Article

# Use of *Bacillus* sp. as a resistance inducer in prickly pear against 'scaly rot'

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## Abstract

'Scaly rot' is one of the most important diseases in prickly pear, for this reason the objective of the present work was to isolate and identify fungi associated with 'scaly rot' and evaluate the ability of bacteria of the genus *Bacillus* sp. isolated to avoid the disease of 'scaly rot' in prickly pear [*Opuntia ficus-indica* (L.) Mill.]. Ten bacteria of the genus *Bacillus* sp. were analyzed in *in vitro* antagonism tests against the pathogen *Neoscytalidium hyalinum*, which was identified as the causative agent of 'scaly rot'. The results of this study demonstrated that all isolates of *Bacillus* sp. significantly decreased ($p \leq 0.05$) the radius of the pathogen in the *in vitro* test, with isolates G11, G21 and G31 standing out. In the *in vivo* resistance induction tests, isolates G11, G21 and G31 significantly decreased ($p \leq 0.05$) the diameter of the lesions caused by *Neoscytalidium hyalinum*. The identification of the species used was carried out through a phylogenetic analysis of the sequences obtained of the regions 16s rRNA (bacteria) and ITS1-5.8s-ITS2 (fungi). Bacterial isolates G11, G21 and G31 were identified as *Bacillus amyloliquefaciens* and all fungi belonging to the genus *Neoscytalidium* sp. were identified as *N. hyalinum*.

**Keywords:** *Bacillus amyloliquefaciens*, *Neoscytalidium hyalinum*, *Opuntia ficus-indica* (L.) Mill., antagonism, resistance induction, scaly rot.

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# Introduction

'Scaly rot' is one of the most important diseases in prickly pear, it is characterized by the appearance of grayish scales of wavy or semi-wavy shape (Souza *et al*., 2010). The scales produced can completely cover the cladode, causing a loss of the photosynthetic surface of the plant with significant losses in production (Souza *et al*., 2010). The causative agents of 'scaly rot' are the fungi *Neoscytalidium hyalinum* and *Scytalidium lignicola*, which can also cause root rot in prickly pear (Souza *et al*., 2017; Feijo *et al*., 2019).

To control these and other phytopathogenic fungi, chemical fungicides are usually used, which are effective, but often have negative effects on the environment and on the health of consumers, in addition, there is always the possibility of the appearance of resistant strains (Spadaro and Gullino, 2004; Deising *et al*., 2008). The problems generated by the use of chemical fungicides have led to the search for alternative control methods such as antagonist fungi and bacteria, of which the bacteria of the genus *Bacillus* sp. can be highlighted (Wilson, 1997; Santoyo *et al*., 2012). Among the antagonistic capacities that *Bacillus* sp. has are competition for space and nutrients, antibiosis and induced systemic resistance (Layton *et al*., 2017).

Induced systemic resistance (ISR) is a state in which the plant's defense capacity is enhanced by the plant's interaction with growth-promoting bacteria (Vallad and Goodman, 2004). These bacteria produce liposaccharides, antibiotics, and siderophores that serve as elicitors of ISR, which are called microbe-associated molecular patterns (MAMPs) (Wiesel *et al*., 2014; Canchignia *et al*., 2015). Once the plant detects the elicitors, it begins to produce jasmonic acid (JA) and ethylene (ET), these promote the synthesis of pathogenicity-related (PR) proteins, allowing the plant to react more efficiently to the attack of a pathogen (Canchignia *et al*., 2015).

It is known that *Bacillus* sp. can induce resistance in different plants and help reduce damage caused by fungi such as *Fusarium* sp., *Alternaria* sp., *Colletotrichum* sp., *Pythium* sp. and *Phytophthora* sp. (Nakkeeran *et al*., 2007; Chowdappa *et al*., 2013; Wang *et al*., 2014; Gond *et al*., 2015). At present, it is unknown whether *Bacillus* sp. is able to induce resistance in prickly pear and prevent the disease of 'scaly rot'. Therefore, the objective of the present work was to isolate and identify fungi associated with 'scaly rot' and evaluate the capacity of bacteria of the genus *Bacillus* sp., to avoid the disease of 'scaly rot' in prickly pear [*Opuntia ficus-indica* (L) Mill.].

