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Research Paper

Native soil bacteria isolates in Mexico exhibit a promising antagonistic effect against *Fusarium oxysporum* f. sp. *radicis-lycopersici*

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Sinaloa state accounts for 23% of Mexico's tomato production. One constraint on this important crop is the *Fusarium* crown and root rot, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, which has been reported to reduce crop yield by up to 50%. In this study, we set out to identify bacterial populations which could be used to control this disease through natural antagonism. Five tomato rhizospheric soil samples were collected, dried for 1-week, and homogenized. Sub-samples were used to prepare an aqueous solution used to isolate microorganisms in pure cultures. Organisms were purified and grown separately, and used to generate a collection of 705 bacterial isolates. Thirty-four percent from this bank (254 strains) was screened against Forl, finding 27 bacteria displaying *in vitro* Forl growth inhibition levels from 5% to 60%. These isolates belonged to the genus *Bacillus* and their 16Sr DNA sequences showed that they are closely related to seven species and they were putatively designated as: *B. subtilis*, *B. cereus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, *B. megaterium*, and *B. pumilus*. One isolate belonged to the genus *Acinetobacter*. Two *B. subtilis* isolates (144 and 151) and one *B. cereus* isolate (171) showed the best antagonistic potential against FCRRT when evaluated on seedlings. Plate and activity assays indicate that these isolates include a diverse repertoire of functional antagonistic traits that might explain their ability to control FCRRT. Moreover, bacteria showed partial hemolytic activity, and future research will be directed at ensuring that their application will be not harmful for humans and effective against Forl in greenhouse or field conditions.

Abbreviations: Forl – *Fusarium oxysporum* f. sp. *radicis lycopersici*; FCRRT – *Fusarium* crown and root rot of tomato

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Introduction

Tomato agriculture and exporting in Mexico's Sinaloa state represent an annual value of >600 million USD. Sinaloa accounts for 23% of the national production (www.siap.gob.mx) and is considered one of the main tomato producing regions in Mexico. One issue concerning this production is that tomato may be severely affected by the fungus *Fusarium oxysporum* Schlechtend: Fr. f. sp. *radicis-lycopersici* (Forl) W.R. Jarvis & Shoemaker, resulting in the *Fusarium* crown and root rot (FCRRT) disease [1]. The disease can strike crops in both greenhouse and field conditions, causing significant loss of production [2]. FCRRT has been reported worldwide in,

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at least, 32 countries [3]. In Sinaloa, FCRRT has resulted in losses that can damage up to 50% of tomato yields [4]. It is thus of great interest to control FCRRT, and several methods have been proposed, including genetically resistant varieties [5], soil solarization [6], crop rotation [7], and chemical control [8]. Chemical control is expensive, but it can also cause environmental pollution and induce pathogen resistance [9, 10], and it is not completely effective. Due to these concerns, a more promising alternative to prevent FCRRT would be the use of bacterial biocontrol agents isolated from the rhizosphere [11]. *Pseudomonas chlororaphis* isolate PCL1391 has been studied for its broad-spectrum antifungal activity. This bacterial strain inhibits the damage caused by FCRRT, and can efficiently colonize the roots. This strain also showed *in vitro* antagonistic effects against *Rhizoctonia solani*, *Botrytis cinerea*, *Pythium ultimum*, *Verticillium albo-atrum*, and *Alternaria dauci* [12]. Kamilova *et al*. [11] showed that preinoculation of the seeds of tomato cv. Carmello with *Pseudomonas fluorescens* WCS365 strain reduces the damage caused by FCRRT by up to 97%. Another microorganism showing biocontrol activity against Forl is *Aporothielavia leptoderma*. This is a ubiquitous saprophytic ascomycete of the *Chetomiaceae* family that was initially isolated from the soils of Guasave, Sinaloa in Mexico [13]. Mexico is the only country in Latin America that allows the entrance of foreign biocontrol products [14]. By contrast, the rules for introducing biocontrol agents are very restrictive in other Latin American countries, whereas the use of native natural enemies is currently encouraged [14]. Applications of native soil microorganisms in the same region where they have been isolated have the advantage that they have co-evolved with plants and other organisms, thus they display adaptive features that might help them to establish successfully in the soil [15]. The aim of this work was to screen a collection of native tomato rhizosphere microorganisms from Sinaloa, in order to find novel soil antagonistic microorganisms against Forl.

