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Antagonistic activity of *Pseudomonas donghuensis* and *Bacillus subtilis* for the management of "Damping off" phytopathogens of the chile crop.

Actividad Antagónica de *Pseudomonas donghuensis* y *Bacillus subtilis* para el manejo de fitopatógenos del "Damping off" del cultivo del chile.

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ABSTRACT

The effectiveness of the *Bacillus subtilis* and *Pseudomonas donghuensis* strains for the control of *Fusarium oxysporum* and *Pythium aphanidermatum*, which cause the drowning of chili seedlings in the greenhouse, was evaluated. Its pathogenicity to cause the disease was verified and antagonism tests were obtained in culture plates with enriched PDA. Through the *in vitro* confrontation of these phytopathogens with three strains of the species *Bacillus subtilis*, it was observed that this sporulate has the capacity to inhibit to a greater or lesser degree (39.35- 56.24 %) the mycelial growth of *F. oxysporum*, but not the from *P. aphanidermatum*, none of the *Bacillus* isolates used in this study had an effect in inhibiting this Oomycete, since it grew faster than the spore-forming bacteria, since *P. aphanidermatum* developed and grew to fill the culture plate in just 36 hours. On the other hand, *P. donghuensis* inhibited the growth of this last phytopathogen by 56 %, however, it did not present an inhibition effect against *F. oxysporum*, so it could be feasible to use mixtures of these two antagonists for the management of this disease and be able to control Damping off problems caused by these phytopathogens.

KEY WORDS: *Bacillus subtilis, Damping off, Fusarium oxysporum, Pseudomonas donghuensis, Pythium aphanidermatum.*

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RESUMEN

Se evaluó la efectividad de *Pseudomonas donghuensis* y *Bacillus subtilis* para el control de *Fusarium oxysporum* y *Pythium aphanidermatum* causantes del ahogamiento de plántulas de chile en invernadero. Se comprobó su patogenicidad para ocasionar la enfermedad y se realizaron pruebas de antagonismo en placas con PDA enriquecido. Mediante la confrontación *in vitro* de estos fitopatógenos con tres cepas de *Bacillus subtilis* se comprobó que este esporulado inhibió en mayor o menor grado (39.35- 56.24 %) el crecimiento micelial de *F. oxysporum*, pero no tuvo efecto alguno para *P. aphanidermatum*, ningún *Bacillus* inhibió a este Oomycete, debido a su rápido crecimiento en la placa de cultivo (36 h de incubación). En contraste *P. donghuensis* inhibió en un 56 % el crecimiento de *P. aphanidermatum*, y no presentó efecto inhibitorio para *F. oxysporum*, por lo que la mezcla de ambos antagonistas pudiera tener potencial para el control de los problemas de ahogamiento ‘‘Damping off” causados por estos fitopatógenos.

PALABRAS CLAVE: *Bacillus subtilis*, Damping off, *Fusarium oxysporum*, *Pseudomonas donghuensis*, *Pythium aphanidermatum*.

Introduction

One of the most common diseases affecting chili seedling production in nurseries and greenhouses is known as “Damping off”. The phytopathogens causing such disease are a complex of fungi that include *Fusarium* spp, *Rhizoctonia solani*, and the oomycetes *Pythium* spp and *Phytophthora capsici* (González *et al*., 2013; Larios *et al*., 2019). This disease is considered cosmopolitan and one of the most important diseases in seedlings, causing losses of up to 100 %. Damage by this disease is observed in pre-emergence, post-emergence, and in the field after transplanting (Reveles-Hernández *et al*., 2010; Hernández- Hernández *et al*., 2018). Symptoms of this disease are; seed rot, hypocotyl necrosis, necrotic strangling at the base of the stem, root rot, wilting, and seedling death (Reveles-Hernández *et al*., 2010). For its control, farmers rely mainly on chemical fungicide applications (Captan, Metalaxyl, Azoxystrobin, among others), where low effectiveness and resistance of phytopathogens are reported, in addition to human toxicity and environmental contamination (Castillo- Reyes *et al*., 2015; Hernández- Hernández *et al*., 2018; Larios *et al*., 2019).

