy and Kim, 2011). The potential of microbial mixtures to treat plants has been studied in the last decade, and it has been shown that mixed microbial combinations may lead to increased, reduced or similar pathogen suppressive effects (Xu et al., 2011).

In the present study, the ability of various strains of rhizobacteria isolated from tomato rhizosphere [(*Bacillus subtilis* str. B2 (KT921327), *B. thuringiensis* str. B10 (KU158884) and *Enterobacter cloacae* str. B16 (KT921429)] and a *P. oligandrum* strain (Po37) isolated from the rhizosphere of grapevine, to colonize, to protect tomato against *R. solani* and to enhance tomato plant growth, was assessed. Accordingly, we determined whether *P. oligandrum* and rhizobacteria strains, applied individually or in combination, were able to reduce the symptoms, *i.e.* root necroses, on two tomato cultivars and to enhance plant growth. Fungal dynamics in the two tomato cultivars grown and the effects of the rhizobacteria-based treatments on indigenous microbial populations colonizing the rhizosphere were also studied using molecular fingerprinting (Single Strand Conformational Polymorphism-SSCP). SSCP will provide information on the shifts that may occur, or not, in the native rhizosphere-bacterial microflora after introduction of the potential biocontrol agents in the plant rhizosphere.

2. Materials and methods

2.1. Pathogenic strain and culture

The pathogenic isolate used was originally isolated from naturally infected tomato plants presenting typical symptoms of Rhizoctonia root rot. *R. solani* culture (Plant Pathology Laboratory of the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia) was grown on Potato Dextrose Agar (PDA) medium amended with Streptomycin sulphate (300 mg/L) (w/v). This strain is well known for its virulence and ability to induce necrosis in the roots (Ouhaibi-Ben et al., 2016b).

For inoculum production, the pathogen was grown on PDA for 5–6 days at 28 °C before use. Then, the mycelium of pathogen cultures, from 10 PDA Petri plates, were macerated using a blender in 1 L of Sterile Distilled Water (SDW). The number of mycelial fragments in suspension was counted using a hemacytometer before being adjusted to 10^8 mycelial fragments/mL for the *in planta* assays.

2.2. Pythium oligandrum strain

The biological control agent *P. oligandrum*, strain Po 37, was kindly provided by Dr. Jonathan Gerbore (BIOVITIS, Saint Etienne Chomeil France).

P. oligandrum P37 was grown on a selective medium for oomycetes, made up of Corn Meal Agar supplemented with Pimaricin, Ampicillin, Rifampicin and Pentachloronitrobenzene (coded CMA-PARP) (Jeffers and Martin, 1986).

Inocula were prepared by the BIOVITIS Company, and the concentration was adjusted to 1×10^4 oospores/mL before being used in the *in planta* assays.

2.3. Tomato-associated rhizobacterial strains

Three rhizobacterial strains (*Bacillus subtilis* str. B10 (KT921327), *B. thuringiensis* str. B2 (KU158884), and *Enterobacter cloacae* str. B16 (KT921429)), preselected in a previous study (Ouhaibi-Ben et al., 2016a), were used in this experiment. These strains were identified using biochemical and molecular tools. They were screened for their hypersensitive reaction (HR) on tobacco leaves to prove their eligibility as potential biocontrol agents; and they were also characterized for PGPR traits (IAA detection, siderophore production and phosphate solubilization) (Ouhaibi-Ben et al., 2016c). Their main characteristics are

provided in Table 1.

The strains stock cultures were maintained at −20 °C in Luria Bertani (LB) broth amended with 15% glycerol.

2.4. In vitro antagonism tests

2.4.1. Screening of whole cells suppressive effects (Rhizobacteria / R. solani)

The antifungal potential of cell suspensions of the 3 selected bacterial isolates was evaluated using the dual culture and the disc diffusion methods on PDA as follows: bacterial suspension (~10^8 cells/mL), grown in Nutrient Broth (NB) medium at 28 ± 2 °C under continuous shaking (150 rpm) for 2 days, was deposited (10 μL) into a well performed using a sterile Pasteur pipette (6 mm in diameter, 3 mm in depth) at one side of the Petri plate (90 mm in diameter). An agar plug (6 mm in diameter) removed from the growing edge of a 5-day-old culture of *R. solani* was placed at the opposite side of the plate.

Control plates were treated with the same volume of SDW (He et al., 2009). Each individual treatment was repeated in triplicate. After 5 days of incubation at 25 °C, the colony diameter of the pathogen was measured. The inhibition rate (IR) of the pathogen was calculated using the formula of Prashar et al. (2013) as follows: GI %= ([(C2-C1)/C2] × 100), where C2: diameter of *R. solani* colony in control plates and C1: diameter of *R. solani* colony in the presence of the selected rhizobacterial isolates.

2.4.2. Assessment of the antifungal activity of P. Oligandrum in vitro

The antagonism of *P. oligandrum* P37 isolate against *R. solani* was tested *in vitro* using the dual culture method as follows: *P. oligandrum* P37 was grown on CMA-PARP (Jeffers and Martin, 1986), and incubated at 25 °C in the dark during 7 days. An agar plug (6 mm in diameter) was placed at the one side of the Petri plate (90 mm in diameter) while another one, colonized by the pathogen (*R. solani* removed from a 5-day-old culture at 25 °C), was placed at the opposite side. Control cultures were challenged by the pathogen only in the PDA medium without transplanting the antagonist. Each individual treatment was performed in triplicate. After 5 days of incubation at 25 °C, the colony diameter of the pathogen was measured. The mycelial growth inhibition rate of the pathogen (IR) was calculated using the formula of Prashar et al. (2013).

Mycelial samples from the interaction region between *P. oligandrum* and *R. solani* were collected, using a piece of Scotch tape, 5 days after inoculation and processed for light microscopy.

2.5. In planta antagonism study

2.5.1. Plant material and experimental design

A total of 135 tomato plants cvs. Rio Grande and Marmande, known for their susceptibility to *R. solani* (Taheri and Tarighi, 2012; Haggag and El-Gamal, 2012; Goudjal et al., 2015) were used for the bioassays. Tomato seeds were surface-sterilized using the method of Cao et al. (Cao et al., 2004). They were cultured in alveolus plates (7 × 7 cm) filled with previously sterilized peat (Floragard Vertriebs GmbH für gartenbau, Oldenburg, Germany), grown under controlled conditions (13/11 h light/dark photoperiod and 21/18 ± 2 °C light/dark temperature) and regularly watered. The plants were used at the two-true-leaf growth stage.

Nine treatments, each applied on 15 plants for both tomato cultivars, were tested: (i) UC: Untreated Control, (ii) RS: plants inoculated with the pathogen *R. solani*, (iii) RSfung: *R. solani*-inoculated plants treated with a commercial fungicide, *i.e.* Previcur®, (iv) Po37: plants inoculated with the biocontrol agent *P. oligandrum* P37, (v) RS + Po37: plants inoculated with *R. solani* and *P. oligandrum*, (vi) B2 + B10 + B16: plants inoculated with the 3 selected rhizobacteria, *i.e. Bacillus subtilis* B2, *B. thuringiensis* B10 and *Enterobacter cloacae* B16, (vii) Po37 + B2 + B10 + B16: plants inoculated with *P. oligandrum* and the 3 selected rhizobacteria, (viii) RS + B2 + B10 + B16: plants inoculated with *R. solani* and the 3 selected rhizobacteria, (ix) Po37 + RS + B2 + B10 + B16: plants inoculated with *R. solani*, *P. oligandrum* and the 3 selected rhizobacteria. The whole experiment was repeated twice.

