SYNERGISTIC INTERACTIONS BETWEEN *PSEUDOMONAS* spp. AND *XANTHOMONAS PERFORANS* IN ENHANCING TOMATO PITH NECROSIS SYMPTOMS

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SUMMARY

Surveys carried out in soil-grown tomatoes in Sicily (Italy) showed that *Pseudomonas* spp. strains were frequently associated with *Xanthomonas perforans*, causal agent of tomato pith necrosis. In this study, 30 *Pseudomonas* spp. strains from symptomatic tomato tissues and International Collections with biocontrol properties, were evaluated for pathogenicity on tomato plants. All *Pseudomonas* spp. strains were able to induce brown vascular discoloration and some of them showed *in vitro* antagonistic activity against *X. perforans*. Selected strains were tested for their effects in enhancing tomato pith necrosis caused by *X. perforans* in co-inoculation experiments. Six strains belonging to *Pseudomonas fluorescens*, *P. putida*, *P. citronellolis* and *P. protegens* species significantly increased pith necrosis and vascular discoloration symptoms when co-inoculated with *X. perforans* on tomato plants. In these co-inoculations, *X. perforans* population density resulted significantly higher than that of *X. perforans* inoculated individually. The results of this work highlight the need for a careful evaluation of biological control agents during the screening procedures and before commercialization to avoid the risk of introducing strains that could be pathogenic on tomato plants.

Keywords: Tomato bacterial diseases, co-inoculations, pith necrosis, *Pseudomonas* spp.

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INTRODUCTION

Several bacterial species cause worldwide pith necrosis and vascular discoloration of tomato plants (*Solanum lycopersicum* L.) associated with leaf yellowing, wilting and premature death of plants. The main species associated to this syndrome is *Pseudomonas corrugata* (Clark and Watson, 1986; Scarlett *et al.*, 1978) and, more occasionally, *P. mediterranea* (Trantas *et al.*, 2015), *P. cichorii* (Trantas *et al.*, 2013; Wilkie and Dye, 1974), *P. fluorescens* and *P. putida* (Dimartino *et al.*, 2011; Lo Cantore and Iacobellis, 2002; Saygili *et al.*, 2004), *P. marginalis* (Kudela *et al.*, 2010) and *P. viridiflava* (Alippi *et al.*, 2003).

Studies focused on commensal/pathogen and pathogen/pathogen interactions that lead to increased plant disease severity represent an important topic for a better understanding of plant diseases (Lamichhane and Venturi, 2015). Plants may be colonized/infected at the same time by more than one commensal/pathogenic species and, while a single microbe infection may not result in disease occurrence, the co-infection with another microbial species may lead to the development of severe disease symptoms due to synergistic interactions.

Tomato pith necrosis is a well-known example of these interactions, in which sometimes more than one bacterial species can be involved in the occurrence of symptoms. The enhanced severity of disease has been reported in co-infections caused by *P. fluorescens* and *P. corrugata* (Molan and Ibrahim, 2007), *P. marginalis* and *P. corrugata* (Kudela *et al.*, 2010), *P. fluorescens*, *P. viridiflava* and *P. corrugata* (Pechtereva *et al.*, 2009), and *P. corrugata* and *P. mediterranea* (Moura *et al.*, 2005).

More recently, *Xanthomonas perforans* was reported as a causal agent of pith necrosis and vascular discoloration on tomato plants (Aiello *et al.*, 2013; Torelli *et al.*, 2015). Interestingly, during the surveys, numerous *Pseudomonas* species (*P. fluorescens*, *P. putida*, *P. marginalis*, *P. citronellolis* and *P. straminea*) were isolated in association with *X. perforans* from symptomatic plants, and some of them, when stem-inoculated, were able to induce tomato vascular discoloration (Aiello *et al.*, 2013). However, no information is available in literature on the synergistic interactions of these *Pseudomonas* species with *X. perforans* in inducing pith necrosis and vascular discoloration symptoms.

Generally, *Pseudomonas* spp. are common inhabitant of the aerial parts of plants, soil and rhizosphere, able to

Table 1. Strains of *Pseudomonas* spp. tested for their *in vitro* antagonistic activity against *X. perforans* and pathogenicity on tomato.

