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Full Length Article

Biocontrol of *Fusarium* spp., Causal Agents of Damping-off in Cotton Plants by Native *Bacillus subtilis* Isolated from *Prosopis juliflora*

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Abstract

The present study was conducted to evaluate the application of native phosphate solubilizing *Bacillus sp.*, isolated from *Prosopis juliflora* rhizosphere in the biocontrol of three different *Fusarium* species. The native *Bacillus sp.*, was further confirmed as *B. subtilis* using the Polymerase Chain Reaction (PCR). The results showed that the phosphate solubilizing ability of *B. subtilis* strain ALICA was tested in liquid medium with 0.5% $Ca_3(PO_4)_2$, and maximum of the value of soluble phosphate was 202.02 µg/mL after 3 days of incubation. On the other hand, the growth inhibition of *Fusarium solani*, *F. equiseti* and *F. oxysporum* were more evident in cell-free medium filtrates with respect to dual medium with a range of percentage inhibition from 51, 66 and 47% after 5 days, respectively. Our result of the DNA-PCR amplification of the lipopeptide genes from *B. subtilis* strain ALICA, showed 94% identity with subtilosin and subtilisin of different *Bacillus spp*. This result turns *B. subtilis* strain ALICA into a potential alternative for the biological control of *Fusarium* species present in the soil of transgenic insect-resistant cotton plants in Mexico. © 2017 Friends Science Publishers

Keywords: *Bacillus subtilis*; Phosphate solubilization; Antifungal; Biocontrol; *Fusarium* spp.; Cotton

Introduction

As result of beneficial impacts of transgenic cotton on yields and pesticide use, the commercial cultivation of *Bacillus thuringiensis* (Bt) cotton has been increasing since in the last 34 years in Mexico, principally in Baja California and the Comarca Lagunera (Terán-Vargas *et al*., 2005). However, diseases caused by fungi represent serious problems of cotton production in Mexico and others countries (Lutfunnessa and Shamsi, 2011; González-Soto *et al*., 2015). In this sense, recent studies showed that *Fusarium* wilt represent the principal disease in soil of transgenic insect-resistant cotton plants in Mexico (González-Soto *et al*., 2015). In general, the strategies for the management of *Fusarium* wilt include the use of resistant cultivars, chemical control and use of natural antagonistic organisms (*e.g*., microorganisms). But, the excessive use of agrochemicals may produce soil pollution with detrimental effects in humans (Djébali and Belhassen, 2010). On the other hand, the biological control represent excellent alternative for control of fusarium wilt in cotton plants due to their low environmental impact. In this sense diverse microorganisms have been reported to have antagonistic effects against different Fusarium species (Sun *et al*., 2011; Sundaramoorthy and Balabaska, 2013). Antagonistic bacteria, specially belonging to the *Bacillus* genus are among the most used biological agents to fight against many plant pathogens (El-hamshary and Khattab, 2008). *Bacillus* species showed different strategies to reduce the infection in the plants by pathogens, for example: activation of genes that participle in the systemic resistance and production of antibiotics (Kloepper *et al*., 2004; Kim *et al*., 2009; Kumar *et al*., 2011; Chen *et al*., 2012).

The study of native microorganisms capable of producing an effective biocontrol of fusarium wilt has focused on isolating mesophilic microorganisms from tropical and subtropical regions (Ruiz-Sanchez *et al*., 2014; Adame-Garcia *et al*., 2015; Fu *et al*., 2015). Nevertheless, research that evaluating the diversity of autochthonous microorganisms in agricultural and native desert soils from Baja California, are scarce. Considering the above the objective of the present investigation was to characterize *Bacillus* spp., isolated of *Prosopis juliflora* rhizosphere a native plants from Northwestern Mexico and evaluate its antagonistic potential against three different *Fusarium* species: *F. oxysporum, F. solani* and *F. equiseti*.