2.5.2. Rhizobacterial inoculum preparation

Before being used, stock cultures were cultured onto Nutrient Agar (NA) medium and incubated at 28 °C for 24 h. One colony of each bacterial isolate was scraped into 300 mL of NB and incubated in a rotary shaker (175 rpm/48 h at 28 °C). After incubation, bacterial cell suspensions containing 10^8 cells/mL were prepared by diluting the obtained bacterial liquid cultures into 1 L of SDW (Wu et al., 2014). Equal volumes of cell suspensions of each strain were mixed and the mixture obtained was used for plant for treatments.

2.5.3. Rhizobacteria and P. Oligandrum co-inoculation

For mixed inoculum preparation, equal volumes of cell suspensions of each bacterial strain from 48 h-old NA cultures were mixed and adjusted to 10^8 cells/mL with SDW. The *P. oligandrum* inoculum was injected alone or mixed with the bacterial suspensions.

For greenhouse assays, 30 mL of each bacterial suspension and/or *P. oligandrum* inoculum mixture was applied as soil drench around the stem of each tomato cultivars Rio Grande and Marmande seedling (two-true-leaf stage), cultivated in honeycombed plates. After one week, 30 mL of pathogen inoculum were drenched at the same level to each seedling. The following day, seedlings were transplanted into pots (20 cm height × 16 cm diam.) filled with 30 mL of pathogen-infected peat (Benchabane et al., 2000; Le Floch et al., 2003). This method of inoculation was used to avoid any trauma of tomato plants following root injury. The negative control (uninoculated control seedlings) were treated with SDW only. The positive control plants were inoculated with *R. solani* and treated with SDW or with a commercial fungicide, *i.e.* Previcur® (Bayer, France, propamocarb hydrochloride 722 g/L) applied at 0.5 mL/L. Uninoculated seedlings challenged with mixed bacterial strains were also used for comparison and for elucidation of their PGP effect.

Each individual treatment was repeated 5 times (5 plants per treatment). Pots were kept under greenhouse conditions (60–70% relative humidity, 13/11 h light/dark photoperiod at 21 ± 2/18 ± 2 °C light/dark temperature) for about 60 days, and watered with tap water when needed.

Table 1
The selected tomato-associated rhizobacteria tested and their main traits.

Molecular identification		PGP[1] traits			
Strains	Accession numbers	HR[2]	3IAA[1]	Phosphatesolubilisation	Siderophoreproduction (mm)
Bacillus thuringiensis B2	KU158884	–	+	+	31.7
Bacillus subtilis B10	KT921327	–	+	+	90.0
Enterobacter cloacae B16	KT921429	–	+	+	90.0

[1]PGP: Plant Growth Promoting traits
[2]HR: Hypersensitive Reaction on tobacco leaves
[3]IAA: Indole-3-Acetic Acid
* (+): Positive reaction, (-): Negative reaction

2.5.3.1. Assessment of the antifungal activity and the plant growth-promoting potential. After two months, fifteen tomato plants were uprooted and washed for removing adhering peat. Root and aerial parts length and fresh weight were measured. Disease severity on roots was also assessed for all tomato plants. Thus, disease severity assessments were made on a scale of 0 to 5 (0 = no symptoms, 1 = 0–25% root browning, 2 = 26–50% root browning, 3 = 51–75% root browning, 4 = 76–100% root browning and 5 = plant wilting) adapted from the work done by Lamsal et al. (2012) on pepper. Root rot disease incidence (DI) was also recorded using the formula: DI % = (Number of plants infected/ Total number of plants evaluated) × 100.

2.5.3.2. Analysis of the microbial communities colonizing the rhizosphere. Sampling and DNA extraction: Root samples were collected from each individual treatment. Fifteen plants were tested per treatment and for each sample, roots were cut into fragments (5 mm in length). After scrambling, 1 g of root segments per sample was subsequently used for microbial and molecular analyses.

DNA was extracted from 60 mg of root tissues with the protocol adopted by Gordon et al. (1997) with slight modifications. Briefly, all the roots samples were kept overnight at − 80 °C then lyophilized for 12 h, prior to DNA extraction. After the addition of 600 µL of the lysis buffer CTAB (1x) to each sample the 2 mL tube was incubated at 65 °C for 1 h. To remove proteins, 400 µL of chloroform-isoamyl alcohol (24 :1, v/v) was added and the tube was shaken at 200 rpm for 10 min. Samples were then centrifuged at 13,000 rpm for 10 min at 4 °C. The aqueous phase was transferred to a new 2 mL tube. Nucleic acids were precipitated by the addition of 330 mL of cold isopropanol then kept at −20 °C overnight. The nucleic acids were recovered by centrifugation at 13,000 rpm at 4 °C during 10 min.

The supernatant was discarded and the DNA pellet was finally washed with 800 µL of ethanol 70%. After centrifugation at 13,000 rpm at 4 °C for 10 min, the ethanol was discarded. Then the DNA pellet was dried and resuspended into 50 µL of SDW. DNA extracts were then quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 10 ng/µl.

PCR–SSCP Analyses. Pairs of primers recognizing the mitochondrial large-subunit rDNA gene (ML1/ML2) (White et al., 1990) for fungi and the 16S rDNA gene (799f/1115r) (Redford et al., 2010) for bacteria were used for fingerprinting analyses using Single-Strand Conformation Polymorphism (SSCP). PCR was performed on DNA extracts from the root samples collected.

DNA was amplified by PCR in a PTC-100 thermocycler (MJ Research, Inc.) in a reaction mixture (25 µL final volume) consisting of 1 µL of DNA template (10 ng/µL), 2.5 µL of Pfu buffer (10x), 2.5 µL of BSA (Bovine Serum Albumin) at 10 µg/µL (BioLabs), 1 µL of dNTP (10 mM), 0.5 µL of each primer, 0.5 µL of Pfu turbo (Stratagene), and 16.5 µL of SDW. For fungi, the cycling parameters were 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min and final extension at 72 °C for 10 min. The cycling conditions for bacteria were as follows: enzyme activation at 95 °C for 2 min, 25 cycles at 95 °C for 45 s; 30 s at 54 °C, 72 °C for 1 min and 72 °C for 10 min. The PCR products were visualized by 2% Tris-Borate-EDTA (TBE) agarose gel electrophoresis prior to SSCP analysis. The lengths of the fragments yielded by amplification were 250 bp and 350 pb respectively for fungi and bacteria.

SSCP analyses were performed on an ABI Prism 3130 genetic analyzer (Applied Biosystems) equipped with four 36-cm-long capillaries. One microliter of a PCR product was mixed with 18.8 µL Hi-Di formamide (Applied Biosystems) and 0.2 µL of the Genescan 400 HD ROX standard internal DNA molecular size marker (Applied Biosystems). The sample mixture was denatured at 95 °C for 5 min, instantly iced (10 min) and then placed onto the instrument. SSCP is based on the electrophoretic mobility of single-stranded DNA fragments. This mobility is different according to their three-dimensional conformation. Samples were allowed to comigrate with the fluorescent size standard (GeneScan 400 ROX) to allow comparison of migration profiles between samples.