Species	Strain	Antagonistic activity[a, c]	Symptoms	Pathogenicity[b,c]	Source of strains[d]
P. fluorescens	**3P2S1**	0.8 b	VD	2.7 bc	Aiello *et al.*, 2013
P. fluorescens	**WCS417**	2.0 a	VD	2.2 c	Van Peer *et al.*, 1992
P. fluorescens	2124	0.0 d	VD	1.9 c	CFBP
P. fluorescens	2129	0.0 d	VD	1.9 c	CFBP
P. fluorescens	G7a	0.0 d	VD	1.8 c	Dimartino *et al.*, 2011
P. fluorescens	1P4S2	0.4 c	VD	1.7 c	Aiello *et al.*, 2013
P. fluorescens	5755	0.0 d	VD	1.2 d	CFBP
P. putida	**P9**	1.5 a	VD	3.6 ab	Andreote *et al.*, 2009
P. putida	**1P1S2**	0.0 d	VD	3.4 b	Aiello *et al.*, 2013
P. putida	**B56**	0.5 c	VD	3.3 b	Wilson *et al.*, 1996
P. putida	**B001**	0.4 c	VD	2.5 c	Park *et al.*, 2011
P. putida	**WCS358**	0.5 c	VD	2.3 c	Geels and Schippers, 1983
P. putida	**06909**	0.5 c	VD	2.1 c	Steddom *et al.*, 2002
P. putida	3.2	0.0 d	VD	2.0 c	Dimartino *et al.*, 2011
P. putida	20.4	0.0 d	VD	2.0 c	Dimartino *et al.*, 2011
P. putida	3.3	0.0 d	VD	2.1 c	Dimartino *et al.*, 2011
P. putida	21.11	0.0 d	VD	2.0 c	Dimartino *et al.*, 2011
P. putida	16.10	0.0 d	VD	1.8 c	Dimartino *et al.*, 2011
P. putida	1.2	0.0 d	VD	1.3 d	Dimartino *et al.*, 2011
P. putida	G6a	0.0 d	VD	0.6 d	Dimartino *et al.*, 2011
P. citronellolis	**1P2S2**	0.0 d	VD	3.7 ab	Aiello *et al.*, 2013
P. citronellolis	G5b	0.0 d	VD	3.1 b	Dimartino *et al.*, 2011
P. citronellolis	6P2S2	0.0 d	VD	2.7 bc	Aiello *et al.*, 2013
P. citronellolis	3P2S2	0.0 d	VD	2.4 c	Aiello *et al.*, 2013
P. citronellolis	5P2S2	0.0 d	VD	2.2 c	Aiello *et al.*, 2013
P. citronellolis	4P2S2	0.0 d	VD	1.7 c	Aiello *et al.*, 2013
P. marginalis	**1P5S1**	0.8 b	VD	1.0 d	Aiello *et al.*, 2013
P. straminea	**2P1S2**	0.3 c	VD	3.0 b	Aiello *et al.*, 2013
P. straminea	4P4S1D	0.2 c	VD	1.8 c	Aiello *et al.*, 2013
P. protegens	**Pf5**	2.0 a	VD	4.5 a	Paulsen *et al.*, 2005

[a]Width of inhibition zone (cm) induced by *Pseudomonas* spp. *in vitro* assay.
[b]Mean data for vascular discoloration length (cm) on stem tissues of 3 replicates, each formed by 16 tomato seedlings cv. Syr Elian.
[c]Values followed by the same letter within a column are not significantly different according to Fisher's least significance difference test (P = 0.01).
[d]CFBP = Collection Francaise de Bacteries Phytopathogens, INRA, Angers, France.
VD = vascular discoloration. The 12 strains used for the co-inoculation experiment with *X. perforans* are reported in bold (see Table 2).

establish endophytic populations in roots, tubers, stems, leaves and other organs, frequently acting as beneficial/antagonistic bacteria and often used in biological control strategies (Lugtemberg and Kamilova, 2009). However, sometimes strains of *P. fluorescens*, *P. putida*, *P. marginalis*, *P. viridiflava* and *P. corrugata* share biocontrol and plant pathogenic activity on one or several hosts. Thus, for unpredictable circumstances, antagonistic bacterial strains able to control fungal or bacterial diseases in one host/pathogen system, could become pathogenic or could "synergistically" interact with other pathogens present in the same or different host/pathogen system enhancing disease severity.

Little or no information is available on the ability of beneficial strains to cause disease on tomato or on their ability to increase the virulence of "main pathogens". For all these reasons, and to avoid the use of "potential" plant pathogens and/or deleterious interaction with tomatoes in biological plant protection, it is necessary to evaluate the risk of each potential biocontrol agent during the screening procedures and before use in the field.