An alternative management option for plant diseases is biological control through the application of antagonistic microorganisms (Hernández- Hernández *et al*., 2018; Larios *et al*., 2019), which include several species of *Bacillus* spp, *Pseudomonas* spp, *Trichoderma* spp, among others (Asaka & Shoda, 1996; Gravel *et al*., 2005), which are not harmful to health and the environment (Larios *et al*., 2019), even do not promote resistance (Espinoza-Ahumada *et al*.,

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2019). These microorganisms act by different modes of action, such as parasitism, competition for space and nutrients, synthesis of antibiotics, or induction of systemic resistance in plants (Chirino-Valle *et al*., 2016; Espinoza-Ahumada *et al*., 2019), in addition, some genera are considered as plant growth promoters (Sivasakthi *et al*., 2014). Among the species that are known for their high capacity to produce low molecular weight antifungal compounds, the species *Pseudomonas donghuensis* stands out, which produces five times more siderophores than the other species, being this species a good option for phytopathogens control (Gao *et al*., 2015), since it has shown to have an antagonistic effect against several phytopathogens (Ossowicki *et al*., 2017). Therefore, this work aimed to test the effectiveness of bacterial isolates (*Bacillus subtilis* and *P. donghuensis*) to inhibit the growth of the causal agents of "Damping off" of chili, as a biological alternative for disease management.

Material and Methods

Experiment location

The research work was carried out in the Microbiology Laboratory of the Department of Agricultural Parasitology of the Universidad Autónoma Agraria Antonio Narro (UAAAN) located at Calzada Antonio Narro 1923, Buenavista, 25315 Saltillo, Coahuila, Mexico.

Biological material

The strains of the antagonistic bacteria were kindly provided by the Microbiology strain of the Parasitology Department of the UAAAN, which consisted of three isolates of the genus *Bacillus* spp (B15, BITV, and BIBT) and one isolate of *Pseudomonas* sp (Pd) recovered from the root of zacatón (*Muhlenbergia macroura*). The isolates were reactivated and purified on Nutrient Agar (AN) and incubated at 28 ± 2 °C for 72 h.

Bacillus spp and *Pseudomonas* sp isolates were molecularly identified based on 16S rDNA gene sequencing. The genes were amplified using a Verity end-point PCR thermal cycler (Applied Biosystems) (Jang-Jih *et al*., 2000) at the facilities of the Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental (LANBAMA) located in San Luis Potosí, Mexico. In addition, the species had already been characterized morphologically by compound microscopy and by biochemical tests.

The phytopathogens isolation was performed from samples of chili serrano seedlings 30 days old, with symptoms of "Damping off" and coming from greenhouses in the agricultural region of Parras de la Fuente, Coahuila, Mexico, during the summer of 2019. For isolation of phytopathogenic fungi, substrate residues were removed from the root ball, the whole plant was washed with potable water under aseptic conditions, and small pieces of roots and stems were cut with a sterile scalpel and disinfected in a 1 % NaClO solution for 3 min, washed three times with sterile distilled water for 1.30 min and allowed to dry inside a laminar flow hood on sterile stratified paper. Once dried, they were transferred to Petri dishes with Potato-Dextrose-Agar (PDA) or V8-Agar (V8-A) culture medium and incubated at 26 ± 2 °C for 3 days. When mycelial growth was

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observed, it was transferred to Agar Water (AA) for 48 hours and purified by the hyphal tip in Petri dishes with PDA and V8-A.

Pathogenicity test

The technique used by Sánchez *et al*. (1975) and modified by Espinoza-Ahumada *et al*. (2019) was used, for which 50 seeds of chili hybrid Platino were germinated in AA medium, disinfected for 3 min in a 1 % NaClO solution and washed in sterile distilled water three times. Five days after sowing, once the hypocotyl had developed, they were transferred in groups of three seeds to Petri dishes with an AA culture medium. When root and cotyledon development was observed (two days later), a culture explant 3 mm in diameter with the mycelium of the purified phytopathogen was inoculated in the center of the Petri dishes. Petri dishes were incubated at 26 ± 2°C with a photoperiod of 12:12 (light: dark). To determine pathogenicity, seedling mortality was evaluated for nine days using a visual scale of severity described by Apodaca *et al*. (2004) with modifications, where 0 = no symptoms, healthy plant, 1 = small necrotic spots on the root or cotyledons, 2 = necrosis at the base of the root, 3 = root necrosed up to 50 %, 4= root or plant completely necrotic. The data obtained were analyzed through an analysis of variance (ANOVA) to stratify the treatments through a comparison of means by Tukey ($p \leq 0.05$) and under a completely randomized design with two treatments (1 = *P. aphanidermatum*, 2 = *F. oxysporum*), absolute control and three replicates.

