

Biocontrol of Chickpea Wilt Caused by *Fusarium oxysporum* f. sp. *ciceris* Race 0 with Plant Growth-Promoting Bacteria

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ABSTRACT

Objective: To evaluate the antagonisms of the plant growth-promoting bacteria, A46, P61 (*Pseudomonas tolaasii*), R44 (*Bacillus pumilus*), BSPS1.1 (*Paenibacillus polymyxa*), and CPPC55 (*Serratia plymuthica*) strains to *Fusarium oxysporum* f. sp. *ciceris* race 0 under *in vitro* and *in vivo* conditions.

Design/methodology/approach: A growing chamber and greenhouse assays were carried out to evaluate the antagonistic effect of the plant growth-promoting bacteria strains against *Fusarium oxysporum* f. sp. *ciceris* race 0 in Petri dishes and chickpea plants, respectively.

Results: The five bacteria strains significantly reduce the pathogen radial growth, and the strains A46, P61, R44 and CPPC55 reduced the chickpea plant disease symptoms and the affectation of different plant morphological traits such as height, stem width, number of leaves and root volume, plus nitrogen and potassium content.

Limitations on study/implications: *Fusarium* Wilt is one of the main causes of chickpea production losses worldwide. The identification and evaluation of promising biocontrol agents are crucial for their inclusion in integrated management programs to the disease.

Findings/conclusions: Chickpea seeds inoculation with plant growth-promoting bacteria strains A46, P61, R44 and CPPC55 are recommended to *Fusarium oxysporum* f. sp. *ciceris* race 0.

Keywords: Control, Fusariosis, *Cicer arietinum*, wilt, PGPB.

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INTRODUCTION

Mexico is in the top ten of main producers of chickpea (*Cicer arietinum* L.) worldwide and in the second place in the Americas with around 111,143 ha cultivated in 2022 (FAOSTAT, 2023). Northwestern region, including Sinaloa, Sonora and Baja California states is the main producer area of chickpea in Mexico, where production reached in 2022 about 74.13% of the total national area cultivated to chickpea (SIAP, 2023).



Different factors affect chickpea yield in Northwestern Mexico such as wilting diseases caused by soil born fungi complex, including *Fusarium oxysporum* (Cota-Barreras *et al.* 2024). For chickpea, eight *F. oxysporum* f. sp. *ciceris* races have been described so far which are grouped according to their symptomatology into yellowing (0 and 1B/C) and wilting pathotypes (1A, 2, 3, 4, 5 and 6) (Jendoubi *et al.* 2017). Five out of the eight races have been reported in Mexico (0, 1B/C, 4, 5 and 6) and two in the Northwestern states (0 and 5) (Velarde-Félix *et al.* 2015; Guerrero-Aguilar, 2015). In this region, wilting by *F. oxysporum* f. sp. *ciceris* causes losses up to 60% (Arvayo-Ortiz *et al.* 2012), and commercial cultivars and breeding lines are susceptible, including race 0 provoking farmer's efforts to manage the disease to not work, needing control alternatives.

The use of plant growth-promoting bacteria is an effective, eco-friendly, and sustainable control strategy to complement or substitute the use of chemical fungicides (Shaikh and Sayyed, 2014). Plant growth-promoting bacteria are known to promote the plant development by producing siderophores, auxins, phytohormones and enzymes, nitrogen fixation, solubilizing phosphate, releasing ammonia or by supplying essential vitamins to plants (Saini *et al.* 2023). Nevertheless, these microbes also exhibit protection against phytopathogenic fungi mediated by volatile metabolites including hydrogen cyanide and ammonia, iron acquisition by siderophores production of antibiotic, exopolysaccharides, production of cell wall degrading enzymes and antioxidant enzymes like superoxide dismutase, catalase, peroxidase and phenols (Chowdhury *et al.* 2020; Khalil *et al.* 2022). In Mexico, different screening studies have been conducted to detect, identify, characterize and test different plant growth-promoting bacteria, strains A46, P61 (*Pseudomonas tolaasii*), R44 (*Bacillus pumilus*), BSPS1.1 (*Paenibacillus polymyxa*), and CPPC55 (*Serratia plymuthica*) have proved to promote different plant growth parameters and nutrients uptake on several crops such as peppers (*Capsicum* spp.), maize (*Zea mays*) and cucumber (*Cucumis sativus*) (Perez-Rosales, 2012; Benítez-Noyola, 2013; Angulo-Castro *et al.* 2018; Castillo-Aguilar *et al.* 2017; Quiroz-Sarmiento *et al.* 2019), however, evaluations against phytopathogenic fungi are limited (Pérez-Rosales 2012; Pineda-Mendoza *et al.* 2018; Florencio-Anastasio *et al.* 2022) and for *Fusarium oxysporum* f. sp. *ciceris* race 0 no study has conducted. Therefore, the goals of the present study were to evaluate the antagonisms of these five bacteria strains to *Fusarium oxysporum* f. sp. *ciceris* race 0 under *in vitro* and *in vivo* conditions.