Thus, studies were carried out to investigate: i) the ability of *Pseudomonas* strains of different origin to act as antagonists against *X. perforans*; ii) their pathogenic behavior when artificially inoculated in tomato plants; iii) their role on pathogenic behavior and symptom expression in co-inoculation experiments with *X. perforans*.

MATERIALS AND METHODS

Bacterial strains. Thirty *Pseudomonas* spp. strains, belonging to *P. fluorescens*, *P. putida*, *P. citronellolis*, *P. marginalis*, *P. straminea* and *P. protegens* species, were used for this study. Eleven strains were isolated from internal stem tissues of tomato plants affected by pith necrosis caused by *X. perforans* (Aiello *et al.*, 2013), nine strains were isolated from internal stem tissues of tomato plants affected by vascular discoloration and necrosis caused by *P. fluorescens* and *P. putida* (Dimartino *et al.*, 2011), and ten *P. putida*, *P. fluorescens* and *P. protegens* strains were from CFBP and from other laboratories (Table 1). *Xanthomonas perforans*

4P2S1 used in this study, causal agent of tomato pith necrosis, was previously isolated and characterized (Aiello *et al*., 2013; Torelli *et al*., 2015).

***In vitro* antagonistic activity of *Pseudomonas* strains against *Xanthomonas perforans*.** *Pseudomonas* strains were tested for their inhibitory activity against *X. perforans* according to Cirvilleri *et al.* (2005) with some modifications. Briefly, the isolates were individually placed on nutrient agar (NA) agar plate and incubated at 27°C. After 48 h, 20 µl aliquots of bacterial suspensions (approximately 1×10^8 CFU ml^{-1}) in sterile distilled water (SDW) were spotted on PDA plates, using two spots per plate. The plates were incubated for 48 h at 28°C. Then, the plates were over sprayed with a suspension containing approximately 1×10^8 CFU ml^{-1} of *X. perforans* and incubated at 27°C for 2-4 days. The presence and size of a clear zone around bacterial strains, showing the inhibitory effect, was scored. All tests were performed twice.

Pathogenicity tests of *Pseudomonas* spp. strains on tomato seedlings. *Pseudomonas* strains were tested for their pathogenicity on 4-week-old tomato seedlings cv. Sir Elyan. Bacterial suspensions (approximately 1×10^8 CFU ml^{-1}) in SDW, prepared by growing the isolates for 48 h at 27°C on NA, were stem-injected into tomato pith tissues at the attachment point of the first true leaf using a sterile hypodermic syringe. SDW served as a control. Plants were covered with plastic bags for 48 h and maintained in a growth chamber at 27°C and 70% relative humidity with a 16 h photoperiod. After 15 days the presence and the length of pith necrosis and vascular discoloration were determined (Table 1). Each treatment was replicated three times, with 16 tomato seedlings per replicate, and the single experiment was carried out twice.

Effect of co-inoculation of *Pseudomonas* spp. and *Xanthomonas perforans* strains on pith necrosis symptoms. To evaluate the contribution of *Pseudomonas* spp. strains in enhancing the severity of pith necrosis symptoms, mixtures (1:1 ratio) of *X. perforans* cells (approximately 1×10^8 CFU ml^{-1}) and *Pseudomonas* spp. strains (approximately 1×10^8 CFU ml^{-1}) (*P. fluorescens* 3P2S1, *P. fluorescens* WCS417, *P. putida* P9, *P. putida* 1P1S2, *P. putida* B56, *P. putida* B001, *P. putida* WCS358, *P. putida* 06909, *P. citronellolis* 1P2S2, *P. marginalis* 1P5S1, *P. straminea* 2P1S2 and *P. protegens* Pf5) were co-inoculated on 4-week-old tomato plants cv. Sir Elyan by stem-injecting bacterial mixtures into pith tissues at the attachment point of the first true leaf as previously described. Each treatment was replicated three times, with 16 tomato seedlings per replicate, and each experiment was performed twice. The same number of tomato plants treated with SDW served as a control. After 15 days, the presence and the length of pith necrosis and vascular discoloration were determined.

To assess population density of *X. perforans* in co-inoculated tomato plants, inoculated stems were cut, surface-disinfected with 1% sodium hypochlorite (NaOCl) solution for three min, rinsed twice with SDW and blot dried on sterile blotting paper. Stems were portioned into segments (1 g), ground in SDW, sonicated in ultrasonic cleaner for 7 min, and serial dilutions in SDW were spread on NA+Rif^{100} using a Spiral Plater Eddy Jet. Colonies were counted after incubation at 28°C for 48–72 h, population data were $\log_{10}$ transformed. All experiments were carried out twice.

