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Research Article

Antifungical Activity of Autochthonous *Bacillus subtilis* Isolated from *Prosopis juliflora* against Phytopathogenic Fungi

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Abstract The ability of *Bacillus subtilis*, strain ALICA to produce three mycolytic enzymes (chitinase, β-1,3-glucanase, and protease), was carried out by the chemical standard methods. *Bacillus subtilis* ALICA was screened based on their antifungal activity in dual plate assay and cell-free culture filtrate (25%) against five different phytopathogenic fungi *Alternaria alternata*, *Macrophomina* sp., *Colletotrichum gloeosporioides*, *Botrytis cinerea*, and *Sclerotium rolfesii*. The *B. subtilis* ALICA detected positive for chitinase, β-1,3-glucanase and protease enzymes. Fungal growth inhibition by both strain ALICA and its cell-free culture filtrate ranged from 51.36% to 86.3% and 38.43% to 68.6%, respectively. Moreover, hyphal morphological changes like damage, broken, swelling, distortions abnormal morphology were observed. Genes expression of protease, β-1,3-glucanase, and lipopeptides (subtilosin and subtilisin) were confirmed their presence in the supernatant of strain ALICA. Our findings indicated that strain ALICA provided a broad spectrum of antifungal activities against various phytopathogenic fungi and may be a potential effective alternative to chemical fungicides.

Keywords *Bacillus subtilis*, Biocontrol, Lipopeptides, Mycolytic enzymes, *Prosopis juliflora*

In the Northwestern Mexico, the fungi are the most common plant pathogens, which cause large losses in the quantity and quality of crops yield and thus cause great economic losses [1]. Control of these fungi has been largely through the application of fungicides, which represents a quick solution to the phytosanitary problems. However, these agrochemicals represent a risk to environmental by the negative effects in the human and animal health [2]. On the other hand, biological control using beneficial autochthonous microorganisms is an eco-friendly alternative to chemical fungicide, which can protect the plant from pathogens by several mechanisms such as antibiosis, parasitism, competition, lytic enzymes, and induced systemic resistance [3, 4]. The term "autochthonous microorganisms" refers to a group of beneficial microbes that are native to the area, thus the name autochthonous. Autochthonous microorganisms play an important role by protecting the normal host from invasion by microorganisms with a greater potential for causing disease [4]. In this context, autochthonous microorganisms, specially belonging to the *Bacillus* genus are among the most used biological agents to fight against many plant pathogens [5]. *Bacillus* species (e.g., *B. subtilis*) displayed diverse strategies to reduce the infection in the plants by pathogens: (1) production of extracellular antibiotics; and (2) production of mycolytic enzymes like chitinase, protease, and β-1,3-glucanase [5, 6]. *Bacillus subtilis* is a species commonly found in the environment, its taxonomy and that of related species has been extensively studied in the last two decades [7]. However, though many different environmental isolates and strains of *B. subtilis* have been described, to date there is no report regarding the identification of native *B. subtilis* isolates from extreme arid condition present in northwestern of Mexico, that elicit significant

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antagonistic activity against fungi that cause plants diseases.

The study of *B. subtilis* capable of producing an effective biocontrol of different phytopathogenic fungi has focused on isolating of several strains from tropical and subtropical regions [7, 8]. Nevertheless, research that evaluating the diversity of autochthonous microorganisms as *B. subtilis* in native soils from Northwestern, Mexico, specifically, Baja California, is scarce. Considering the above the present study was carried out to evaluate the antagonistic potential of *B. subtilis* strain "ALICA" previously isolated from *Prosopis juliflora* rhizosphere a native plants from northwestern of Mexico against *Alternaria alternata*, *Macrophomina* sp., *Colletotrichum gloeosporioides*, *Botrytis cinerea*, and *Sclerotium rolfesii*.