MATERIAL AND METHODS

The *Fusarium oxysporum* f. sp. *ciceris* race 0 (FOC 0) isolate used was provided by Dr. Sixto Velarde-Félix (INIFAP, Culiacán, Sinaloa) with isolate number 130CS and accession number KJ000583. The PGPB strains *Pseudomonas tolaasii* "R46" (KY933652) and "P61" KY933651, *Bacillus pumilus* "R44", *Paenibacillus polymyxa* "BSPS1.1", and *Serratia plymuthica* "CPPC55" (KX259564) were provided by the Colegio de Postgraduados, Montecillo, México.

The fungal isolate was prepared in Petri dishes (90×15 mm) using the portion inclusion method (Leslie and Summerell 2006). Mycelium disks of 1-cm in diameter cut with a perforating punch were placed in the center of Petri dishes supplemented with PDA-Difco® culture medium (39 g L⁻¹), incubated at 25 °C for 7 days in complete darkness

inside a Friocel[®] growth chamber (Landa *et al.*, 2006), the final conidia suspension of the isolate was adjusted to 1×10^6 spores/mL. On the other hand, the bacterial strains were prepared in Petri dishes without division (90×15 mm) with Difco[®] nutritional culture agar (23 g L^{-1}) under aseptic conditions in a laminar flow hood. A sample of each strain was taken following the streak plate method using a sterile bacteriological loop. Dishes were incubated at 28°C for 24 h for their development under darkness in Friocel[®], the final concentration for each strain was adjusted to 1×10^8 CFU/mL.

A growing chamber assay (25°C) was carried out to evaluate the antagonistic effect of the five PGPB strains against FOC 0 in Petri dishes with the dual culture technique (Ezziyyani *et al.*, 2004). For this, a 1-cm diameter disk of the PDA-grown phytopathogen was placed at one end of the plate and, after 72 h, the bacterial strain was sown in the center of the plate using a thick loop forming a vertical barrier (1.0 cm thick); a control dish with only the fungal inoculum was also included. The bacterial inhibitory capacity was determined after seven days. A digital Vernier was used to measure the pathogen radial growth (cm), subsequently, the inhibitory percentage of radial growth (IPRG) was calculated with the formula from Ezziyyani *et al.* (2004).

$$IPRG = (R1 - R2) / R1 \times 100$$

where: $R1$ = larger *Fusarium* growth radii and $R2$ = smaller *Fusarium* growth radii.

A completely randomized design was used with four replications per treatment where each challenging disk was considered as replication. The experiment was performed twice.

An *in vivo*-greenhouse experiment ($25\text{--}30^\circ\text{C}$ and $50\text{--}60\%$ HR) was carried out to evaluate the effect of the five PGPB strains to control FOC 0 in chickpea plants (Blanco Sinaloa-92). Disinfected seeds (2.5% sodium hypochlorite) were submerged in a 1×10^8 CFU/mL bacterial inoculum for 5 min. Inoculated seeds were germinated and grown individually in polystyrene trays of 128 cavities (autoclaved peat and vermiculite [2:1]). Each plant (3 true leaves) received 15 mL of fungal inoculum (1×10^6 spores mL^{-1}). To fertilize the plants, a Steiner solution (0.5 ds/m and pH 6.5) was used with a daily dose of 50 mL per plant. Plants symptoms were recorded every 15 days after the inoculation (DAI) until 75 DAI using the Fusariosis wilt visual scale (0-4) proposed by Marlatt *et al.* (1996), where: 0 = asymptomatic plant; 1 = mild wilting symptoms, leaf chlorosis, growth delay; 2 = moderate chlorosis symptoms, wilting, growth delay; 3 = severe chlorosis symptoms, wilting or growth delay; 4 = dead plant, completely wilted. The disease severity index (Chiang and Bock, 2022).