2.6. Statistical analyses

Data were submitted to one-way variance analysis (ANOVA) using Statistical Package for the Social Sciences software (SPSS, version 16.0). Each of the *in vitro* or *in vivo* experiments was repeated twice yielding similar results. Therefore, one representative trial of each experiment is reported. Data were analyzed according to a completely randomized design where 9 treatments were tested and each individual treatment was replicated 15 times (*i.e.* 15 plants per individual treatment). The means were separated using the Student-Newman-Keuls test to identify significant pair-wise differences at $P \leq 0.05$. Correlations between Rhizoctonia Root Rot severity and plant growth parameters were analyzed using the bivariate Pearson's test at $P < 0.01$.

SSCP patterns were aligned with the StatFingerprints program (version 1.3) (Michelland et al., 2009) and studied by principal component analysis (PCA) using R software (version 2.15.2).

3. Results

3.1. In vitro antagonism tests

3.1.1. Antifungal activity of the rhizobacteria against R. Solani

ANOVA revealed significant differences in the colony diameter of *R. solani* depending on the treatments tested. As shown in Table 2, the 3 rhizobacteria induced a significant reduction ($P \leq 0.05$) in the mycelial growth of *R. solani*, as compared to the untreated control. After 5 days of incubation at 25 °C, pathogen growth was inhibited by 60, 57 and 54% respectively with *B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16. This assay also showed that all the tested bacterial strains led to the formation of inhibition zones when dual cultured with *R. solani*. The diameter of this zone was about 5, 6 and 11 mm for *B. subtilis* B10, *B. thuringiensis* B2 and *E. cloacae*, respectively (Table 2).

3.1.1.1. In vitro antagonism activity of P. Oligandrum and light microscopy observations. The antagonism activity of *P. oligandrum* was tested against *R. solani.* The ANOVA revealed a significant difference ($P \leq 0.05$) in the colony diameter of *R. solani* when challenged with the antagonistic oomycete. Data from the dual culture assay given in Fig. 1 showed that *P. oligandrum* significantly ($P \leq 0.05$) inhibited the mycelium growth of *R. solani*, as compared to the untreated control. In presence of *P. oligandrum*, the pathogen growth was reduced by 48%, after 5 days of incubation at 25 °C. After 7 days of incubation, the macroscopic observations revealed that mycelia of *R. solani* were overgrown by *P. oligandrum* that rapidly colonized the entire plate. At the microscopic level, examination of samples from the interaction region between *P. oligandrum* and *R. solani* showed that five days after inoculation, the antagonist multiplied abundantly and coiled around *R. solani* hyphae (Fig. 2a). These observations indicated that coiling of the antagonistic oomycete around the pathogen was an early event preceding hyphal

Table 2
Effects of diffusible and volatile metabolites released by tomato-associated rhizobacteria against *Rhizoctonia solani* after 5 days of incubation at 25 °C.

Antagonistic treatments	Isolates	Colony diameter (mm)	Growth inhibition (%)	Inhibition zone(mm)
Untreated control	C	90 a	0	0c
B. thuringiensis	B2	35.33b	60.75	6.33b
B. subtilis	B10	38.33b	57.41	5.67b
E. cloacae	B16	41.00b	54.44	11.67 a

For each column, values followed by the same letter are not significantly different according to Student–Newman–Keuls test (at $P \leq 0.05$).

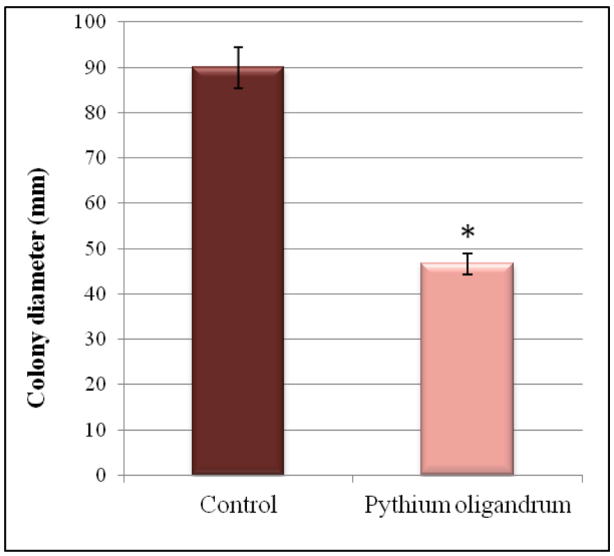

Fig. 1. *In vitro* inhibition of *Rhizoctonia solani* mycelial growth induced by *Pythium oligandrum* Po37 after 5 days of incubation at 25 °C as compared to the untreated control. *: Indicates means that are, within the trial, significantly different between the two treatments at $P < 0.05$ (ANOVA).

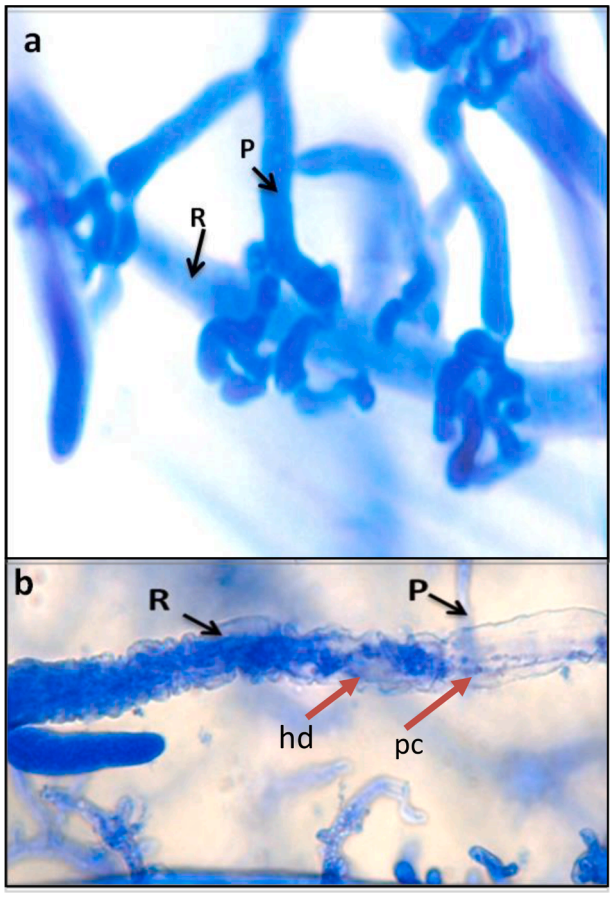

Fig. 2. Light micrographs of the hyphal interactions between *Pythium oligandrum* Po37(P) and *Rhizoctonia solani* (R) in dual cultures. Coiling of a hypha of *Pythium oligandrum* around a hypha of *Rhizoctonia solani* (a). Hyphal side branches of *P. oligandrum* Po37 interacting with a hypha of *R. solani*. An appressorium and infection pegs formed by the mycoparasite at sites of interaction (b). P: *Pythium oligandrum* P37; R: *Rhizoctonia solani*; hd: hyphal damage and pc: penetration site.

damage. Consequently, the hyphae of *P. oligandrum* were closely appressed to host hyphae and started penetrating cells of *R. solani* that look like empty hyphae (Fig. 2b).

3.2. *In planta antagonism study: Ability of antagonist treatments to suppress Rhizoctonia root Rot disease*

Two months post-treatment with the antagonists, disease severity was assessed on tomato plants inoculated with *R. solani,* based on the presence of root browning. All tomato plants not challenged with the pathogen, remained symptomless, while for infected plants, the disease incidence ranged from 33 to 100% for cv. Marmande and from 53 to 100% for cv. Rio Grande (Table 3). Assessed for their ability to suppress disease severity, all treatments tested had significantly (ANOVA at $P \leq 0.05$) decreased the Rhizoctonia Root Rot symptoms as compared to *R. solani* inoculated and untreated controls (Fig. 3). The rhizobacterial mixture, tested alone or in combination with *P. oligandrum*, was found to be more effective in suppressing the disease than the fungicide, on both cultivars (Table 3).