# Materials and methods

## Study area and sample collection

The study areas were a prickly pear orchard in the municipality of Gómez Farias, Tamaulipas with coordinates 23° 09' 37.64" north latitude, 99° 09' 12.23" west longitude, and also the hydroponic modules of the Technological Institute of Victoria City with coordinates 23° 45' 16.94" north latitude, 99° 10' 01.76" west longitude. The cladodes of the prickly pear belonging to the species *Opuntia ficus-indica* with symptoms of 'scaly rot' were obtained by random sampling at each of

the sites and each individual sample were placed in polyethylene bags labeled with the collection data. Twenty samples were obtained for each of the sites, which were transported to the Laboratory of Microbiology and Biotechnology of the Division of Postgraduate Studies and Research of the Technological Institute of Victoria City for later use.

**Isolation of fungi and bacteria**

For the isolation of fungi, fragments (1 cm x 1 cm) of the prickly pears that presented the symptoms produced by 'scaly rot' were cut. These fragments were disinfected with 70% alcohol for one minute, then rinsed with distilled water and left to dry on filter paper. Once dried, they were placed in Petri dishes with potato dextrose agar (PDA) culture medium and incubated for two weeks at 30 °C.

The bacteria were isolated with the plate dilution technique, for this one gram of prickly pear roots was macerated in a mortar and mixed with 9 ml of saline solution. Serial dilutions were then performed until a dilution factor of $1 \times 10^6$ was obtained. From this solution, 100 µl was placed in Petri dishes with PDA culture medium to later spread it with a glass loop. The fungi were used for monosporic cultures (Valiente and Pavone, 2013) and the bacteria were reseeded from a single colony with the bacteriological loop and reseeded in a Petri dish with PDA culture medium.

**Identification of the genus of fungi and bacteria**

The fungi were identified to genus through the taxonomic keys proposed by Watanabe (2010); Phillips *et al*. (2013). For this, slides were prepared with the adhesive tape technique to later be observed in the optical microscope, using the 40x objective. The bacteria were identified by biochemical tests and microscopic observation using the 100x objective and those that were Gram (+), bacillary and catalase (+) were identified as of the genus *Bacillus* sp. (De Vos *et al*., 2009; Ruiz-Sánchez *et al*., 2014).

**Pathogenicity tests**

To determine which isolated fungi generate the same symptoms observed in the field, pathogenicity tests were carried out and for this, first sterile toothpicks were placed on colonies of the isolated fungi and left in incubation at 37 °C until the fungi grew on them and covered them completely. They were then cut into 1 cm fragments and used to inoculate prickly pear cladodes previously disinfected with 70% alcohol. Each cladode was inoculated with five toothpick fragments and three cladodes per isolated fungus genus were inoculated, after which they were placed in moisture chambers for a month.

After this period of time, it was observed which of the infected prickly pears presented the same symptoms observed in the field and from these, the fungus was reisolated from the lesions to confirm that it is the same with which it was inoculated (Swart *et al*., 2003). Once identified which of the genera of the isolated fungi causes the disease, pathogenicity tests were carried out again with all the fungi of this genus and the percentage of incidence was calculated with the number of lesions caused by the pathogen.