Materials and methods

Soil samples

Five rhizospheric soil samples (representing silty clay loam [sand 17.4%, clay 37.5%, and lime 44.9%]) were collected from one commercial tomato (*Solanum lycopersicum* cv. Gabriela) field at Agrícola del Rancho in Guasave, Mexico during March 2006. Sampling consisted of taking a soil core 0–30 cm in depth. Each sample was dried at room temperature (25 °C) for 1-week and passed through a 1 mm mesh to eliminate large particles. Samples were homogenized according to O'Brien *et al*. [16] and stored at 4 °C for further use.

Microorganism isolation

A sub-sample was taken from each soil sample to prepare an aqueous homogenate solution. Organisms were isolated and purified after serial dilutions by plating on Luria Bertani (LB) agar medium. Plates were incubated at 25 °C. Seven hundred and five colonies were purified, grown separately, and used to generate a small microorganism collection; two specimens per isolate were cryopreserved at −70 °C, in LB liquid medium containing 15% glycerol [17].

Viability test of the microorganism collection

Two hundred thirty one isolates from the bacterial bank were thawed out on LB plates and incubated at 25 °C for 24 h. The isolate was considered viable only if growth was restored [17]. In 2010, all 705 bacterial isolates were thawed out to confirm viability of the whole bank.

Forl culture

The Forl isolate used in this work was obtained from tomato stem. Pathogenicity assays were performed on tomato cv. Rio Grande [18]. Plants exhibited typical symptomatology of FCRRT, and the re-isolated fungus was molecularly identified as Forl.

In vitro antagonistic bioassays against Forl

Screening for *in vitro* antifungal activity against Forl was performed on water-agar plates. A plug of 1 cm in diameter containing the fungus was placed in the center of the plate, and bacterial isolates were streaked at a distance of 3.5 cm from the fungus [19]. Four bacterial isolates were evaluated per plate, and three replicate plates were prepared. The plates were incubated at 25 °C [20] for 17 days. Bacterial isolates inhibiting mycelial growth were selected for molecular identification and used for *in planta* assays.

Molecular identification of Forl antagonistic bacteria

The bacterial isolates characterized *in vitro* as Forl antagonists were grown in pure culture on LB plates for 24 h at 25 °C. Genomic DNA was extracted in DNAzol (Invitrogen, Cat. No. 10503-027, USA) as described by the manufacturer. Genomic DNA was used for molecular identification using the polymerase chain reaction (PCR) technique. Universal oligonucleotides F2C (5′-AGAGTTTGATCATGGCTC-3′) and C (5′-ACGGGCGGTGTGTAC-3′) were used for 16S rDNA amplification [21]. The PCR mixture contained 1 µl of eluted DNA extract, 1× reaction

buffer, 1 mM $MgCl_2$, 0.5 mM of each primer, 500 µM each deoxynucleotide triphospate (dNTP), and 0.5 U of Taq DNA polymerase (Invitrogen, Cat. No. 10966-030) in a total volume of 25 µl. The DNA templates were first subjected to an initial denaturation step at 95 °C for 4 min. The subsequent cycles consisted of 1 min denaturation step at 95 °C, 1 min annealing step at 55 °C, and 2 min extension step at 72 °C. After a total of 30 cycles, there was a final 5 min extension at 72 °C. The PCR was performed using a MyCicler thermocycler (Bio-Rad, Cat. No. 580BR-2592, CA, USA). Products were visualized by 1% agarose gel electrophoresis in 0.5× Tris-acetate–EDTA (TAE) buffer and stained with ethidium bromide, using a Chemidoc photo documentation system (Bio-Rad) to verify the product size.

PCR cloning

The PCR fragments were inserted into the pGEM-T kit Easy Vector System II (Promega, Cat No. 157348, Madison, WI, USA) as described by the manufacturer. Ligation reactions were incubated at room temperature overnight. Transformation of circularized plasmids into *Escherichia coli* JM-109 competent cells was performed following the procedure described by Sambrook *et al*. [22].