Determination of *in vitro* antagonism

The test was carried out by the dual confrontation technique between antagonist and phytopathogen in plates with Potato Nutrient Broth Agar (ANCP), for this purpose, the plates were marked in four cardinal equidistant points, in each one a roast of each antagonist strain was placed, after 24 or 72 hours a disk-shaped explant with mycelium of each phytopathogen was placed separately and incubated at 26 ± 2 °C until the mycelium of the control filled the culture plate. The experiment was conducted under a completely randomized design with 4 treatments (B15, BITV, BIBT, and Pd) and a control, with 4 replicates per treatment for each phytopathogen (*Fusarium* and *Pythium*). Radial growth of the phytopathogen (mm) in confrontation with each bacterial strain was measured, and this was transformed to percentage inhibition (%I), using the formula %I= (C-T)/Cx100 described by Castillo- Reyes *et al*. (2015), where C is the diameter of the control and T is the treatment. Data were subjected to analysis of variance (ANOVA) and Tukey's mean comparison test ($p \leq 0.05$), with the statistical program InfoStat version 2019.1.2.0.

Results and Discussion

Identification and characterization of *Bacillus* spp and *Pseudomonas* sp.

Bacterial species were identified based on their 16S rRNA gene sequencing. Species of the genus *Bacillus* named B15, BITV, and BIBT showed 91.91, 98.36, and 96.15 % similarity to *B. subtilis* species with GenBank accession keys of MK616213.1, MH619505.1, and KY010584.1, respectively. Although strain B15 coincides with a percentage of 91.91 % with the species *B.*

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subtilis, this percentage is considered low, in addition, this strain in previous studies presented a higher percentage (94.12 %) of similarity with the species *Bacillus pumilus* when morphological and biochemical tests were performed (Ordaz, 2004), thus the morphological and biochemical identification is more acceptable. The three strains (B15, BITV, and BIBT) presented a typical form of double bacillus with endospore production (Figure 1A, B, and C), Gram+ staining, and positive catalase (Sosa *et al*., 2005).

The bacterium of the *Pseudomonas* genus showed a 96.12 % similarity with the species *Pseudomonas donghuensis* with access code MK883145.1. This bacterium isolated from the root of *M*. *macroura* and by streak sowing in phosphate agar (NBRIP) (Flores *et al*., 2014) presented a yellow translucent colony formation characteristic of the genus *Pseudomonas* spp. In the beginning, the isolation presented irregularity to solubilize tricalcium phosphate. This bacterium presented itself as an individual vegetative bacillus or in pairs, non-sporulating (Figure 1D), Gram- (Figure 1E) with polar flagella and positive catalase. In AN at 48h of incubation, their colonies generate a yellow pigment (Figure 1F), while in King B medium they do not produce fluorescence and their colonies show irregular borders. Gao *et al*. (2012) first reported this species of *Pseudomonas*, later named *Pseudomonas* HYS, Gao *et al*. (2015) classified it as a new species named *P*. *donghuensis* isolated from the water of Donghu Lake of Wuhan in China, other researchers report its isolation from tomato rhizosphere (Ossowicki *et al*., 2017), cotton (Tao *et al*., 2020) and agricultural soil (Ágaras *et al*., 2018). The traits observed by *P*. *donghuensis* isolated in this research are similar to those described by Ágaras *et al*. (2018) for the species *P*. *donghuensis* SVBP6, but there are differences with respect to those reported by Gao *et al*. (2015) which pointed out that *P*. *donghuensis* HYS develops fluorescence on King A and B agar, while the strain in the present study did not produce fluorescence on King B medium, which coincides with Ágaras *et al*. (2018).

Identification of the species responsible for “Damping off”.

A fungus and an oomycete responsible for “Damping off” were isolated from chili seedlings. The phytopathogenic fungus showed septate hyphae, globose chlamydospores (Figure 2Aa), short phialides (Figure 2Ab) where kidney-shaped bicellular microconidia are inserted (Figure 2Ba), curved canoe-shaped macroconidia of three (Figure 2Bb and Cb) or four septa (Figure 2Ca), with foot cell (Figure 2Bc), besides the formation of sporodochium (Figure 2D). These observed microscopic morphological characteristics corresponded to the genus *F*. *oxysporum* according to the specialized taxonomic keys of Leslie and Summerell, (2006).