### *In vitro* antagonism tests

To find out which of the bacteria have the potential to control pathogens, *in vitro* dual culture tests were carried out, placing in the center of a Petri dish with sterile PDA medium a fragment of the pathogenic fungus obtained with a 0.5 cm diameter punch from a three-week-old colony of the fungus. To then inoculate the isolated bacteria by means of a bacteriological loop, these were placed in a circular way around the fungus in the outermost part of the dish, then they were left in incubation at 37 °C for a week or until the control completely covered the dish, 15 repetitions were made for each possible combination (Mohammadi *et al*., 2017). Once the incubation time has passed, the radii of the pathogen in confrontation and the controls were measured and used to calculate the percentage of radial inhibition (PIRG) (Reyes-Ramírez *et al*., 2011) using the following formula: $PIRG= \frac{RP-RC}{RC} *100$. Where: RP= radius of the pathogen in confrontation; RT= radius of the control pathogen.

### DNA extraction

For the extractions, spore suspensions of the fungi were first inoculated in 500 ml flasks with 250 ml of liquid culture medium (sucrose 20 g $L^{-1}$, peptone 5 g $L^{-1}$, yeast extract 5 g $L^{-1}$ and sterile distilled water 1l) and were left in incubation for one week at 32 °C. After the incubation time, the culture media were filtered, then the recovered mycelium was placed in 1.5 ml microtubes. The extraction method with DNAzol® was used to purify the DNA, following the protocol proposed by Guo *et al*. (2005) with some modifications, consisting of macerating the sample with a plastic pestle after adding the DNAzol® and increasing the incubation time to 45 min. For the bacteria, test tubes were first inoculated with LB culture medium and incubated at 32 °C for two days. DNA extractions were performed using the DNAzol® kit, for this, the manufacturer's instructions were followed.

Once the extractions of both fungi and bacteria were made, they were visualized. This was done by mixing 2 µl of sample and 2 µl of run blue buffer containing the stain for DNA SYBRGreen®. Then the samples were placed in a 1% agarose gel in a 14 OWL® electrophoresis chamber (8 cm x 15 cm) with SB buffer x1 (boric acid-NaOH) at a voltage of 100 for 15 min, after this the gel was placed in a UV light transilluminator and photodocumented using the Logitech webcam program.

### PCR-sequencing

The amplification of the regions ITS1-5.8s-ITS2 of the nuclear DNA of the fungi and the 16S rRNA of the bacteria was carried out by PCR with the primers ITS1 (5'- TCCGTAGGTGAAC CTGCGG3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) for fungi, and primers Y1 (5'-CCAGCAGCCGCGGTAATACG-3') and Y2 (5'- ATCGG(C/T) TACCTTGT TACGACTTC-3') for bacteria (Lu *et al*., 2000). Each PCR reaction contains 21 µl of mix (Water 14.7, Buffer 10x 2.5, DNTPs 25mM, 0.25 µl, BSA 25x 1 µl), 2 µl of DNA, 2 µl of primers ITS1-4 for fungi or Y1-Y2 for bacteria and 0.3 µl of TaqPol for a final volume of 25.3 µl.

The PCR reaction was carried out in a BIO-RAD T-100TM thermal cycler, for this the following temperature cycles were used for the fungi: pre-denaturation of 5 min at 94 °C, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 30 s and extension of 1 min at 72

°C and final extension of 5 min at 72 °C (Moo-Koh *et al*., 2017), for bacteria the cycles used were as follows: pre-denaturation of 10 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 59.9 °C for 1 m and extension of 2 min at 72 °C and final extension of 1 min at 72 °C (Lu *et al*., 2000).

After obtaining the amplified products of both the ITS 1-2 region and the 16 S rRNA by PCR, they were sent to the faculty of science of the National Autonomous University of Mexico (UNAM, for its acronym in Spanish) for sequencing. The sequences obtained were edited with the BIOEDIT program and compared with sequences from the Gene Bank database of the National Center for Biotechnology Information using Basic Local Alignment Search Tool (BLAST). In addition, the CLC Sequence Viewer 8 program was used to perform a phylogenetic tree with the UPGMA method with which the sequences obtained were compared with Gene Bank sequences.