Sequence analysis

The amplified cloned fragments were sequenced with an ABI Prism 3100 Automated Sequencer at the National Laboratory of Genomics (Langebio, CINVESTAV-Irapuato, Mexico). Plasmid sequences were first analyzed with the DNASTAR software to eliminate the vector sequences, then with the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) package available on-line [23] using the BLAST-N program and the Mega Blast algorithm.

Hemolysis assays on human blood

Bacterial isolates were grown in 5 ml of LB medium at 25 °C for 24 h, at 250 rpm. One milliliter of each culture tube was taken and transferred to a 1.5 ml Eppendorf tube, centrifuged twice at 13,000 rpm for 5 min and the supernatant was transferred to a new tube. Fifty microliters were taken and placed in circular wells 5 mm in diameter, previously made using a cork borer, in blood agar plates. The plates were stored at 37 °C for 24 h. Complete hemolysis (or β-hemolysis) was observed as a clear zone around the well in the blood agar medium (indicating complete breakage of erythrocyte, whereas partial hemolysis (or α-hemolysis) was observed as a dark-green coloration around the well indicating the partial damage of erythrocytes. Bacteria with γ-hemolysis do not exhibit any alteration of color or opacity in the medium indicating the lack of hemolysis.

In vitro antagonism seedling assays

Twenty-one isolates selected from the *in vitro* antagonistic bioassay were used for this experiment. Tomato seeds (cv. Missouri) were surface-disinfected in 70% EtOH solution for 2 min, then 0.5% sodium hypochlorite for 10 min, and washed three times in copious sterile distilled H_2O for 5 min. Selected bacterial isolates were grown on LB plates, at 25 °C for 24 h. With a sterile toothpick, a subsample of the isolate was transferred to a 250 ml Erlenmeyer flask, containing 50 ml of liquid LB, and allowed to stir at 25 °C and 200 rpm for 16 h. Bacteria were recovered by centrifugation at 9,000*g* for 5 min using a microfuge (Beckman, Microfuge 8), and the pellet was resuspended in 5 ml of sterile distilled H_2O. The volume was adjusted to obtain 1.0 O.D. at 600 nm absorbance. Tomato seeds were soaked in the bacterial suspension for 2 h at 25 °C. A plug of PDA containing Forl mycelium was placed on the plate center of the water-agar medium. Eight seeds per plate were placed surrounding the fungus inoculum and plates were set up in triplicate for each treatment [24]. The boxes were incubated for 1-week using a 16 h light/8 h darkness photoperiod, 25/20 °C, inside a growth chamber (Binder, KBW 400, Germany).

Damage level was measured using a previously reported table of severity [25]. Disease severity was scored on a scale of 0–5, where 0 = absence of damage in root and crown, 1 = slight brown necrotic tissue in roots, 2 = extensive brown necrosis of vascular tissue in roots, 3 = slight brown necrosis of vascular tissue and cortex in the crown, 4 = extensive brown necrosis of vascular tissue and cortex in the crown, and 5 = completely brown crown and root system indicating complete necrosis. A completely randomized experimental design was used. Severity scale values obtained were subjected to a normality test using the Shapiro Will test and a Bartlett's test to confirm variance homogeneity. Data were parametric and severity scale data was subjected to statistical analysis of variance (ANOVA) to detect differences between treatments, and to a Tukey test ($p = 0.05$) for mean comparisons. Analyses were performed using the Statistical Analysis System 9.0 (SAS Institute, Cary, NC). The data obtained in this bioassay were converted in percentage of severity [26] according to Apodaca-Sanchez *et al*. [27]. The bioprotective effect assay against FCRRT in seedlings was performed twice.