MATERIALS AND METHODS

Microorganisms. *B. subtilis* strains "ALICA" (GeneBank No. KX137176) used in the present study was previously isolated from rhizosphere soil of *P. juliflora* [9]. The plant pathogenic fungi *A. alternata*, *Macrophomina* sp., *C. gloeosporioides, Botrytis cinerea*, and *S. rolfesii* were kindly supplied by Dra. Rosalba Troncoso-Rojas from the Research Center in Food and Development (CIAD-Hermosillo, Mexico).

Enzymes assay. For the production of enzymes, the strain *B. subtilis* ALICA was inoculated in 100-mL Erlenmeyer flasks containing 50 mL of synthetic broth medium with (%): K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.01, NaCl 0.1, $(NH_4)_2SO_4$ 0.7, and lyophilized fungal mycelium 0.1. After 72-hr incubation at 30°C with rotary agitation 150 rpm, the culture was centrifuged at 10,000 rpm for 10 min at 4°C. The crude supernatant was used as enzyme source.

Chitinase assay. The chitinase activity was measured as described by Brants and Earle [10], 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside [4-MU-β-(GlcNAc) 3] (Sigma, St. Louis, MO, USA) solution was prepared as a substrate at a concentration of 1.3 µM in 50 mM sodium phosphate buffer pH 7. The reaction mixture containing 10 µL of enzyme solution, 5 µL of the substrate, and 35 µL of 50 mM sodium phosphate buffer pH 7 was performed at 37°C for 20 min. One hundred fifty microliters of 0.2 M Na_2CO_3 was added to stop the enzymatic reaction. Fluorescence after the enzyme activity was measured by using a TBS-380 Mini-Fluorometer (Tuner Biosystems, Promega, Madison, WI, USA) at 325 nm excitation and 446 nm emission wavelengths.

Enzyme assays were performed in triplicates. One unit of enzyme activity (U) was defined as the amount of enzyme able to release 1 ng of 4-methylumbelliferone per minute.

β-1,3-Glucanases assay. The β-1,3-glucanase activity was determined by measuring the reducing sugars released from laminarin as a substrate using 1% dinitrosalicylic acid (DNS) method [11]. Five hundred microliters of supernatant was mixed with 500 µL 0.5% laminarin substrate which dissolved in 50 mM acetate buffer, pH 6.0. After incubation at 40°C for 60 min the enzyme was inactivated by adding 1 mL of a 1% DNS solution and heating the preparation in a boiling water bath for 10 min. The absorbance was measured at a wavelength of 540 nm. The amount of reducing sugar released was calculated using glucose as standard. One unit of β-glucanase activity is defined as the amount of enzyme required to produce 1 µg of glucose in 1 min under the experimental conditions used.

Detection of protease enzyme production. Protease activity was performed on skimmed milk agar (SMA) which prepared using a 10% (w/v) stock solution of skimmed milk powder autoclaved at 115°C for 10 min while agar solution made separately and autoclaved at 121°C for 20 min. The tow solutions were mixed to a final concentration of 1% skimmed milk, while still hot, and poured into Petri plates. Tested strain spotted on SMA plates and incubated 3 days at 30°C, the formation of clear zone around the colony was considered as protease positive.

Protease assay. Overnight preculture cultivated in Nutrient Broth medium was inoculated at 1% in protease production media (50 mL in 100-mL Erlenmeyer flasks) contained (% w/v): glucose 0.1, peptone 1, yeast extract 0.02, $MgSO_4$ 0.01, $CaCl_2$ 0.01, K_2HPO_4 0.05 (pH 7.0) and incubated 72 hr at 30°C on a rotary incubation shaker (150 rpm). After incubation, culture was harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cell free supernatant was used as the enzyme source for protease assay and the protease activity was determined as described by Jayashree *et al.* [12]. One milliliter of enzyme solution was added to 1 mL 2% (w/v) casein solution (dissolved in 50 mM phosphate buffer with pH 7) and incubated in water bath at 37°C for 20 min. The reaction was terminated with the addition of 2 mL of 0.4 M trichloroacetic acid. After 30 min stand at room temperature, the reaction mixture was centrifuged at 10,000 rpm for 10 min. Aliquots of 0.5 mL supernatant were mixed with 2.5 mL of 0.4 M Na_2CO_3 and 0.75 mL of Folin-Ciocalteu's phenol reagent : water (1 : 3 v/v) and incubated at room temperature for 30 min in dark condition. The optical density of the solutions was determined at 660 nm and compared against a tyrosine standard curve. One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 µg of tyrosine per minute under the standard assay conditions. Specific activity was expressed as units/mg protein.