### Resistance induction tests in prickly pear

Once it was determined which are the most effective bacterial isolates to control the pathogen *in vitro* and which are the isolated fungi with the highest percentage of pathogenicity, resistance induction tests were performed. For this, three prickly pear cladodes were placed in black bags for plants with peat moss as a substrate until they rooted, a total of eight bags were prepared. They were then inoculated with the chosen bacteria that were previously grown in 500 ml Erlenmeyer flasks with 250 ml of liquid culture medium containing sucrose (20 g $L^{-1}$), peptone (30 g $L^{-1}$), yeast extract (7 g $L^{-1}$), KH2PO4 (1.9 g $L^{-1}$) and MgSO4 (0.45 g $L^{-1}$). The roots were inoculated with 10 ml of culture and 72 h later the cladodes were inoculated with the selected fungi (Swart *et al*., 2003).

### Experimental design

The *in vitro* and *in vivo* antagonism experiments consisted of 15 repetitions per treatment and were performed using a completely randomized experimental design. All data obtained passed the tests of normality and homogeneity of variance and were compared with a one-way analysis of variance (Anova) and a Tukey comparison of means, both with a significance value of 0.05. All statistical analyses were performed with Excel 2016.

## Results and discussion

### Isolated fungi and bacteria

It was possible to isolate and identify a total of 33 fungi for both sites, 20 in the hydroponic module of the ITCV and 14 in the orchard in Gómez Farías. *Neoscytalidium* sp. was the most common genus at both sites as it is an abundant fungus as a pathogen in places with tropical climates, as mentioned by Souza *et al*. (2010); Souza *et al*. (2017). The genus *Neoscytalidium* sp. presented dark green colonies and they formed chain spores, cylindrical with a septum characteristic of the genus (Phillips *et al*., 2013). Other fungi isolated from prickly pear cladodes were *Fusarium* sp., which is considered the causative agent of 'tip rot' and for causing necrotic lesions in prickly pear (Swart and Kriel, 2002; Ammar *et al*., 2004; Souza *et al*., 2010).

The genus *Alternaria* sp., also isolated, causes the disease known as 'golden disease' and 'dry rot' and can cause necrotic lesions in prickly pear (Swart and Kriel, 2002; Swart *et al*., 2003; Méndez *et al*., 2007; Faedda *et al*., 2016). In addition, fungi of the genus *Curvularia* sp. were isolated, which is associated with circular brown to dark lesions with a velvety appearance (Flores *et al*., 2013). Finally, fungi of the genus *Schizophyllum* sp. were obtained., but this genus is not associated with any disease.

Moreover, 10 bacteria were isolated at both sampling sites, all belonging to the genus *Bacillus* sp., since all presented the typical characteristics of the genus, such as being catalase positive, and Gram positive, the colonies had irregular edges and when observed under the microscope, they were bacillary in shape (De Vos *et al*., 2009; Ruiz-Sánchez *et al*., 2014). Bacteria belonging to the genus *Bacillus* sp. were used in *in vitro* antagonism tests.

## Molecular identification of fungi and bacteria

The sequences obtained from the isolates of *Neoscytalidium* sp. were compared in BLAST and isolates M7, M9, G81 and G112 had a similarity of 100% with the species *Neoscytalidium dimidiatum*, on the other hand, the sequences of M2, M3, M4, M5, M6 M8, M10, G42, G43, G11, G311 have a 100% similarity with sequences of the species *Neoscytalidium hyalinum*. When performing the phylogenetic analysis of the sequences and comparing them with sequences obtained from GenBank of the species *N. hyalinum* (MK387852.1 and MK387853.1), *N. dimidiatum* (KP132488.1 and KP132489.1), *N. novaehollandiae* (NR_111260.1 and MH863173.1) and *N. orchidacearum* (KY933091.1), it was observed that they were mostly related to sequences of fungi *N. hyalinum* and *N. dimidiatum*, so the isolated fungi were considered to belong to these species (Figure 1).