Tests for functional antagonistic traits

Eight antagonistic isolates were screened by plate assays for phosphate solubilization [28], indole-3-acetic acid (IAA) secretion indicating plant growth promotion [29], and siderophore production [30], as well as chitinase [31], glucanase [32, 33], and protease [34] activities. Tests were conducted at 25 °C. Briefly, for IAA production evaluation, single colonies were grown in LB broth for 24 h and the supernatants were treated with Salkowsky reagent according to Bric *et al.* [29]. After several minutes, IAA production was identified by a color change in the supernatant from clear to pink. To detect phosphate solubilizing bacteria, the strains were streaked onto Pikovskaya's medium [28]. Strains that induced a clear zone around the colonies after 1-week were considered positives. Siderophore production was determined after 1-week by the chrome azurol S agar assay [30] and was considered positive on the basis of color change in the medium from blue to orange. The chitinase assay was performed on colloidal chitin agar medium, and chitinase activity was identified by the formation of a clear zone around the bacterial cells after 5 days of growth. For each isolate tested, every assay was performed in triplicate. The endo β-1, 4-glucanase *in vitro* activity was measured as described previously [32] using salicin as the substrate and the medium supernatant from the bacterial cultures grown for 48 h. The reducing glucose released in the enzymatic reaction was then determined by recording the absorbance at 540 nm. One unit (U) of glucanase activity was defined as the amount of reducing sugars equivalent to 1 µmol of glucose per minute produced per ml of bacterial supernatant. The protease activity was tested in skimmed milk agar (SMA) with commercially available non-fat milk according to Jones *et al.* [34]. The strains were streaked onto SMA and the plates were incubated for 1-day. The protease activity was identified by the formation of a clear zone around the bacterial colonies.

Results

Viability test of the microorganism collection

All collected isolates were thawed in October 2010, after 4 years of generating the collection. Six hundred twenty five isolates (89%) were viable, whereas 80 isolates (11%) were not.

In vitro screening of putative *Bacillus* and *Acinetobacter* isolates as potential antagonists against Forl

Among the viable samples used for the *in vitro* antagonistic assays, 27 bacterial isolates were observed to inhibit Forl. Typical results in Petri plates are shown in Fig. 1. The isolates were classified in three arbitrary categories according to their inhibition of Forl growth: low (5–18% growth reduction), medium (24–31% growth reduction) and high (34–60% growth reduction) antagonistic activity. Isolates 102 and 537 had the lowest percentage of inhibition (5%), whereas isolates 144 and 145 exhibited the highest inhibition (60%; Table 1).

Among the isolates, 26 samples belonged to the genus *Bacillus* and only one to the genus *Acinetobacter* (No. 208). The similarity of the sequences analyzed from the 26 isolates ranged from 87% to 100%. Phylogenetic analysis of the 16S rDNA region (data not shown) indicated that the *Bacillus* isolates are closely related to seven different species. We have putatively designated these isolates with these species names in our study, although more extensive work would be necessary to conclude whether these designations are accurate (i.e., DNA probe-hybridization, genome sequencing).

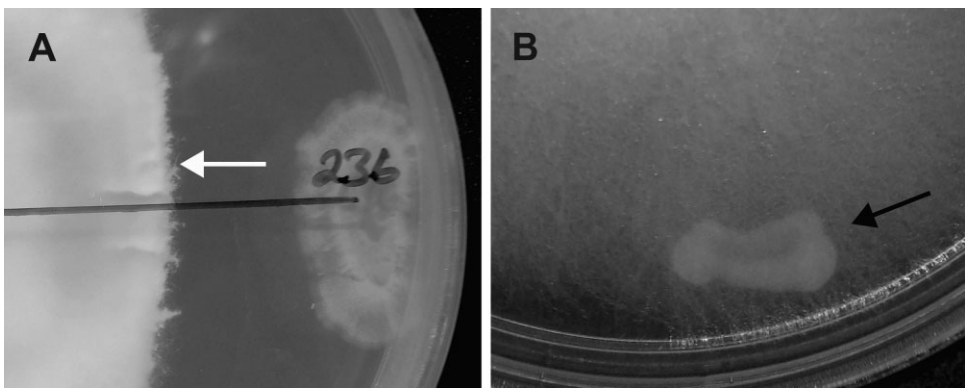

Figure 1. *In vitro* antagonism bioassays against Forl. (A) Isolate 236 inhibits Forl growth. White arrow indicates the arrest of fungal growth. (B) Isolate 212 displayed no antagonistic activity against Forl. Black arrow points to the bacterial colony. Photographs were taken 17 days after inoculation of both plates with Forl and dark-incubation at 25 °C.

Table 1. Isolates from the microorganism collection showing an *in vitro* antagonistic effect against Forl in antagonism bioassays.