Quantitative assay of protein. Protein content of the enzymes solution (supernatant) was determined by using the Bradford method and bovine serum albumin as a standard.

Antifungal assay. The efficacy of *B. subtilis* strain "ALICA" was tested against *A. alternata*, *Macrophomina* sp., *C. gloeosporioides*, *Botrytis cinerea*, and *S. rolfesii* on potato

Table 1. Sequences of primers used in the study

Genes	Primer name	Sequence	Reference
Subtilosin	Sbo1F	5′-TCGGTTTGTAAACTTCAACTGC-3′	[15]
	Sbo1R	5′-GTCCACTAGACAAGCGGCTC-3′	
Subtilisin	Qk1F	5′-CTTAAACGTCAGAGGCGGAG-3′	[15]
	Qk1R	5′-ATTGTGCAGCTGCTTGTACG-3′	
Protease	22mer (F)	5′-CATATGTTTGGGTACTCTATGG-3′	[16]
	25mer (R)	5′-GGATCCTTATTGGCCGGGAACGGAA-3′	
β-1,3-glucanase	β-glu (F)	5′-AATGGCGGTGTATTCCTTGACC-3′	[6]
	β-glu (R)	5′-GCGCGTAGTCACAGTCAAAGTT-3′	

dextrose agar (PDA) medium using dual plate method. Ten-day-old culture agar discs (6 mm) of pathogens were disposed at the center of Petri dishes and the bacterial strain was streaked in a square form around the agar disc at 2 cm distance from the center and incubated at 28°C until mycelial growth had fill plates containing the control (without bacteria). The reduction of fungal growth was monitored by measuring the diameter in millimeter of the colony. The percentage of growth inhibition of tested fungi was calculated according to the formula given by Trivedi *et al.* [13], growth inhibition (%) = (R1 − R2/R1) × 100, where, R1 = radial growth of control and R2 = radial growth of the fungus in dual culture. At the end of the incubation period, the morphological changes of fungal mycelium were studied microscopically under optical microscope (40× magnification).

Antifungal activity of cell-free supernatants of *B. subtilis*. Bacteria were grown on Nutrient Broth at 30°C, with constant shaking at 150 rpm and after 3 days of incubation, cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatant was passed through a 0.22-µm membrane to obtain cell-free filtrate. The antifungal activity of cell-free supernatant of *B. subtilis* strain "ALICA" on the growth of five pathogenic fungi was tested in Petri dishes containing the PDA medium. Twenty-five milliliters of the culture filtrate mixed with 75 mL of the PDA medium was poured into Petri dishes. Thereafter, an inoculum (6 mm diam.) of each fungus was inoculated at the center of PDA plate and incubated at 28°C until the mycelia colony of the control had grown to almost fill the plate. Relative inhibition of fungal growth was evaluated by the percentage reduction in the growth of the mycelia in comparison to that on control plates without culture filtrate as described earlier.