Biofilm formation. Bacterial strains were grown at 27°C for 48 h in Luria Bertani (LB) broth (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} sodium chloride) used as a common nutritional medium or on XVM2 (20 mM NaCl, 10 mM $(NH4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% casamino acid) culture medium to simulate apoplastic conditions (Astua-Monge *et al*., 2005). Bacterial adhesion to an inert surface was measured by a polypropylene 96-well plate assay as previously described (Parafati *et al*., 2015) with some modifications. After static bacterial growth in LB and XVM2 media for 3 and 72 h at 27°C, the medium was removed, plates rinsed with SDW and stained with 0.3% crystal violet (CV) for 15 min. Excess stain was removed by rinsing the plates with SDW, residual CV was solubilized by the addition of 200 µl of 20:80 acetone:ethanol, and absorption quantified using a microplate reader set at A570 nm wavelength. Absorption values (ODs) for each strain are the means of three readings of five wells from three assays.

Droplet collapse. Bacteria were grown in culture tubes containing 5 ml of KB broth (King *et al*., 1954) for 1 day with shaking (200 rpm) at 27°C. Cells were removed by centrifugation and 10 µl of supernatant was placed on parafilm (Pechiney Plastic Packaging; Neenah, WI, USA). The flattening of droplets measured as an increase in the diameter of the droplets, was assessed 1 and 5 days post inoculation. There were five replicate droplets for each culture supernatant. Experiment was repeated once.

Swarming motility. Swarming motility was evaluated on soft agar (0.6% wt/vol) in KB medium (King *et al*., 1954). Three-microliter samples of a bacterial suspension (OD_{600} of 0.2) from cultures grown 24 h in KB broth were spotted onto the agar surface at the center of each plate with three replicates per treatment. Plates were incubated at 20°C and colony diameter (mm) was measured at 1 and 5 days post inoculation. The experiments were done twice.

Statistical analysis. All data obtained from the *in vitro* and *in vivo* experiments were subjected to an analysis of variance (ANOVA) using the STATISTICA package software (Version 10, Statsoft Inc., Tulsa, OK, USA) according

Fig. 1. Vascular discoloration following artificially inoculation on tomato plants cv. Sir Elyan with: (a) *Pseudomonas fluorescens* WCS417; (b) *Pseudomonas marginalis* 1P5S1; (c) *Pseudomonas putida* P9; (d) *Pseudomonas straminea* 2P1S2; (e) *Pseudomonas protegens* PF5; (f) *Pseudomonas citronellolis* 1P2S2.

to a parametric approach with the factorial treatment structure and interactions and for treatments arranged in a randomized complete block design (RCBD). Since the treatment (bacterial species) × trial interactions were not significant in each experiment for all variables (pathogenicity, *in vitro* antagonistic activity and pathogen population) they were combined as mean data among repeated trials presented in the tables. In the post-hoc analyses, the corresponding mean values were subsequently separated by Fisher's least significant difference test (P = 0.01).

RESULTS

***In vitro* antagonistic activity of different *Pseudomonas* species and strains against *Xanthomonas perforans*.** In this experiment 18/30 of *Pseudomonas* spp. strains did not show antagonistic activity against *X. perforans*, whereas 12 strains inhibited the growth of *X. perforans* with inhibition zones between 0.2 and 2 cm variables depending on the tested *Pseudomonas* strain (Table 1). In detail, *P. fluorescens* WCS417, *P. putida* P9 and *P. protegens* Pf5 showed the highest *in vitro* antagonistic activity, whereas *P. fluorescens* 1P4S2, *P. putida* strains B56, B001, WCS358 and 06909, and *P. straminea* strains 2P1S2 and4P4S1D showed the lowest inhibitory activity. Intermediate inhibition values were recorded for *P. fluorescens* 3P2S1 and *P. marginalis* 1P5S1.

