Selection of growth promoters in the culture of potato (*Solanum tuberosum* ssp. *andigena*) using endophyte bacterias *Bacillus* type

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Abstract

Bio-products that promote potato growth were isolated from native potato plants from the high and cold areas of the Bolivian Andes. Thirty nine bacterial isolates were incubated *in vitro* and thirty of them were identified as *Bacillus.* To test the promotion of growth, the identified *Bacillus* were inoculated in potato Huaycha variety, and grew under greenhouse conditions. A complete randomised design with 6 repetitions was done in two block stages, and the following responses were measured: plant height, root size, root weight, root volume, number of tubers, tuber weight and tuber yield. Eleven isolated (13, 19, 27, 32, 38, 45, 46, 47, 138, 146 and 150) were selected for enhancing the development of the phenotypic characteristics of the crop. *In vitro* evaluations resulted in nine nitrogen fixing bacteria, five phosphate solubilizing bacteria, and three indol-acetic acid (IAA) producing bacteria. Isolates 27 and 146 were the only strains with the three functions (N fixing, P solubilising, and IAA producing), and all of the strain tested corresponded to the genus *Bacillus*.

In a second stage, we reassessed the best performed isolates (19, 146, 45, 47, and 150) in the greenhouse, including a commercial inoculum of *Bacillus subtilis* as positive control. Isolated 45 showed the greatest results in plant height and tuber development, yielding 3 t/ha of potatoes.

Keywords: Fixers, solubilizers, isolated, PGPR, endophytes.

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Introduction

Potato (*Solanum tuberosum*) is the fourth crop of world importance after wheat, rice and corn. It represents the economical, cultural and food security of vast populations for its nutritional quality in protein, carbohydrates, potassium and B6 vitamin. In 2008, UNESCO recognised the importance of potato by declaring it the International Year of the Potato (Pérez, 2008).

In Bolivia, the importance of potato cultivation have a historical, social, economic and nutritional side. Currently, potato production involves more than 200,000 families that grow a total of 129,230 ha, in 7 of the 9 departments of the country. The potato annual production is 827,690 t with an average yield of 5.6 t/ha (Crespo and Bellot, 2003).

Fertilisers increases yields nevertheless its intensive use caused alterations in the environment and human health. Actually, the demands of the population for healthy agricultural products, is one of the reasons why there is a need for strategies to reduce their side effects, and ensure plant yield at a relatively low cost and without environment or soil fertility deterioration.

The demand of the Bolivian producers of technological alternatives that help to reduce water, fertilisers and pesticides consumption is big. Nowadays, the need for agricultural or agrochemical alternatives generate the use of microbiological and other resources available in the country to develop biopesticides and biofertilizers. This type of technology can be used in organic agriculture or in conventional production, reducing in this way the use of agrochemicals (Ortuño et al., 2010).

Bacillus bacteria have high thermal tolerance, rapid development in liquid medium, and highly resistant spores formation. In addition to that, they promote plant growth, which make them appropriate for the formulation and development of viable and stable products that promote plant growth.

The search of sustainable management strategies together with the above mentioned, lead us to select endophytic *Bacillus* bacteria, isolated from native potatoes, to work as promoters of plant growth, and contribute as agroecological alternatives (bio-inputs) in the cultivation of potatoes. Therefore, the objetive of this work was to sort out from native potatoes, the best endophytic *Bacillus*-type bacteria, and use it in the Huaycha variety of potato crop to further develop a bio-inputs capable of promoting plant growth.

Materials and Methods

This work was carried out in two stages, which are described below:

First stage

Isolation of endophytic bacteria from native potatoes field

To isolate the strains, the liquid culture medium TSB (Triptona Soya Agar) was prepared. Six millilitres was added in test tubes with a dispenser, which were then autoclaved for 15 min at 121 °C at 20 psi Subsequently, in a laminar flow chamber under aseptic conditions, each bacterium was inoculated into a properly identified test tube. Then, they were taken to an orbital shaker in constant movement of 100 rpm, for 1 week, at room temperature.