The identification and report of the oomycete corresponded to *Pythium aphanidermatum*, such work was published before the present research and was reported as an aggressive and fast-growing strain that causes “Damping off” (Jiménez- Pérez *et al*., 2022). The hyphae were ascertained to be coenocytic toruloid (Figure 3Aa), smooth spherical terminal oogonia (Figure 3Aa, Ba), aplerotic oospores (Figure 3Bb), diclinous or monoclinous antheridia (Figure 3Ac, Bc), and filamentous and irregular sporangia (Figure 3Ca), as shown in Figure 3 (Van der Plaats-Niterink, 1981; Watanabe, 2010; Jiménez-Pérez *et al*., 2022).

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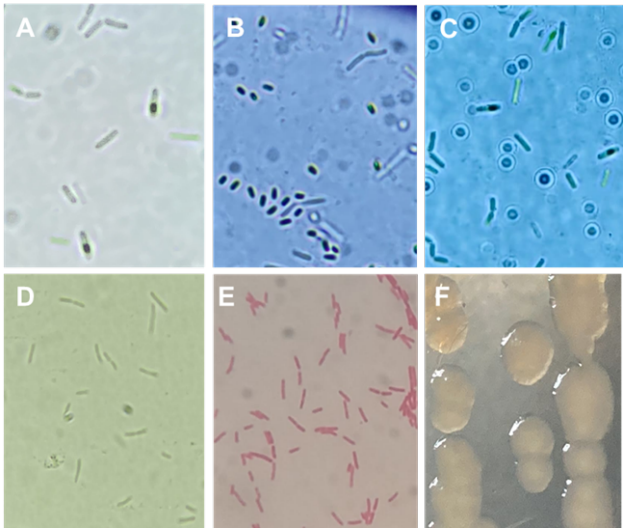

Figure 1. Morphological characteristics of *Bacillus subtilis* and *Pseudomonas donghuensis*. A, B and C) vegetative cells and endospore production of *B. subtilis* (A- B15, B- BITV and C- BIBT). D) *P. donghuensis* vegetative cells, B) Gram stain. C) Bacterial colony in AN culture medium.

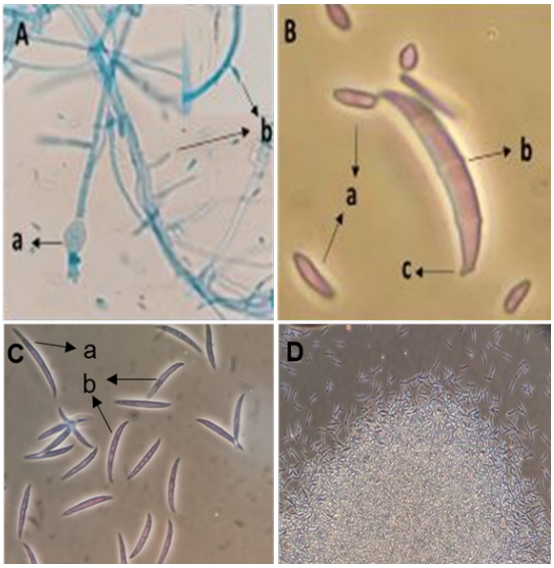

Figure 2. Microscopic observation of *Fusarium oxysporum*. A) a- Chlamydospore globosa and b- Phialides, B) a- Microconidia, b- Macroconidia with three septa and c- foot cell. C) a- Macroconidia with four septa and b- with three septa. D) Sporodochium.

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Figure 3. Microscopic observation of *Pythium aphanidermatum*. A) a- coenocytic toruloid hyphae, b- oogonium and c- antheridium, B) a- oogonium, b- aplerotic oospore and c- antheridium. C) a- sporangia.

