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Activity of Plant Growth Promoting Rhizobacteria (PGPRs) in the Biocontrol of Tomato Fusarium Wilt

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Abstract

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The potential of *Pseudomonas fluorescens* PF15 and *Pseudomonas putida* PP27 to protect tomato plants against Fusarium wilt under greenhouse conditions was evaluated. *In vitro* antagonism showed a significant inhibition of the pathogen growth (47%) revealed by PF15. However, PP27 presented a 10% rate of the mycelium inhibition. An *in situ* experiment was conducted with split-root design for induced systemic resistance (ISR) and without split-root design to measure both ISR and antagonistic activities. Fluorescent *Pseudomonas* revealed a delay in the onset of symptoms and slower kinetics of disease progression compared to the pathogen control. McKinney's index, which measures the severity of the disease, was reduced by 37–72%, and the levels of infection (incidence) by 7–36%.

Keywords: *Pseudomonas fluorescens* PF15; *Pseudomonas putida* PP27; *Fusarium oxysporum* f.sp. *lycopersici*; Induced Systemic Resistance

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans, is one of the most prevalent and damaging diseases of tomato, causing considerable losses (Ramaiah *et al.* 2015). The fungus invades plant vascular tissues and induces severe wilting of the foliage by blocking xylem transport and impeding the movement of water. Controlling the vascular wilt pathogen is difficult for several reasons; the pathogens live deep in the interior of their host plants, many vascular wilt pathogens are soil-borne and produce persistent resting structures that are able to survive for long periods of time in the absence of host plants (Suárez-Estrella *et al.* 2007; Ji *et al.* 2008; Yadeta & Thomma 2013). There are not any efficient chemical treatments that exist to cure infected plants. Management with chemical fungicides causes serious environmental problems and they are toxic to non-target organisms as well (Ramaiah *et al.* 2015). Elicitation of plant's defence by plant growth-promoting rhizobacteria (PGPRs) has received increasing attention in recent years. *Pseudomonas* spp. known as PGPRs have been shown to trigger systemic resistance in plants, often referred to as induced systemic resistance (ISR) (Van Loon *et al.* 1998; Pieterse *et al.* 2000, 2014; Bakker *et al.* 2007). ISR improves the plant's defence mechanisms, is not specific and can protect plants against a broad spectrum of pathogens (Van Peer *et al.* 1991; Pieterse *et al.* 2000, 2014). Induced systemic resistance is based on the recognition between specific elicitors of rhizobacteria and receptors (Van Loon *et al.* 2008). Elicitors of induced resistance can be either components of the bacterial cell surface or metabolites excreted by PGPRs (Van der Ent *et al.* 2009). ISR reduces the sensitivity of plants to pathogens and is phenotypically similar to systemic acquired resistance (SAR) (Van Loon *et al.* 1998; Van Wees *et al.* 1999). Simultaneous activation of

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SAR and ISR provides enhanced defensive capacity compared to each single resistance.

Priming is a common feature of systemic resistance elicited by rhizobacteria that increases the responsiveness of the plant's immune system and allows it to more effectively express its own defence reactions (Beckers & Conrath 2007). Priming for defence may combine the advantages of enhanced disease protection and low costs. Induced resistance can entail costs due to the allocation of resources of defensive products (Van Hulten *et al.* 2006).

The aim of this study was to evaluate the ability of two PGPR strains and their combination to protect tomato plants against Fusarium wilt by induced systemic resistance. For this purpose, two experimental designs were carried out; firstly, the split-root design where the biocontrol agents (PGPRs) with the pathogen were applied at the root level and kept physically separated. Secondly, a non-split-root experiment design was used to compare the antagonistic activity that ensures contact between the PGPR strains and the pathogen.

MATERIAL AND METHODS

Microorganisms and inoculum preparation. *Pseudomonas fluorescens* PF15 and *Pseudomonas putida* PP27 were used for induced systemic resistance and growth assays. PF15 and PP27 are Algerian PGPR strains and were provided by the Laboratory of Protection and Agro-Resources Valorisation in Algeria. They were isolated from the rhizosphere of tomato plants; their ability to release DAPG (2,4-diacetylphloroglucinol) and siderophores had been proved in previous studies (Benchabane *et al.* 2000; Boukerma 2012). The PGPR strains also increased the total protein and proline in tomato plants (Boukerma 2012). PF15 and PP27 were grown overnight at 28°C in King B medium. Bacterial cells were collected and resuspended in 10 mM $MgSO_4$ and adjusted to a concentration of 10^8 CFU/ml with the spectrophotometer (Shiwadzu). The absorbance was calculated: $A_{600} = 1$ corresponding to 5×10^8 CFU/ml, then the concentration of the bacterial inocula was adjusted and delivered to the seedlings (Vijayan *et al.* 2006). The inoculation was performed 3 days before the pathogen challenge application.