Results, shown in Table 3, indicated that disease incidence was reduced by 74 to 92% on cv. Marmande plants compared to 52% obtained using the fungicide control. For cv. Rio Grande plants, disease severity decrease ranged between 72 and 86% versus 49% obtained using the fungicide control. The three-strain mixture treatment alone exhibited significantly higher effectiveness in decreasing disease severity than when combined with *P. oligandrum*-based treatments.

3.3. *Plant growth-promoting ability*

The rhizobacterial strains mixture, tested alone or combined with *P. oligandrum*, was also screened for its ability to promote the growth of pathogen-free tomato plants. The plant growth-promoting (PGP) abilities were assessed based on various growth parameters on plants (i) infected or not with *R. solani*, (ii) treated with a fungicide and (iii) not treated. ANOVA analysis revealed that the plant height, the aerial part and roots fresh weights, recorded 60 days post-treatment, varied significantly ($P \leq 0.05$) (Tables 4, 5 and 6). These variations depended on the antagonistic treatment tested and the tomato cultivar. The

Table 3
Rhizoctonia Root Rot-suppressive ability of a three-strain bacterial consortium mixed or not with *Pythium oligandrum* Po37 compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars.

Tomato cultivars	Marmande		Rio Grande	
Antagonistic treatments tested	DI* (%)	DS**	DI (%)	DS
Untreated control	0	0 d	0	0 e
P. oligandrum Po37	0	0 d	0	0 e
B2 + B10 + B16 [a]	0	0 d	0	0 e
Po37 + B2 + B10 + B16	0	0 d	0	0 e
R. solani-inoculated control	100	4.47 a (0) [c]	100	4.33 a (0)
R. solani + Po37	80	1.13c (74.72)	67	1.20c (72.28)
R. solani + B2 + B10 + B16	33	0.33 d (92.61)	53	0.60 d (86.14)
R. solani + Po37 + B2 + B10 + B16	80	0.87c (80.53)	60	0.87 cd (79.91)
R. solani + Fungicide[b]	100	2.13b (52.34)	100	2.20b (49.19)

*DI: Disease incidence; **DS: Disease severity
[a]B2: *Bacillus thuringiensis* str. B2 (KU158884); B10: *B. subtilis* str. B10 (KT921327) and B16: *Enterobacter cloacae* str. B16 (KT921429).
[b]Fungicide-based treatment using Previcur®.
[c]Values in brackets (in %) indicate the percentage of decrease in disease severity as compared to the inoculated and untreated control plants.
For each column, values followed by the same letter are not significantly different according to Student–Newman–Keuls test (at $P \leq 0.05$).

Fig. 3. The rating scale adopted to estimate the disease severity. 0 = no symptoms, 1 = 0–25% root browning, 2 = 26–50% root browning, 3 = 51–75% root browning, 4 = 76 100% root browning and 5 = plant wilting.

Table 4
Plant height increment obtained using three tomato-associated rhizobacteria, applied as a consortium, mixed or not with *Pythium oligandrum* Po37, compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars.

Tomato cultivars Antagonistic treatments tested	Marmande Plant height (cm)	%[c]	Rio Grande Plant height (cm)	%
Untreated control	92.93b	0.00	28.13 d	0.00
P. oligandrum Po37	115.67 a	19.66	55.47 d	49.28
B2 + B10 + B16[a]	112.00 a	17.02	51.80 bc	45.69
Po37 + B2 + B10 + B16	116.93 a	20.52	69.80 a	59.69
R. solani-inoculated control	50.40c	0.00	6.00f	0.00
R. solani + Po37	94.93b	46.91	47.73c	87.43
R. solani + Po37 + B2 + B10 + B16	122.80 a	58.96	52.40b	88.55
R. solani + B2 + B10 + B16	109.60 a	54.01	53.93b	88.87
R. solani + Fungicide[b]	85.07b	40.75	21.13 e	71.60

[a]B2: *Bacillus thuringiensis* str. B2 (KU158884); B10: *B. subtilis* str. B10 (KT921327) and B16: *Enterobacter cloacae* str. B16 (KT921429).
[b]Fungicide-based treatment using Previcur®.
[c]Values (in %) indicate the percentage of increase in plant height as compared to *R. solani*-inoculated and untreated controls.
For each column, values followed by the same letter are not significantly different according to Student–Newman–Keuls test at $P \leq 0.05$.

Table 5
Enhancement of aerial part fresh weight obtained using three tomato-associated rhizobacteria, applied as consortium, mixed or not with *Pythium oligandrum* Po37, compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars.

Tomato cultivars Antagonistic treatments tested	Marmande APFW[c] (g)	%[d]	Rio Grande APFW (g)	%
Untreated control	32.53 d	0.00	29.13b	0.00
P. oligandrum Po37	60.67 a	46.38	29.40b	0.92
B2 + B10 + B16[c]	64.47 a	49.54	50.37 a	42.18
Po37 + B2 + B10 + B16	66.53 a	51.10	30.67b	50.01
R. solani-inoculated control	13.93 e	0.00	3.27 e	0.00
R. solani + Po37	48.00c	70.98	17.93c	81.76
R. solani + B2 + B10 + B16	57.07b	75.59	30.20b	89.17
R. solani + Po37 + B2 + B10 + B16	57.73 a	75.87	20.06c	83.69
R. solani + Fungicide[b]	30.00 d	53.57	9.53 d	65.68

[a]B2: *Bacillus thuringiensis* str. B2 (KU158884); B10: *B. subtilis* str. B10 (KT921327) and B16: *Enterobacter cloacae* str. B16 (KT921429).
[b]Fungicide-based treatment using Previcur®.
[c]APFW: Aerial Part Fresh Weight.
[d]Values (in %) indicate the percentage of increase in the aerial part fresh weight as compared to *R. solani*-inoculated and untreated controls.
For each column, values followed by the same letter are not significantly different according to Student–Newman–Keuls test at $P \leq 0.05$.

Table 6
Enhancement of root growth obtained using three tomato-associated rhizobacteria, applied as consortium, mixed or not with *Pythium oligandrum* Po37, compared to fungicide and untreated controls, noted 60 days post-planting in two tomato cultivars.

Tomato cultivars Antagonistic treatments tested	Marmande [c] (g)	%[d]	Rio Grande RFW (g)	%
Untreated control	3.13 cd	0.00	2.80 ef	0.00
P. oligandrum Po37	4.40 bc	28.86	4.27c	34.43
B2 + B10 + B16[a]	6. 00 a	47.83	5.80 a	51.72
P37 + B2 + B10 + B16	5.47 ab	42.78	5.13b	45.42
R. solani-inoculated control	0.80 e	0.00	0.60 g	0.00
R. solani + Po37	2.20 d	63.63	2.73 ef	78.02
R. solani + B2 + B10 + B16	6.33 a	87.36	3.53 d	83.00
R. solani + Po37 + B2 + B10 + B16	3.00 d	73.33	3.27 de	81.65
R. solani + b	2.20 d	63.63	2.33f	74.24

[a]B2: *Bacillus thuringiensis* str. B2 (KU158884); B10: *B. subtilis* str. B10 (KT921327) and B16: *Enterobacter cloacae* str. B16 (KT921429).
[b]Fungicide-based treatment using Previcur®.
[c]RFW: Root Fresh Weight.
[d]Values (in %) indicate the percentage of increase in the root fresh weight as compared to *R. solani*-inoculated and untreated control.
For each column, values followed by the same letter are not significantly different according to Student–Newman–Keuls test at $P \leq 0.05$.

biocontrol agent relative effects, on each parameter, were detailed below.