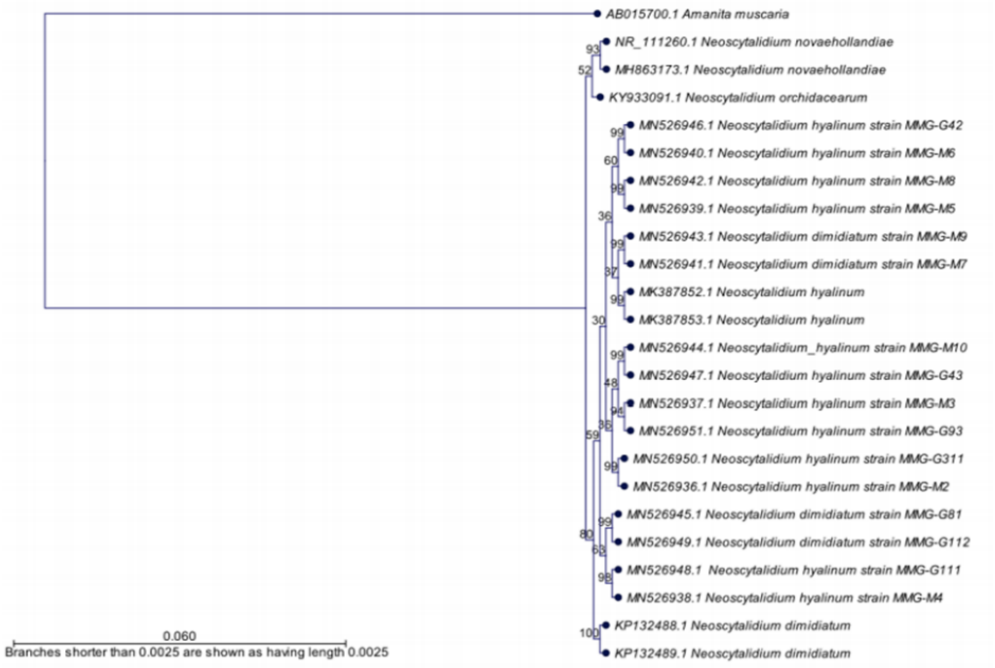


**Figure 1. Phylogenetic tree of *Neoscytalidium* sp., based on sequences of the ITS 1-2 region, where *Amanita muscaria* (AB015700.1) was used as an external group (AB015700.1).**

Originally, both *N. dimidiatum* and *N. hyalinum* were part of the genus *Scytalidium* sp., but currently these and other species are classified in the genus *Neoscytalidium* sp., which is a mixture between *Scytalidium* sp. and *Fusicoccum* sp., in addition, both *N. hyalinum* and *N. dimidiatum* are currently considered the same species (Phillips *et al*., 2013; Machado *et al*., 2014).

The phylogenetic analysis was performed with the sequences of the 16s rRNA of the bacteria with higher percentages of inhibition of *in vitro* tests, sequences G11, G21 and G31 were highly related to sequences of *B. amyloliquefaciens* (NR_041455.1 and NR_116022.1), considering that the isolated bacteria belong to species *B. amyloliquefaciens* (Figure 2).