Pathogenicity test

In the pathogenicity test, chili seedlings were inoculated with culture explant with mycelium of *F. oxysporum* (Figure 4B) at four ddi caused necrotic spots on the root, which developed and grew until completely necrotizing the seedlings at nine ddi, in addition, abundant growth of white mycelium was detected that completely covered the seedlings, causing 100 % mortality of the seedlings (Figure 4E). In chili seedlings inoculated with *P. aphanidermatum* (Figure 4C), at three ddi they showed necrosis, roots colonized by mycelium, cotyledons with necrotic spots, and at five ddi the seedlings showed abundant mycelial growth, causing 100 % mortality of the seedlings (Figure 4F). In controls, normal root, stem, and cotyledon growth were observed, with no signs or symptoms of necrosis (Figure 4A-D). According to the severity scale of Apodaca *et al*. (2004), the level of damage observed in the cotyledons, hypocotyl, and root of seedlings inoculated with *P. aphanidermatum* and *F. oxysporum* is the highest level (4), since they caused the death of the seedlings, while in the controls there was no damage. Therefore, it is suggested that *P. aphanidermatum* and *F. oxysporum* species were the causal agents of “Damping off” and death of chili seedlings.

The results of this study coincide with the reports of other authors on the ability of these phytopathogens to cause “Damping off”, such as Gravel et al. (2005) who reported *P. aphanidermatum* and *P. ultimum* as etiological agents of “Damping off” and seed rot in tomato seedlings, as well as González et al. (2013), who report the phytopathogens *Pythium* and *Fusarium* in tomato seedlings. While Sánchez *et al*. (2015) report *F. oxysporum* as the causal agent of “Damping off” in onion seedlings. Similarly, Rivera-Jiménez et al. (2018) reported that F. oxysporum caused the death of 49.54 % of chili poblano seedlings in 30 days of the test under greenhouse conditions, in contrast to the present work that was performed under in vitro conditions and where higher mortality of seedlings in less time (9 ddi) was observed.

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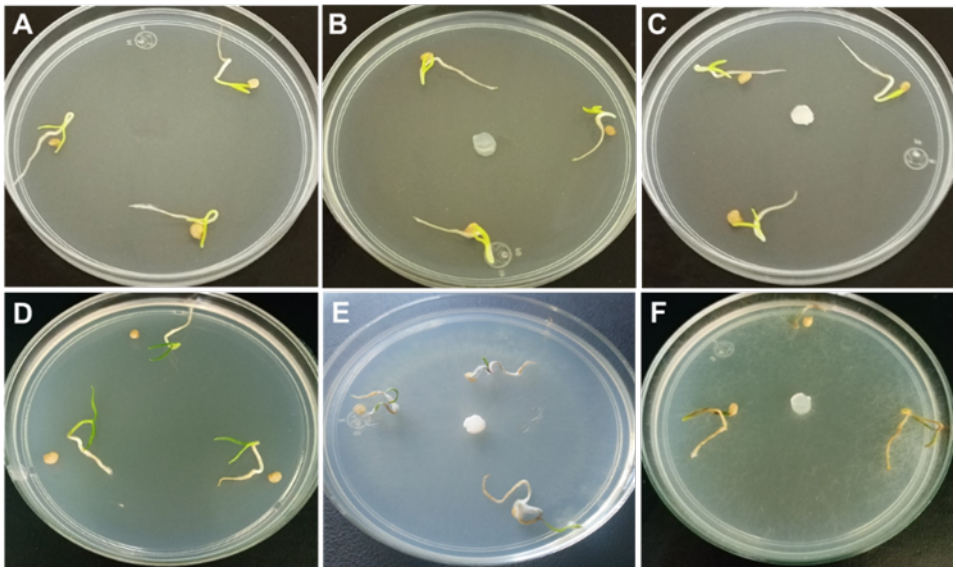

Figure 4. Pathogenicity test: A) Control treatment. B) Treatment inoculated with *F. oxysporum.* C) Treatment inoculated with *P. aphanidermatum.* D) Control treatment at nine ddi. E) Treatment inoculated with *F. oxysporum* at nine ddi. F) Treatment inoculated with *P. aphanidermatum* at five ddi.

Determination of *in vitro* antagonism

In the confrontations of *F. oxysporum* with *Bacillus subtilis* and *P. donghuensis* strains, a significant statistical difference ($p \leq 0.05$) was observed in the percentage of inhibition. All *Bacillus* strains showed inhibitory capacity, with B. *subtilis* (BITV) having the highest efficiency in inhibiting mycelial growth of *F. oxysporum*, at 56.24 % (Figure 5C), followed by *B. subtilis* (BIBT) with 49.62 % (Figure 5A) and the *B. subtilis* isolate (B15) with the lowest inhibition (39.35 %) (Figure 5B). The *P. donghuensis* strain (Pd) did not inhibit the growth of *F. oxysporum* (Figure 5D) during the six days of the experiment, which is the time of the control to grow on the entire culture plate (Figure 5E) (Table 1).