3.3.0.1. Plant height

Plant height variations according to each treatment and tomato cultivars are illustrated in Table 4.

For disease-free plants, height increase, as compared to the untreated control, ranged from 19 to 20% and from 45 and 59% respectively for the cv. Marmande and the cv. Rio Grande. On both cultivars, the highest PGP effect was obtained using the combination of *P. oligandrum* and the three rhizobacterial strains (Po37 + B2 + B10 + B16).

Regarding *R. solani*-inoculated plants, treatments with the three-strain mixture (*B. subtilis* B2, *B. thuringiensis* B10, and *E. cloacae* B16) and/or *P. oligandrum* significantly ($P \leq 0.05$) enhanced plant height. For cv. Marmande, height increase ranged from 46 to 58% as compared to 40% achieved using the commercial fungicide Previcur®, with the mixed treatment (Po37 + B2 + B10 + B16) being the most efficient (58%). For cv. Rio Grande, height increment varied from 87 to 88% as compared to 71% obtained with the chemical-based treatment, with the three-strain mixture (B2 + B10 + B16) exhibiting the highest PGP and disease suppression effect (88%).

3.3.0.2. Aerial parts fresh weight

Aerial Parts Fresh Weight (APFW), noted 60 days post-planting, varied significantly ($P \leq 0.05$) according to the treatment and the

tomato cultivars. Results given in Table 5 indicate that for disease-free cv. Marmande plants, APFW significantly increased compared to the untreated control, by 46 to 51%. The highest PGP effect was achieved using the three strains mixed with *P. oligandrum*. For disease-free cv. Rio Grande plants, this growth parameter increment ranged from 0.92 to 42%. The rhizobacterial mixture (B2 + B10 + B16) exhibited the highest PGP effect.

Data shown in Table 5 reveal that plants treated with *B. thuringiensis* B2, *B. subtilis* B10 and *E. cloacae* B16 mixture alone or in combination with *P. oligandrum* led to significant increase in APFW of both cultivars, as compared to *R. solani*-inoculated and untreated control plants. For cv. Marmande, APFW increment ranged from 70 to 75% compared to 53% obtained using the fungicide-based treatment. Regarding cv. Rio Grande, this parameter was enhanced by 81 to 89%, compared to 65% recorded on Previcur®-treated plants.

3.3.0.3. Root fresh weight

Root Fresh Weight (RFW), measured 60 days post-planting, varied significantly ($P \leq 0.05$) depending on the treatments tested and the tomato cultivars grown as illustrated in Table 6. For disease-free cv. Marmande plants, RFW was significantly improved, compared to the untreated control, by 28 to 47% when plants were treated with the mixed bacterial strains alone or with *P. oligandrum*. The highest PGP effect was observed when plant roots were inoculated with the three-strain mixture only (47%). For disease-free cv. Rio Grande plants, root growth increment ranged from 34 to 51%, with the bacterial mixture treatment (B2 + B10 + B16) exhibiting the highest PGP effect.

Regarding *R. solani*-inoculated plants, the three rhizobacterial strains, applied alone or in combination with *P. oligandrum*, significantly ($P \leq 0.05$) enhanced root development as compared to *R. solani*-inoculated and untreated control. Their PGP effect varied according to the tomato cultivar grown. While for cv. Marmande, RFW increased by 63 to

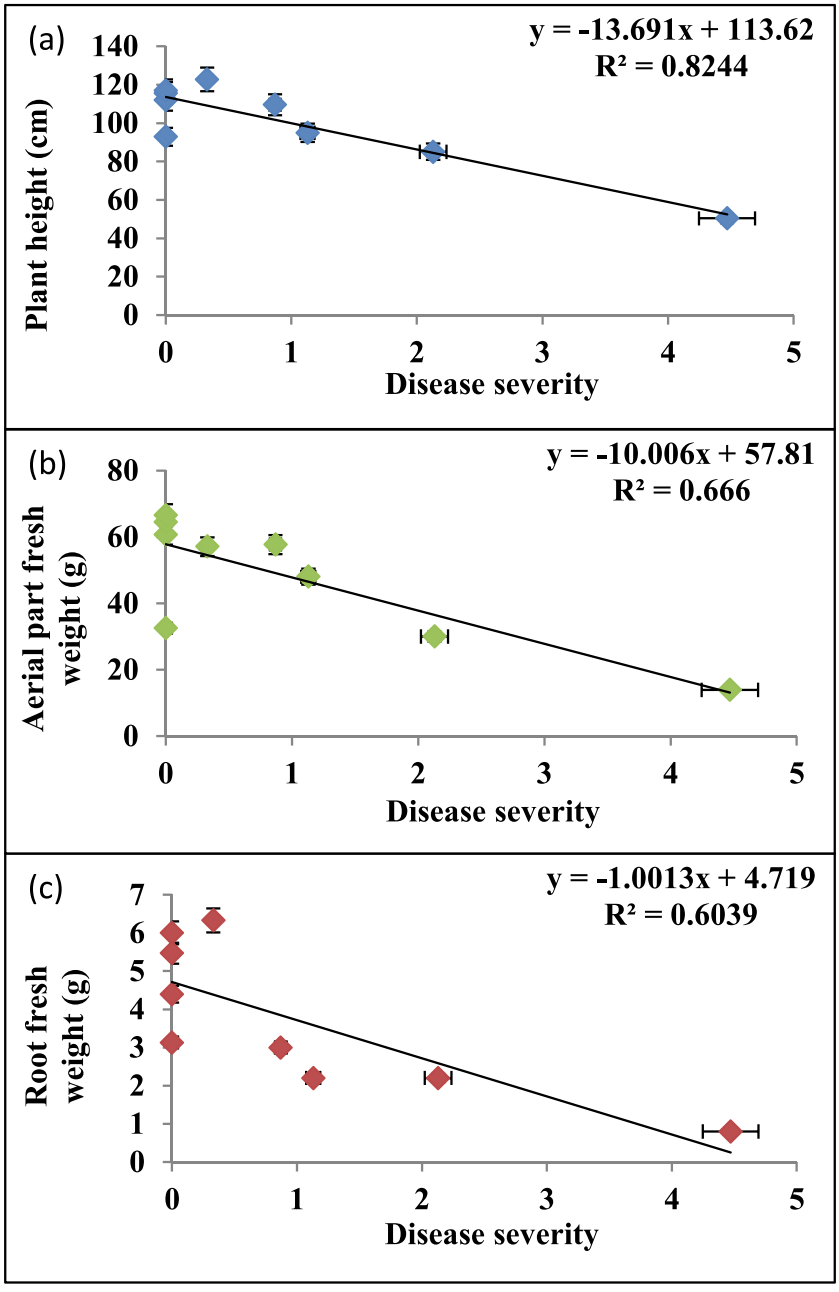

Fig. 4. Correlation between Rhizoctonia Root Rot severity and plant growth parameters (a, b, c) for the cv. Marmande. (a): Plant height, (b): Aerial part fresh weight and (c): Root fresh weight. Correlations analyses were performed using bivariate Pearson's test at $P \leq 0.01$.

