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In vitro antagonistic activity of native bacteria isolated from soils of the argentine Pampas against *Fusarium tucumaniae* and *Fusarium virguliforme*

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The aim of the present study was to evaluate indigenous PGPR (Plant growth promoting rhizobacteria) previously isolated from Argentina's soybean fields for their *in vitro* antagonistic effects on the control of *Fusarium tucumaniae* and *F. virguliforme*, in two separated *in vitro* assays. In assay 1, the bacteria that showed the highest significant (P < 0.05) *F. tucumaniae* mycelial growth inhibition were strains *Bacillus subtilis* 54 (70%), *B. cereus* 13 (44%), *B. cereus* 7 (44%) and *Chryseobacterium vietnamense* 110 (42%). Despite their antagonistic activity, the strains identified as *Stenotrophomonas malthophilia* and *B. cereus* were not included in any further experiments, because of their potential hazard. In assay 2, strains 54, 110 and *Pseudomonas fluorescens* 9 and 115 were tested against *F. tucumaniae* and *F. virguliforme*. In this study, native bacterial strains isolated from Argentine Pampas were tested for the first time against these pathogens. All four bacterial strains significantly inhibited mycelial growth of *F. virguliforme*. Further studies on the effects of these strains on the growth of soybean plants and on the Sudden Death Syndrome (SDS) control will uncover the mechanisms and *in vitro* antagonism potential of these bacterial isolates.

Key words: Antagonism, plant growth promoting rhizobacteria (PGPR), *Fusarium*, *Pseudomonas*, *Bacillus*, *Chryseobacterium vietnamense.*

INTRODUCTION

Soybean is the main crop in Argentina. In the last seasons, the area planted to soybean in Argentina was about 20 million hectares per year (Carmona et al., 2015). Sudden Death Syndrome (SDS) is a soybean

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disease caused by at least four *Fusarium* species, but in Argentina *F. tucumaniae* and *F. virguliforme* are the predominant. These are soil-borne pathogens commonly found in Argentina's pampas region and are important causes of crop losses (Scandiani et al., 2010). The fungus infects soybean roots and, under appropriate conditions, toxin-dependent symptoms develop in the aerial tissues after flowering and during pod fill, leading to rapid necrosis (Hartman et al., 2015). Under monoculture and no-till conditions in the argentine Pampas region the presence of SDS has intensified (Scandiani et al., 2010). Because of the difficulty in obtaining resistant soybeans varieties, the impossibility of fungicides to move towards the roots basipetally, wide host range of the pathogen and its ability to survive in the soil with resistance structures (chlamydospores), common management strategies such as genetic resistance, seed treatment with fungicides and crop rotation do not provide adequate control of SDS (Scandiani et al., 2010). In this context, biological control appears as an alternative and interesting tool.

Plant growth promoting rhizobacteria (PGPR) have been widely reported and recognized to have the potential for PGP and for their ability to antagonize the growth of fungal pathogens in crops such as maize, rice, potato, wheat and canola (Siddiqui et al., 2006). In soybean, PGPR were successfully tested against *Macrophomina phaseolina* (Simonetti et al., 2015) and *Pythium ultimum* (León et al., 2009).

Biocontrol PGPR are able to antagonize phyto-pathogenic fungi by different mechanisms (Siddiqui et al., 2006), including antibiosis, competition, mycoparasitism, degrading enzymes or induced resistance (Ahmad et al., 2008). PGPR could produce antibiotics or secrete lytic enzymes such as glucanases, proteases, cellulases and chitinases that degrade disease-causing fungi cells (Someya et al., 2007). Antibiotics produced by PGPR include volatile antibiotics (hydrogen cyanide, aldehydes, alcohols, ketones, and sulfides) and nonvolatile antibiotics such as polyketides (diacetyl phloroglucinol; 2,4 diacetylphloroglucinol and mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives) and phenylpyrrole antibiotic (pyrrolnitrin) (Dilantha Fernando et al., 2005). PGPR could also antagonize by competition, for example by siderophore production. In addition, siderophores produced by PGPR could contribute to enhanced plant growth.