Identified putative species	Hemolysis type	% Inhibition	Isolate number
Acinetobacter sp.	γ	18	208
Bacillus sp.	γ	14	187
B. amyloliquefaciens	β	18	225
B. cereus	α	10	171
B. cereus	β	5	102
B. licheniformis	γ	12	231
B. megaterium	β	35	705
B. pumilus	β	48	168
B. subtilis	α	60	144
B. subtilis	α	60	145
B. subtilis	α	28	151
B. subtilis	α	34	162
B. subtilis	α	34	163
B. subtilis	α	31	167
B. subtilis	β	34	164
B. subtilis	β	28	179
B. subtilis	β	26	180
B. subtilis	β	18	188
B. subtilis	β	17	207
B. subtilis	β	14	230
B. subtilis	γ	15	40
B. subtilis	γ	28	150
B. subtilis	γ	34	160
B. subtilis	γ	12	185
B. subtilis	γ	30	236
B. subtilis	γ	12	258
B. thuringiensis	α	5	537

α, partial hemolysis; β, complete hemolysis; γ, no hemolysis. % Inhibition after 17 days of incubation at 25 °C.

The most common Forl antagonistic putative species was *B. subtilis* with 18 isolates, followed by *B. cereus* with two isolates; single isolates were identified for *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, *B. megaterium*, *B. pumilus*, and *Bacillus* sp. (Table 1).

Hemolysis assays

Out of the 27 isolates selected for the hemolysis test, a partial α-hemolysis was detected for eight strains (Nos. 144, 145, 151, 162, 163, 167, 171, and 537), complete β-hemolysis in ten isolates (Nos. 164, 168, 179, 180, 188, 207, 225, 230, 102, and 705) and nine isolates showed γ-hemolysis, or no hemolysis (Nos. 40, 150, 160, 185, 187, 208, 231, 236, and 258).

In vitro biocontrol seedling assays allowed selection of potential Forl antagonists

Twenty-one out of 27 isolates showing *in vitro* antagonistic effect were tested on tomato seedlings. The assay included isolates causing high, medium, and low inhibition of Forl mycelium growth. We included potential antagonists with medium or low levels of Forl inhibition, since a previous work showed that isolates exhibiting the best inhibitory response *in vitro* will not necessarily be the best *in planta*, and vice-versa [35].

Seventeen days after Forl inoculation, negative control (seed + H_2O) seedlings showed no FCRRT (Fig. 2A), while inoculated tomato seedlings showed a marked browning coloration accompanied by root growth arrest (Fig. 2B). At this stage, bacteria-treated plants showed a different level of root growth when compared to the negative control (Fig. 2C and D).

The most successful *in planta* antagonists were the isolates 144 (*B. subtilis*), 151 (*B. subtilis*), and 171 (*B. cereus*; Fig. 3). They showed only slight brown root coloration (Fig. 2C). Isolate 144 also showed a high percentage of Forl mycelium growth inhibition *in vitro* (60%), whereas isolates 151 and 171 showed 28% and 10% inhibition, respectively (Table 1). Seeds inoculated with isolates 144, 151, and 171 displayed moderate percentages of FCRRT severity, with an average of 39%, 46%, and 45%, respectively. These values are statistically different from the pathogen-treated control (seed + Forl), whose disease severity percentage was 89% (Fig. 3).

All other isolates either possessed a low capacity to prevent seedling FCRRT symptoms, or did not show any effect (Fig. 3).

Possible antagonistic traits employed by some of the bacterial isolates tested on seedling assays

Eight isolates were tested for several functional antagonistic assays (Table 2). They exhibited different activities that could explain the mechanisms by which these isolates might cause inhibition of Forl growth. The three antagonists showing Forl inhibition in seedlings (144, 151, 171) exhibited diverse activities such as phosphate solubilization, IAA secretion, siderophore production and chitinase, endo-β-1, 4-glucanase, and protease activities.

Discussion

In the present study, a microorganism collection containing 705 bacterial isolates was screened for antagonistic activity against Forl. This collection was made in 2006, and 1-year later 254 isolates were thawed out and viability was 95% (data not shown). When all isolates were thawed out 4 years later, in October 2010 [36], the viability was 89%. The percentage of survival is comparable to that reported in similar studies [17, 37].