Gene expression of biocontrol genes. Total RNA was extracted from the overnight culture of *B. subtilis* strain "ALICA" cells grown in nutrient broth media, according to the method of Méndez *et al.* [14]. After treatment of the RNA with DNAse I (Fermentas), cDNA was synthetized using iScript-cDNA-synthesis-kit (Bio-Rad, Hercules, CA, USA). Reverse transcription PCR amplifications of the subtilosin, subtilisin, protease, and β-1,3-glucanase genes were carried out using gene-specific primers (Table 1). PCR was carried out in 30 µL reaction volume containing 1 µL 10 mM dNTPs, 3 µL 10× PCR buffer, 2.5 µL 50 mM $MgCl_2$, 0.3 µL (5 units/µL) of Taq polymerase (Bio-Rad), 1.2 µL (10 mM) of each primer, and 2 µL template cDNA. Amplification was performed in a DNA thermal cycler (Bio-Rad) and consisted of an initial denaturation at 94°C for 5 min; followed by 35 cycles; denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, extension at 72°C for 1 min with final extension was at 72°C for 7 min. PCR fragments were analysed by agarose gel electrophoresis and ethidium bromide staining and changes in gene expression levels were assessed by visual inspection of band densities. The image analysis software ImageJ (ver. 1.33u, freely available at http://rsb.info.nih.gov/ij/) was used for semi-quantitative analysis of the subtilosin, subtilisin, protease, and β-1,3-glucanase gene expression and expressed in arbitrary units. In order to confirm that there was no significant contamination in the total RNA preparation, we synthesized first-strand DNA and performed control reactions in the absence of reverse transcriptase, for which we did not find bands.

Statistical analysis. Statistical Package for the Social Sciences (SPSS) software ver. 21 (IBM Corp., Armonk, NY, USA) was used to analyze the significant differences between different treatments using one-way ANOVA and *post-hoc* Tukey's honest significant difference test ($p < 0.05$). Results are indicated with the average of the values determined ± standard deviation (SD).

RESULTS

Mycolytic enzymes. Quantification of lytic enzymes of *B. subtilis* ALICA used in this study produced various cell wall degrading enzymes chitinase, β-1,3-glucanase, and protease. Results were recorded according to the U/mg protein produced in the enzyme assays. Chitinase and β-1,3-glucanase activities were induced using a lyophilized fungal mycelium amended medium. Protease enzyme production was detected preliminarily as formation of a clear zone around the colony on skim milk agar medium (Fig. 1), then quantified using standard methods. Data showed that *B. subtilis* ALICA produced significant activities of chitinase, β-1,3-glucanase, and protease which were 138,

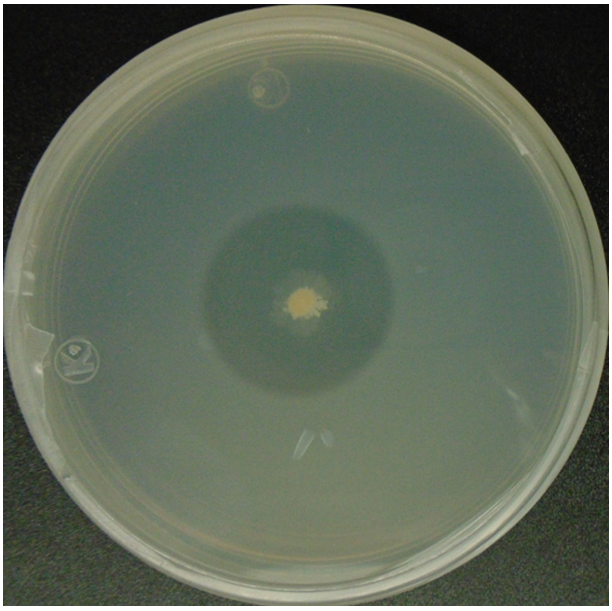

Fig. 1. Qualitative assay for protease activity on skimmed milk agar plate.

211.4, and 345.7 U/mg protein, respectively.

