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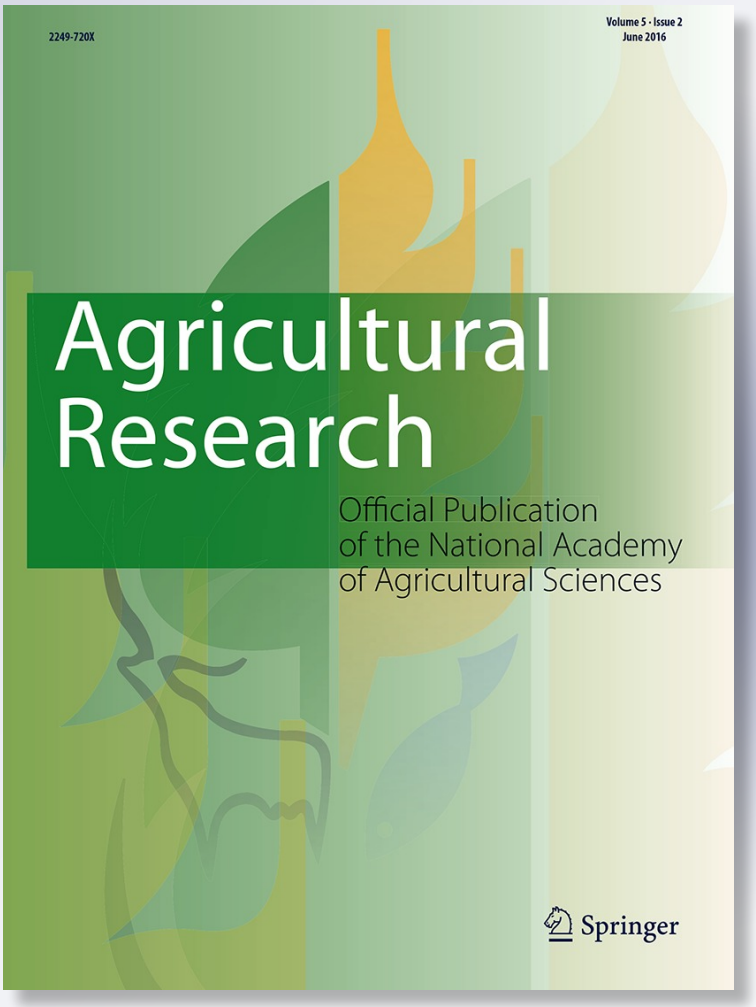

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FULL-LENGTH RESEARCH ARTICLE

Biocontrol of *Rhizoctonia solani* in Tobacco (*Nicotiana tabacum*) Seed Beds Using *Pseudomonas fluorescens*

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Abstract *Pseudomonas fluorescens* isolates were screened from the rhizosphere soil of tobacco-growing fields of Andhra Pradesh and Karnataka. Antagonistic activity of isolates was observed against *Rhizoctonia solani*. In order to obtain a fluorescent *Pseudomonas* strain having capacity to reduce the disease symptoms produced by *R. solani*, six *P. fluorescens* strains with moderate and high fungal growth inhibition capacity were tested in vitro. Tobacco seed beds pre-inoculated with *R. solani* at 10^8 cfu ml^{-1} concentration were treated with six promising isolates of *P. fluorescens* obtained from tobacco rhizosphere. Despite the differences found in the dynamics of colonization and colonization capacity, all evaluated strains induced tobacco growth and reduced disease symptoms produced by *R. solani*. Present investigation clearly indicates that establishment of *P. fluorescens* strains in tobacco rhizosphere is a feasible alternative for the management of *R. solani* symptoms. *P. fluorescens* when applied as a biocontrol agent on tobacco seed beds showed appreciable increase in the biometric parameters of tobacco seedlings.