There are few reports on the antagonistic effect of bacterial isolates on soybean *Fusarium* species causing SDS (Xing and Westphal, 2007; Agaras et al., 2012). It is reported that the chance of finding bacterial strains effective for biocontrol increases if the isolates are obtained from pathogen suppressive soils and from the same environment in which they will be used (Cook and Baker, 1983). The aim of the present preliminary study was to evaluate indigenous PGPR previously isolated from soy fields in Argentina's pampas region for their *in vitro* antagonistic effects on the control of *F. tucumaniae* and *F. virguliforme*.

MATERIALS AND METHODS

Ten PGPR strains that were previously isolated and identified by Simonetti et al. (2015) for their *in vitro* antagonistic capacity against *M. phaseolina*, were tested for their *in vitro* inhibitory capacity against the fungal pathogen *F. tucumaniae* (Table 1).

The fungal strains used in this study were originally isolated from infected soybean plants showing SDS root rot and provided by Centro de Referencia de Micología (CEREMIC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (Table 1).

Assay 1

All bacterial strains were tested for their ability to inhibit the mycelial growth of *F. tucumaniae*149-12. Each bacterial isolate was streaked as a band on the edge of a PDA 90-mm diameter plate and incubated for 24 h at 28 ± 2°C. Then, a 6 mm diameter mycelial disc of *F. tucumaniae* 149-12 was taken from the margin of a growing colony and placed onto the centre of previously inoculated potato-dextrose-agar (PDA) plates. The Petri dishes were sealed by parafilm and incubated at room temperature in the dark. Plates containing only the fungal mycelial plug were maintained as control.

Assay 2

The bacterial strains that did not show genetic relationship with potentially hazardous bacteria in the assay 1 were tested for their ability to inhibit the growth of *F. virguliforme* 101-03 and *F. tucumaniae* 149-12 using *in vitro* dual-culture assay (Simonetti et al., 2012). Each bacterial isolate was prepared in nutrient broth (NB) and incubated for 48 h at 28 ± 2°C in order to use them in stationary phase. Fungi were maintained on PDA at 24 ± 2°C for one week. A 6 mm diameter mycelial plug was taken from the margin of a growing colony and placed centrally in a Petri dish containing PDA medium. Two drops (2 µL) of each bacterial culture previously prepared were placed in a straight line 3 cm away from the center of the plate and drops of sterile water served as control.

All these experiments were performed in triplicate. After incubation period of 11 days at 24 ± 2°C, mycelium growth inhibition was calculated as $I=[(C-T)/C]\times100$, where C is the mycelium diameter in control, and T is the mycelium diameter in bacteria-inoculated plates.

Data were analyzed using analysis of variance and differences between means were tested using Tukey test with an overall risk level of 5%.

RESULTS

In assay 1, the bacteria that showed the highest significant ($P < 0.05$) *F. tucumaniae* 149-12 mycelial growth inhibition were strains 54 (70%), 7 (44%), 13 (44%), 110 (42%), 125 (34%), 123 (32%), 116 (31%) and 48 (30%) (Figure 1A and B). Despite their antagonistic activity, the strains identified as *S. malthophilia* (48) and *B. cereus* (7, 13, 116, 123 and 125) were not included in any further experiments because of their genetic

Table 1. Identification of PGPR and fungal strains used in the antagonistic trials.

PGPR strain ID	Strain genus/species	Geographic origin	
		Location	GPS Coordinates
7	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
9	*Pseudomonas fluorescens*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
13	*Bacillus cereus*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
48	*Stenotrophomonas maltophilia*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
54	*Bacillus subtilis*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
110	*Chryseobacterium vietnamense*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
115	*Pseudomonas fluorescens*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
116	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
123	*Bacillus cereus*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
125	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
FungalStrain ID	**Strain genus/species**	**Geographic origin**	
		Location	**GPS Coordinates**
149-12	*Fusarium tucumaniae*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
101-03	*Fusarium virguliforme*	San Pedro, Buenos Aires	33°41'42.79"S, 59°42'0.60"O

PGPR bacteria strains were previously isolated and identified by Simonetti et al. (2015).

relationship with potentially hazardous bacteria (Bottone, 2010; Brooke, 2012). For this reason only strains 9, 54, 110 and 115 were used in assay 2.

