Colegio de Postgraduados

Bioprospecting of rhizobacteria with antagonistic activity against *Fusarium* spp., a parasite of cucumber (*Cucumis sativus*)

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ABSTRACT

Objective: To identify bacteria of genus *Bacillus* which, isolated from the tomato and cucumber rhizosphere, have an antagonistic effect against *Fusarium* spp. isolated from cucumber plants in Culiacan, Sinaloa.

Design/Methodology/Approach: Both the *in vitro* and *in vivo* antagonisms of rhizobacterial isolates against *Fusarium* spp. on cucumber plants were evaluated. Bacteria with the highest antagonistic effect were identified based on their morphological and molecular characteristics.

Results: Isolates FA15 and FA16 showed the highest *in vitro* biological efficacy against *Fusarium* spp., with 50.0% and 61.36% inhibition of mycelial growth, respectively. Rhizobacterium FA15 achieved the highest biological efficacy (88.89%) against *Fusarium* spp. in cucumber plants, while rhizobacterium FA16 recorded a 59.27% efficacy. The morphological and molecular characterization of isolates FA15 and FA16 confirmed a 100% molecular identity between FA15 and Bacillus velezensis and FA16 and *B. subtilis*.

Study Limitations/Implications: The rhizobacteria identified in this study inhibited the mycelial growth of the phytoparasite. Therefore, further studies about these rhizobacteria should be carried out to determine the potential antibiosis that may cause the inhibitory effect.

Findings/Conclusions: During the search for native beneficial rhizobacteria, two bacteria that exercise a biologically-effective control over *Fusarium* spp were identified in Culiacan: *Bacillus velezensis* and *B. subtilis*. This finding offers an opportunity in the agricultural biotechnology field to study beneficial native species that could provide an alternative to the use of chemicals.

Keywords: Antagonism, agricultural biotechnology, biological effect, cucumber.

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INTRODUCTION

When the physicochemical characteristics of the soil and the exudates resulting from the physiological activity of plants are favorable, fungi and bacteria (part of the diverse microorganisms found in the rhizosphere) play a key role in the growth, nutrition, and health of plants [1]. Rhizobacteria found around root tissues have beneficial potential: they

can establish a symbiotic relationship with plants and work as antagonists of soil-borne phytopathogens [2]. The benefits of rhizobacteria include the improvement of nutrient availability and absorption; this antagonistic activity is also the result of hyperparasitism, antibiosis, and the competition with phytopathogenic organisms for space [3,4]. The rhizobacteria from genus *Bacillus* can form endospores as survival structures [5], which facilitates their use in stable commercial formulas [6].

Mexico is one of the most important producers of horticultural produce around the world. In particular, the state of Sinaloa produces a high volume of cucumber (*Cucumis sativus*) [7]. However, several phytosanitary problems have a severe impact on cucumber production, mainly as a consequence of the activity of phytopathogens. These pathogens include fungi from genus *Fusarium* [8], which cause withering, root rot, and plant death. Diseases caused by fungi from genus *Fusarium* are also considered soil-borne diseases with high pathogenic potential, whose resistance structures survive on the soil for several years [9,10].

Contemporary agricultural practices employed in the management of *Fusarium* in cucumber crops are highly-dependent on the use of synthetic fungicides. Additionally, the indiscriminate use of these compounds has created resistance among phytopathogenic organisms, as well as public health problems [11]. In this context, the use of rhizobacteria from genus *Bacillus* has become increasingly important for plant health management, particularly as a green and sustainable strategy for the protection of cucumber crops. Therefore, the aim of this research was to isolate rhizobacteria of genus *Bacillus* from the horticultural rhizosphere in Culiacan and use them as antagonists of the *Fusarium* spp. that impact cucumber plants.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from January 12 to January 24, 2022, in the two agricultural plots of the Facultad de Agronomía of the Universidad Autónoma de Sinaloa (UAS). These plots house experimental tomato (*S. lycopersicum*) and cucumber (*C. sativus*) crops, both in the fruit production phenological stage. The tomato plot is located at 24° 37' 30.95" N and 107° 26' 35.75" W, while the cucumber plot is located at 24° 54' 50.71" N and 107° 37' 26.44" W. The cultivation of both crops has involved a low use of synthetic agro-inputs. The roots of 15 plants with soil-traces were collected from each plot, at a depth of 6 to 12 cm; the plants were randomly selected in a zigzag path. The roots and soil collected were placed in individual plastic bags; they were then labelled and sent to the Horticultural Diseases Laboratory of the Facultad de Agronomía, where they were kept at 4 °C, until they were processed.