Up to 11.7% of the microorganisms tested in our study (27 out of 231) showed antagonistic activity against Forl. Although still not well characterized, the presumptive

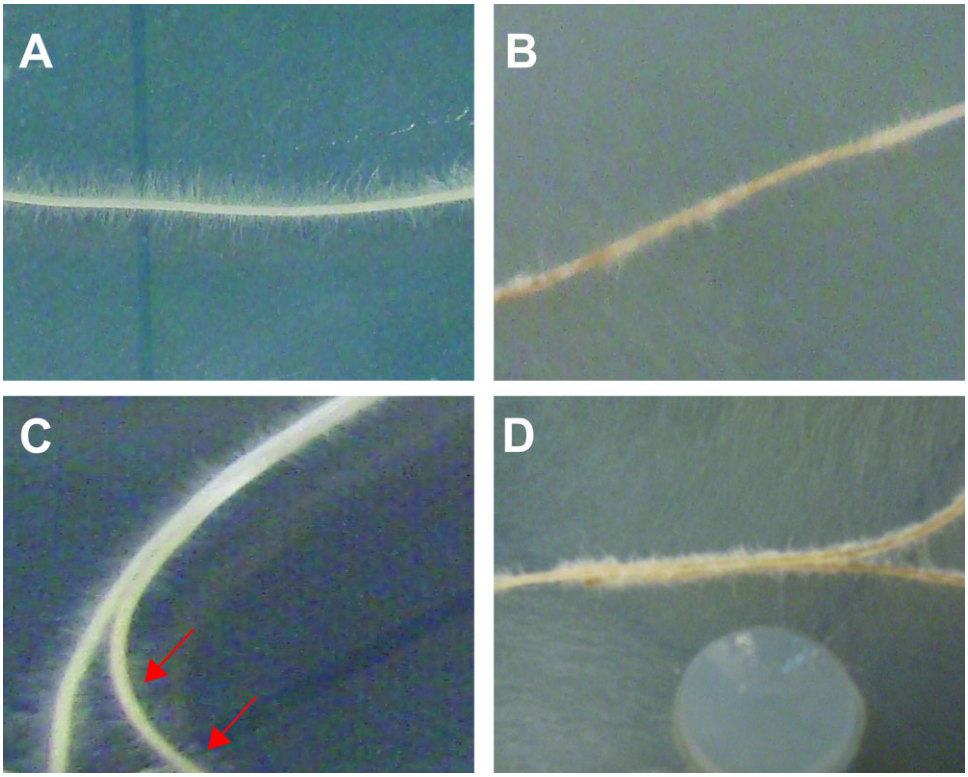

Figure 2. Symptoms observed in roots of tomato var. Missouri 17 days after inoculation with Forl in *in vitro* seedling assays. (A) Negative control (seed + H_2O), disease severity 0. (B) Positive control (seed + Forl) exhibiting completely necrotic tissue, disease severity 5. (C) Isolate 144, showing fewer root hairs than the negative control and a very slight brown coloration, disease severity 1 (red arrows). (D) Isolate 705, root tissue is completely necrotic (brown color), and the root also exhibits profuse mycelial growth on the surface, disease severity 5 (in white color).