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Materials and Methods

Isolation of Microorganisms

Rhizosphere sections from *Prosopis juliflora* were collected in Baja California, México. One gram of soil sample was diluted in sterile distilled water (9 mL). The dilutions were homogenized by agitation and then were serially diluted up to 10^{-6} dilution. 1 mL from each dilution of $10^{-5}$and 10^{-6} were spread on specific medium (Pikovskaya´s Agar) and incubated at 30 ± 2°C for 3 days. Finalized the period of incubation, the phosphate solubilizing bacteria were selected based on the zone of clearing around the colonies. The colonies showing clear zone were picked and restreaked onto Pikovskaya's Agar plates. The isolate showing maximum clear halo zone of P-solubilization was designated as *Bacillus* sp., strain ALICA and stored in 30% glycerol at -20°C for further studies.

Solubilization Efficiency of *Bacillus* sp., Strain ALICA

For estimating solubilization efficiency, the selected *Bacillus* sp., strain ALICA was spot inoculated at the center of Pikovskaya's agar media in triplicates and incubated at 30± 2°C. Diameters of colony and solubilization halo were measured after 1, 2, 3, 4, 5, 6 and 7 days. The solubilization index (SI) was evaluated according to Zhao *et al.* (2014); where SI = diameter of solubilization halo/diameter of colony.

Quantification of Soluble Phosphate and pH in Liquid Media

The determination of phosphate solubilization was realized in flasks with 100 mL of Pikovskaya's broth (pH 7.0) with 1 mL of phosphate solubilizing bacteria at 10^8 CFU/mL, approximately. Autoclaved un-inoculated Pikovskaya's broth served as control. The flasks were incubated for 7 days at 30°C on a shaker at 130 rpm. 10 mL of homogenized suspensions were sampled at days 0, 1, 2, 3, 4, 5, 6 and 7 post-incubation to follow the concentration of released soluble phosphorus and the pH changes. The cultures were collected by centrifugation (10,000 rpm) for 6 min at room temperature. Phosphate in culture supernatant was estimated using "QuantiChrom™ Phosphate Assay Kit (DIPI-500)" (Hildebrand *et al.*, 2009).

Molecular Identification of Strain ALICA by Amplification of 16s rDNA

Bacillus sp., strain ALICA was cultivated in 20 mL of Nutrient Broth media at 30°C in agitation 130 rpm for 24 h. DNA was isolated using the method proposed by Mendez-Trujillo *et al.* (2013). The polymerase chain reaction (PCR) was implemented with 2 µL DNA previous obtain from strain ALICA as PCR template and 1.2 µL (10 mM) of each specific primer 27f/1495r according to Zhang *et al.* (2013). The PCR reaction was carried out using the program proposed by Mendez-Trujillo *et al.* (2013). The fragment of 16S rDNA was visualized under a UV transillumination imaging system. For sequencing, PCR product was sent to a laboratory of GENEWIZ® (South Plainfield, NJ 07080-USA) with forward primer 27f. The 16s rDNA gene sequences were compared with available nucleotide chains of bacterial lineage in NCBI GenBank and the neighbor-Joining tree was constructed using the MEGA7 program.

PCR Screening for Antibiotic Genes in Strain ALICA

Detection of the genes that encode for the production of antibiotics such as subtilosin and subtilisin was done by PCR using gene-specific primers. The subtilosin (*Sbo*) and subtilisin (*Stn*) specific primer used were: *Sbo1* Forward (5´-TCGGTTTGTAAACTTCAACTGC-3´) and *Sbo1* Reverse (5´-GTCCACTAG ACAAGCGGCTC-3´) and *Stn* Forward (5´-CTTAAACGTCAGAGGCGGAG-3´) and *Stn* Reverse (5´-ATTGTGCAGCTGCTTGTACG-3´). A total volume of PCR reaction 30 µL: 1 µL 10 mM dNTP, 3 µL 10x PCR buffer, 2.5 µL 50 mM $MgCl_2$, 0.3 µL (5 units/µL) of Taq polymerase (BIORAD, USA), 2.4 µL (10 mM) of both primers and 2 µL template (20 ng of DNA). Amplification was performed in a DNA thermal cycler (BIORAD, USA) according to Mendez-Trujillo *et al.* (2013). The samples were electrophoresed in a 1.5 % (w/v) agarose gel containing ethidium bromide and purified as earlier described. The PCR products obtain were purified and analyzed using software provided by GenBank.

