INTRODUCTION

Tomato (Solanum lycopersicum L.) is within the most economically important vegetable crops around the world. Nevertheless, the fruit is highly perishable, and large postharvest losses have been reported (Arah et al., 2016). Among the factors responsible for these losses are infections produced mainly by fungi. After harvest, tomato fruit is frequently infected by Alternaria alternata, the causal agent of the alternaria rot disease. This disease is characterized by the appearance of black, large, leathery, and sunken spots on tomato fruit (Lemma et al., 2014). Applying chemical fungicides is a common practice to control this disease; however, these compounds can affect human health and the environment (Damalas and Eleftherohorinos 2011).

Biological control using beneficial microorganisms offers a practical and economical alternative for the controlling of phytopathogens. Bacterial strains produce compounds that directly inhibit the growth of fungal or suppress diseases by inducing host defenses (Köhl et al., 2019). In this regard, bacterial metabolites instead of chemical fungicides can be a smart choice to control diseases during postharvest. Some Bacillus species belong to a group of plant growth-promoting rhizobacteria (PGPR), and some of them have been applied in agriculture as antagonist agents (Aloo et al., 2019). The antagonistic activity of Bacillus is accompanying with the synthesis of various antimicrobial metabolites such as enzymes, peptides, and volatile organic compounds (Caulier et al., 2019; Guo et al., 2014). Thus, several
studies focused on the biological control of diverse phytopathogens, including Alternaria species, have shown that metabolites produced by Bacillus, may provide an environmentally friendly alternative to toxic fungicides (Tozlu et al., 2018).

Major aspects of biocontrol are bioprospecting for new active bacterial isolates as well as understanding the mechanisms of pathogen antagonism for their future improvement and broader use. Recently, B. atrophaeus has emerged as a new biological agent against phytopathogenic fungi. The main performance was obtained with the use of metabolites produced by B. atrophaeus B5 against two virulent strains of Colletotrichum gloeosporioides, reducing the severity and incidence of anthracnose disease on harvested soursop and avocado fruit (Guardado-Valdivia et al., 2018).

The genus Brevibacterium is also considered a new promising biocontrol agent, with a potential of Plant Growth Promoting Rhizobacterium (PGPR) and biocontrol; nevertheless, at the moment only a few species and strains as biocontrol agents, have been studied (Ahmed et al., 2015; Chopra et al., 2020; Mohamad et al., 2018). Certainly, the biological control using new bacterial strains provides an eco-friendly alternative to fungicides, therefore it is important bioprospecting for new strains. Based on the above, this research aimed to evaluate the antifungal activity of metabolites produced by B. atrophaeus B5 and a new Brevibacterium strain against A. alternata, both in vitro and in vivo on tomato fruit during postharvest.

**MATERIALS AND METHODS**

**Fungal phytopathogen, bacterial antagonist and plant material**

The virulent strain A. alternata strain AA5 and bacterial strains used in this study were provided by the Biotechnology Laboratory of the Instituto Tecnologico de Tepic, Mexico. Previously, bacterial strains were obtained from rhizosphere samples of field-grown crops from several regions of Nayarit, Mexico. Briefly, the soil samples were homogenized in distilled water, and the serial dilution method was made. Aliquots of each dilution were spread on King broth (KB) medium and incubated at 25°C for 48 h. Bacterial isolates were partially screened for growth inhibition of phytopathogenic fungi (data not shown). Strains with antagonistic activity against fungal phytopathogens, including B5 and B7 strains, were preserved for further experimentation. A. alternata was cultured on potato dextrose agar (PDA) at 28°C for 7 days. Bacterial strains were cultured on KB at 28°C for 2 days. All strains were maintained at 4°C. Tomato fruits (Solanum lycopersicum L.) cv. Saladette were visually selected based on uniform shape, size, color, stage of mature: mature green, and the lack of fungal diseases or mechanical damage. Fruits were collected in Santiago Ixcuintla, Nayarit, Mexico, and transported to the laboratory.

**Preparation of fungal spore suspensions and bacterial cell-free supernatant**

To prepare the fungal spore suspensions, A. alternata was inoculated on a PDA plate and incubated for 7 days at 28°C. The fungal conidia were harvested by adding sterile water and stirring with a glass rod and then subsequently filtering the liquid to remove the mycelia with degreasing cotton. Spore concentration was adjusted to 1×10^6 spores/ml by microscopic counting in a hemocytometer.

To prepare the cell free supernatant (CFS), bacterial cells were grown in KB medium at 25°C for 24 h. CFS was obtained by centrifugation of bacterial culture at 5,000 x g for 15 min at 20°C. We usually use 0.20 µm filter (Millipore Corp., Bedford, MA, USA) to prepare cell-free culture.