Dual culture assay. The *B. subtilis* strain "ALICA" illustrated certain degree of antagonism in the dual-plate assays to all of the tested phytopathogenic fungi, *A. alternata*, *Macrophomina* sp., *C. gloeosporioides*, *Botrytis cinerea*, and *S. rolfesii* (Figs. 2 and 3A). The results revealed considerable reductions in the growth of fungal mycelia as affected by *B. subtilis* strain ALICA. The results of the analysis of variance showed significant differences ($p < 0.05$) of growth inhibition among the different pathogens. The maximum growth inhibition was 86.3 ± 0.69% for *B. cinerea* and the minimum inhibition was 51.36 ± 1.32% for *C. gloeosporioides.* During antagonism a damaged mycelia of tested fungi was observed under optical microscope. Morphological changes like damage, broken, swelling, distortions abnormal morphology and the material of the cell let out were found in the fungal mycelium grown with *B. subtilis* ALICA comparing to the control (Fig. 3B). Moreover, spores formation of pathogens was not observed in dual plates.

Inhibitory effects of *B. subtilis* ALICA cell-free culture filtrate on pathogens growth. The antifungal activity of *B. subtilis* ALICA cell-free culture filtrate against several phytopathogenic fungi is summarized in Figs. 2 and 3A. It was remarkable that cell-free culture filtrate at 25% in PDA plates decrease significantly ($p \leq 0.05$) the growth of all target fungi. In general, the effect of culture filtrate on mycelial growth inhibition was less effective than dual culture for all tested fungi except *A. alternata,* which was more affected by cell-free culture filtrate as compared to dual culture. The highest significantly inhibition (68.6 ± 0.69%) using cell-free culture filtrate was achieved for *A. alternata,* followed by *S. rolfesii* (58.8 ± 1.2), *B. cinerea* (47.86 ± 1.3%), *Macrophomina* (43.53 ± 1.1%), and *C. gloeosporioides* (38.43 ± 2.9%) was the least affected by cell-free culture filtrate of *B. subtilis* strain "ALICA" as in the case of dual culture assay.

Gene expression of biocontrol genes. As illustrated in Fig. 4A and 4B, the PCR amplification of biocontrol genes subtilosin, subtilisin, protease and β-1,3-glucanase in *B. subtilis* showed that all of the four genes have been expressed. Subtilosin and subtilisin gene expression showed one specific band at around 360 and 672 bp, respectively.

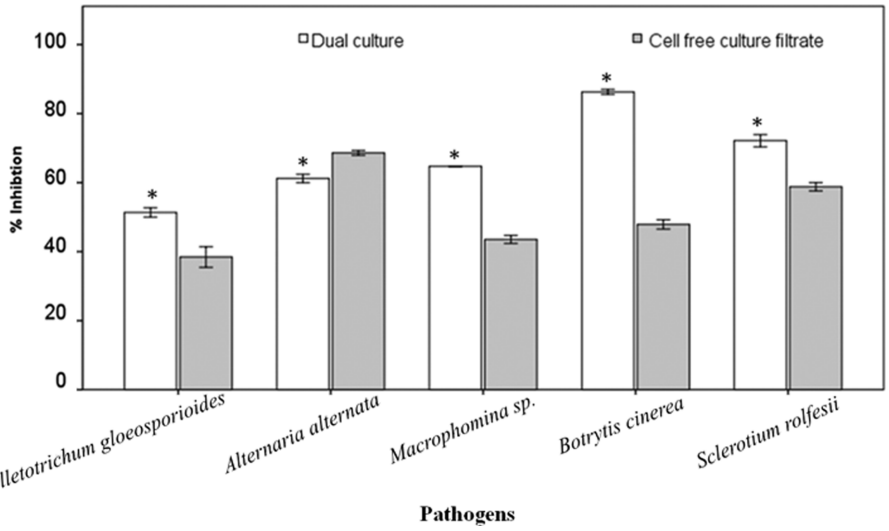

Fig. 2. Effect of *Bacillus subtilis* ALICA and its cell-free culture filtrate on mycelial growth of phytopathogenic fungi. The values are expressed as mean ± SD of the four replicated samples. Asterisk indicates statistically significant differences between treatments ($p < 0.05$).