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Table 1. Percentage of *in vitro* inhibition of the treatments against *Fusarium oxysporum* six days after the confrontation.

Treatments		
Strain	**Code Id**	**Means**
Bacillus subtilis	BITV	56.24A
Bacillus subtilis	BIBT	49.62B
Bacillus subtilis	B15	39.35C
Pseudomonas donghuensis	Pd	0.00D
Control	Control	0.00D

*Means with a letter in common are not significantly different ($p > 0.05$).

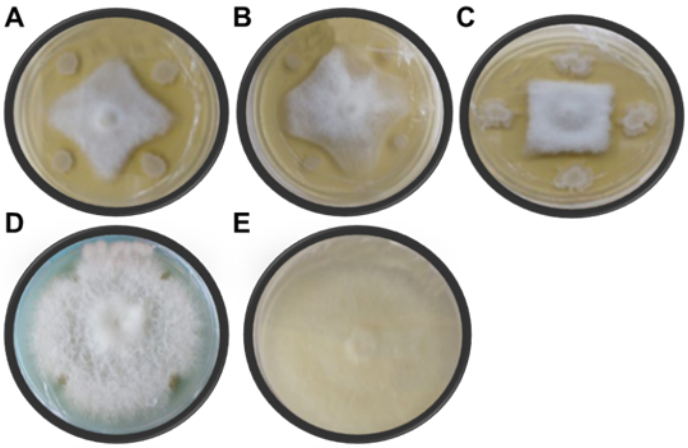

Figure 5. *In vitro* antagonism of the different strains of *Bacillus* spp., against *F. oxysporum* at six days of confrontation in comparison with the control. A) BIBT, B) B15, C) BITV, D) Pd, and E) Control.

For the challenge against *P. aphanidermatum*, the treatments were carried out 72 hours before inoculating the phytopathogen, since this oomycete showed a very fast mycelial growth by developing in the whole culture plate in only 32 hours after incubation (Fig. 6- E). Unlike the *F. oxysporum* assay, none of the *B. subtilis* strains showed inhibition against this oomycete (Fig. 6- A-C), only the *P. donghuensis* strain inhibited 56 % of the mycelial growth of *P. aphanidermatum* (Fig. 6- D) (Table 2).

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Table 2. Percentage of *in vitro* inhibition of the different treatments against *Pythium aphanidermatum* at 32 hours of confrontation.

Treatments		
Strain	**Code Id**	**Means**
Pseudomonas donghuensis	Pd	56.00A
Bacillus subtilis	BITV	0.00B
Bacillus subtilis	BIBT	0.00B
Bacillus subtilis	B15	0.00B
Control	Control	0.00B

*Means with a letter in common are not significantly different ($p > 0.05$).

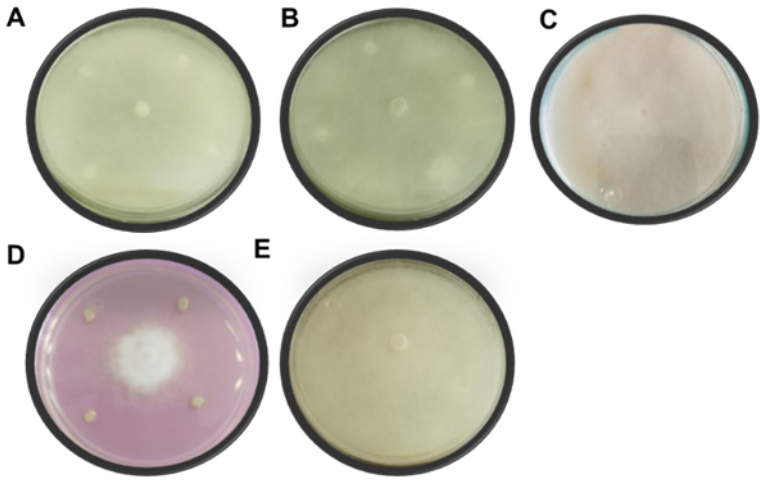

Figure 6. *In vitro* antagonism of the different strains against *P. aphanidermatum* at 32 hours after confrontation. A) BIBT, B) BITV, C) B15, D) Pd, and E) Control.