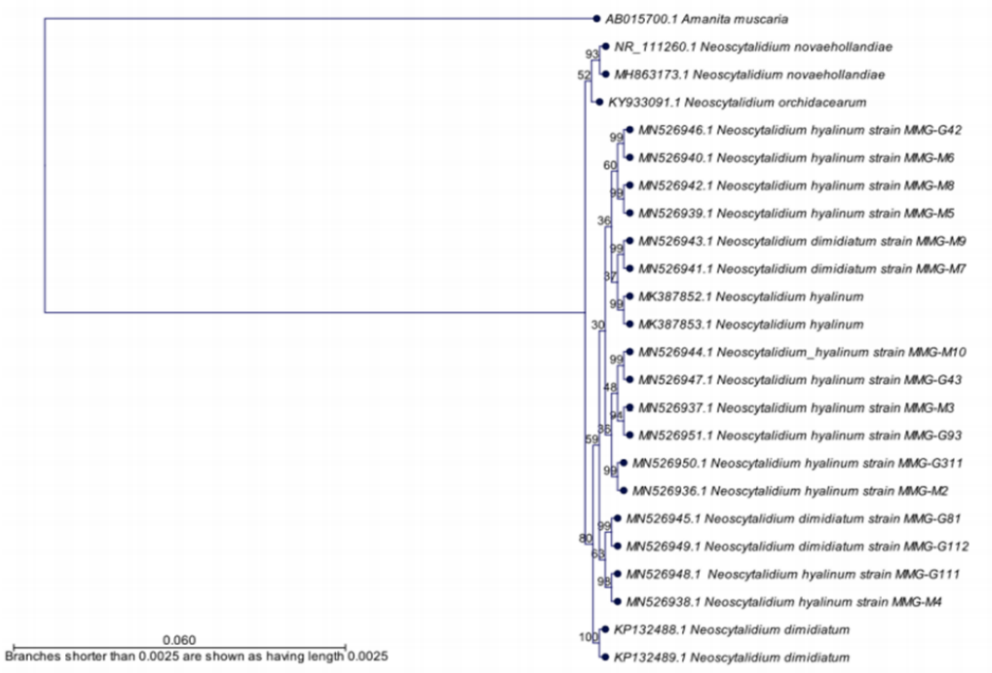


**Figure 2. Phylogenetic tree of *Bacillus* sp., based on sequences of the 16s region of rRNA, where *Escherichia* coli was used as an external group (NR_024570.1).**

## Pathogenicity tests and incidence percentages

In pathogenicity tests, fungi of the genus *Neoscytalidium* sp. caused the appearance of brown spots that turned to a darker color, which are the initial symptoms of 'scaly rot' (Souza *et al*., 2010; Feijo *et al*., 2019). Of the different isolates of *Neoscytalidium* sp., the G111 isolate presented a higher incidence with 93.33% and the isolates M10 and G93 had the lowest incidence percentages with 33.33%.

These differences are given by the ability of the plant to detect pathogens through proteins in the membranes of its cells called R proteins that serve to detect proteins produced by the pathogen known as Avr proteins (Ordeñana, 1998; Wiesel *et al*., 2014). In case there is a compatibility between the R proteins of the plant and the Avr proteins of the pathogen, a hypersensitivity reaction that causes programmed cell death and systemic acquired resistance as a defense in the whole plant is triggered, both mechanisms prevent or reduce the damage caused by the pathogen (Ordeñana, 1998; Durrant and Dong, 2004; Forouhar *et al*., 2005; Días, 2012).

### *In vitro* antagonism

The radii obtained from the dual culture tests (Figure 3 and 4) were compared with an Anova test (0.05), showing significant differences among treatments ($p \leq 0.05$). When comparing the treatments using the Tukey test (0.05) (Table 1), it was observed that all bacteria significantly reduce the radius of the pathogen when comparing with the control and among the treatments, the G11 bacterium obtained significant differences with respect to the rest of the treatments, since it achieved the highest percentages of inhibition of the pathogen with an average of 50.09%. Likewise, the isolates G21 and G31 showed significant differences with most treatments, except each other, both have a mean percentage of inhibition of 41.84% and 41.73%, respectively. All bacterial isolates caused halos of inhibition because the bacterium releases antibiotics into the environment, with the most common being surfactins, iturins and fengycins (Villarreal *et al*., 2018).