Pathogenicity tests of *Pseudomonas* spp. strains on tomato seedlings. All tested *Pseudomonas* strains induced brown vascular discoloration symptoms on stem-inoculated tomato seedlings cv. Sir Elyan (Fig. 1). The mean length of lesions, recorded 15 days after inoculation, was variable depending on the bacterial strain (Table 1). Lesions slightly increased upwards to the apex and downwards to the roots and their length ranged from 0.6 to 4.5 cm. Nineteen strains caused more evident discoloration lesions (between 2 and 4.5 cm) whereas 11 strains induced faintly observable discoloration lesions (length values less than 2 cm). Strains for each of the six species examined (*P. fluorescens*, *P. putida*, *P. citronellolis*, *P. marginalis*, *P. straminea* and *P.*

Table 2. Influence of co-inoculation of 12 *Pseudomonas* spp. strains with *Xanthomonas perforans* on pith necrosis symptoms and relative *X. perforans* population densities on inoculated stem tissues of tomato.

Species and strain	Pathogenicity[a, c]	*X. perforans* population[b, c]
X. perforans 4P1S2	4.67 cd	7.17×10^7 b
X. perforans 4P1S2 + *P. straminea* 2P1S2	3.97 d	6.11×10^6 c
X. perforans 4P1S2 + *P. putida* 06909	4.53 cd	7.22×10^7 b
X. perforans 4P1S2 + *P. putida* B56	4.57 cd	7.18×10^7 b
X. perforans 4P1S2 + *P. putida* WCS358	4.93 bc	7.93×10^7 b
X. perforans 4P1S2 + *P. marginalis* 1P5S1	5.00 bc	8.09×10^7 b
X. perforans 4P1S2 + *P. fluorescens* WCS417	5.10 bc	8.19×10^7 b
X. perforans 4P1S2 + *P. putida* B001	5.77 ab	8.23×10^7 b
X. perforans 4P1S2 + *P. putida* 1P1S2	6.03 a	8.01×10^8 a
X. perforans 4P1S2 + *P. citronellolis* 1P2S2	6.10 a	8.59×10^8 a
X. perforans 4P1S2 + *P. putida* P9	6.16 a	8.34×10^8 a
X. perforans 4P1S2 + *P. fluorescens* 3P2S1	6.50 a	8.94×10^8 a
X. perforans 4P1S2 + *P. protegens* Pf5	6.66 a	8.46×10^8 a

[a] Mean data for stem pith necrosis length (cm) of 3 replicates, each formed by 16 tomato seedlings cv. Syr Elian.
[b] Population density expressed as Log $_{10}$ CFU/g of plant tissue.
[c] Values followed by the same letter within a column are not significantly different according to Fisher's least significance difference test (P = 0.01).

protegens) showing ability to cause vascular discoloration symptoms were selected for the further studies.

Effect of co-inoculation of *Pseudomonas* spp. and *Xanthomonas perforans* strains on pith necrosis symptoms. Length of pith necrosis lesions caused by *X. perforans* was significantly affected by the co-inoculated *Pseudomonas* spp. strains (Table 2, Fig. 2). These data clearly showed that six *Pseudomonas* spp. strains, *i.e. P. putida* strains B001, 1P1S2 and P9, *P. citronellolis* 1P2S2, *P. fluorescens* 3P2S1 and *P. protegens* Pf5, significantly enhanced tomato pith necrosis symptoms if compared with the control. Otherwise, co-inoculation with *P. straminea* 2P1S2 provided the lowest pathogenicity value among tested *Pseudomonas* spp. strains (statistically significant data), although this value did not significantly differ from those provided by *X. perforans* inoculated alone. The co-inoculations with *P. putida* 06909, B56 and WCS358, *P. marginalis* 1P5S1 and *P. fluorescens* WCS417 induced stem-pith necrosis lesions similar to those caused by *X. perforans* inoculated alone (Table 2).

Population density of *X. perforans* in stem-inoculated tomato tissues was also influenced by co-inoculated *Pseudomonas* spp. strains. Significantly highest *X. perforans* population densities (ranging from 8.01 to 8.94×10^8 CFU) were found in the co-inoculations with *P. putida* strains 1P1S2 and P9, *P. citronellolis* 1P2S2, *P. fluorescens* 3P2S1, and *P. protegens* Pf5, that caused the most severe pith necrosis symptoms in co-inoculation (Table 2). On the contrary, the lowest value of *X. perforans* population and the lowest pathogenicity value were found in the co-inoculation with *P. straminea* 2P1S2 (statistically significant data if compared to control). Intermediate values of *X. perforans* population (from 7.18 to 8.23×10^8 CFU) were detected for the remaining *Pseudomonas* spp.-*X. perforans* combinations that were similar to those recorded in *X. perforans* inoculation alone (Table 2).