Isolation, selection, and purification of rhizobacteria

The microorganisms were isolated according to the methodology proposed by Posada *et al.* (2016) [6], with minor modifications. The individual samples were processed as follows: 50 g of roots with soil traces were put inside a 1,000-mL beaker, into which 400 mL of sterile distilled water had been previously poured. An orbital shaker was used to shake the

mixture at 200 rpm for 60 minutes, keeping the temperature at 26±1 °C. Subsequently, a 1 mL aliquote was poured into a 12 mL test tube which contained 9 mL of sterile distilled water. Serial dilutions were carried out until a 10^{-8} dilution was achieved, 100 μL of which were distributed in a Petri dish with a nutrient agar medium. The dishes with the root and soil suspension were kept under lab conditions, at 26±1 °C for 48 h. Rhizobacteria colonies from the dishes with culture medium that formed an antibiosis halo were chosen; this halo limited the development of other nearby microorganisms and its macroscopic morphology (color, rim, texture, and elevation) was similar to bacteria from genus *Bacillus* [12,13].

The rhizobacteria colonies selected were isolated and purified in a nutrient agar medium and preserved at 4 °C in a phosphate buffer, until they were used.

Phytoparasitic organism

The phytoparasitic fungi was obtained from the strain repository of the Horticultural Diseases Laboratory of the UAS – Facultad de Agronomía. The strain was isolated from cucumber plants and identified as member of *Fusarium* spp. The phytopathogenic fungi was activated in the Potato Dextrose Agar (PDA) culture medium and incubated at 26±1 °C until it was used.

***In vitro* antagonisms of rhizobacteria against *Fusarium* spp.**

The dual culture technique was employed. Initially, a *Fusarium* spp. mycelium was allowed to grow in a 0.6-cm cylinder with a culture medium for 120 h. The cylinder was then placed in the middle of a Petri dish with PDA. Subsequently, 10 μL of a rhizobacteria suspension (3×10^8 UFC, according to the McFarland standards) were poured around the cylinder in which the mycelia had grown, 2.2 cm apart from each other, in the four cardinal points [14]. They were incubated at 26±1 °C. Sterile distilled water replaced the bacterial suspension in the cardinal points of the control treatment. The study consisted of a completely random design and eight repetitions per treatment (each Petri dish was considered to be a repetition). The growth of the mycelia of the fungi on the culture medium was measured in every dish when the mycelia in the control treatment reached 2.2 cm (7 days after the start of the treatment). The mycelia growth data determined the biological efficacy of the inhibition (Eterbarian *et al.*, 2005 [15]), according to the following formula:

$$n = a\frac{b}{a}\times 100$$

Where n=biological efficacy (%); a=radial growth of the control; and b=radial growth of the pathogen.

Antagonistic efficacy against *Fusarium* spp. in cucumber plants

Cucumber cv. "Poinsett 76" seeds were sown in polystyrene trays with 128 holes, using peat as substrate and vermiculite as cover. Once the seedlings had emerged and

they had two true leaves, the plants were inoculated, immersing their leaves for 3 min in a water suspension with a 3×103 mL^{-1} concentration of *Fusarium* spp. propagules [16]. Subsequently, the inoculated plants were transplanted to the center of a 3 kg plastic pot with 2.3 kg of a chromic vertisol [17] and peat moss mixture (7:3). Immediately afterwards, 10 mL of a water suspension rhizobacteria at a 1×108 mL^{-1} concentration were added around the neck of the plants. The seedlings were watered by hand daily (250 mL per pot). The damage severity caused by *Fusarium* spp. was determined using the symptom scale proposed by Marlat *et al.* (1996) [18] (Table 1). Additionally, the damage severity percentage was determined using the equation proposed by Ley *et al.* (2018) [19]:

$$\%SD = \sum\left[\frac{GD \times NP}{EM \times TP}\right]\times100$$

Where $\%SD$=damage severity (%); GD=damage degree; NP=number of damaged plants; EM=maximum damage degree in the severity scale; and TP=number of plants in the treatment.

The efficacy of the control was determined with the following equation (Ley *et al.*, 2018):

$$\%EB = \frac{100 - SD\,del\,tratamiento}{SD\,del\,control}\times100$$

Where $\%EB$=percentage of control efficacy; *SD del tratamiento*=damage severity mean per treatment; and *SD del control*=damage severity mean of the control. The study was established under greenhouse conditions, with a completely randomized design, consisting of eight treatments with seven repetitions per treatment. Each plot with a plant was considered as a repetition.