Fusarium oxysporum f.sp. *lycopersici* MUCL 43876 strain (FOL) provided by the Mycotheque of the Catholic University of Louvain, Belgium was used for challenge inoculation. The fungal suspension was prepared first by growing FOL on PDA medium (potato dextrose agar, ingredients are: 200 g potato, 20 g dextrose, 15 g agar, 1000 ml distilled water) (Jonsthon & Booth 1983) and incubating it at 25°C for 7 days. Afterwards, the mycelium was scraped off the plates into a liquid nutrient medium (potato dextrose) and grown at 25°C for 8 days. The final suspension was calibrated with sterile 10 mM $MgSO_4$ to a concentration of 10^6 conidia/ml with a Malassez cell by counting the conidia (De Lapeyre *et al.* 2008).

Antagonism in vitro. Antagonistic activity against FOL was studied on three nutrient media: PDA (potato dextrose agar), medium favourable for the development of FOL, King B – medium favourable for the development of *Pseudomonas* spp., and mixed medium consisting of equal parts of the two media (King B+PDA). Four spots of bacterial culture (10^8 CFU/ml) were spread equidistantly on the plates (28 mm from the centre). A mycelial agar plug of 5 mm in diameter from a 7 day-old culture of FOL grown on PDA medium was placed in the centre of the plate. Control plates that had not been inoculated with bacteria were also prepared. After 7 days of incubation at 25°C, the inhibition percentage of fungal growth was measured as the ratio between the diameter of pathogen growth inhibition and the diameter of FOL growth (alone) (Benchabane *et al.* 2000).

Experimental design. The induction of systemic resistance was evaluated in the tomato *Solanum lycopersicum* L. plant (cv. Marmande), susceptible to *Fusarium oxysporum* f.sp. *lycopersici*. Certified seed was provided by the Technical Institute of Agriculture and Industry in Algeria. The disinfection was carried out by soaking the tomato seeds in 1% sodium hypochlorite (NaClO) for 10 min, and then rinsing them three times with sterile distilled water. The experiment was arranged in three randomised blocks, with each block composed of 14 treatments of 10 plants per block (30 plants per treatment). The seeds were sown on peat (2/3) mixed with sterile soil (1/3); the seedlings were cultivated in a glass greenhouse (28°C). The plants were watered regularly and supplied with Hoagland's solution once a week. The experiments were repeated twice.

The experiments were done in two designs: split-root and non-split-root. The split-root system was used for the assay of induced systemic resistance and involved the division of the root system into two equal parts, and growth in two separate compartments as described by Van Peer (1991) and Van Loon *et al.* (1998). Bacterial strains were applied in one side and FOL in the other. Seven treatments were defined in the split-root sys-

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tem, T–: healthy control, treated with sterile 10 mM $MgSO_4$ in both sides; T+: pathogen control, plants inoculated with FOL in both sides; T1, T2, and T3: plants inoculated with PF15, PP27 or their combination, respectively, in one side and challenged with FOL in the other side; T4 and T5: plants inoculated with PF15 or PP27, respectively, in both sides. The same treatments were defined in a non-split-root design; the pathogen and biocontrol agents were inoculated together and at the same time.

Evaluations. Disease evaluations began when plants were 6 weeks old and extended up to 12 weeks (*n* = 30). To estimate the disease progression, it has been based on the scale adapted for Fusarium wilt of herbaceous plants as defined by Fiely *et al.* (1995) and Benchabane *et al.* (2000). The rating scale contains 5 levels of symptoms: (0) no symptoms, (1) unilateral yellowing, (2) generalised yellowing, (3) unilateral wilting, longitudinal and unilateral discoloration of the stem, (4) wilting widespread, (5) dead. Two disease evaluations were calculated: the disease incidence (infection level) and McKinney's index (severity) (McKinney 1923; Manikandan *et al.* 2010).

For chlorophyll quantification, leaf samples were collected two weeks after the pathogen inoculation (5 leaves, 3 replicates for each treatment). The assay was performed following the methodology described by Arnon (1949): 0.5 g of leaf powder were mixed with 5 ml of acetone (80%), after centrifugation at 4000 rpm for 5 min, extracts were filtered and absorbance was measured at 649 and 665 nm wavelengths. Total chlorophyll content (chlorophyll a and b) was calculated and expressed as µmol/g leaf weight. The chlorophyll index was expressed as the ratio between the total chlorophyll content in the stressed treatment divided by the total chlorophyll content in the control (Kanawapee *et al.* 2012).