Biofilm formation. Bacterial strains formed differing amounts of biofilm on an inundated polypropylene surface depending both on days after inoculation and media used (Fig. 3). All bacterial strains formed more biofilm when grown in XVM2 medium (Fig. 3B) than in LB medium (Fig. 3A). On both media, the highest biofilm formation was formed after growth for 72 h. The highest biofilm formation response in XVM2 was measured for *P. putida* WCS358, *P. fluorescens* 3P2S1, *P. protegens* Pf5 and *P. putida* P9. All the remaining strains produced an intermediate amount of biofilm.

Droplet collapse. Culture supernatants of *P. straminea* 2P1S2, *P. putida* 1P1S2, *P. citronellolis* 1P2S2, *P. fluorescens* 3P2S1, and *P. protegens* Pf5 exhibited the greatest surfactant activity in the droplet-collapse assay after 1 and 5 days post inoculation as assessed by the diameter of droplets on the waxy surface (Fig. 4). Culture supernatants of the remaining strains exhibited no surfactant activity 1 day post inoculation, and high or intermediate levels of surfactant activity only 5 days post inoculation. *P. citronellolis* 1P2S2, *P. fluorescens* 3P2S1, *P. putida* 1P1S2 and *P. protegens* Pf5, showing the highest surfactant activity, enhanced tomato pith necrotic symptoms when co-inoculated with *X. perforans* (Table 2).

Swarming motility. *P. protegens* Pf-5 and *P. putida* P9 exhibited the highest swarming motility 5 days post inoculation (Fig. 5) whereas *P. putida* WCS358, *P. fluorescens* WCS417, *P. putida* B001 and *P. citronellolis* 1P2S2 exhibited intermediate swarming motility. All the remaining strains did not swarm. *P. protegens* Pf5 and *P. putida* P9, showing the highest swarming motility, enhanced tomato pith necrotic symptoms when co-inoculated with *X. perforans* (Table 2).

Fig. 2. Pith necrosis symptoms resulting from artificially co-inoculation of *Xanthomonas perforans* on tomato plants cv. Sir Elyan with: (a) *Pseudomonas marginalis* 1P5S1; (b) *Pseudomonas protegens* PF5; (c) *Pseudomonas fluorescens* WCS417; (d) *Pseudomonas citronellolis* 1P2S2; (e) *Pseudomonas straminea* 2P1S2; (f) *Pseudomonas putida* P9.

DISCUSSION

Interactions between microorganisms during infection phase are known to influence phytopathogenic behavior, although the extent of such interactions and their potential effects on the ability to colonize and to enhance pathogenicity are little investigated and should be analyzed case-by-case (Llama-Palacios *et al.*, 2002; Lamichhane and Venturi, 2015).

A well-known case of these interactions is the tomato pith necrosis, in which more than one bacterial species can be involved in the occurrence of symptoms. In detail, different *Pseudomonas* species have been frequently associated to this disease in combination among them and other bacterial species (Dimartino *et al.*, 2011; Malathrakis and Goumas, 1987; Molan and Ibrahim, 2007; Pekhtereva *et al.*, 2009). More recently, *X. perforans* has been reported as a new causal agent of pith necrosis and vascular discoloration on tomato plants together with *Pseudomonas* strains (Aiello *et al.*, 2013). *X. perforans*, as well as other Xanthomonads causing bacterial spot of tomato and pepper (BSTP), are pathogens with marked tissue specificity restricted to mesophyll (Vauterin *et al.*, 1995). The reasons that promoted the association of *X. perforans* with vascular and pith syndrome, as well as the way used by the pathogen to reach the pith in natural infections, are still not known. In this regard, the occurrence of injuries and wounds in the root system could have a role on the promotion of tissue penetration and vascular infections. Another possibility is that some cultural practices such as green pruning may influence the penetration, spread and severity of bacterial infections. More likely, epiphytic or endophytic bacteria present at time of infection could provide a way for the penetration and could influence pathogen behavior and disease severity.