In vitro selection of Bacillus type bacteria

The protocol of Pérez et al., (2004) was used for the *in vitro* selection of *Bacillus* bacteria. In a laminar flow chamber under aseptic conditions, 1 ml of sample of the bacterial strains were isolated, were dispensed in properly identified eppendorf tubes, then subjected to a thermal shock for 15 min at 80°C to eliminate bacteria which are not *Bacillus* type. The TSA (triptone soy agar) culture medium was prepared one day in advance and dispensed in Petri dishes. Petri dishes were divided into 16 identified quadrants. In a flow chamber at aseptic

conditions, each sample from the eppendorf tubes was seeded with a metal handle. Subsequently, the plates were incubated at 28°C for 48 hours. Those who presented growth of colonies in the middle were selected as *Bacillus*.

Greenhouse test evaluation of the selected strains

A mixture of sand, organic matter, clay and rice husk in a ratio of 1: 1: 1: 2 was used as substrate and sterilized with water vapor at 121°C for 120 min at 1.5 atmospheres of pressure.

Greenhouse disinfection was done by cleaning the inns, floor, pots, labels and everything necessary to use. For the test we used pots of 2 Kg capacity with sterile substrate and humidity at its field capacity.

The bacterial inoculum 500 µL of the strains isolated in TSB liquid culture medium, avoiding all possible contamination or splashes from one pot to another.

Treatments consisted of 30 bacterial isolates from the cepary, a positive control of the commercial bacterium *Bacillus subtilis* (Bs), and a blank control without inoculation.

Pots of 2 Kg with a single plant with the treatments were in a randomized block design, with six repetitions. The effect of light entering only from one side of the greenhouse was blocked.

A cluster analysis was performed to group the strains of the first stage and select them for further analysis.

Second stage

Functional evaluation of bacteria with the greatest potential of plant growth promoters were selected in the first stage.

Supplementary in vitro tests

Supplementary *in vitro* tests were performed on the selected bacteria from the first stage. Qualitative tests were carried out considering three functional mechanisms: Biological nitrogen fixation, phosphate solubilization and production of indole acetic acid (IAA).

Biological nitrogen fixation

The determination of endophytic nitrogen-fixing bacteria was performed based on the protocol used by Dion and Magallón (2009). Solid Burk medium was prepared 24 hours before use, autoclaved for 20 min at 121 °C and 20 Psi, and dispensed in petri dishes under aseptic conditions in a laminar flow chamber. The plates with the Burk medium were divided and identified for each of the selected strains. In each of the divisions, bacterial isolates were seeded with a metal handle, and incubated for 48 hours at 30 ° C. Moreover, positive and negative controls were added. The positive control was a strain of *Paenibacillus sp* obtained from the laboratory collection of PROINPA fundation, known for its Nitrogen fixation properties, and the negative control was a sterile petri dish with Burk medium.

Phosphate solubilisation

The protocol of Nautiyal (1999) was used for the determination of bacterial strains capable of solubilizing phosphate. Initially, the National Botanical Research Institute's phosphate growth medium (NBRIP) was prepared with Tricalcium Phosphate and bromophenol blue as a pH indicator. Once autoclaved and dispensed in petri dishes, each bacterial strain was seeded by extension in each quadrant at a laminar flow chamber. As a positive control a strain of phosphate solubilizer *Bacillus pumilus* was used and for the negative control no strain was sown in the NBRIP medium. Subsequently, the plates were incubated for 48 hours at 30°C.

Production of indole acetic acid (IAA).

IAA is a secondary metabolite produced by some bacteria in the stationary period of growth. To determinate the production of IAA, each strain was sown in tubes containing autoclaved TSB liquid medium

with 5 mM of L-Tryptophan sterilized by a 0.2 µm filter.
Tubes were incubated in an orbital shaker at 28°C and 100 rpm for 7 days. A positive control of *Bacillus subtilis* known for its production of IAA was added, as well as a negative control, in which no strain was sown in the culture medium.

After 7 days of incubation, 1000 µl of sample was taken from each test tube and transferred to Eppendorf tubes. Then, they were centrifuged at 5000 rpm, for 5 minutes. To reveal the IAA production in the samples, the Salkowski solution was prepared 24 hours prior the test. For the test, the bacterial broth supernatant was extracted, placing on an Elisa plate containing the Salkowski reagent. Then, the plate was left in absolute darkness for 15 minutes. Finally, those who turned light pink to reddish tones were reported as positive for IAA production.