87% as compared to 63% noted on fungicide-treated plants, on cv. Rio Grande, root growth enhancement, achieved using antagonistic-based treatments, ranged from 78 to 83% as compared to 74% obtained using Previcur®.

3.4. Correlation between Rhizoctonia root Rot severity and plant growth parameters

For cv. Marmande, Pearson's correlation analysis revealed that plant height was significantly ($P \leq 0.01$) and negatively related to disease index ($r = -0.713$; $P = 2.815E-22$) (Fig. 4a). indicating that increased Rhizoctonia Root Rot severity led to plant stunting. The same findings were also noted between Rhizoctonia Root Rot severity index and APFW ($r = -0.657$; $P = 4.314E-18$) (Fig. 4b) and RFW ($r = -0.552$; $P = 3.765E-12$) (Fig. 4c).

Pearson's correlation analysis revealed that, for cv. Rio Grande, a significant ($P \leq 0.01$) correlations between disease severity and growth parameters was also noted. This analysis indicated that the reduced Rhizoctonia Root Rot severity, recorded on tomato plants and achieved using rhizobacteria-based treatments applied alone or in combination with *P. oligandrum*, was related to the decrease in *R. solani* colonization potential leading consequently to the registered plant growth promotion (Fig. 5).

3.5. Genetic structure of the fungal and bacterial communities that colonize the rhizosphere of the tomato plants

The SSCP profiles of the 54 samples that represent rhizobacteria (27) and fungi (27) status from the root samples collected from cv. Marmande and Rio Grande tomato plants, revealed complex microbial communities (data not shown) based on the number of peaks and the relative height of the baseline. Principal Component Analyses (PCAs) were studied to

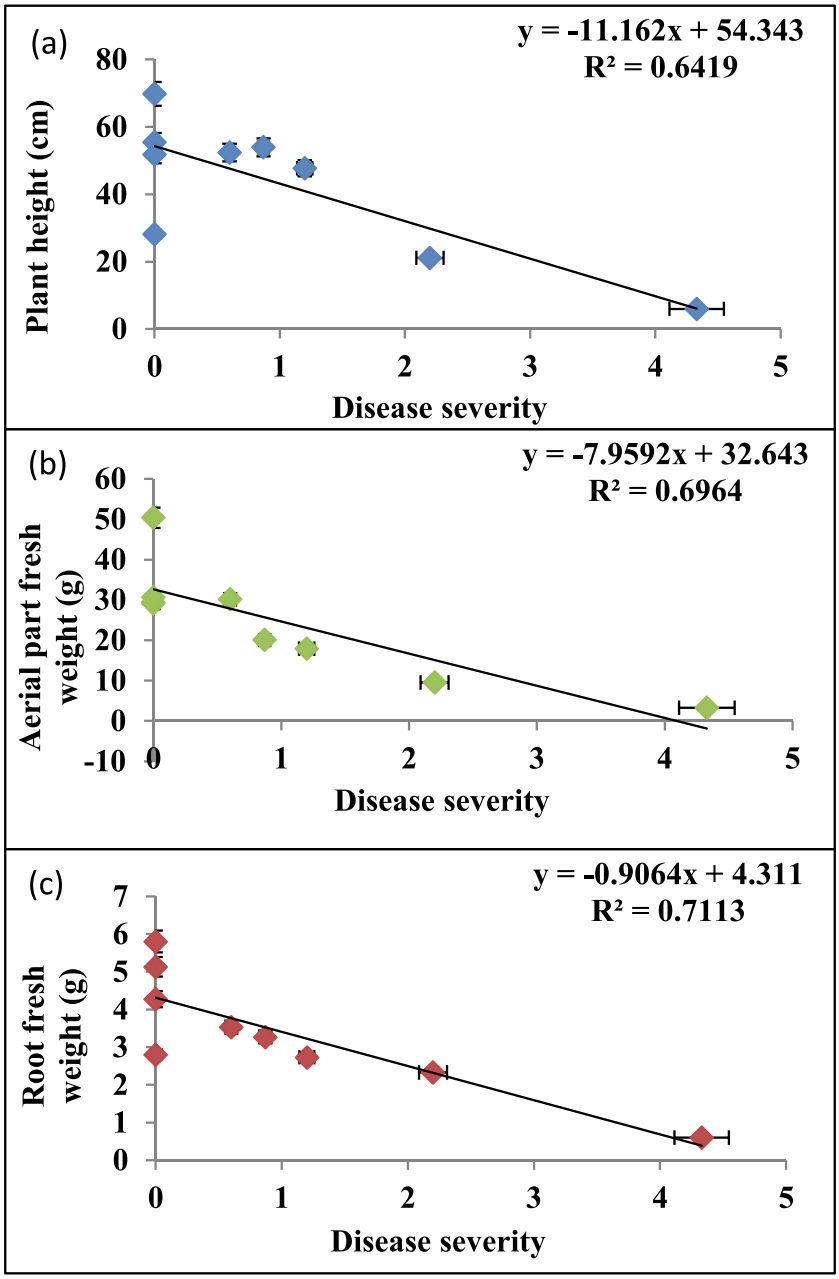

Fig. 5. Correlation between Rhizoctonia Root Rot severity and plant growth parameters (a, b, c) for the cv. Rio Grande. (a): Plant height, (b): Aerial part fresh weight and (c): Root fresh weight. Correlations analyses were performed using bivariate Pearson's test at $P \leq 0.01$.

assess the genetic structure of the rhizosphere bacterial and fungal communities on the roots of both tomato varieties inoculated with *R. solani* and treated with a mixture of rhizobacterial strains applied in combination or not with *P. oligandrum*.

The distributions of the samples on the principal plans generated by PCAs of fungal communities are represented in Fig. 6. Differences in the genetic structure of the fungal communities were observed with the different treatments and varied upon tomato cultivars grown. PCAs eigenvalues indicate that the first two principal components, Dim1 and Dim2, explain 53.94% of total fungal variability, and the ellipses do not overlap. Our results allowed two main types of community structure to be delineated: the first one was associated with the presence of *P. oligandrum* and the second, with the other treatments.

PCA was carried out to compare the genetic structure of the bacterial communities in terms of tomato cultivars grown and treatments applied. The distribution of samples on the principal plan generated by the PCA is represented in Fig. 7. There were no differences in SSCP profiles of the bacterial communities between the various treatments applied on roots of tomato plants. The genetic structure of the bacterial community changed only with the tomato cultivars. PCA eigenvalues indicated that the first two principal components, Dim1 and Dim2, explained 67% of the total data variance, and the ellipses do not overlap. This suggests that the native bacterial communities were not significantly impacted by the various microbial-inoculations.

4. Discussion

Microorganisms that grow in the rhizosphere can play an important role in plant defense and are promising sources of biocontrol agents (Fan et al., 2017). Understanding the mechanisms of biological control of plant diseases through the interactions between antagonists and pathogens may allow us to select the most effective biocontrol agents and to manipulate the soil environment to create conductive condition for successful plant protection (Nega, 2014).

In our previous studies, three bacterial strains identified as *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327) and *E. cloacae* B16 (KT921429), were selected because, following their root colonization, a decrease in Sclerotinia Stem Rot severity and an increase in plant growth was observed (Ouhaibi-Ben et al., 2016d). Taking advantage of this finding, the present study was aimed at gaining more insight into the relationship established between tomato roots and *P. oligandrum*, combined or not, with these 3 rhizobacteria. This combination of antagonists was used in order to evaluate their capacity to coexist in the tomato rhizosphere, to suppress Rhizoctonia root rot in two tomato cultivars, *i.e.* Marmande and Rio Grande, and to promote plant growth.