$$(DSI)(\%) = \left[\sum \frac{(\text{class frequency} \times \text{score of rating class})}{(\text{total number of observations})} \times (\text{maximal disease index}) \right] \times 100$$

On the other hand, the mean area under disease progress curve (AUDPC) was calculated using the formula of Pandey *et al.* (1989),

$$AUDPC = D \left[\frac{1}{2}(Y_1 + Y_k) + (Y_2 + Y_3 + \dots + Y_{k-1}) \right]$$

where D =time interval; Y_1 =first DSI; Y_k =last DSI; and Y_2, Y_3, Y_{k-1} =intermediate DSI.

Besides the visual damage, plant growth parameters such as plant height (cm), stem thickness (mm), number of leaves, and root volume (cm³) were assessed in every evaluation. Additionally, in the last evaluation, five plants were randomly selected from each treatment and the content of the nitrogen, phosphorus, and potassium (mg kg⁻¹) was determined as follows: nitrogen was determined through the semi-micro Kjeldahl procedure (Etchevers, 1987). Phosphorus was determined through colorimetry of molybdophosphoric acid complexes reduced with ascorbic acid (AOAC, 1980). Potassium was determined through flame photometry according to Rodríguez and Rodríguez (2015). The handling of the sample included dehydrating at 70 °C, 24 h, grinding and weighing 0.5 g of dry matter from all the organs of the plant (root, stem and leaves).

All data were analyzed with the XLSTAT statistics software (Addinsoft, 2019). Data generated from both experiments was combine since no statistical difference was exhibited ($P \leq 0.05$). The data was subjected to Shapiro-Wilk and Barlett tests to verify the statistical assumptions of normality and homogeneity of variances, respectively. The radial growth from the *in vitro* assay did not meet these statistical assumptions, therefore, a nonparametric variance analysis was used. In the *in vivo* assay, the damage scale and plant phenotype indicators (plant height, stem thickness, number of leaves and root volume) met the statistical assumptions, therefore, a parametric variance analysis was used. On the other hand, the content of the nitrogen, phosphorus, and potassium did not meet these assumptions. For the multiple comparison among treatments, an ANOVA and the Tukey mean test were used in the parametric variables to determine the significance among treatments ($P \leq 0.05$). For the non-parametric factors, the Kruskal-Wallis and the Dunn median tests were used ($P \leq 0.05$).

RESULTS AND DISCUSSION

The efficacy of the five PGPB *P. tolaasii* “A46” and “P61”, *B. pumilus* “R44”, *P. polymyxa* “BSP1.1”, and *S. plymuthica* “CPPC55” was observed to reduce *Fusarium oxysporum* f. sp. *ciceris* race 0 (FOC 0) radial growth *in vitro*. The five bacteria exhibited a statistical difference with the control but not between them ($H = 14.000$; $df = 5$; $P = 0.016$), indicating a strong inhibition action against the pathogen with a range from 40.92 to 58.51% (Table 1) (Figure 1).

These results indicate an antibiotic ability by the strains; A46 and P61 have been reported to produce siderophores while BSP1.1 and R44 do not, and CPPC55 has not been tested (Angulo-Castro *et al.*, 2018; Pineda-Mendoza *et al.*, 2019), suggesting the involvement of a different compound. Further studies need to be carried out in order to identify it. These results partially agree with those reported by other authors such as Pérez-

Table 1. Inhibition of *in vitro* mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* races 0 by the five plant growth-promoting bacteria strains on PDA media.