Keywords Antagonism · Fluorescent *Pseudomonas* · Rhizobacteria · *Rhizoctonia solani* · Tobacco · Biocontrol agent

Introduction

Tobacco (*Nicotiana tabacum* L.), an important commercial crop earning sizable foreign exchange and internal revenue, is susceptible to several fungal diseases in nursery [27]. Leaf being the end product of tobacco, field planting of vigorous and healthy seedlings is the main prerequisite for successful establishment in the field. *Rhizoctonia solani* Kuhn [teleomorph: *Thanatephorus cucumeris* (A. B. Frank)] Donk is a widespread and an ecologically diverse soil-borne fungus, causing different types of diseases in many plant species [8]. It causes root rot, stem rot, fruit and seed decay, damping-off, foliar blight, stem canker and crown rot in various crops [10, 15]. Among the soil-borne fungal diseases of tobacco nurseries, root rot, caused by *R. solani*, is the most common disease [24]. Majority of work done on plant disease biocontrol relate to soil-borne diseases using either bacteria or fungal antagonists [3, 6, 18]. *Pseudomonas* sp. is ubiquitous in agricultural soils, well adapted to growing in the rhizosphere. *Pseudomonas* possesses many traits that make them well suited as biocontrol and growth-promoting agents [33, 34]. The inoculation of seeds or roots with fluorescent *Pseudomonas* to increase plant vigor and productivity in tobacco has been a widely used practice [28]. Investigation into the cause of the beneficial effect of this kind of bacteria has implicated them in the control of a wide range of root phytopathogens, among which *R. solani*, *Pythium aphanidermatum* and *Fusarium oxysporum* can be singled out [27]. The mechanisms suggested for achieving such inhibition include: production of antibiotics, iron-chelating compounds, hydrolytic enzymes and biosurfactants [16], competition for favorable nutritional sites [4, 30], induction of systemic resistance [2, 13] and even due to their action as mycorrhization helper bacteria [25]. However, all disease suppressive mechanisms exhibited by rhizobacteria are essentially of real value only when these bacteria can

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successfully establish themselves in the root environment [14]. The fluorescent *Pseudomonas* can be found in the rhizosphere where microflora colonization depends on characteristics such as soil texture, rhizosphere pH, temperature, soil matric potential, soil water flow, plant species and even plant genotype [26].

Present focus on management of plant diseases has been shifted from chemical pesticides to more ecofriendly biopesticides in order to reduce environmental pollution and to minimize the risk of development of pesticide-resistant strains of plant pathogens. Many bacteria have the potential to reduce crop losses through biocontrol mechanism [19]. Bacteria isolated from the rhizosphere and belonging to a wide variety of genera have the potential to suppress diseases caused by a diversity of soil-borne plant pathogens [1].

Present study was undertaken to develop the most effective biocontrol treatment for the plant pathogenic fungi *R. solani* in tobacco seed beds as an attempt to identify fluorescent *Pseudomonas* strains, useful for the suppression of *R. solani* in tobacco rhizosphere. Fluorescent *Pseudomonas* strain collection was obtained from a previous study [27]; nine isolates of *P. fluorescens* were selected and evaluated in terms of their antagonistic activity against *R. solani* in vitro conditions. Then, six of those isolates were selected in order to analyze: (a) the root colonization dynamics, (b) growth promotion capacity and (c) their antagonistic activity against *R. solani* in tobacco seed beds during 2010 and 2011.

Materials and Methods

Isolation of the Pathogen

For isolation of the pathogen, tobacco seeds at 0.5 g/m^2 bed were sown thickly in pots containing farm soil collected from nursery site under glasshouse conditions. After sowing, the pots were kept under shade and watered daily to create conditions for expression of disease symptoms caused by *R. solani*. After 25 days, seedlings showing root rot symptoms were collected [22] and the pathogen, *R. solani*, was isolated by tissue segment method [20] on potato dextrose agar medium (PDA). It was purified by single hyphal tip method and maintained in potato dextrose agar slants.