Antagonistic Activities of Dual Plate Assay

Antagonistic activity of *Bacillus* sp., strain ALICA was performed by the dual cultures method according to Elkahoui *et al.* (2012), against the following phytopathogenic fungi: *F. oxysporum*, *F. solani* and *F. equiseti*. Mycelial disc (6 mm diameter) of the fungal pathogen was inoculated at the center of a 9 cm diameter of PDA plate and the bacterial isolate was streaked at a distance of 2 cm from the center in a square pattern around the fungal disc. A fungal disc from each tested fungus was centered in PDA plate without bacteria served as control. All of treatments were done in triplicate. The inoculated petri dishes were incubated at 28 ±2°C and radial growth of the fungal colonies was recorded at 5 days. Data were expressed as growth inhibition (%) according to formula proposed by Trivedi *et al.* (2008).

Antifungal Activity of Cell Free Supernatant

The *Bacillus* sp., strain ALICA was inoculated into Erlenmeyer flask 200 mL containing 100 mL of nutrient broth media and incubated on a rotary shaker (130 rpm) 3 days at 30°C. The bacterial growth (Log phase)

was collected by centrifugation at 10,000 rpm for 6 min. The supernatant previously obtained was filtrate using 0.22 µm membrane filter. To assess the effect of bacterial culture filtrate on the growth of *F. oxysporum, F. solani*, and *F. equiseti* the culture filtrate was added to warm sterilized potato dextrose agar medium at rate of 25% and poured into petri dishes (Abdulkareem *et al.*, 2014). After solidification, a plug of mycelium of each pathogenic fungus (6 mm in diameter) was positioned at the center of the PDA dishes. PDA dishes without culture filtrate were inoculated with each fungus as control. Dishes were grown at 30°C for 120 h. Growth inhibition of each fungus was recorded in triplicate.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) and the means were compared using post-hoc Tukey's HSD test with $P \leq 0.05$ being accepted as significant. The statistical software used was SPSS (Version 21). Results are reported as means ± SD (standard deviation).

Results

Isolation of Phosphate Solubilizing Bacteria

In the present study, the isolated bacterium ALICA showed significant ($P<0.01$) increase of solubilization index (2.38±0.09) during the firsts 72 h of exposure to PVK agar media (Fig. 1). However, within 96 to 168 h after exposure, solubilization index values were significantly ($P \leq 0.440$) lowest, 1.34 ± 0.02, compared with levels observed in the first 72 h of the experiment (Fig. 1).

Quantitative Estimation of Phosphate Solubilization and pH in Liquid Media

The levels of soluble phosphorus and pH in liquid Pikovskaya's medium that contained known amounts of insoluble phosphate were analytically measured at different points during of the 7 days growth period at 30ºC. Our results showed that *Bacillus* sp., strain ALICA, significantly changed ($P<0.01$) the amount of P solubilized with the time. Whereas, the concentration of solubilized phosphate gradually increased from 1 to 3 days and decreased thereafter (Fig. 2). On the other hand, pH medium gradually decreased in the first 3 days and then slightly increased (Fig. 2). A significant negative correlation ($r = -0.954$; $p < 0.01$) between P concentration and pH was observed. The maximum P-solubilization by *Bacillus* sp., strain ALICA (206.02±5.91 µg/mL) was recorded at the 3th day when the pH reached the lowest value (pH= 5.0±0.0, $p < 0.01$). The pH value and soluble-P concentration in the un-inoculated control flasks remained almost the same as in day zero (pH=7±0.0, P= 16.14±1.34 µg /mL).