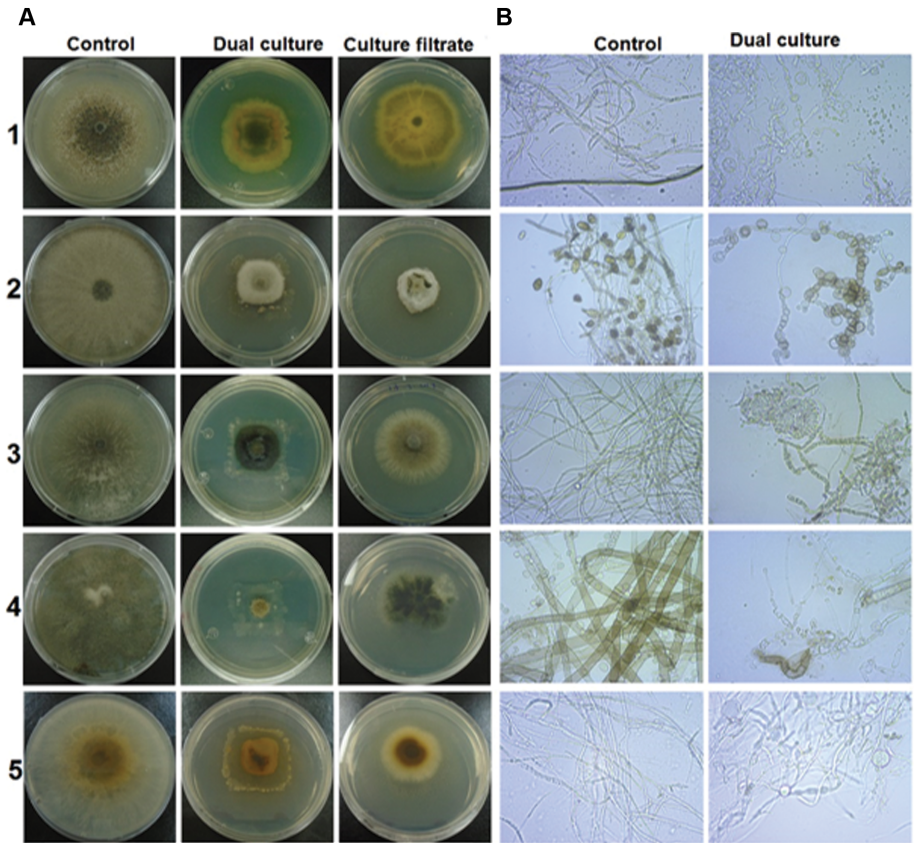

Fig. 3. A, Antifungal activity of *Bacillus subtilis* ALICA and its cell-free culture filtrate against *Colletotrichum gloeosporioides* (1), *Alternaria alternata* (2), *Macrophomina* sp. (3), *Botrytis cinerea* (4), and *Sclerotium rolfesii* (5); B, Morphological changes of fungal mycelia upon interaction with *B. subtilis* ALICA in dual culture plates.