**Phenotypical and physiological characterization of strain B7**

The cell morphology of strain B7 was examined by light microscopy after Gram-staining and spore staining. The utilization of different carbon sources and the assessment of catalase and oxidase activities were determined by standard methods (Selvakumar et al., 2011). The sources of carbon used were gelatin, glycerol, sucrose, glucose, fructose, mannitol, lactose, galactose, and maltose.

**Molecular characterization by 16S rDNA gene analysis**

DNA extraction from bacterial strains was done as previously described (Green and Sambrook 2012). For molecular identification of the strain B7, gene amplification by PCR was carried out using the partial gene 16S rDNA. Universal primers 27Fw (5’AGAGTTTGTGATCMTGGCTCAG-3’) and 1492Rw (5’TACGACTTCTTGGTCAAC-3’) (Kadyan et al., 2013), and sequencing analysis were used. PCR products were sequenced by Macrogen Inc. (Seoul, Korea) and compared to the DNA sequences available in the National Center for Biotechnology Information (NCBI) using the program BLASTN (Nucleotide Basic Local Alignment Search Tool). DNA sequences from the NCBI nucleotide database were aligned with the ClustalW program in MEGA 7.0 (Kumar et al., 2016). A phylogenetic relationship was created using the nearest neighbor data analysis method with 1,000 bootstrap replicates.
Effect of the bacterial suspension

To evaluate the antagonistic effect of bacterial suspension culture of *B. atrophaeus* B5 and strain B7, dual-culture assays were made. To this, a 7 day-old mycelia disks (5 mm diameter) of the fungus were placed in the center of plates containing PDA. Four drops of bacterial suspension (1x10^7 CFU) were spotted around the fungal inoculums at a distance of 3 cm at the same time of pathogen inoculation. Plates were incubated at 28°C for 7 days and then, the antagonistic effect of test strains on *A. alternata* was observed. As a control, PDA inoculated with the pathogen alone was used.

Effect of CFS on mycelial growth and spore germination

Assays for inhibition of mycelial growth were made as previously described (Balouiri et al., 2016). The CFS from *B. atrophaeus* B5 and strain B7 cultures were evaluated. The PDA medium incorporating CFS at a concentration of 20% (v/v), was inoculated with a 7 day-old mycelia disks (5 mm diameter) at the center and was incubated at 28°C. Control plates containing PDA mixed with KB (20%; v/v) or PDA without CFS were included. No difference in mycelial growth between controls was observed. After an incubation period of 7 days, radial mycelial growth of *A. alternata* (T) was measured using a Truper Vernier caliper, until the fungi growth in the control dishes containing PDB medium supplemented with 20% of KB or PDA without CFS (C) was almost complete. The inhibition of the radial growth of the fungal colony was daily evaluated and the percentage of growth inhibition (I) was calculated using the formula: $I (%) = \frac{(C-T)}{C} \times 100$. Tests were carried out in triplicate.

Conidial germination assays were made as previously described (Guardado-Valdivia et al., 2018). To this, 20 µL of fungal spore suspension were mixed with potato dextrose broth (PDB) medium supplemented with 20% of CFS. Then, treated suspension was kept at 28 °C. After 24 h, 100 conidia were analyzed. As a control, a PDB medium supplemented with 20% of KB or PDB alone was used. The germ tube length was observed in light microscope. A spore was considered germinated when the germ tube length was 1.5 times the spore. The experiment was repeated three times.

Efficacy of CFS against alternaria rot disease

Evaluation of CFS on control of alternaria rot disease during postharvest was carried out on tomato fruit. Prior to the experiments, the surface of tomatoes was disinfected by immersion in 1% NaOCl for 1 min, rinsed with distilled water, and allowed to air-dried at 25°C. Tomato fruits were soaked in CFS for 1 min, taken out and placed in trays, and stored at 25°C. As controls, tomatoes were soaked in distilled water or KB medium. After 2 h, treated tomatoes were divided into six groups. The first group was wounded (3 mm deep and 3 mm in diameter) nine times at three sides of each fruit with a sterile punch. This group was not inoculated fungal spore suspension. The second group was wounded as described above and was inoculated with 15 µl of a fungal spore suspension. Both groups were stored at 25°C for 10 days.

The first, second and third group; soaked in CFS, distilled water or KB medium respectively, were wounded (3 mm deep and 3 mm in diameter) nine times at three sides of each fruit with a sterile punch. These groups were not inoculated with fungal spore suspension. The fourth, fifth and sixth group, soaked in CFS, distilled water or KB medium, were wounded as described above and were inoculated with 15 µl of a fungal spore suspension. All fruit were placed in trays and stored at 25°C. Every day, the lesion diameter on each tomato fruit wound was observed during the incubation period.