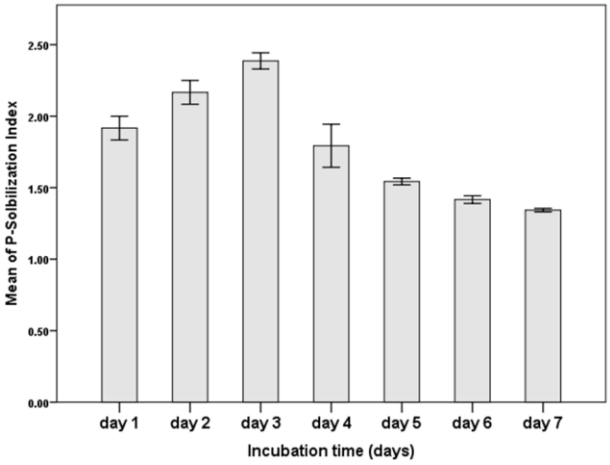

Fig. 1: Solubilization index of $Ca_3(PO_4)_2$ in solid Pikovskaya's medium for bacterial isolate ALICA obtained from Mesquite rhizosphere, during 7 days of incubation at 28±2 °C. Error bars represent the standard errors of the means

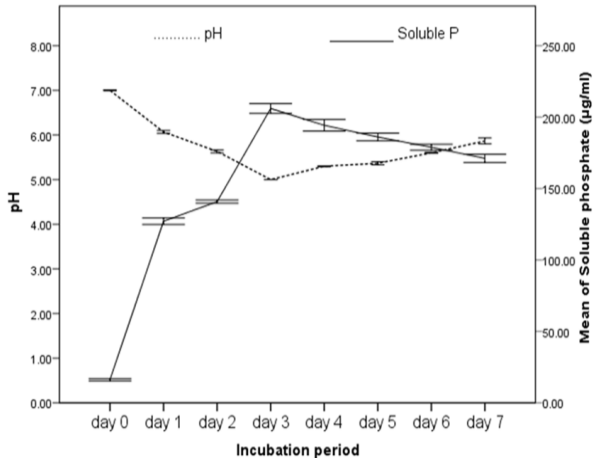

Fig. 2: Soluble phosphorus (µg/mL) and pH in liquid medium Pikovskaya during 7 days of incubation with bacterial isolate ALICA in the presence of $Ca_3(PO_4)_2$. Error bars represent the standard errors of the means

Molecular Identification of Isolate ALICA Based on its 16S rDNA Gene Sequence

The partial 16S rDNA sequence analysis was employed to identify the *Bacillus* sp., strain ALICA to the genus and specie levels. A 1096-bp PCR product from the 16S rDNA gene was amplified from the genomic DNA. Subsequent sequence analysis showed that strain ALICA shared 94% identity with *B. subtilis* strains deposited in GenBank. The gene sequence of ALICA was submitted to GenBank, NCBI data base and accession number was assigned (KX137176). Using DNA sequence of strain ALICA a neighbor joining tree was constructed and the result showed high similarity

with members of the genus *Bacillus* (Fig. 3).

PCR Screening for Antibiotic Genes of Strain ALICA

In the present study, *Sbo* and *Stn* genes were identified by PCR analysis and sequenced from *Bacillus* strain ALICA (Fig. 5). *Sbo* sequence (360 pb) showed homology to the subtilosin A gene sequences present in *B. amyloliquefaciens* strain MZ-40 (92%), B. *subtilis* strain B3 (92%) and *B. subtilis* strain IP (92 %) with accession numbers FJ151507, DQ452520 and JN118836, respectively. In this way, BLAST analysis revealed that *Stn* (672 pb) matched closely with subtilisin gene presents in *B. subtilis* clone pGXAA2011 (97%), *B. mojavensis* strain A21 (98%) and *B. subtilis* strain DTQ-DR23 (90%) with accession numbers AY953434, KC02060 and EF061456, respectively.

Antagonistic of Dual Plate Assay and Cell Free Culture Filtrate Antagonistic

In the present study, the results of dual plate assay demonstrated that *Bacillus* sp., strain ALICA had antagonistic effect on *F. oxysporum*, *F. solani* and *F. equiseti* after 5 days incubation (Fig. 4). The results revealed that strain ALICA showed broad spectrum antagonism against *F. solani* (47%), *F. equiseti* (45%) and *F. oxysporum* (32%), the maximum inhibition (%) in radial growth varied from 32 to 47 after 5 days of incubation (Fig. 4B and Table 1). With respect to effect of supernatant on radial growth of *Fusarium*. The results showed that the growth inhibition of *Fusarium* species were highly effective in cell-free culture filtrates as compared to dual culture (Fig. 4C). In this respect, our data showed that the greatest inhibition (%) in radial growth of *F. solani* (51%), *F. equiseti* (66%) and *F. oxysporum* (47%), caused by strain ALICA were documented after 5 days of incubation on PDA supplemented with cell-free culture filtrate (Table 1).