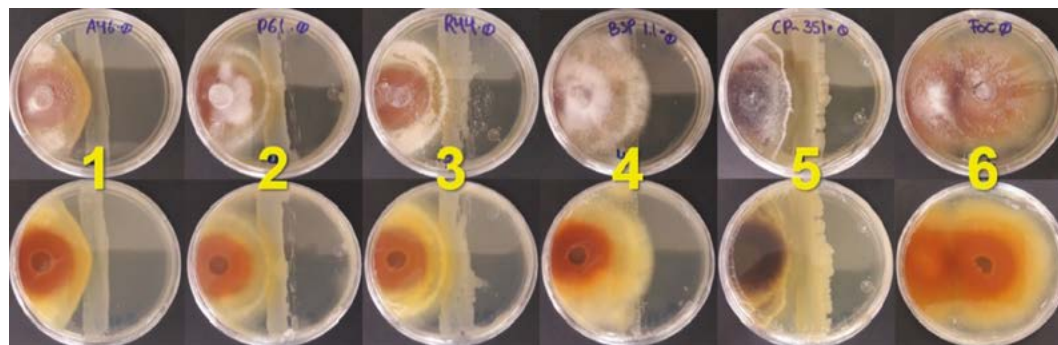
Strain	Pathogen growth (cm)	IPRG (%)*
A46 (<i>Pseudomonas tolaasii</i>)	3.87±0.64 b**	48.45
P61 (<i>Pseudomonas tolaasii</i>)	3.96±0.40 b	47.28
R44 (<i>Bacillus pumilus</i>)	3.76±0.32 b	49.88
BSP1.1 (<i>Paenibacillus polymyxa</i>)	4.43±0.09 b	40.92
CPPC55 (<i>Serratia plymuthica</i>)	3.11±0.08 b	58.51
Control	7.50±0.08 a	/

* IPGR = inhibition percentage of radial growth = $\frac{[control - treatment]}{control} \times 100$.

**=Means with different letter in columns indicate significant difference according to Dunn's median test ($P \leq 0.05$).

Rosales (2012) who determined strain A46 inhibiting *F. oxysporum* and *Alternaria* sp. growth by 36.52 and 53.83% while strain P61 did 20.05 and 55.14%, respectively, and R44 did not inhibit anything at all on both pathogens. On the other hand, Pineda-Mendoza *et al.* (2018) reported 36 and 44% inhibition to *Rhizoctonia solani* by strains A46 and P61, respectively, and Florencio-Anastasio *et al.* (2022) 13.0, 20.5 and 44.7% to *F. oxysporum* f. sp. *cubense* by strains P61, R44 and BSP1.1, respectively. This variation among the results of these studies indicates that the strains have a different respond according to the plant pathogen, needing an individual pathogen by pathogen evaluation. To the best of our knowledge, this is the first time evaluating this bacteria species and strains to FOC 0.

In the *in vivo* assay, the significant differences among strains and the control started after 45 days post FOC 0 inoculation (DAI), nevertheless, in this evaluation no strain was statistically different from the control but between them, R44 exhibited the lowest disease severity index (DSI), and CPPC55 and P61 the highest. In the 60 DAI evaluation, strains A46, P61 and R44 exhibited the lowest disease, and in the 75 DAI strains P61, R44 and CPPC55 did. On the mean area under disease progress curve (AUDPC) calculated with the five evaluations, A46, P61, R44 and CPPC55 significantly differed from the control and only BSP1.1 did not (Table 2).

**Figure 1.** Inhibition of *in vitro* mycelial growth of dual culture against FOC-0 in Petri dishes with PDA-Difco medium® at 25 °C for 7 days in total darkness in growth chamber (Friocel®). Where: 1=FOC 0 vs. A46; 2=FOC 0 vs. P61; 3=FOC 0 vs. R44; 4=FOC 0 vs. BSP1.1; 5=FOC 0 vs. CPPC55; 6=FOC 0.

These results indicate that FOC 0 has a destructive effect on chickpea plants and the inoculation of chickpea seeds with PGPB can reduce the disease severity caused by the pathogen, however, there is variation among bacteria. Diseased plants may show affections through the reduction of morphological traits and nutrients content, and on the other hand, PGPB role is to improve morphological plant traits and nutrients content, in the current experiment morphological parameters such as plant height, stem width, number of leaves and root volume plus nitrogen (N), phosphorus (P) and potassium (K) content was compared 75 DAI in order to evaluate the PGPB strains on inoculated FOC 0 chickpea plants. Strain A46 had higher N and K content in comparison with the control; strain P61 showed a higher plant height, N and K content; strain R44 exhibited a higher plant height, stem width and root volume; strain CPPC55 had a higher plant height, number of leaves, N and K content in comparison with the control (Table 3).