At the end of the experiment, the plants were harvested, the aerial parts were separated from the roots, fresh and dry weights were determined.

Data analysis. One-way analysis of variance was carried out to evaluate bacterial effects. When differences were significant, the multiple range test and the Kruskal-Wallis test were performed with the Statgraphics plus 5.1 (1992) for Windows software.

RESULTS

Antagonism in vitro. *Pseudomonas fluorescens* PF15 showed the highest inhibition of FOL growth

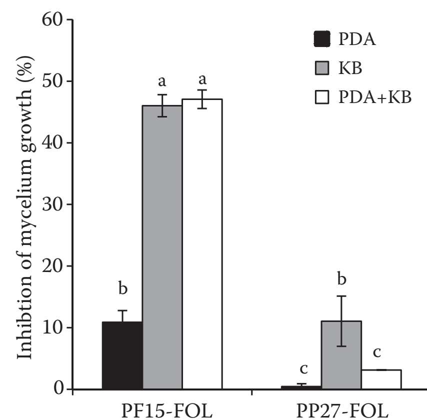

Figure 1. Antagonistic activity of *Pseudomonas fluorescens* PF15 and *Pseudomonas putida* PP27 on *Fusarium oxysporum* f.sp. *lycopersici* (FOL)

PDA – potato dextrose agar medium; KB – King B medium; growth inhibition was expressed as the level of reduction of the FOL mycelium growth relative to the control (not inoculated with PF15 or PP27); bars indicate standard errors and different letters indicate significant differences ($P < 0.05$)

(47%) in the King B and mixed (PDA+King B) media. However, *Pseudomonas putida* PP27 revealed a low reduction of mycelium growth on King B medium (10%) compared to PF15 (Figure 1).

The effect of PGPRs on tomato wilt disease. Plants treated with fluorescent *Pseudomonas* and challenged with the pathogen show a delay in the onset of symptoms and slower kinetics of disease progression in split-root and non-split-root designs compared to the pathogen control; a rapid evolution was observed mainly on the earliest days of the disease development (Figure 2). Despite the progression of disease incidence, the severity of Fusarium wilt was less increased. After 41 days of challenging, plants inoculated with PF15 and PP27 showed a reduction of McKinney's index (severity) by up to 72%; however, 36% of the diseased plants (plants that expressed at least one symptom on the rating scale) were observed (Figure 2).

All treatments showed a similar rate of disease suppression; nevertheless, treatments made in the split-root design presented the best bioprotection compared to those without split roots. Fluorescent *Pseudomonas* reduced significantly the Fusarium wilt symptoms and revealed lower classes of symptoms than the pathogen control (Figure 3). Plants in the non-split-root design showed symptoms of unilateral wilting while plants in the split-root system revealed generalised yellowing compared to the pathogen control where all plants died (Figure 3).

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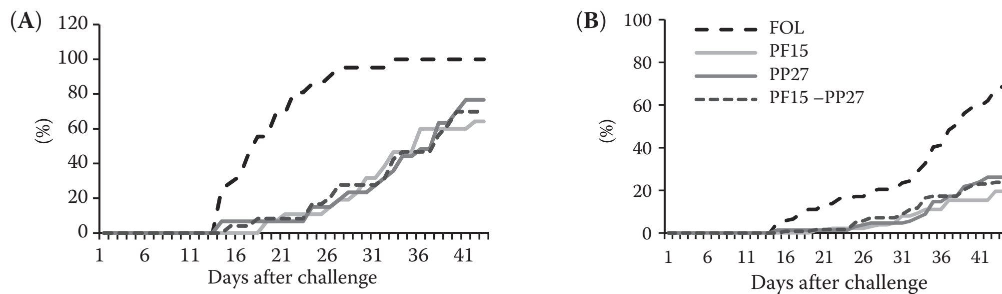

Figure 2. Disease incidence (**A**) and McKinney's index (**B**) of Fusarium wilt in tomato plants grown in a split-root design

Plants treated with PGPR strains *Pseudomonas fluorescens* PF15*, Pseudomonas putida* PP27 or their combination and challenged with *Fusarium oxysporum* f.sp. *lycopersici* (FOL); data are means of 30 replicates for each treatment; protection of tomato plants is expressed as a reduction of the intensity of Fusarium wilt evolution relative to uninoculated plants, during 41 days of the disease following being under greenhouse conditions

Chlorophyll content. The ratio between stressed plants and healthy control showed an increase of chlorophyll pigments in plants treated with PF15, PP27 or their combination. However, the control presented a low value of chlorophyll index (Figure 4).