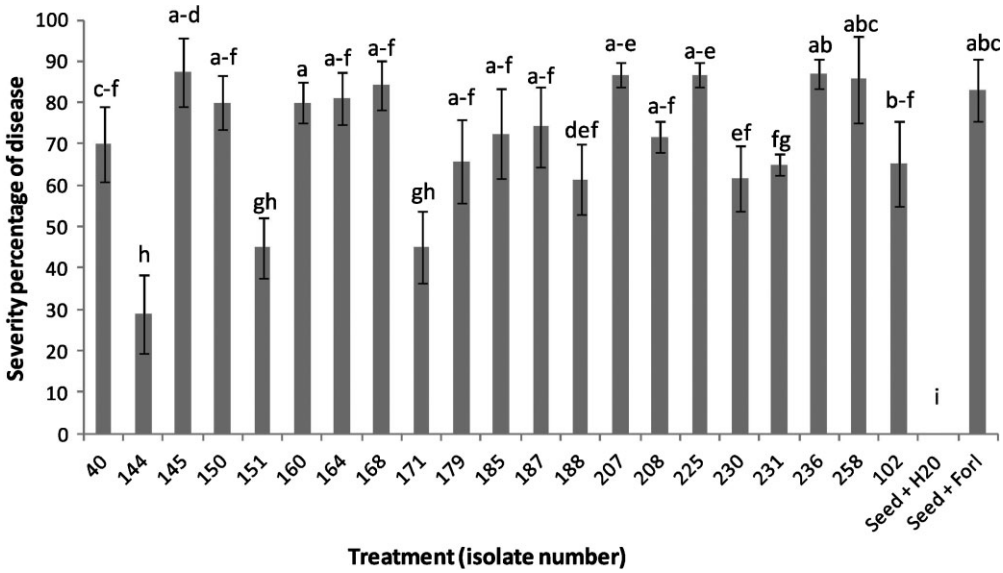

Figure 3. Percent of disease severity in seeds of tomato cv. Missouri inoculated with bacterial isolates and Forl, 17 days after inoculation. Means with same letters indicate no statistical difference. Tukey $p = 0.05$.

Table 2. Analysis of functional traits of antagonistic bacterial isolates against *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

Isolate number	Phosphate	IAA	Siderophore	Chitinase	β-endo-1,4-glucanase[a]	Protease
40	−	+	+	−	+	+
102	−	−	−	+	+	+
144	−	+	−	−	+	+
151	−	−	+	−	+	+
171	+	−	−	+	+	−
188	−	−	+	+	+	+
230	+	−	+	−	+	+
231	+	+	−	−	+	−

[a]β-endo-1,4-glucanase was measured in an *in vitro* enzymatic assay, the isolates indicating positive (+) for this assay exhibited similar levels of enzymatic activity (1.3–1.6 µmol glucose min^{-1} ml bacterial $supernatant^{-1}$).

mechanisms by which these isolates inhibit Forl mycelial growth may include diverse strategies. For instance, there could be competition for nutrients [38], as we observed that several of the isolates tested are able to grow aggressively which suggests they may scavenge some nutrients in the culture medium required by the fungus. This can result in growth arrest when the fungus reaches the nutrient depletion zone surrounding the bacteria [39]. In the plate assays, several tested isolates exhibited the ability to produce siderophores, which may suggest iron chelation. Depletion of this nutrient could make this essential element inaccessible to Forl, thereby causing its growth inhibition in the *in vitro* antagonistic assay.

Antibiosis is another mechanism possibly used by the antagonistic microorganisms identified in this study. In antibiosis, the antagonistic bacteria produce substances such as hydrolytic enzymes or secondary metabolites that inhibit fungal growth [40]. Bioassay observations indicate that the fungus growth was inhibited before contacting the bacterial colonies (Fig. 1). Overlapping of the antagonistic bacterial colonies and the plant pathogen was not observed, which excludes mycoparasitism and suggests that the inhibition mechanism may be antibiosis. Although antibiotic production was not evaluated in this work, we found that some of the isolates inhibiting Forl growth *in vitro* exhibited enzymatic activities that could degrade either the cell wall (chitinase and endo-β-1,4-glucanase) or membranes (proteases; Table 2).

To avoid and/or reduce FCRRT disease progression, plant growth promoting rhizobacteria (PGPR) populations in the soil may induce plant growth by assisting plant nutrition, either by solubilizing phosphorous from the soil [41] and making it available to the plant, or by increasing the iron uptake via siderophores [42, 43]. Bacteria can also produce phytohormones such as IAA that can directly induce plant roots to develop faster or with a different architecture (i.e., lateral root patterning), leading to a larger root volume which is more resistant to fungal attack [44]. Several *Bacillus* isolates studied here are able to produce IAA, and/or solubilize phosphate and siderophores (Table 2). Siderophore-producing bacteria possibly act dually in the rhizosphere, on the fungus (depriving it of iron), and also by providing iron to the plant root system [45]. Although several functional antagonistic traits of the bacterial isolates were preliminary explored in the present work, the exact mechanisms by which these bacteria function within the rhizosphere remain to be determined.