In the literature, a promising antagonistic strain for biocontrol agent is usually reported as having broad-spectrum antimicrobial activities (Xu et al., 2011; Sun et al., 2017; Zhang et al., 2017). In this study, *B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16 were therefore evaluated *in vitro* for their antagonistic potential against another pathogen, *R. solani*. They were able to reduce the pathogen mycelial growth by more than 50%. These results agree with previous studies reporting the capacity of strains from these bacterial species to control various fungal plant pathogens, including by producing diffusible metabolites (Fiddaman and Rossall, 1994; Liu et al., 2011; Ouhaibi-Ben et al., 2016c).

Dual interactions in co-cultures showed that *P. oligandrum* significantly reduced *R. solani* mycelial growth (48%) and, it is speculated that the diffusion of its metabolites into the culture medium led to the formation of an inhibition zone. Antibiosis has been already identified as a common feature in some *P. oligandrum*-fungal interactions. Bradshaw-Smith et al. (1991) observed a growth rate reduction of two major footrot pathogens of peas, *Phoma medicaginis* var. *pinodella* and *Mycosphaerella pinodes*, due to volatile antibiotics produced by *P. oligandrum*. In another study by Benhamou et al. (Benhamou et al., 1999) transmission electron microscopy observations revealed severe damages in *Phythophthora megasperma* cells (*i.e.* retraction and aggregation of the host cytoplasm) presumably associated with a release of diffusible compounds that infiltrated the pathogen hyphae. This was observed despite the lack of physical contact between *P. oligandrum* and *Phythophthora megasperma* hyphae. Our microscopic observations showed an infection pegs formed by the mycoparasite at the interaction site level. The hyphae of *R. solani* looked frequently like empty shells. A similar coiling around or penetrating into the hyphae of *R. solani* was confirmed through light micrographs observations. Benhamou et al. (1999) observed, using light and electron microscopy, that hyphae of *P. oligandrum* established close contact with the plant pathogen *R. solani* by frequent coiling around the hyphae early during parasitism. Brozova (2002) showed that *P. oligandrum* and *R. solani* interaction resulted in disorganization of most of the cell cytoplasm of the pathogen (80%), whereas the *P. oligandrum*-cell wall looked well preserved.

Biocontrol agents (BCAs) applied individually to plants can display a good biocontrol potential, however, plant protection is frequently variable from one year to another, due to changing environmental related issues. Hence, to improve stability of the released microbes in different agricultural fields, the use of microbial consortia is advocated. It is generally assumed that these consortia are better prepared to face the challenges of different soil and environmental conditions as they partially mimic the soil microbial communities (Le Floch et al., 2009; Sundaramoorthy et al., 2012; Sarma et al., 2015).

So, in order to assess a new biocontrol strategy, the study reported here relied on the association of the antagonistic oomycete *P. oligandrum*, with the 3 rhizobacteria, *i.e. B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16.

Our *in planta* experiments indicate that the three-strain consortium, mixed or not with *P. oligandrum*, were shown to be a promising alternative to the commercial fungicide Previcur®. This fungicide is routinely used to protect horticultural crops and particularly in suppressing Rhizoctonia Root Rot on pot-grown tomato. Previous studies already reported that rhizobacteria and *P. oligandrum* were more effective than fungicides in increasing plant defense against *R. solani* (Brozova, 2002; Kurzawinska and Mazur, 2008; Scherwinski et al., 2008; Naureen et al., 2015).

The three-strain consortium tested (*B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16) and their combination with *P. oligandrum* protected tomato plants from *R. solani* infection. Their disease-suppression effect against *R. solani* was significant on both cultivars used as compared to control plants. The efficacy of these BCAs differed depending on the bioagents used and the cultivar grown. Results showed that strains mixture, applied as a substrate drench, exhibited significantly higher effectiveness in decreasing disease severity as compared to other treatments. For combined data of both cultivars, *i.e.* Marmande and Rio Grande, disease suppression ranged from 79 to 80% with the combination of *P. oligandrum* and the three rhizobacteria, compared to 86–92% achieved using the 3 strains only, and to 72–74% using *P. oligandrum* alone. Hence, this research suggested that the combination of *P. oligandrum* with rhizobacteria species had a critical role in antagonism against *R. solani* and improved biocontrol efficacy.

The current trend is to mix BCAs of diverse microbial species having plant growth-promoting activities to achieve desired agricultural outcomes. A comprehensive understanding on the mechanisms that govern recruitment and activity of the selected microbes may open up opportunities to utilize the phenomenon in increasing crop productivity (Berendsen et al., 2012). According to the literature, in many cases and based on some disease and growth indicators, combinations of biocontrol agents were found to be more effective than single one in decreasing diseases incidence and severity and in increasing plant growth (Nandakumar et al., 2001; Sundaramoorthy et al., 2012; Bahramisharif and Rose, 2019), suggesting thus synergistic or antagonistic interactions among mixed BCAs as cited by Le Floch et al. (2009) and Patel et al. (2014). In fact, a combination of antibiotic-producing strains has additive or synergistic effects and therefore will result in a better control of