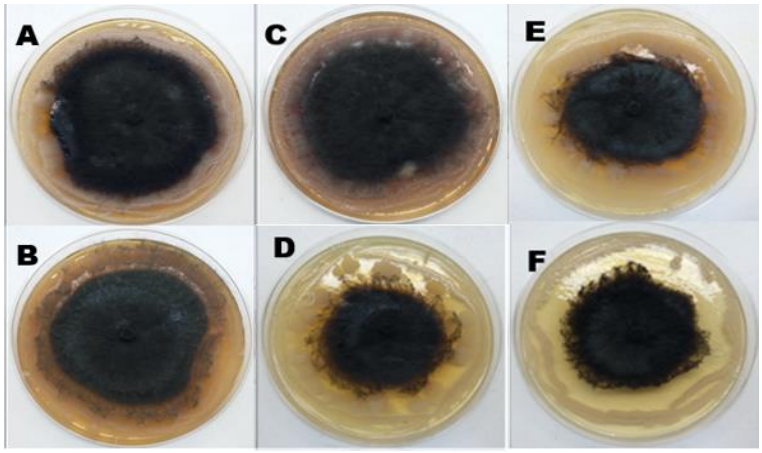


**Figure 3. *In vitro* antagonism tests by dual culture. A) G111vBMN3; B) G111vBMN21; C) G111vBMN22; D) G111vG11; E) G111vG21; and F) G111vG31.**

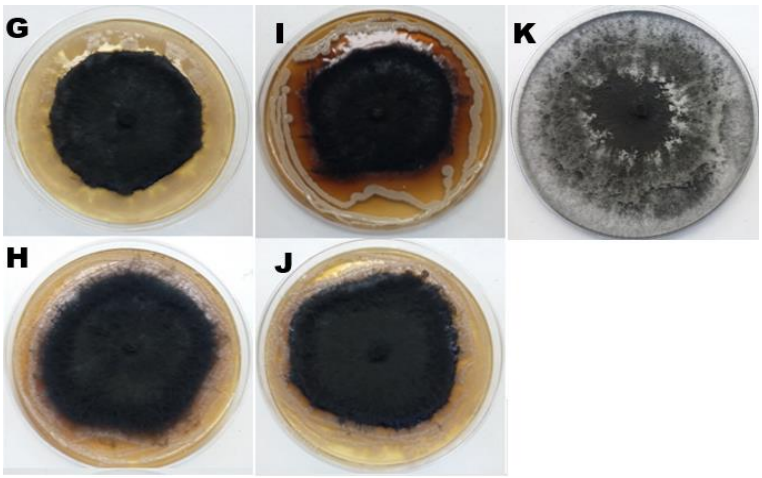


**Figure 4. *In vitro* antagonism tests by dual culture. G) G111vG51; H) G111vG52; I) G111vG53; J) G111vG54; and K) G111 control.**

**Table 1. Results of the Tukey test (0.05) of *in vitro tests*, treatments with the same letter have no significant differences.**

| Treatments | Averages |
|---|---|
| Control (G111) | 4.24 ±0.053 a |
| G51 | 2.83 ±0.053 b |
| NM3 | 2.95 ±0.074 c |
| MN22 | 2.84 ±0.059 b |
| G52 | 2.93 ±0.074 c |
| G21 | 2.46 ±0.069 d |
| G54 | 2.7 ±0.056 e |
| G53 | 2.79 ±0.054 b |
| G11 | 2.11 ±0.065 f |
| MN21 | 2.83 ±0.057 b |
| G31 | 2.47 ±0.06 d |

The differences in inhibition percentages observed in *in vitro* tests may be due to the amount of antibiotics produced by bacteria, for example *Bacillus subtilis* allocates 5% of its genome to produce antibiotics, while *Bacillus amyloliquefaciens* allocates 10% of its genome to the production of these (Chowdhury *et al*., 2015; Gond *et al*., 2015).