This study reports for the first time the synergistic interactions between *X. perforans* and six different *Pseudomonas* spp. on tomato plants. Herein, we demonstrate how the severity of symptoms induced by *X. perforans* may increase when co-inoculated with 6 strains belonging to *Pseudomonas* spp. from different origin (Aiello *et al.*, 2013; Andreote *et al.*, 2009; Dimartino *et al.*, 2011; Geels and Schippers, 1983; Paulsen *et al.*, 2005; Park *et al.*, 2011; Steddom *et al.*, 2002; Van Peer and Schippers, 1992; Wilson *et al.*, 1996). Among these strains, *P. fluorescens* 3P2S1, *P. protegens* Pf5 and *P. putida* P9 and B001, showed both an antagonistic activity and ability in inducing vascular discoloration when individually inoculated on tomato, whereas

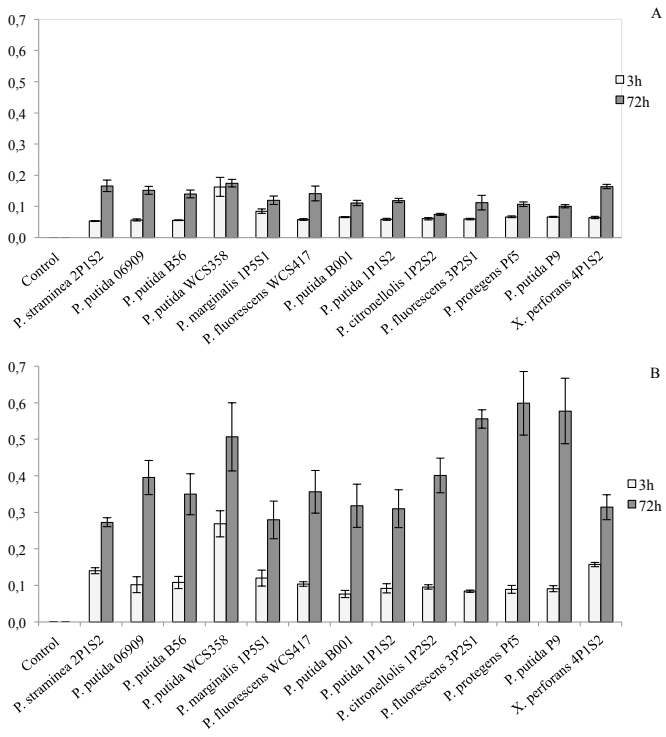

Fig. 3. Biofilm formation by bacterial strains on polypropylene surface, after growth for 3 and 72 h on LB (A) and XVM2 (B) media, quantified by absorbance (at A570 nm wavelength) of crystal violet stain (OD). Data are the mean values of two repeated assays and bars represent the standard error (SE) from the mean of three measurements from five different wells.

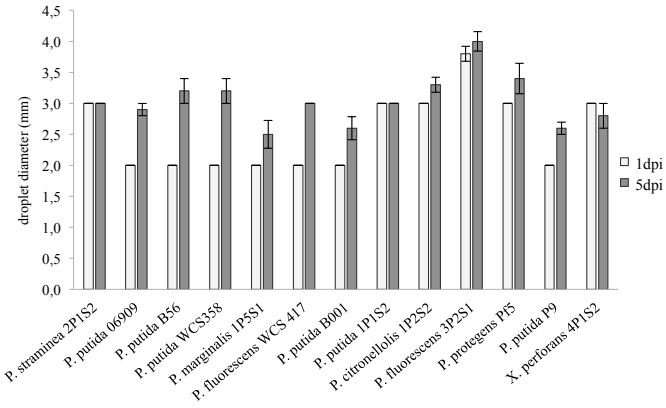

Fig. 4. Surfactant activity by *Pseudomonas* spp. and *Xanthomonas perforans* 4P1S2 determined on KB broth by using the droplet-collapse assay. Mean diameter of droplets from three replicate are shown. Bars on the columns represent the standard error of the mean (SE).

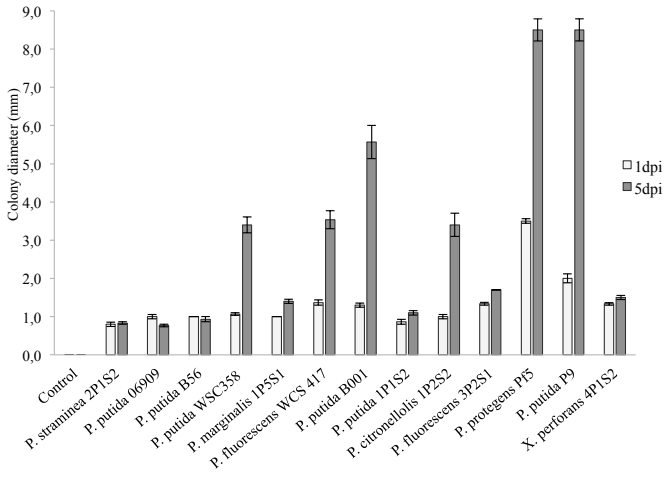

Fig. 5. Swarming motility on modified KB (0.6% agar) plates incubated at 20°C for 1 and 5 days post inoculation. Mean diameter of colonies from three replicate are shown. Bars on the columns represent the standard error of the mean (SE).