These results indicate that chickpea plants inoculated with these four PGPB can besides reducing FOC 0 symptoms, can reduce the plants morphological traits affections in major agronomic parameters linked to yield such as plant height, stem width, number of leaves

Table 2. Effect of the plant growth-promoting bacteria strains inoculation on chickpea seeds on the *Fusarium oxysporum* f. sp. *ciceris* race 0 disease severity index (DSI %) and the area under disease progress curve (AUDPC).

Strain	Disease severity index (%)			AUDPC
	45 dai	60 dai	75 dai	
A46 (<i>Pseudomonas tolaasii</i>)	16.7 ab*	46.7 b	57.5 ab	3313 b
P61 (<i>Pseudomonas tolaasii</i>)	21.7 a	48.3 b	51.7 bc	3187 b
R44 (<i>Bacillus pumilus</i>)	13.3 b	47.5 b	53.3 bc	3168 b
BSP1.1 (<i>Paenibacillus polymyxa</i>)	16.7 ab	49.2 ab	57.5 ab	3388 ab
CPPC55 (<i>Serratia plymuthica</i>)	25.0 a	50.0 ab	50.0 c	3190 b
Control	16.7 ab	55.0 a	64.2 a	3769 a
P	0.042	0.003	0.001	0.001

*=Means with different letter in columns indicate significant difference according to Tukey's mean test ($P \leq 0.05$); dai=days after inoculation.

Table 3. Response of chickpea plants morphological traits and nutrient content of five previous seed inoculated growth-promoting bacteria strains 75 days after *Fusarium oxysporum* f. sp. *ciceris* race 0 inoculation.

Strain	Plant morphological traits				Nutrient content		
	PH (cm)	SW (mm)	NL	RV (cm ³)	N	P	K
A46 (<i>Pseudomonas tolaasii</i>)	31.2±4.5 b	2.6±0.4 ab	20.5±2.9 b	2.5±1.0 ab	6.6±1.0 ab	2.0±0.3 a	15.6±0.9 a
P61 (<i>Pseudomonas tolaasii</i>)	33.6±3.2 a	2.5±0.3 ab	20.4±1.9 b	2.4±0.8 ab	6.2±1.9 b	2.1±0.2 a	12.3±0.5 a
R44 (<i>Bacillus pumilus</i>)	33.3±2.3 a	2.7±0.4 a	21.0±2.8 ab	2.7±0.8 a	5.0±1.0 bc	1.9±0.3 a	10.6±2.3 ab
CPPC55 (<i>Serratia plymuthica</i>)	35.7±2.8 a	2.7±0.5 ab	22.5±2.7 a	2.5±0.7 ab	8.4±0.9 a	1.8±0.9 a	13.7±0.5 a
Control	30.1±3.9 b	2.5±0.3 b	20.7±2.0 b	2.2±0.8 b	4.1±0.8 c	1.8±0.2 a	9.8±0.6 b
P	0.001	0.038	0.008	0.010	0.001	0.384	0.001

PH=plant height; SW=stem width; NL=number of leaves; RV=root volume; N=nitrogen; P=phosphorus; K=Potassium. Means with different letter in plant morphological traits columns indicate significant difference according to Tukey's mean test ($P \leq 0.05$) and in nutrient content indicate significant difference according to Dunn's median test ($P \leq 0.05$).

and root volume, plus nutrient content including N and K. To the best of our knowledge, this is the first *in vivo* assay designed to evaluate these strains as biocontrol agents.

CONCLUSIONS

PGPB strains A46, P61 (*Pseudomonas tolaasii*), R44 (*Bacillus pumilus*), BSPS1.1 (*Paenibacillus polymyxa*), and CPPC55 (*Serratia plymuthica*) significantly reduces mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* race 0. *Fusarium oxysporum* f. sp. *ciceris* race 0 has a destructive effect on chickpea plants and the inoculation of chickpea seeds with PGPB can reduce the disease severity caused by the pathogen, however, there is variation among PGPB. Strains A46, P61, R44 and CPPC55 reduce the chickpea plant disease symptoms and the affectation of different plant morphological traits such as height, stem width, number of leaves and root volume, plus nitrogen and potassium content.

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