Isolation of Bacterial Antagonists

Bacterial antagonists, rhizobacteria were isolated from the rhizosphere soil of tobacco crop as described by [32]. Nine native bacterial antagonists of ICAR-Central Tobacco Research Institute, Rajahmundry were coded as CTRI Pf isolates. These nine isolates were used under in vitro conditions for the management of root rot disease in tobacco seedlings caused by *R. solani.*

In vitro *R. solani* Growth Inhibition Assays

For in vitro *R. solani* growth inhibition assays, the methodology of [5] with modifications was used to determine fungal growth inhibition capacity of *P. fluorescens* isolates. Bacterial colonies grown for 48 h were streaked on the edges of PDA plates and incubated at 25 °C for 72 h. A 0.5-cm^2 plug of a 6 days old *R. solani* culture was inoculated in the middle of the plate. Finally the plates were incubated at 25 °C for 6 days and scored for inhibition of fungal growth by measuring the halo of growth daily in millimeters. The fungi inoculated with a non-antagonistic strain of *Escherichia coli* were used as negative control. This assay was done as a completely randomized design (CRD), having five replicas and two repetitions. The isolates used for this test were: CTRI Pf-2, CTRI Pf-5, CTRI Pf-6, CTRI Pf-9, CTRI Pf-11, CTRI Pf-15, CTRI Pf-18, CTRI Pf-21 and CTRI Pf-24. The results were analyzed using ANOVA.

Characterization of *P. fluorescens* antagonism against *R. solani* in tobacco seed beds

For characterization of *P. fluorescens* antagonism against *R. solani* in tobacco seed beds, nine *P. fluorescens* isolates characterized, presenting none (CTRI Pf-2, CTRI Pf-11, CTRI Pf-18,), moderate (CTRI Pf-5, CTRI Pf-6, CTRI Pf-24,) and high (CTRI Pf-9, CTRI Pf-15, CTRI Pf-21,) fungal growth inhibition in vitro, were screened and six were selected for the evaluation in field conditions. Colonies with natural resistance to rifampicin were selected in order to identify the *Pseudomonas* isolates of interest and distinguish them from the natural population present in the soil. Selected isolates presented total sensitivity at concentrations of 20 μg ml^{-1} or higher. No changes, in growth curves, colony morphology, duplication time, fluorescence pattern or in vitro antagonism, were found after the antibiotic selection using a concentration of 20 μg ml^{-1}.

The methodology reported by [31] and [11] was used for bacterial suspension preparation. Check treatment maintaining the same amount of cfu ml^{-1} (10^8 cfu ml^{-1}) was prepared. The production of *R. solani* was carried out by inoculation in sterile wheat grains (previously soaked for 24 h in tap water), with 1 cm^2 piece of mycelia grown in PDA. The wheat grains were then incubated at 25 °C for 3 weeks. *Rhizoctonia solani* inoculum was added to soil as previously described by [7]. Pathogen was inoculated to the soil 1 day before sowing.

Earthen pots, containing approximately 20 kg of sterilized soil (soil texture sandy clay loam (sand 89 %-silt 5 %-clay 8 %), pH: 6.0), were used to carry out root colonization and antagonism tests. The soil was sterilized in two cycles of 1 h at 121 °C and 15 psi. Tobacco seeds of *N. tabacum* variety VT-1158 were inoculated by submersion in the bacterial suspension. The untreated control and the treatment with *R. solani* alone were submerged in sterile 0.1 M $MgSO_4$.

A CRD having seven replicas was used for the evaluation of the seven treatments (six *P. fluorescens* isolates and the mix of isolates), against *R. solani*. A negative control (with neither pathogen nor antagonist), a disease marker (pathogen without antagonist) and a check were used. Disease presence was determined by the evaluation of diseased seedlings, sclerotia formation.

Root Colonization

Fluorescent *Pseudomonas* population of tobacco rhizosphere resistant to rifampicin (20 μg ml^{-1}) was determined as cfu g^{-1} of root in each one of these samples, following the methodology reported by [11].