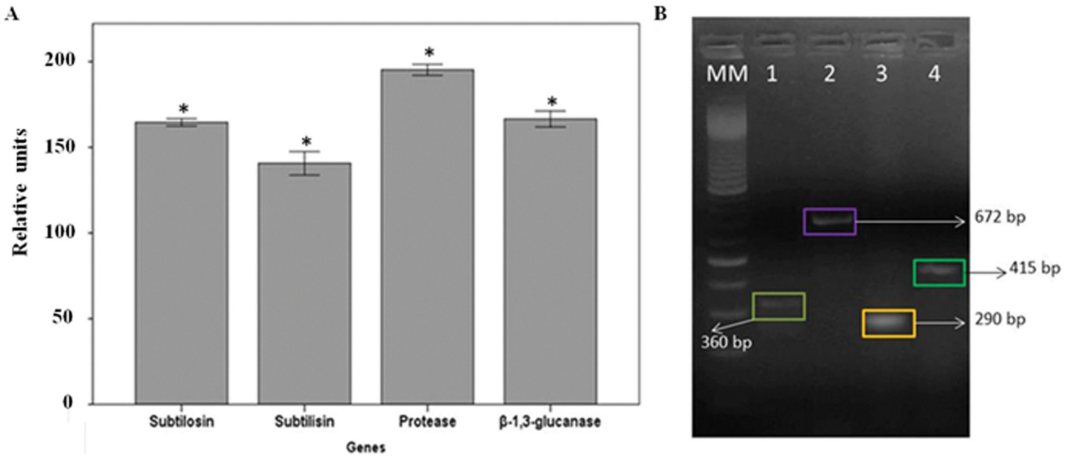

Fig. 4. Biocontrol genes quantification, using a reverse transcription PCR assay expressed in arbitrary units. A, B, PCR products visualized in 1% agarose gel electrophoresis and stained with ethidium bromide of *Bacillus subtilis* strain ALICA: lines 1 and 2, subtilosin (360 bp) and subtilisin (672 bp) gene; 3 and 4, protease (290 bp) and β-1,3-glucanase (415 bp). The values are expressed as mean ± SD of the four replicated samples. Asterisk indicates statistically significant differences between treatments ($p < 0.05$).

On the other hand, protease gene amplification showed one specific band at around 290 bp length and amplification of β-1,3-glucanase gene was also specific on the 415 bp in *Bacillus subtilis* strain "ALICA."

DISCUSSION

In the present study, one new *Bacillus subtilis* strain "ALICA" isolated from the rhizosphere of *P. juliflora*, in Baja California,

Mexico, with antagonistic activities against wide range of phytopathogenic fungi was evaluated. In the present study *B. subtilis* strain "ALICA" had a strong antagonistic activity against *C. gloeosporioides*, *Alternaria alternata*, *Macrophomina* sp., *Botrytis cinerea*, and *S. rolfesii* showing strong inhibitory activity against mycelial growth. These results contrast with previous report [5] in which the inhibition of growth of phytopathogenic fungi by another *B. subtilis* strains was significantly minor compared with our results obtain in the present study.

On the other hand, mycelial growth inhibition of five test fungi by the bacterial culture filtrate and the hyphal damages with no noticeable parasitism following the bacterial treatment as viewed by microscopy, suggest that bacterial antibiotics produced by *B. subtilis* strain "ALICA" might be responsible for the inhibition of the pathogen growth. In this sense the gene expression of subtilosin and subtilisin, genes from "ALICA" support the hypothesis that antibiosis is the major action mode that exhibits this strain. In this context, recent studies have shown that the reason for antagonism between *Bacillus* spp. and fungi may be the production of antifungal lipopeptides such as surfactin, subtilosin, or subtilisin [17, 18]. On the other hand, *B. subtilis* strain "ALICA" expressed two hydrolytic enzymes, β-1,4-glucanase and protease, which possibly degrade the contents of the fungal cell wall, such as β-1,3-glucan and glucosidic bonds. Therefore, it is assumed that the cell wall lysis of the pathogenic fungi is due to the coordinated action of hydrolytic enzymes and antifungal lipopectides, such as subtilosin, subtilisin, protease and β-1,3-glucanase. Similarly, Zhang *et al.* [19] reported the inhibition of growth of *Gaeumannomyces graminis* var. *tritici* by *B. subtilis* Z-14 culture filtrate, indicating that the antifungal effects of *B. subtilis* Z-14 may be due to the secretion of diverse compounds in the culture filtrate. Finally, the strain "ALICA" used in the present study was isolated from the rhizosphere of *P. juliflora*, obtained in Baja California, Mexico, where the phytopathogenic fungi has been present in last decades but has never been efficiently controlled. In this sense, "ALICA" could represent an effective biocontrol agent against *C. gloeosporioides*, *A. alternata*, *Macrophomina* sp., *Botrytis cinerea*, and *S. rolfesii*. However, these studies were conducted under *in vitro* conditions and further studies are required to investigate whether these results hold true under field conditions.

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