Discussion

In this work, one new *B. subtilis* strain (ALICA) from *Prosopis juliflora* rhizosphere with capacities of P-solubilization and antagonistic activities against *Fusarium* species was successfully identified. The solubilization index and soluble P concentrations of isolate ALICA fluctuated during incubation days with maximum values at 3 days. These results are in line with those obtained by Jena and Rath (2013). The fluctuation observed in the solubilization index of strain "ALICA" during the period of incubation with Pikovskaya's agar and broth medium may be due to the varying type, amount, and diffusion rates of diverse organic compounds secreted by phosphate solubilizing microbes as previously mentioned by Li *et al*. (2015). In this concern, the fluctuations in solubilization index are due to the fact that this bacterium was in a state of phosphate deficiency and starvation which explains the rapid

Table 1: *In-vitro* inhibition of fungal growth by bacterial isolate ALICA on the basis of dual culture and culture filtrate techniques

Pathogens	Incubation days	Percentage inhibition of radial growth	
		Dual culture	Culture filtrate
F. solani	5	47.87 ± 1.67	51.40 ± 1.28
F. equiseti	5	45.83 ± 0.95	66.03 ± 1.10
F. oxysporum	5	32.57 ± 1.27	47.87 ± 1.07

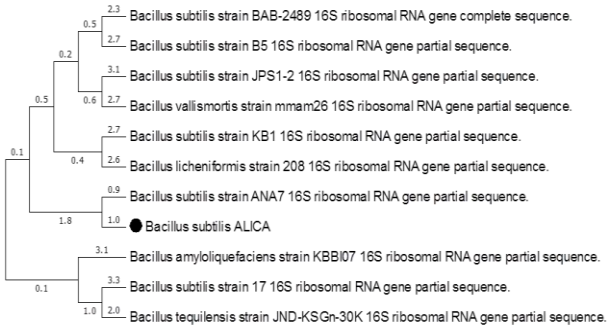

Fig. 3: Phylogenetic tree of bacterial isolate ALICA, based on partial 16S rDNA gene sequence. The dendrogram was constructed by the neighbor-joining method, using the MEGA 7 program

assimilation of tricalcium phosphate (TCP) within the first hours of incubation (Mardad *et al*. 2013). Our findings showed a negative relation between pH values and levels of soluble phosphorus in the culture media. This suggested the activation of metabolic products as organic acids that facility the conversion of insoluble phosphate to soluble form (Zhang *et al*., 2013). In this regard, Marra-Leandro *et al*. (2015) proposed that organic acids biosynthesis cause one drop in pH values due to the release of protons and this reaction is an elementary principle of phosphate solubilization. This acidification caused by the production of organic acids by bacteria, which through their carboxylic groups chelate the cations (mainly Ca) bound to phosphate converting them into the soluble forms, and the negative relationship between pH and soluble-P indicates the significant role of these organic acids in mineral P solubilization (Chen *et al*., 2006).

Therefore microorganisms as strain ALICA which decrease the medium pH during growth could be considered as efficient P solubilizer according to Li *et al*. (2015). On the other hand, the obtained result showed that in both assays (dual-culture and cell free supernatant), strain ALICA significantly inhibited the growth of pathogenic fungi such as: *F. solani*, *F. equiseti*, and *F. oxysporum* in comparison with control. Similarly, Ashwini and Srividya (2014) found that *B. subtilis* inhibited fungal growth of *Alternaria* (3) spp. (55%), *Colletotrichum gloeosporioides* (57%), *Phytophthora capsici* (55%), *Rhizoctonia solani* (42%), *F. solani* (42%), *F. oxysporum* (40%) and *Verticillium* sp. (36%). This result may be