The dynamics of root colonization of the isolates was proposed as a CRD with seven treatments using a control (inoculated with 0.1 M $MgSO_4$). Three replicas and five sampling points each were analyzed per treatment. A CRD having 2 × 8 factorial structure, with 16 treatments (six isolates, the bacterial mix and the control with and without *R. solani*) and three replicas, was carried out to determine the possible influence of *R. solani* on *P. fluorescens* isolates colonization. The values were collected at the end of the culture cycle (day 60) and transformed logarithmically (log 10) for the root colonization analysis.

Seedling Production and Growth Promotion

Seedling production and growth promotion of healthy transplantable seedlings were evaluated after harvesting by taking the number and average weight of seedlings produced per m^2 seed bed. At this point, dry weight (in g) was also determined for plant growth promotion in the presence and absence of pathogen. The dry weight was determined by leaving the fresh plants in an oven at 40 °C for 6 days. These assays were carried out as a CRD with 16 treatments and three replications. The *T. viride* isolate was used as a positive biological control [9]. Data were subjected to analysis of variance. Since there is no seasonal variation and similar trend was observed in both the years, data were presented as such.

Table 1 *Rhizoctonia solani* inhibition by different *P. fluorescens* isolates after 6 days of growth in vitro conditions

Strains	% inhibition of growth
CTRI Pf-2	11.07
CTRI Pf-11	17.53
CTRI Pf-18	24.06
CTRI Pf-5	61.00
CTRI Pf-6	63.83
CTRI Pf-24	59.03
CTRI Pf-9	81.67
CTRI Pf-15	79.00
CTRI Pf-21	83.30
S.Em ±	0.65
CD at 5 %	1.91
CV %	2.61

Results

Pseudomonas fluorescens Antagonist Activity Against *R. solani* In Vitro

Six of the nine *P. fluorescens* isolates evaluated inhibited in vitro growth of *R. solani*. The most significant inhibition occurred with the strains Pf-9, Pf-15 and Pf-21 (79–83.3 %). Then, there were three more strains with a moderate activity showing 59.03–63.83 % of growth inhibition and finally three more strains with little activity against *R. solani* (Table 1). From the nine isolates, six were chosen to be evaluated in terms of colonization capacity, plant growth promotion and antagonism against *R. solani* in seed beds. Out of nine three of those isolates presented little (Pf-2, Pf-11 and Pf-18), two moderate (Pf-5, Pf-6 and Pf-24) and two high (Pf-9, Pf-15 and Pf-21) antagonistic activity in vitro conditions against *R. solani*. There was a significant variation among different isolates of *P. fluorescens* in terms of antagonistic activity of *R. solani*. Among the isolates Pf-9 and Pf-21 did not show significant differences between them and were superior to the rest of the isolates followed by Pf-15 with 79 % inhibition. The next in ranking with considerable inhibition were Pf-5, Pf-6 and Pf-24 with inhibition ranging from 59 to 63 %. The least inhibition was recorded in the range of 11.07–24.06 in Pf-2, Pf-11 and Pf-18.

P. fluorescens isolates showed variation in the dynamics of root colonization pattern at various stages of root growth at 20d, 30d, 40d, 50d and 60d. Isolates with moderate and high inhibition activity were chosen for the study along with *T. viride* as positive control, *E. coli* as negative control and *R. solani* as a marker. Population density of each

Table 2 Root colonization dynamics presented by the six analyzed *P. fluorescens* isolates