The disease incidence (%) in each treatment was measured by the ratio between the number of symptomatic injuries and the number of total injuries in the tomato fruit. *In vivo* experiments were conducted in a completely randomized design with three replications per treatment, each replication consisting of ten tomatoes.

Statistical analysis

Uni-factorial statistical designs were applied to internal severity and incidence determination. Ten fruits were used per replicate. Analysis of variance (ANOVA) was performed using STATISTICA version 8.0 High-Performance Analytical Software Solutions. Differences between means of data were compared by the least significant differences (LSD). Differences at P <0.05 were considered to be significant. All the experiments were repeated three times.

RESULTS AND DISCUSSION

Phenotypical, physiological and molecular identification of strain B7

Microscopic examination has shown that strain B7 was Gram-positive tiny rods in a chain, non-endospore former. Colonies growing on LB agar were circular creamy white with a glistening surface. Strain B7 utilized various carbon sources such as glucose, sucrose, fructose, maltose, and mannitol; however, the best-used carbon source was glucose. Additionally, it was positive for oxidase and catalase activity as well as gelatin hydrolysis. The 16S rDNA sequence amplified from strain B7 was compared using NCBI Blast. The sequence showed 100% homology to the 16S rDNA gene of *Brevibacterium frigoritolerans* strain DSM 8801.
Based on phenotypical, physiological, and molecular analysis of the 16S rDNA gene sequence, strain B7 was identified as *Brevibacterium frigoritolerans*. Several studies referred to *B. frigoritolerans* as an entomopathogen, mainly of *Anomala dimidiata* and *Holotrichia longipennis* (Selvakumar et al., 2011). Recently, few reports about biocontrol traits for this species have been done. Thus, it was reported two strains of *B. frigoritolerans*, one of them was able to stimulate the growth of *Triticum aestivum* HD2967 seedlings under greenhouse conditions, and the other one, suppressed Fusarium stalk rot of maize (Batool et al., 2019; Meena et al., 2017). In accord, *B. frigoritolerans* displays biocontrol traits that require to be investigated.

**In vitro** antifungal activity of bacterial strains on *A. alternata*

To evaluate the antagonism of bacterial strains against the growth of *A. alternata*, we conducted a dual culture plate test. The antifungal activity of extracellular bacterial metabolites contained in the CFS was analyzed by test inhibition assays. *B. atrophaeus* B5 displayed a strong antagonistic activity against the mycelia growth after seven days post-inoculation, unless *B. frigoritolerans* B7, which showed less antagonistic activity than strain B5 (Fig. 2A). Similar results were obtained when extracellular bacterial metabolites were evaluated. The CFS from *B. atrophaeus* B5 was more efficient inhibiting the mycelia growth of *A. alternata* than CFS from *B. frigoritolerans* B7 and the control (Fig. 2B). Concerning to spore germination, CFS from *B. atrophaeus* B5 and *B. frigoritolerans* B7 strains displayed antifungal activity. Although *B. frigoritolerans* did not show an efficient inhibition of the mycelia growth compared with *B. atrophaeus* B5, their metabolites were more effective in reducing spore germination (Table 1). These results indicate that the antifungal metabolites produced by B5 and B7 strains show a different biocontrol mechanism each other. Some studies have reported that several fungal plant pathogens could be controlled by metabolites, such as O-anisaldehyde and lipopeptides, produced by *B. atrophaeus* strains (Guardado-Valdivia et al., 2018; Zhang et al., 2013). In agreement with our results, lipopeptides produced by *B. atrophaeus* B5 could participate in the inhibition of mycelial growth and spore germination of *A. alternata*. Regarding *B. frigoritolerans*, metabolites with antifungal activity have not yet been identified.

**Evaluation of preventive treatment applied to fruit**

Preventive treatments were done by first applying the treatment to tomatoes and then exposing treated fruit to the pathogen by inoculating with a fungal spore suspension. As controls, KB medium and distilled water were used. During all assays, we did not observe differences between...
control treatments. In tomato fruits treated with KB medium, spores of *A. alternata* were able to germinate on the tomato fruit wound sites, causing large, dark, sunken areas with concentric rings, characteristic of alternaria rot disease. Conversely, fruits treated with CFS were resistant to *A. alternata* (Fig. 3A). Visual quality was preserved in all tomato fruits treated with bacterial CFS, KB, or water without spore inoculation, thus a possible deleterious effect of the preventive treatment with CFS was discarded (Fig. 3B).

After day ten of storage, fruits inoculated with the pathogen showed a mean lesion diameter of 2.5 cm; however, tomatoes treated with CFS from *B. atrophaeus* B5 and *B. frigoritolerans* B7 displayed 0.55 cm and 0.33 cm of the mean lesion; respectively. Statistical analysis revealed a significant difference between supernatants, being more efficient supernatant produced by *B. frigoritolerans* B7 (Fig. 4A). In general, preventive treatments using CFS obtained from both strains effectively avoided the fungus establishment in fruit tissues compared to control (*P* <0.05).