Most of the 27 identified isolates belong to the genus *Bacillus*. The putative species identities include *B. subtilis*, *B. megaterium*, *B. cereus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, and *B. thuringiensis*. Only one isolate, *Acinetobacter* sp., belongs to a different genus. Multiple *Bacillus* species have been already reported to possess antagonistic capacities [46–50]. In our previous work characterizing 268 prokaryotic rDNA clones from the same soil used in this study, the genus *Bacillus* represented 7.5% of the total [51]. The species present included: *B. amyloliquefaciens*, *B. azotoformans*, *B. lentus*, *B. licheniformis*, *B. psychrosaccharolyticus*, *B. pumilus*, *B. subtillis*, and *Bacillus* sp., several of which overlap with some of the putative species identified in the present study. *Bacillus subtilis* has previously been reported to exhibit different mechanisms of phytopathogenic fungus biocontrol, such as iron (siderophore secretion) [52] and production of various antibiotic compounds (i.e., surfactin, iturin, and fengycin) [39].

Four identified species (*B. subtilis*, *B. megaterium*, *B. cereus*, and a species of *Acinetobacter*) have recently been reported as antagonistic organisms against *Fusarium oxysporum* f. sp. *ciceris* race 5 [46], *F. verticillioides* [47], *F. oxysporum* f. sp. *radicis-lycopersici* [48], and *F. graminearum* [49]. Beneficial effects of inoculation on three crops (maize cv. GS2; pigeon pea cv. P 921; wheat cv. HD 2285) with a strain of *B. cereus* have been reported, and more recently it has been shown that this species is an

endophyte of maize able to grow in the vascular tissue of the root system [53]. The strain *B. cereus* 28-9 produces a chitinase (ChiCW) that is effective against the fungal pathogen *Botrytis elliptica* in lilly plants [50]. De la Vega *et al*. [34] reported that a purified 66-kDa chitinase from *B. thuringiensis* subsp. *aizawai* exhibits lytic activity against the cell walls of six phytopathogenic fungi and inhibited the mycelial growth of *Fusarium* sp. and *Sclerotium rolfsii*.

Bacillus amyloliquefaciens, *B. licheniformis*, and *B. thuringiensis* show antagonistic capacity towards *Alternaria brassicae* and *Erwinia carotovora* [54], and also insects [55]. Recently, a 36-kDa chitinase from *B. thuringiensis* subsp. *colmeri* was observed to completely inhibit the spore germination of *R. solani* and *B. cinerea* [56].

In our study, putative isolates of *B. thuringiensis* (Nos.102 and 537) showed the lowest percentage of Forl inhibition *in vitro* (5%), while most isolates of *B. subtilis* (Nos. 144, 145, 151, 160, 163, 164, 167, 179, and 236), exhibited moderate to high inhibition levels of FCRRT (Table 1). This suggests that *B. subtilis* could represent a promising biocontrol agent against Forl.

Our results confirm previous findings indicating that bacterial isolates can differentially respond to microorganisms, whether *in vitro* or *in planta* [35]. Nevertheless, *in vitro* Petri dish assays are fast and unexpensive, and allow continuing the selection process to the plant level.

To avoid the use of potential human pathogens in biological plant protection, it is necessary to evaluate the risk of each biological control agent [57]. An important feature displayed by many human pathogens is blood hemolysis. Hemolytic activity is associated with a 107-kDa extracellular protein known as hemolysin [58]. This enzyme plays a role in the release of iron from red blood cells, making it available for invading pathogens [59]. Many pathogenic bacteria secrete soluble proteins, which can damage the plasma membrane of eukaryotic cells [58]. The activity of these proteins is directed against erythrocytes. The most effective FCRRT antagonistic isolates were α-hemolytic. Although these isolates revealed partial hemolysis, other confirmatory tests are necessary, as proposed by Zachow *et al*. [60] before concluding that these strains are pathogenic to humans. Accordingly, a compromise must be found between the best application results against FCRRT [61, 62] and minimizing the potential negative effects on human health. One alternative might be to only use the metabolites produced by the bacteria to inhibit the pathogen [63].

With these concerns in mind, our group is currently testing some of the isolates selected in the greenhouse. The objective of this ongoing research is directed at selecting the best native bacterial isolates with the potential to be used in tomato fields for biological control of FCRRT.

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