### *In vivo* resistance induction tests

The diameters obtained in the experiments were used to perform a one-way Anova test (0.05), where it was found that they have significant differences ($p \leq 0.05$). When comparing the treatments with the Tukey test (0.05) (Table 2), the bacteria were shown to significantly reduce the damage caused by the pathogen. During induced resistance, the size of lesions caused by the pathogen is decreased due to the accumulation of PR proteins such as glucanases and chitinases (Wiesel *et al*., 2014; Canchignia *et al*., 2015). When comparing between treatments, bacteria G11, G21 and G31 showed no significant differences in reducing the damage caused by the fungus *N. hyalinum*.

**Table 2. Results of the Tukey test (0.05) of the resistance induction test, treatments with the same letter have no significant differences.**

| Treatment | Average |
|---|---|
| Control (G111) | 4.27 ±0.069 a |
| G11 | 0.39 ±0.075 b |
| G21 | 0.47 ±0.072 c |
| G31 | 0.45 ±0.07 bc |

The G11 bacterium obtained an average value of 0.39 cm, in the treatment with the G21 bacterium an average of 0.47 cm was achieved, in the treatment with the G31 bacterium an average of 0.45 cm was reached and finally, the average of the diameters of the lesions of the control treatment was

4.27 cm. The genes that are activated in the prickly pear when resistance induction occurs are unknown (Figure 5). In corn plants inoculated with *B. subtilis*, the genes PR-1 and PR-4 are activated, while in lettuce plants when inoculated with *B. amyloliquefaciens*, the genes PR-1 and PDF1.2 are activated (Chowdhury *et al*., 2015; Gond *et al*., 2015). This activation causes an increase in the production of defense-related enzymes such as peroxidase, polyphenol oxidase and superoxide dismutase, which decrease or prevent damage caused by plant pathogens (Ongena *et al*., 2005; Chowdappa *et al*., 2013).

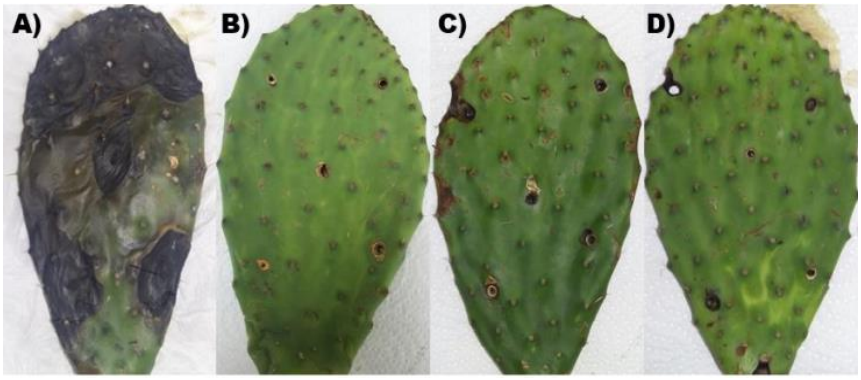


**Figure 5. Resistance induction tests. A) control treatment inoculated with *N. hyalinum* (G111); B) treatment with G11 bacterium vs *N. hyalinum*; C) treatment with G21 bacterium vs *N. hyalinum*; and D) treatment with G31 bacterium vs *N. hyalinum*.**

## Conclusions

The isolated bacteria belonging to the genus *Bacillus* sp. managed to reduce the diameter of the pathogen *N. hyalinum* (G111) in the *in vitro* tests by antibiosis, with the isolates G11, G21 and G31, identified as *B. amyloliquefaciens*, standing out. The results obtained in the *in vivo* tests show that isolates are able to reduce the damage caused by *N. hyalinum* (G111). But, although the isolates of *B. Amyloliquefaciens* manage to induce resistance in prickly pear, it is unknown which are the antibiotics that the isolates G11, G21 and G31 produce and which PR genes they manage to activate in prickly pear, so future research should be focused on which are the antibiotics that the isolates G11, G21 and G31 produce and the PR genes they induce in prickly pear.

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