Several studies demonstrate the antagonistic capacity of the genus *B. subtilis* against various phytopathogens, for example, Mejía- Bautista *et al*. (2016) report varied inhibition ranges (21.28- 71.70 %) against two strains of *Fusarium* (*F. equiseti* and *F. solani*) in confrontation with 10 strains of *Bacillus* spp (4- *B. subtilis*, 1- *B. cerus*, 1- *B. amyloliquefaciens* and 4- *Bacillus* spp) being the strains of *B. subtilis* CBMT51 and CBRF8 which exhibit the highest inhibitory activity with inhibition percentages of 71.70 % and 69.92 % against *F. equiseti* and *F. solani*, respectively, while in the present study, inhibition ranges from 39.35 to 56.24 % were obtained with the three *Bacillus* strains against *F. oxysporum*. Khedher *et al*. (2020) reported inhibition of *F. oxysporum*

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of 54.7 % using *B. subtilis*, this result is similar to the present work (56.24 %) using the strain of *B. subtilis* (BITV). Sosa *et al*. (2005), confronting 17 strains of *Bacillus* spp., demonstrated their inhibitory capacity against several phytopathogens, being *P. aphanidermatum* one of them, contrasting with our research where none of said three strains of *Bacillus* managed to inhibit the oomycete at 32 h that lasted the experiment, unlike the work of Sosa *et al*. (2005) in which the *P. aphanidermatum* strain presented a slow growth of 72 h to fill the Petri dish. Some authors mention that the inhibition generated by *Bacillus* spp is due to the ability to produce some antibiotics (Iturin A and surfactin) and lytic enzymes (Asaka & Shoda, 1996; Sosa et al., 2005).

The present results obtained with *P. donghuensis* are similar to those proposed by Gao *et al*. (2015) who suggest this species is a candidate for biological control due to its high capacity for synthesis of siderophores, which is greater than other strains (Gao *et al*., 2012). There are reports of *P. donghuensis* antagonism against some phytopathogens, for example, Ossowicki *et al*. (2017) report growth inhibition of *Rhizoctonia solani*, *Fusarium culmorum*, *Verticillium dahlia*, and *Pythium ultimum* with *P. donghuensis* strain P482. This same strain (P482) was also shown to be effective against bacteria such as Dickeya solani and *Pseudomonas syringae* pv. *syringae* (Matuszewska *et al*., 2021). Tao *et al.* (2020) inhibited *V. dahliae* with *P. donghuensis* strain 22G5. Similarly, Muzio *et al*. (2020) reported the inhibition of *Macrophomina phaseolina* by *P. donghuensis* SVBP6. The cited authors attribute the antagonistic capacity of *P. donghuensis* to its high capacity for siderophore synthesis, especially of the non-fluorescent siderophore 7-hydroxytyropolone, which has not been reported in other *Pseudomonas* species. At present, there are still few studies carried out with this species of *Pseudomonas* against phytopathogenic fungi, having only reports with the strains mentioned above called P482, 22G5, and SVBP6, such is the case that at the present day, there are no results of a strain of *P. donghuensis* capable of inhibiting *P. aphanidermatum* and without inhibitory activity against *F. oxysporum* as observed in this work.

Conclusions

The genera *F. oxysporum* and *P. aphanidermatum* isolated from chili seedlings with symptoms of the disease and pathogenicity are confirmed as causal agents of “Damping off”.

The inhibitory effect of the antagonistic bacteria depends on the species and type of phytopathogen against which it is confronted since the isolates of *B. subtilis* exhibit the ability to inhibit the mycelial growth of Fusarium, but not that of *Pythium*, and in contrast, *P. donghuensis* inhibited the oomycete, but not the deuteromycete. Therefore, for future experiments, it is suggested to use mixtures of both antagonists formulated for more efficient control of “Damping off” disease.

Author Contributions

Original research idea and project fund manager, Gabriel Gallegos Morales (Autor 2), development of the methodology and experimental validation, Omar Jiménez Pérez (Autor 1), software and data management, Epifanio Castro del Angel (Autor 5); analysis of results, Cesar

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Espinoza Ahumada (Autor 4); manuscript preparation and adjustments, Juan Manuel Sánchez Yañez (Autor 6); and Francisco Daniel Hernández Castillo (Autor 3) for the morphological characterization of phytopathogenic fungy and final reviewer of the paper.

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Ethical Statement

The published information was presented to the postgraduate academy as part of the thesis degree of the Master's in Agricultural Parasitology.

Informed Consent Statement

Not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

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