In assay 2 (Table 2), all four bacterial strains significantly inhibited mycelial growth of *F. virguliforme* 101-03. Strain 110 (*C. vietnamense*) exhibited the highest inhibition on the mycelial growth of *F. virguliforme* 101-03 (31.78%) (Figure 1D). On the other hand, the only strain that significantly inhibited mycelial growth of *F. tucumaniae* 149-12 was strain 54 (*B. subtilis*) (44%) (Figure 1C) and strains 9 and 115 (*P. fluorescens*) showed no significant effect, in accordance with assay 1. However, these strains (9 and 115) significantly inhibited mycelial growth of *F. virguliforme* 101-03 (22.48 and 19.4%, respectively).

DISCUSSION

Altogether, these results suggest that strain 54 (*B. subtilis*) displays antifungal features mainly towards *F. tucumaniae* 149-12, one of the causing agents of soybean SDS. The original contribution of this study is the isolation and testing of bacteria originating from the Pampas region.

These findings are in accordance with those of Xing and Westphal (2007), who found antagonism of *B. subtilis* against 12 isolates of *F. virguliforme*. On the other hand, our results differ with those found by Agaras et al. (2012) where *Pseudomonas* strain SMMP3 antagonized the growth of several pathogenic fungi, including the *F. tucumaniae* isolate CCC 132-11.

Because good results obtained *in vitro* cannot always be dependably reproduced under field conditions, these *in vitro* results should be confirmed by *in planta* experiments. In this way, Agaras et al. (2012) carried out both greenhouse and field trials using soybean seeds inoculated with *Pseudomonas* strain SMMP3. This inoculation treatment caused a reduction of SDS ratings (incidence, severity) and a decrease in the AUDPC (area under disease progress curves) values. Nevertheless, these effects were not statistically meaningful (P>0.05). This may be due to the many factors that affect the effectiveness of the bacteria in natural conditions (Badri et al., 2009). Isolated bacterial strains should be rhizospheric competent, able to survive and colonize in the rhizospheric soil (de Souza et al., 2015).

Further studies on the effects of this strain on the growth of soybean plants and on the SDS control will uncover the mechanisms and potential of this bacterial isolate. However, it has been previously described that most cases of naturally occurring biological control result from mixtures of antagonists, rather than from high populations of a single antagonist (Myresiotis et al., 2012). Moreover, root-infecting *Fusarium* species attack soybean seedlings in the first developmental stages, thus additional tests as for example seed treatment with multiple strain inoculation might be required to improve the degree of SDS control.

Conflict of Interests

The authors have not declared any conflict of interests.