Strains	20 days	30 days	40 days	50 days	60 days
Pf-9	8.23	8.03	7.96	8.00	8.17
Pf-15	7.60	7.07	7.03	7.03	6.76
Pf-21	7.80	7.53	7.74	7.60	7.67
Pf-5	7.27	6.46	6.53	7.70	6.80
Pf-6	6.90	6.87	7.13	6.97	6.77
Pf-24	6.46	6.70	6.27	6.26	6.33
Control (+ve)	4.07	5.00	5.26	5.10	5.10
Control (−ve)	3.97	4.17	5.30	5.30	4.93
Marker	3.50	3.96	3.90	4.30	4.40
S.Em ±	0.13	0.11	0.09	0.11	0.14
CD at 5 %	0.38	0.32	0.29	0.34	0.42
CV %	0.52	0.44	0.40	0.47	0.58

Root colonization of *P. fluorescens* in the presence and absence of *R. solani*

Table 3 Root colonization of tobacco seedlings by *P. fluorescens* isolates In the presence and absence of *R. solani*

Strains	Log 10 per g of fresh root	
	With *R. solani*	Without *R. solani*
Pf-9	8.57	7.10
Pf-15	8.30	7.17
Pf-21	8.07	6.73
Pf-5	7.16	6.90
Pf-6	7.37	7.40
Pf-24	6.86	6.40
Combined mix	7.50	8.40
Control	5.93	5.73
S.Em ±	0.09	0.09
CD at 5 %	0.28	0.26
CV %	0.39	0.36

Antagonistic effect of *P. fluorescens* against *R. solani* in tobacco seed beds

isolate is different from one another at different intervals (Table 2).

At the time of pulling of tobacco seedlings from seed beds for transplanting them in the field (60d), all isolates showed higher cfu g^{-1} of root in the presence of *R. solani* compared to the treatments without the pathogen. The combined treatment showed a colonization pattern different to that expressed by the other treatments. It was best in the absence of the pathogen, surpassing other treatments by more than 1.0 logarithmic units, while colonization diminished in the presence of the pathogen by 0.9 logarithmic units, placing it among those treatments having the least colonizing effect (Table 3). Statistically significant differences were found between isolates in terms of root colonization. In the presence and absence of *R. solani*, the Pf-9 and Pf-15 isolates showed the greatest root colonization, surpassing other isolates.

All tested isolates of *P. fluorescens* significantly reduced the severity of the disease in the seedlings grown in seed beds (Fig. 1a). The seedlings collected from all the seed beds inoculated with *Pseudomonas* isolates showed much less incidence of disease (Table 4). *P. fluorescens* application also prevented severe levels of disease by comparison with the disease marker, in which severe disease infestation and sclerotia formation were observed (Fig. 1b). In the same way, *T. viride* application significantly reduced the symptoms of the disease and did not allow severe levels to be expressed.

Inoculation of *P. fluorescens* on tobacco seed beds in the absence of pathogen increased the number of healthy transplantable seedlings and dry weight significantly in comparison with the untreated control and the disease marker (Table 5). The Pf-9 and Pf-15 *P. fluorescens* isolates and *T. viride* positive control presented the highest yield increase, of around twofold in comparison with both, the untreated control and the disease marker. The other isolates although had lower seedling production rates and seedling dry weight gain were significantly greater than the control. It is important to mention that despite the differences in number of healthy transplantable seedlings and dry weight between isolates, the presence of *R. solani* did not affect those values within treatments. In the presence of the pathogen, the inoculation of *P. fluorescens* and *T. viride* avoided the development of disease in seedlings. The number of healthy transplantable seedlings obtained was more in case of seed beds inoculated with the isolates Pf-9 and Pf-21, followed by Pf-15. The treatment with combined mix yielded the highest number of healthy transplantable seedlings superior to the individual isolates, which in turn were superior to that obtained by control

Fig. 1 **a** Healthy seedbeds treated with*P. fluorescens* **b** Tobacco seed beds affected by *R. solani*