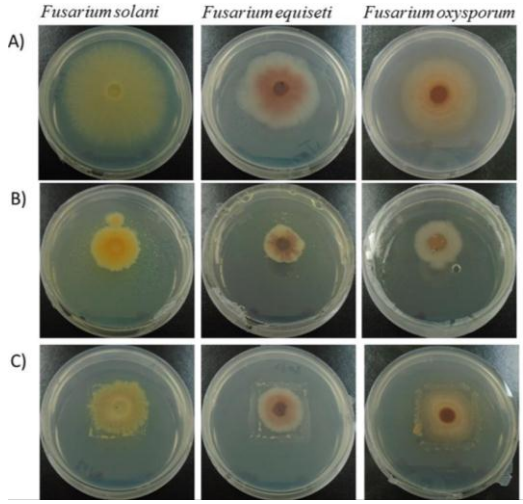

Fig. 4: Growth inhibitions of *F. solani*, *F. equiseti* and *F. oxysporum* by the treatment without bacteria (A), with bacterial isolate ALICA (B) and cell free filtrate (C) in dual culture assay

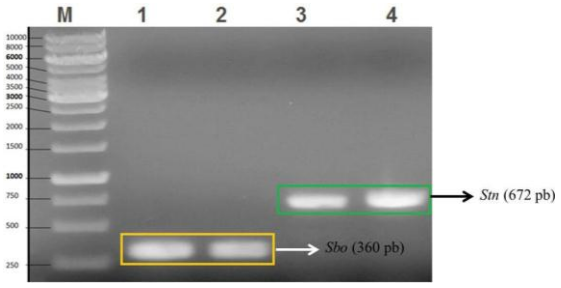

Fig. 5: PCR products of subtilosin (*Sbo*) and subtilisin (*Stn*) genes for duplicate from *B. subtilis* strain ALICA. Lane MM = molecular marker; lines 1 and 2: subtilosin (*Sbo*) gene; lines 3 and 4: subtilisin (*Stn*) gene

recognized that growth inhibition of pathogenic fungi by *B. subtilis* might be due to the synthesis of antimicrobial compounds.

In addition to, *B. subtilis* probably suppresses the growth of pathogenic fungi as results of antifungal substances or by colonizing micro-sites faster than fungi (Elkahoui *et al*., 2012). In this concern, Gao *et al.* (2015) reported that *Bacillus* species secrete proteins with antifungal activity; therefore the inhibitory effect is mainly due to the antifungal substances. *Bacillus* strains could produce a wide range of antifungal compounds, such as lytic enzymes, antifungal peptides and nutrient depletion (Berg *et al*., 2001; Zhao *et al*., 2013). Overall, there are several mechanisms to inhibit fungal growth by *B. subtilis*. However, our results demonstrate that this inhibition is probably due to production of antifungal compounds in the culture filtrate, because the cell free culture filtrates capable to suppress the fungal growth.

In this sense, the identification of antifungal antibiotic in microorganism is important for determining its competence to be a good biocontrol agent for plant diseases (Zhao *et al*., 2014). Our result of the PCR amplification of the lipopeptide genes from DNA of *B. subtilis* strain ALICA, showed the presence of subtilosin (*Sbo*) and subtilisin (*Stn*) (Fig. 5), which showed the highest degrees of similarity to homologous sequences previously identified in other biocontrol strains when compared with GenBank sequences. This is important because the mainly restrictions of biological control is the use of a large quantity of "exotic" microorganisms with high inconsistency in the efficacy of one test to another and the negative effect on native microbial communities (Pereira *et al*., 2009). In this sense considering the antimicrobial assays results of *B. subtilis* strain ALICA, this new strain could be applied as bio-fertilizer and bio-fungicide based on positive effect showed *in vitro* in desert ambient as Northwestern Mexico.

Conclusion

Our present investigation concludes that *B. subtilis* ALICA is potential phosphate solubilizing bacteria and biocontrol agent against phytopathogens. It showed antifungal effects on three different *Fusarium* species *in vitro*. However, tests in the field are required to complete this work, in addition to evaluate other plant growth promoting features, extraction of antifungal compounds, mycolytic enzymes and the effect of biotic and abiotic factors on this strain.

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