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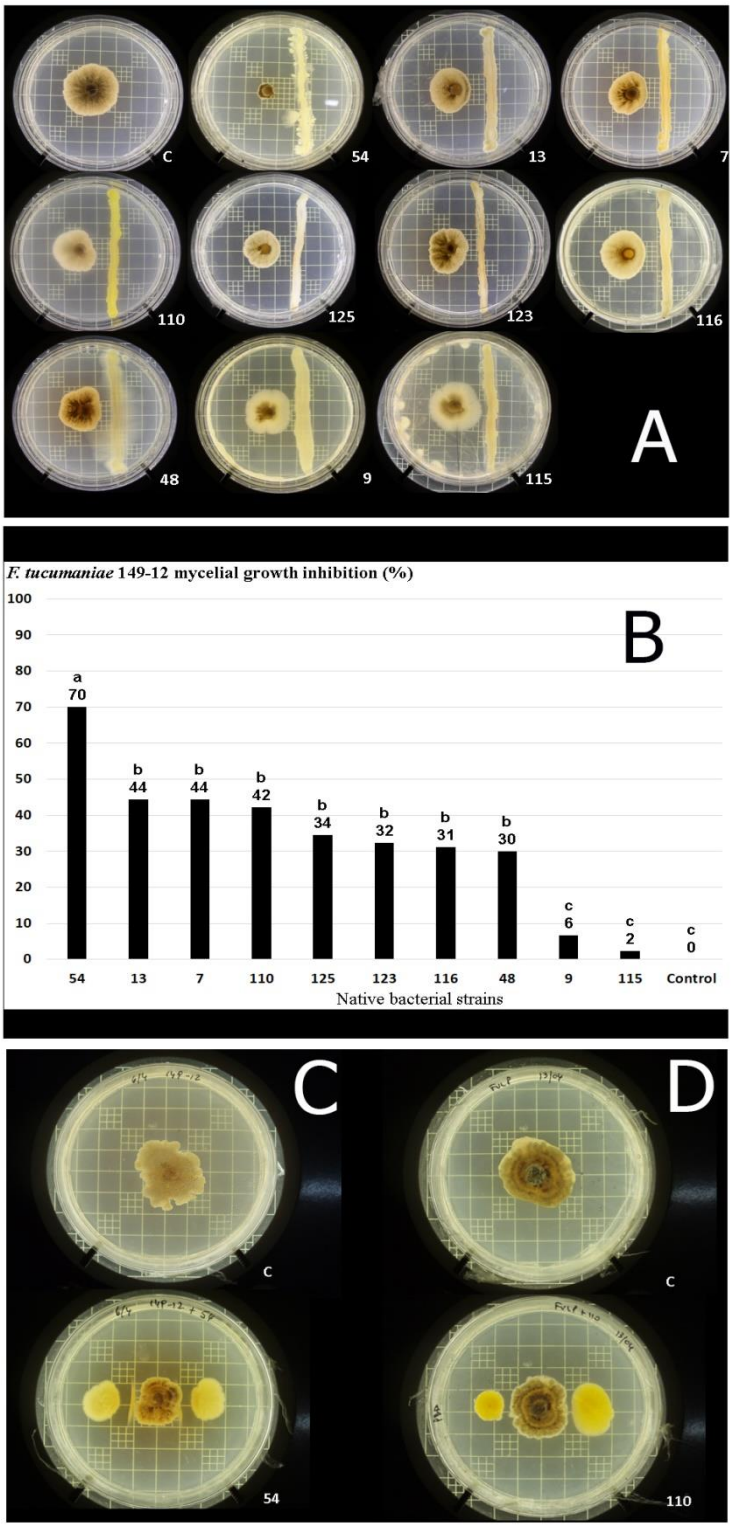

Figure 1. Photographs (A) and figure (B) of the bacteria strains tested for their ability to inhibit the mycelial growth of *F. tucumaniae* 149-12, in assay 1 (columns with different letters are significantly different P<0.05); Photographs of *B. subtilis* strain 54 and its control, tested for their ability to inhibit the mycelial growth of *F. tucumaniae* 149-12 (C) in assay 2; and *C. vietnamense* strain 110 and its control, tested for their ability to inhibit the mycelial growth of *F. virguliforme* 101-03 (D) in assay 2.

Table 2. *F. tucumaniae* and *F. virguliforme* mycelial growth inhibition caused by four selected strains of native bacteria isolated from soils of the argentine Pampas, in assay 1 and 2.

Trial	Treatment (Strain ID)	Mycelial diameter (mm)	Standard deviation	Mycelial growth inhibition (%)
1	Control (*F. tucumaniae* 149-12)	15.0^{c}	1.0	-
	115 (*P. fluorescens*)	14.7^{c}	0.6	2.2
	9 (*P. fluorescens*)	14.0^{c}	1.0	6.7
	54 (*B. subtilis*)	4.5^{a}	1.3	70.0
	110 (*C. vietnamense*)	8.7^{b}	1.2	42.2
2	Control (*F. tucumaniae* 149-12)	25.0^{b}	2.6	-
	115 (*P. fluorescens*)	20.0^{b}	1.0	20.0
	9 (*P. fluorescens*)	22.3^{b}	3.2	10.7
	54 (*B. subtilis*)	14.0^{a}	1.0	44.0
	110 (*C. vietnamense*)	25.3^{b}	1.2	-1.3
	Control (*F. virguliforme* 101-03)	43.0^{c}	2.0	-
	115 (*P. fluorescens*)	34.7^{ab}	2.1	19.4
	9 (*P. fluorescens*)	33.3^{ab}	2.1	22.5
	54 (*B. subtilis*)	35.3^{b}	2.5	17.8
	110 (*C. vietnamense*)	29.33^{a}	1.53	31.8

Means with different letters within a column are significantly different (P<0.05).

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