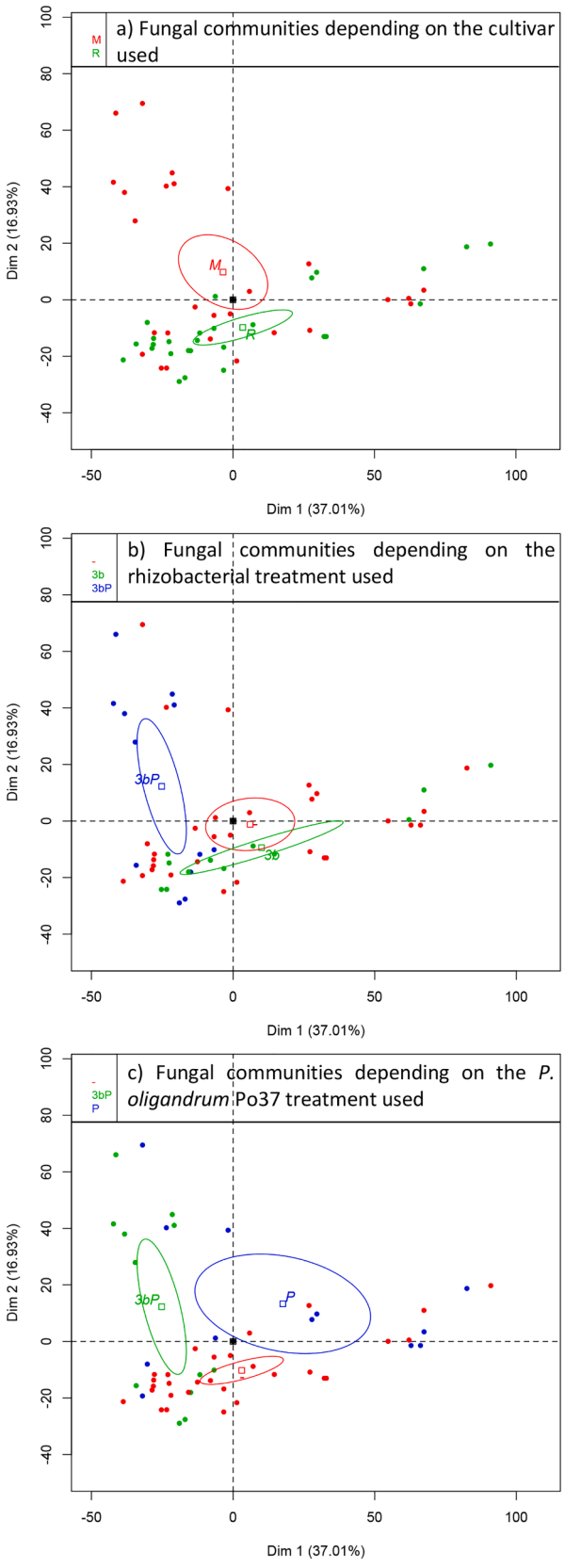

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Fig. 6. Distribution of the fungal communities on the principal planes defined by the first two axes obtained in the principal component analysis (PCA) of SSCP profiles, depending on the cultivar (a) and the treatments used (b and c). (a) The colors used here represent the profiles obtained depending on the tomato cultivars (R: Rio Grande (green)) and M: Marmande (red)). (b) The colors used here represent the profiles depending on the rhizobacterial treatment used (3b: 3 tomato-associated rhizobacteria, applied as consortium (green); 3bP: 3 tomato-associated rhizobacteria, applied as consortium, mixed with *Pythium oligandrum* Po37 (blue); and -: untreated plants (red)). (c) The colors used here represent the profiles obtained depending on the *P. oligandrum* Po37 treatment used (3bP: 3 tomato-associated rhizobacteria, applied as consortium, mixed with *P. oligandrum* Po37 (green); P: *P. oligandrum* Po37 alone (blue); and -: untreated plants (red)). The variation (%) explained by each PCA axis is given in brackets. Ellipsoids draw the center of factors with 95% confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diseases (Choudhary and Johri, 2009; Bouizgarne and Maheshwari, 2013; Dey et al., 2014). Besides, Szczech and Shoda (2004) proved the enhanced consistency of disease resistance using mixtures of *B. subtilis* RB14-C and *Burkholderia cepacia* BY strains against *R. solani* of tomato plants under growth chamber conditions.

To achieve an appropriate and effective biological control of *R. solani*, an important prerequisite for a BCA is to be able to colonize and persist on the roots in order to induce resistance and/or trigger other protection mechanisms, *e.g.* antibiosis, competition for nutrients and space, mycoparasitism etc. Following BCA inoculation, a shift may also potentially occur within native microbial community structure, as reported by Buyer et al. (Buyer et al., 2010). We have verified this point by using Single Strand Conformation Polymorphism (SSCP) to determine the structure and dynamics of the fungal and bacterial communities colonizing the rhizosphere of the two tomato cultivars. Depending on the Marmande or Rio Grande cultivars, there was a variation in rhizosphere fungal community. Besides, the SSCP-results also clearly indicate that the fungal microflora changed over the treatments.

As for the bacterial communities, SSCP showed that, depending on the root samples, they were different depending on grown tomato cultivars. However, no difference was observed in rhizosphere bacterial communities following inoculation of plants by rhizobacteria and/or *P. oligandrum*, or the pathogen. Thus, the different treatments used did not significantly affect native bacterial communities. The only differences were obtained when the native bacterial microflora of the two tomato cultivars were compared. This demonstrate that native fungal and bacterial communities respond differently when microbial-BCAs are inoculated in the rhizosphere. This finding was also observed by (Vallance et al., 2009) and 2012), but they did not reported shifts in the native fungal community of the rhizosphere after root inoculation by *P. oligandrum*, whereas they observed a transient shift in the native bacterial community (Vallance et al. 2012).

The synergistic effect obtained with BCA-mixtures offer multiple beneficial effects including plant-growth promotion and yield enhancement. Marimuthu et al. (Marimuthu et al., 2013) and Liu et al. (Liu et al., 2018) demonstrated that co-inoculation of a biocontrol agent and a bio-phytostimulator is considered as a positive approach in plant health management and in improving the yield parameters. Interestingly, in our study, an increase in root weight and plant height, the aerial part and roots fresh weights, was seen with all rhizobacteria-based treatments, used alone or as consortium with *P. oligandrum*, relative to the untreated controls (uninoculated and pathogen-free). These results are in agreement with the studies of Sandramoothy et al. (2012) and Liu et al. (Liu et al., 2018) that showed an increase in plant growth due to combined applications of BCAs. This amelioration could be attributed to increased availability of nutrients due to phosphate solubilization; providing protection to plants from diseases by producing antibiotics and siderophores and production of plant hormones like Indole-3-Acetic Acid (IAA). Previous works have also demonstrated the additive plant

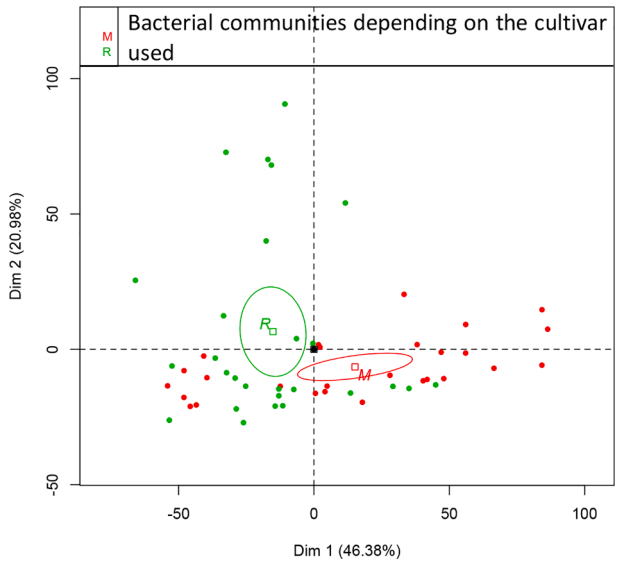

Fig. 7. Principal Component Analysis (PCA) of the bacterial communities colonizing the rhizosphere of two tomato cultivars (R: Rio Grande (green)) and M: Marmande (red)) based on SSCP profiles. The variation (%) explained by each PCA axis is given in brackets. Ellipsoids draw the center of factors with 95% confidence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth effect using combined PGPR (plant growth rhizobacteria) such as *Pseudomonas fluorescens*, *B. subtilis* and *B. amyloliquefaciens*. Interestingly, in our study, a synergistic effect between *B. subtilis*, *B. thuringiensis* and *E. cloacae* with *P. oligandrum* was observed for the first time.

In conclusion, the magnitude of these results was greater with mixtures of biocontrol strains than with the individual one. According to our results, the best alternative approach is the use of combination of strains with different modes of action on suppression of pathogen infection (*R. solani*) and on plant growth promotion. To our knowledge, this is the first report of rhizobacteria strains (*B. thuringiensis* B2, *B. subtilis* B10, and *E. cloacae* B16) mixed with the BCA-oomycete, *P. oligandrum*, exhibiting both antifungal activity and growth-promoting potential onto tomato seedlings.

Considering the community-based living style of PGPR strains, the current trend is to mix BCAs of diverse microbial species having plant growth-promoting activities to achieve desired agricultural outcomes (Sarma et al., 2015) and (Mehmood et al., 2018). The types of relationship established by the four antagonistic microorganisms and the plant warrant thorough examination. Therefore, it has become of great importance to develop comprehensive understandings on the microbial components of a consortium so that desired benefits can be provided to plants especially under the pathogen challenged conditions. It will be interesting to see whether microbes in consortia apart from managing plant diseases could also engineer the metabolic pathways in plants to obtain some other desired benefits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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