Identification of the bacteria

The bacteria were identified observing their morphology (cell shape, colony, and Gram stain) [13] and using molecular techniques. A rhizobacteria culture was grown for 48-72 hours before it was used. The DNA of the rhizobacteria was amplified through a Polymerase Chain Reaction (PCR) using the gene 16S

Table 1. Severity of the damage caused by *Fusarium* spp.

Scale value	Damage Description
0	Symptomless plant
1	slight chlorosis, wilting or stunting
2	Moderate chlorosis, wilting or dwarfing of the plant
3	Severe chlorosis, wilting or dwarfing of the plant
4	Dead plant

of the DNAr. The FD2 (5'-AGAGTTTGATCATGGCTCAG-3') and RP1 (5'-TACCTTGTTACGACTTCACC-3') universal initiators were used for this purpose, amplifying a 1,500 pb fragment with a T100™ Thermal Cycler (Singapore). According to the methodology proposed by Ley *et al*., (2018) [19], the following temperatures and times were used: enzyme activation at 95 °C for 5 min, followed by 30 cycles, including a denaturation at 94 °C for 1 min, an alignment step at 56 °C for 1 min, and an extension at 72 °C for 1 min. A final extension at 72 °C was carried out for 10 min, once the cycles were over.

The resulting fragments were visualized in a Powerpac™ chamber (Bio-Rad), through an electrophoresis process, using a 1% agarose gel. Likewise, the fragments were purified, sequenced, and compared with the nucleotide sequence available in the database of the National Center for Biotechnology Information (NCBI).

Statistical analysis

The resulting data were subjected to an analysis of variance (ANOVA), using the Minitab 19 statistical software. Likewise, the means were compared using Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Seven rhizobacteria were isolated from the experimental plots. They were then studied and codified as: FA11, FA12, FA13, FA14, FA15, FA16, and FA17.

In vitro antagonism of rhizobacteria isolates against *Fusarium* spp.

Table 2 shows the inhibitory effect of seven bacterial isolates on the *in vitro* growth of mycelia of *Fusarium* spp. The growth of the mycelia of the fungi under study was impacted by rhizobacteria, except for isolate FA11. Only isolates FA15 (1.35 cm) and FA16 (1.1 cm) recorded a significantly lower growth of the mycelia ($p \leq 0.05$) than in the control fungi.

Regarding the percentage of biological efficacy, only isolates FA15 and FA16 recorded a significant inhibition of mycelial growth ($p \leq 0.05$). Meanwhile, the percentage obtained with isolate FA15 was 11.36% higher than the percentage reported with isolate FA16 (Table 2).

Table 2. Inhibitory effect of rhizobacteria on the *in vitro* growth of the mycelium of *Fusarium* spp.

Treatments	Mycelial growth of *Fusarium* sp. *in vitro*	
	Mycelial growth (cm)	Biological effectiveness of inhibition (%)[A]
FA11	2.20±0.0 a	0±0 d
FA12	2.14±0.05* ab**	2.84±2.35 cd
FA13	2.11±0.06 ab	3.97±2.91 cd
FA14	2.08±0.04 ab	4.55±1.6 cd
FA15	0.85±0.09 d	61.36±4.21 a
FA16	1.10±0.08 c	50.00±3.43 b
FA17	2.14±0.05 ab	2.84±2.35 cd
Control	2.20±0.0 a	0±0 d

*Standard deviation. **Means not sharing a letter are significantly different according to Tukey ($P \leq 0.05$).

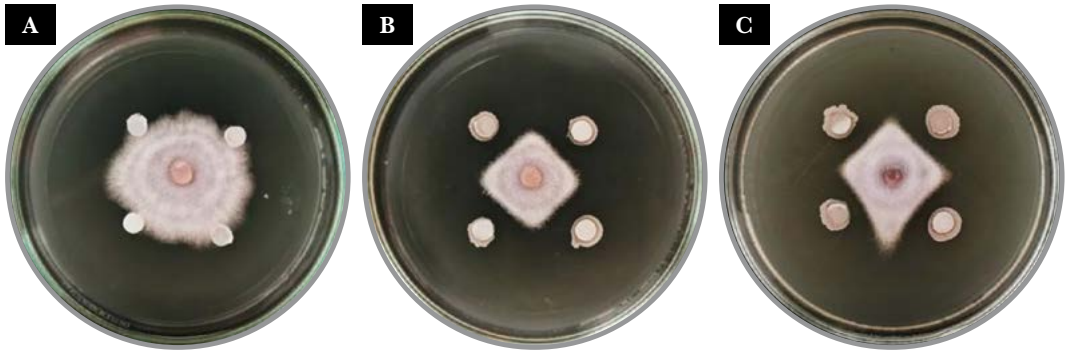

Figure 1. Inhibition of the mycelial growth of *Fusarium* spp., using a rhizobacteria cell suspension: A) control (*Fusarium* spp.), B) FA15 *vs. Fusarium* spp., and C) FA16 *vs. Fusarium* spp.