Growth enhancement. The ability of fluorescent *Pseudomonas* to enhance tomato growth as measured by shoot fresh and dry weight was evaluated (Figure 5). PF15 and PP27 applied alone strongly improved shoot dry and fresh weight. However, challenged plants showed growth levels similar to the healthy control. PP27 showed the best enhancement of tomato growth in the split-root design.

DISCUSSION

The results presented in this paper describe the abilities of the biocontrol activity of the two strains PF15 and PP27 against Fusarium wilt. *Pseudomonas fluorescens* PF15 showed the best inhibition of mycelium growth in the King B medium (the *in vitro* assay). This medium is deficient in iron that promotes pyoverdine synthesis by fluorescent *Pseudomonas* for chelating ferric ions, causing a reduction of its availability to the pathogen (Eyquem *et al.* 2000). These results suggest that the main mechanism of the PF15 strain is siderophore production in the medium poor in ferric ions (King B), but on the mixed medium and PDA, cumulative actions (antibiosis, parasitism) can be the origin of the antagonistic effect. However, the PP27 strain revealed a suppression of pathogen growth only on the King B medium, which explains that the main mechanism expressed *in vitro* of this strain is siderophore production.

Plants treated with fluorescent *Pseudomonas* (PF15, PP27 or their combination) showed the late onset of symptoms, lesser rating scale and significant bioprotection against *Fusarium oxysporum* f.sp. *lycopersici* (FOL). The specific resistance of plants is expressed as all or nothing (resistance/sensitiv-

Figure 3. Symptoms of Fusarium wilt on tomato plants treated with PGPR strains and challenged with *Fusarium oxysporum* f.sp. *lycopersici* (FOL) compared to the pathogen control: (**A**) Symptoms of Fusarium wilt on tomato plants treated with *P. fluorescens* PF15 and challenged with FOL. The severity of Fusarium wilt is less developed as shown in the above figures; the root and vascular tissues maintained in good health and shape; (**B**) Symptoms of Fusarium wilt for the inoculated untreated control presented by the pathogen control. Total destruction of the roots and vascular tissues was observed which was the cause of the rapid yellowing and wilting of leaves

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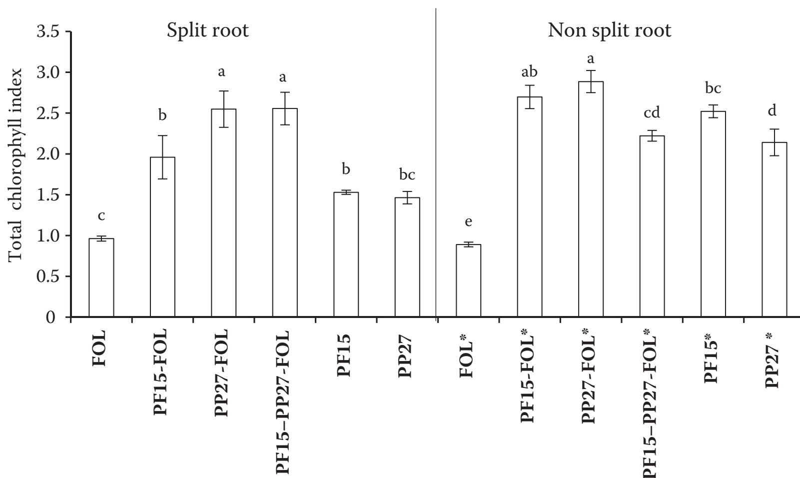

Figure 4. Total chlorophyll index expresses the ratio between stressed tomato plants (plants treated with *Pseudomonas fluorescens* PF15, *Pseudomonas putida* PP27 or their combination and challenged with *Fusarium oxysporum* f.sp. *lycopersici* /FOL/) and healthy control

Bars indicate standard errors and different letters indicate significant differences ($P < 0.05$)

ity), while the partial or quantitative resistance is marked by modulations in the kinetics and intensity of the disease development (Young 1996). The split-root system provided no physical contact between pathogen and PGPRs and promoted the interaction of each microorganism when either pseudomonads or FOL were applied to the root. This allowed observing the ability of *P. fluorescens* PF15 and *P. putida* PP27 to induce systemic resistance and it is the only mechanism that can explain disease suppression. In this context, similar observations have already been recorded by several studies (Ongena *et al.* 2000; Verhagen *et al.* 2010).