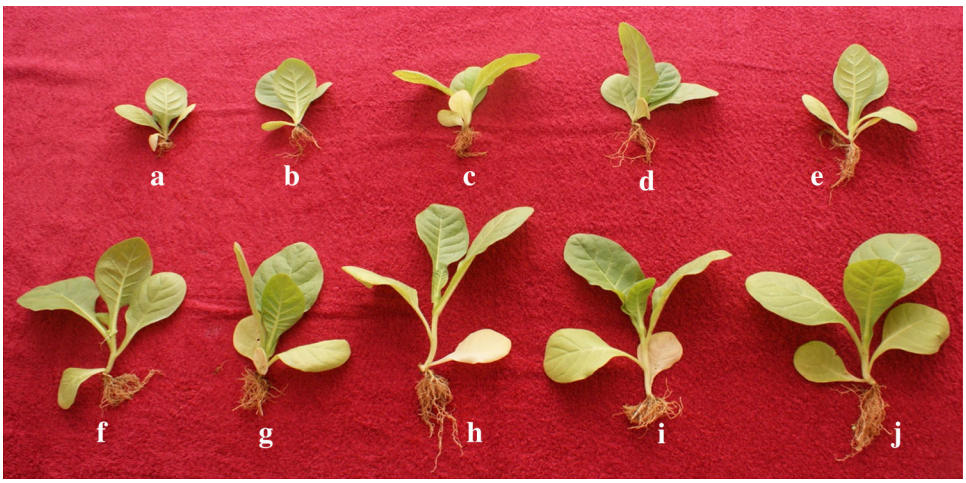

Fig. 2 Treatment variation in the growth of tobacco seedlings due to the effect of *P. fluorescens* isolates against *R. solani*. **a** Marker, **b** Control, **c** Pf 5, **d** Pf 6, **e** Pf 24,**f** Pf 9, **g** Pf 15, **h** Pf 21, **i** *T. viride*, **j** Combined mix

Table 4 Effect of *P. fluorescens* isolates against *R. solani* in tobacco seedbeds

Strains	Diseased seedlings (%)	Sclerotia formation (%)
Pf-9	15.0	11.0
Pf-15	14.7	12.0
Pf-21	16.3	14.0
Pf-5	25.6	19.0
Pf-6	32.0	20.3
Pf-24	32.7	22.3
Combined mix	11.6	15.3
T. viride	15.7	13.6
Marker	69.0	59.3
Control	93.0	92.6
S.Em ±	1.53	1.33
CD at 5 %	4.54	3.95
CV %	6.22	5.41

Yield of healthy transplantable seedlings and growth promotion due to the activity of *P. fluorescens* in the presence and absence of *R. solani*

plants (Fig. 2). Dry weight of the seedlings was highest with the combined mix treatment followed by Pf-9 and Pf-21 (Table 5).

Discussion

Main aim of the study of soil microorganisms is to identify and manipulate natural microbial communities in the rhizosphere. In order to advance in this direction more studies have to be conducted in the structure and dynamics of microbial populations in the rhizosphere of plants [14]. Hence, research efforts were concentrated to identify the more representative species of fluorescent *Pseudomonas* in tobacco rhizosphere, in order to improve the chances of selecting the most successfully established bacteria of this type, for tobacco rhizosphere [29]. Earlier reports showed *P. fluorescens* was the dominant species in the rhizosphere and rhizoplane of tobacco [23]. This finding leads to take up the current study where six isolates of *P. fluorescens*

Table 5 *N. tabacum* seedling production and growth promotion after inoculation with *P. fluorescens* isolates in seedbeds after 60 days

Strains	No. of transplantable seedlings per m^2 bed		Dry weight of the seedlings (g)	
	R. solani	No *R. solani*	*R. solani*	No *R. solani*
Pf-9	235	382	3.80	6.72
Pf-15	227	378	3.70	6.63
Pf-21	240	373	3.75	6.57
Pf-5	220	343	3.55	5.73
Pf-6	223	355	3.58	5.65
Pf-24	204	350	3.57	5.77
Combined mix	275	370	3.83	6.90
T. viride	222	346	3.56	5.83
Control (-ve)	196	207	3.32	4.33
Marker	55	302	2.68	4.63
S.Em ±	6.10	8.78	0.04	0.08
CD at 5 %	18.14	26.10	0.13	0.26
CV %	5.04	4.60	2.11	2.53