Efficacy of the antagonism of the FA15 and FA16 rhizobacteria against *Fusarium* spp. on cucumber plants

The efficacy of the antagonism against *Fusarium* spp. showed that plants treated with isolates FA15 and FA16 recorded a significantly lower severity of the disease ($p \leq 0.05$) than plants that were only inoculated with *Fusarium* spp. (Table 3). The severity of the damage on plants that were only inoculated with *Fusarium* spp. was 81.6% and 53.6% higher than plants treated with isolates FA15 and FA16, respectively. Consequently, the biological efficacy of FA15 was significantly higher ($p \leq 0.05$) than with isolate FA16: isolate FA15 had a 28% greater biological efficacy.

Identification of isolates FA15 and FA16

Colonies of FA15 and FA16 bacteria had similar shape and color; both colonies had a creamy consistency, a central elevation, and a mucus-like texture. FA16 colonies were smaller (1.5 mm) than FA15 colonies (2.0 mm). Both isolates recorded Gram-positive stains. After they were compared with the sequences previously reported in the Gen Bank database (NCBI), the sequences obtained from isolates FA15 and FA16 using the PCR molecular techniques showed a 100% identification with *Bacillus subtilis* and *Bacillus velezensis*, respectively. The sequences were deposited in the database, with accession numbers PP862827 (FA15) and PP862812 (FA16).

According to Phung and Dao (2024) [20], biodiversity conservation and the minimization of the negative environmental impact, among other factors, have placed

Table 3. Efficacy of the antagonism of isolates FA15 and FA16 against *Fusarium* spp. on cucumber plants.

Treatments	Antagonism on *Fusarium* sp. in cucumber plants	
	Severity of damage (%)	Biological efficacy (%)
FA15	14.82±13.36* c**	88.89±13.86 a
FA16	42.82±27.82 b	59.27±28.85 b
Fusarium sp.	96.42±9.45 a	---
Control (without fungus)	0±0 c	---

*Standard deviation. **Means not sharing a letter are significantly different according to Tukey ($P \leq 0.05$).

agricultural sustainability at the core of worldwide discussions. Therefore, further research should focus on beneficial microorganisms that can be used as part of an environmental, profitable, and sustainable agricultural strategy [21]. This interest has increased as a consequence of the efficacious promotion of plant growth of some bacteria and their role as biological control agents against various phytopathogens [2]. According to Tejera-Hernández *et al.* (2011) [22] efficacious beneficial bacteria must have >50% antagonistic efficacy against phytopathogen microorganisms. The FA15 and FA16 rhizobacteria chosen for this study had a >50% *in vitro* antagonistic activity against *Fusarium* spp. The morphological characteristics of these rhizobacteria belong to genus *Bacillus* [12,13], several species of which are known to have an antagonistic effect on phytopathogens [23]. The morphological characteristics of isolates FA15 and FA16 match the molecular identification, determining that isolates FA15 and FA16 belong to *Bacillus velezensis* and *B. subtilis*, respectively. The antagonistic effects of these isolates match the results obtained by Hasan *et al.* (2024) and Tian *et al.* (2023) [3,24]. This research proved that the *B. velenzesis* and *B. subtilis* rhizobacteria have biological efficacy against *Fusarium* spp. on cucumber plants. *B. velezensis* and *B. subtilis* can produce several highly-biodegradable cyclical lipopetides (including iturins, surfactins, and fengycins) with antimicrobial activity at a low concentration [5,24].

CONCLUSIONS

Two rhizobacteria with antagonistic efficacy against the *Fusarium* spp. pathogen that damages cucumber were isolated in Culiacán, Sinaloa. The rhizobacteria were identified as *Bacillus velezensis* and *B. subtilis*, both of which had a significant inhibition effect on the mycelia of *Fusarium* spp. Additionally, they proved to have potential as biological control agents on cucumber plants. This is an outstanding opportunity for the agricultural biotechnology industry to find native species that could be used as an alternative to chemical products.

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