Challenged plants can express several defence mechanisms: the production of reactive oxygen species (Yoshioka *et al.* 2008), antimicrobial compounds, lytic enzymes, and pathogenesis-related (PR) proteins (Van der Ent *et al.* 2009). Several studies confirm that the prior application of fluorescent *Pseudomonas* strengthens the cell wall, which limits the invasion of pathogens in plant tissues (Benhamou *et al.* 2000).

The protection level conferred by such strains was not statistically different from their combination. This result can be explained by the lack of compatibility between the two bacterial strains. However, it has been suggested that the combination of PGPRs could be more effective than their individual application (Saravanakumar *et al.* 2008). ISR depends on the bacterium-host interaction, *P. fluorescens* WCS374r triggered ISR in radish but cannot prime *Arabidopsis*. It has been shown that *P. putida* WCS358r induced systemic resistance in *Arabidopsis* but not in radish, which shows that the ability of pseudomonads to trigger systemic resistance depends on the plant genotypes. In *Arabidopsis*, ISR expression varies among ecotypes; Landsberg erecta and Columbia are susceptible to *P. fluorescens* WCS417r; however, RLD and Wassilewskija are not (Van Wees *et al.* 1997).

Disease suppression in the non-split-root design may be due to ISR and/or antagonistic actions of *Pseudomonas.* The antagonism is a competition for nutrients and space. Antagonistic PGPRs act on pathogens by inhibiting germination and/or sporulation and/or by interfering with pathogenicity elements (Haas & Défago 2005). Fluorescent *Pseudomonas* synthesise various lytic enzymes, β-1,3-glucanase and β-1,4-glucanases and lipases, which causes the cell wall lysis of the pathogen (Denton 2007).

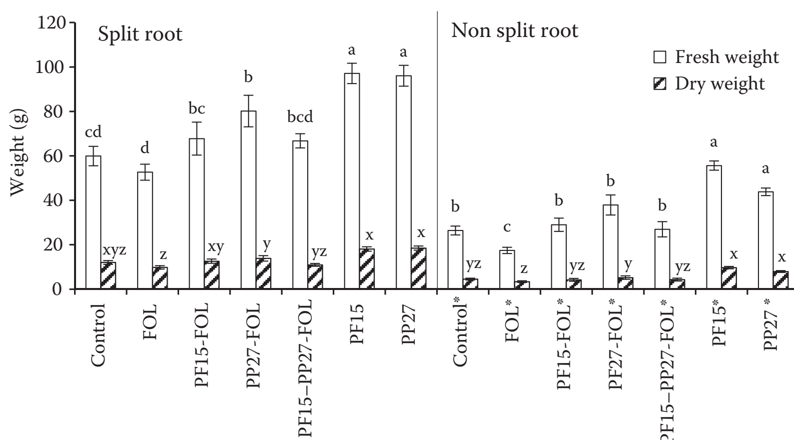

Figure 5. Fresh and dry weight of shoots of tomato plants treated with *Pseudomonas fluorescens* PF15, *Pseudomonas putida* PP27 or their combination and challenged with *Fusarium oxysporum* f.sp. *lycopersici* (FOL)

Bars indicate standard errors and different letters indicate significant differences ($P < 0.05$) in fresh weight (a– d) and dry weight (x–z)

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Pseudomonas fluorescens PF15 and *Pseudomonas putida* PP27 enhanced tomato growth significantly and allowed challenged plants to maintain a similar growth level as the healthy control, which can decrease immunity costs. Immunity costs are high for individuals, leading to decreased resources available for fitness. Negative correlations between growth rate and resistance to disease represent a long-known phenomenon (Smedegaard-Petersen & Tolstrup 1985). It is generally understood that induced resistance evolved to save energy under pathogen or insect-free conditions, although costs still arise when defences are activated following the attack (Walters & Heil 2007). Induced systemic resistance could be associated with growth enhancement or decrease, depending on the actions of applied strains and the response of the plant host.

Challenged plants showed symptoms on a scale between 2 and 3 (wilting leaves), and total chlorophyll content was higher compared with control plants (pathogen and healthy control). This reveals the positive effect of applied PGPRs on plant physiology regardless of the development of the disease. Fluorescent *Pseudomonas* strains can increase the total chlorophyll content in plants (Farhan *et al.* 2010). The ability of *Pseudomonas* sp. to enhance the total chlorophyll level in plants can be related to their supply of some nutrients such as nitrogen and phosphorus (Hameed & Farhan 2007).

The results presented in this paper showed the ability of *P. fluorescens* PF15 and *P. putida* PP27 to protect tomato against Fusarium wilt. Disease suppression was due to the induction of resistance and/or the antagonistic effect in the host plant.

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