were selected to identify their capacity of reduction of *R. solani* symptoms and the growth promotion of tobacco on seed beds. Despite the lack of correlation between results of in vitro and in vivo antibiosis of fluorescent *Pseudomonas* against several soil-borne pathogenic fungi [17], the in vitro antagonistic capacity of these bacteria has been continuously selected for screening purposes [13, 14, 27]. The success of that strategy for the prediction of antagonistic activity against *R. solani* in the rhizosphere of tobacco was evaluated by application of *P. fluorescens* isolates with little, moderate and high activity against *R. solani* under in vitro condition. These results suggest that the association of *P. fluorescens* isolates with tobacco rhizosphere and its antagonistic capacity against *R. solani* are determined by the colonization capacity of the bacteria, more than the production of active compound against a pathogenic agent. The results obtained in this study show that *P. fluorescens* acts as a plant growth promoter of tobacco by reducing the disease symptoms caused by *R. solani* and increasing seedling production and plant dry weight. Although similar results have been reported for the related crops using *P. fluorescens* [11], this is the first report of this kind of results for the management of tobacco nurseries. The dynamics of root colonization of six *P. fluorescens* isolates is presented here to understand the effect of this bacterium on tobacco. The population density obtained for each isolate varies differently throughout the crop cycle. Such variation is related to the phenological changes of the plant as it is deduced from the relation between the seedling growth phase (day 60) and the increase shown by the cfu of all *Pseudomonas* isolates. However, the variations found overall crop cycle between the different strains show that the association dynamics is a strain-specific phenomenon, which is not well understood [11, 14].

Populations of the rifampicin-resistant *Pseudomonas* for all the treatments (except for the combined treatment) were greater on tobacco rhizosphere infected with *R. solani* than on roots without the pathogen (Table 3). This effect probably resulted from the increased availability of root exudates released through lesions incited by the root pathogen [27]. This finding supports the contention of Mazzola 1998 [12]. Despite the *R. solani* establishment, the increase in seedling production in all plants inoculated with a single strain and the increase in plant weight induced by all the isolates were not affected by the presence of the pathogen. The lower population obtained by the combined treatment in the presence of the pathogen suggests a decrease in the colonization capacity of the combination mix under these conditions. That effect was probably due to the expression of different antagonistic compounds by some or each isolates, affecting the colonization of the artificial community. Such compounds usually provide a selective advantage over rhizosphere colonist, even if they are from the same or related species [23]. The above-mentioned results also explain why the mixture of *P. fluorescens* strains tested in this study was not superior in diminishing the severity of the *R. solani* symptoms, in comparison with the results obtained for each strain alone. Other studies using combinations of fluorescent *Pseudomonas* have revealed the benefits of that strategy to provide greater control of different plant pests [21]. The present investigation clearly indicates that other combinations of the evaluated *Pseudomonas* strains or even some strains with *T. viridi* isolate can improve the results obtained in this study. Other strain combinations should

improve results by broad spectrum of antifungal metabolites or mechanisms beyond those produced by *P. fluorescens*. Nevertheless, compatibility between these biocontrol agents must first be evaluated [27].

The results of the present study suggest that the utilization of *P. fluorescens* for the control of *R. solani* is a promising strategy for disease management. This confirmation is supported by the fact that all tested *P. fluorescens* isolates reduced the disease severity in tobacco seed beds. Such reduction was evident due to the decrease in the number of affected seedlings, decrease in the number of sclerotium formation and disappearance of the presence of severe disease symptoms on the seed beds. Under the conditions tested, the isolates Pf-9, Pf-15 and Pf-21 can be considered for a next level of evaluation as a promising isolates due to their good performance in terms of colonization, plant growth promotion and the reduction of the *R. solani* disease symptoms.

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