P. putida 1P1S2 and *P. citronellolis* 1P2S2 did not show *in vitro* antagonistic activity but were moderately pathogenic when inoculated individually. Accordingly, five of these pseudomonad strains revealed a good ability in increasing *X. perforans* population.

Similar data were recently reported for *P. syringae* pv. *syringae* strains that, when co-inoculated with *P. syringae* pv. *actinidiae*, reached a higher density than in inoculations performed singly (Petriccione *et al.*, 2017).

Based on these data, antagonistic activity of Pseudomonads (and their antimicrobials produced) seems not be related with their ability to enhance *X. perforans* virulence when both bacterial species occurred on the same tomato plant. Nevertheless, some *Pseudomonas* spp. strains well-known in literature as beneficials/antagonists (Paulsen *et al.*, 2005; Andreote *et al.*, 2009; McSpadden Gardener, 2007; Park *et al.*, 2011; Weller, 2007) and evaluated in our study, revealed their ability both in causing disease symptoms alone and in enhancing pith necrosis infections on tomato plants.

Altogether, all tested *Pseudomonas* spp. strains were able to induce vascular discoloration when inoculated individually. *Pseudomonas* species are well distributed into soil and plant rhizosphere as also opportunistic microorganisms (Bradbury, 1986). Although several *Pseudomonas* species have been also reported as pathogens on different hosts (Castello *et al.*, 2017; Dimartino *et al.*, 2011; Zhang *et al.*, 2014; Zhang *et al.*, 2016), our findings represent the first clear evidence about pathogenicity on tomato of antagonistic *Pseudomonas* species.

In plant pathogenic bacteria, biofilms may play an important role in early stages of colonization and infection (Guttenplan and Kearns, 2013) and represent a protective mechanism of growth that allows bacterial cells to survive under unfavorable environmental conditions. Moreover, biofilm formation also contributes to the virulence of phytopathogenic bacteria through various mechanisms, including blockage of xylem vessels, increased resistance to plant antimicrobial compounds, and/or enhanced colonization of specific habitats (Bogino *et al.*, 2013; Mansfield *et al.*, 2012). The present study underlined the differences in biofilm formation strictly depending on *Pseudomonas* spp. strain and on growth media. Lower biofilm formation occurring on rich (LB) than on minimal media (XVM2) confirmed previous findings reported by Rigano *et al.* (2007).

Less biofilm formation after growth in LB medium may be interpreted as a response to nutrient richness and stress absence (Petrova and Sauer, 2012), whereas other medium simulating apoplast conditions (Rico *et al*., 2009) induced a higher biofilm formation because it has scarce nutrient availability. Positive relation between high biofilm formation and enhanced pith necrosis symptoms in co-inoculated tomato tissues was detected for *P. citronellolis* 1P2S2, *P. fluorescens* 3P2S1, *P. putida* P9 and *P. protegens* Pf5. It is well-known as in phytopathogenic bacteria LPs play a crucial role during the early stages of host-interaction, for the virulence development and for auto-aggregation and biofilm formation (Bogino *et al*., 2013; Lau *et al*., 2009). Swarming motility and surfactant activity of *Pseudomonas* species varied widely depending on the tested strain. Bacterial motility is well-known as a factor involved into penetration mechanism by wounds, competitive colonization and dispersal (Danhorn and Fuqua, 2007). Higher swarming motility was observed for *P. protegens* Pf5 and *P. putida* P9, that enhanced pith necrotic symptoms caused by *X. perforans*. Our data suggest that biofilm formation, swarming motility, and surfactant activity could be involved in synergistic interactions for tomato pith necrosis.

In the light of our studies, it is necessary to evaluate the potential risks related to single Pseudomonads prior to large-scale application as BCAs to avoid the use of 'potential' pathogens alone and in synergistic interactions with other microorganisms, resulting in an increase of tomato bacterial diseases. Consequently, the use of commercial fertilizers containing well-known BCAs microorganisms but not characterized under phytosanitary profile should be discouraged.

Synergistic interactions between microbial pathogens in host represent a crucial step for the understanding of microbial pathogenesis and relative setting-up of effective disease control strategies.

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