Concerning disease incidence, low rates of incidence were recorded at 10 days compared to control. Thus, 7% of incidence was registered in tomatoes treated with *B. atrophaeus* B5 CFS; but, the best incidence control was achieved by the CFS from *B. frigoritolerans* B7, displaying 1% of incidence after 10 days post-inoculation (Fig. 4B). These results agree with those obtained during spore germination assays, where *B. frigoritolerans* was more efficient than *B. atrophaeus*. These results are important due to the effectiveness of the treatments to prevent the fungal infection by *A. alternata* on tomato fruits during postharvest, demonstrating the potential of *B. frigoritolerans* B7 as a biocontrol agent against this fungal phytopathogen.

Undoubtedly, the effectiveness of preventive treatments for controlling fungal decay on tomato is related to the production of bacterial metabolites. It was reported that *B. atrophaeus* B5 controls *C. gloeosporioides* producing fengycin, iturin, surfactin, and unknown lipopeptides that contribute to their antifungal activity (Guardado-Valdivia et al., 2018). In agreement with our results,
FIG. 3 Effect of CFS against alternaria rot disease: (A) Preventive treatment to control alternaria rot disease in tomato fruit and (B) treatments without fungal inoculation.

FIG. 4 Lesion diameter and incidence of alternaria rot disease: (A) Effect of CFS on lesion diameter in tomato fruit inoculated with *A. alternata* and (B) disease incidence graphic. Bars represent means from replicates and error bars represent standard errors. Mean values and LSD intervals (*n = 10*). Different letters (a and b) indicate significant differences (*P < 0.05*) for each treatment.
these metabolites are also effective against A. alternata, revealing a versatile ability to reduce the development of several phytopathogens fungal species.

Concerning B. frigoritolerans, at the moment there are no reports about antifungal metabolites produced by this species. In an effort to detect lipopeptide biosynthetic genes coded into B. frigoritolerans B7 genome, we conducted PCR analyses using primers directed to fenD, ituC, and srfA4 genes encoding for fengycin, iturin, and surfactin, respectively (Alvarez et al., 2012; Mora et al., 2011). As a result, we did not yield any amplification product using DNA from B. frigoritolerans B7 as a template (data not shown), supporting the idea that this strain does not produce these compounds. Several species of Brevibacterium are able to produce lipopeptides. For instance, a marine strain of B. aureum produces brevifactin, which was chemically characterized and their structure was different from other lipopeptides, including surfactin, fengycin, iturin, or bacillomycin produced by Bacillus spp. (Seghal-Kiran et al., 2010). According to this, we considered it feasible that B. frigoritolerans B7 may be producing brevifactin or other unknown antifungal metabolites.

Several studies have demonstrated that metabolites obtained from antagonistic bacteria prime the expression of defense responses in harvested fruits through the induced systemic resistance (ISR), resulting in an enhanced level of induced resistance against fungal infection. (Ongena et al., 2007; Waeuthongrak et al., 2014). According to our results, we can therefore propose that metabolites produced by strains B5 and B7 inhibited conidial germination and could be stimulating the defense systems of the tomato fruit during postharvest. Further analysis of the mode of action of the metabolites produced by B. atrophaeus B5 and B. frigoritolerans B7 is currently being undertaken in our laboratory to understand their mechanism and improve their use.

**CONCLUSIONS**

The future phytopathogen management will depend strongly on biocontrol, because it is the cheapest, sustainable, and environmentally safest system, with additional benefits for growers and consumers. Bacterial metabolites are gaining great interest due to their potential to provide quality and safety benefits. This fact is important because the use of viable cells in agro-alimentary products may be restricted, mainly when biocontrol agents are not determined to be Generally Recognized As Safe (GRAS). The use of metabolites produced by B. atrophaeus B5 and B. frigoritolerans B7 represents a new approach to reduce the use of chemical pesticides to preserve tomato fruits of quiescent infections and control fungal decay on postharvest stage. Some Brevibacterium species have been recognized as GRAS, for their potential to produce compounds used in the health and food industries. However, more efforts should be done to further validate the currently available outcomes, deepen the knowledge on the most valuable metabolites, and to improve their efficacy by setting up effective formulations, application protocols, and integrated strategies.

**ACKNOWLEDGEMENTS**

The authors thank to the Consejo Nacional de Ciencia y Tecnología (CONACYT) for the scholarship granted to Lizeth Guardado-Valdivia. We are thankful to Dr. Bravo for technical support.

**REFERENCES**