Biochemical characterization of bacterial strains

The protocol of Pérez et al. (2004) was used for the biochemical characterization of the selected bacterial strains. These tests were: amylase, catalase, potassium hydroxide (KOH) and Gram staining. All the strains were inoculated in TSA medium and incubated for 48 hours at 30°C.
For the amylase test, the agar-starch medium was prepared, autoclaved and the bacterial strains were sown under a laminar flow chamber, one strain per quadrant of the Petri dish. Then, they were placed in the incubator at 30°C for 24 hours. After this time, iodine was added and the strains that showed transparent halos around the colony were recorded as positives.
For the catalase test, the bacterial sample was placed in a slide with the help of a sterilized toothpick, then a drop of 3% hydrogen peroxide (H2O2) was added. Those marked as positive were the bubbling ones.
For KOH test, a bacterial sample was placed on a disinfected slide and then, a drop of 10% KOH was added with a micropipette and mixed with a sterile toothpick. The positive ones were those with a viscous thread raised with the toothpick.
For Gram staining, the standard protocol for Gram staining was used. After the extension, fixation, colouring and drying, the *Bacillus* were observed under a microscope. The strains that looked red were recorded as Gram (-) and the strains that showed the violet color were recorded as Gram (+).

Multiplication of bacteria

The selected bacteria were multiplied on a small scale. Six ml of liquid TSB culture medium was prepared, autoclaved for 15 min at 121 °C at 20 psi and added to test tube. Subsequently, under a laminar flow chamber, each of the isolates, the positive control and a tube with only medium as blank control were inoculated. The test tubes were taken to an orbital shaker at 100 rpm for 7 days at room temperature.

Inoculum standardization

The inoculum was standardized at a concentration of 1 × 108 spores/ml. Bacterial concentration was observed in a Neubauer chamber. At the greenhouse, 2 kg with sterile substrate were used in pots, where one seed was planted. Then, 500 µL of bacterial solution was inoculated into the seed with the help of a micropipette.
The bacteria selected from the first stage, together with the positive and negative control, were evaluated with the same experimental design (complete random with 6 repetitions) used in the first stage. The experimental unit consisted of a 2 kg pot with a single plant.
In the greenhouse, the following variables were evaluated in both phases: plant height, root length (cm), root weight (g), root volume (cc), number of tubers, tuber weight (g), and the weight of tubers (t /ha) as a measure of yield.
The data of each qualitative variable, of the first and second stage, were analyzed with the SAS PROC MIXED, after verification of the assumptions of normal distribution and

homogeneity of variances under a statistical model in randomized block design, where the effect of natural light presented in a part of the greenhouse was blocked. Then, comparison of Tukey multiple range means (p = 0.05) was made. To select the strains with similar characteristics, the means of all the response variables were used, which were submitted to the CLUSTER analysis according to the maximum distance method using the SAS PROC CLUSTER.

Results and Discussion

The observed results are presented in two stages. In the first stage, *Bacillus*-type bacterial strains were selected *in vitro* and at the greenhouse. In the second stage, the selected strains of the first stage were evaluated *in vitro* and greenhouse.

First stage

In vitro selection of Bacillus type bacteria

Thirty nine endophytic bacterial isolated from native potatoes were used, 30 of them were positive for the *Bacillus* genus and presented optimal growth in the TSA medium. These 30 were evaluated in the first stage in the greenhouse. The genus *Bacillus* forms highly resistant endospores at high temperatures (80ºC) giving it a very important competitive advantage in the soil as they must adapt to sudden changes in temperature (Petersohn et al., 2001).

Greenhouse Selection

Plant height

Plant height varies significantly (p=0.05) with the inoculation of the different isolates. The plants inoculated with isolates 12, 146, 19, 20, 34, Bs and To were the most favoured with inoculation, compared to the rest of the inoculated plants (11, 13, 137, 138, 145, 150, 1b, 2, 24, 27, 29, 3, 32, 38, 3a, 45, 46, 47, 48, 49, 51, 52, 56, 6 and 9), which presented a less developed plant height (Figure 1). The isolated 6 was the least developed with a plant height of 32.83 cm.plant.

The use of microorganisms or rhizobacteria that promote plant growth can cause a number of beneficial effects on plants, especially on the growth of roots and aerial part, due to an increase in the availability and uptake of nutrients such as phosphorus, nitrogen, potassium and micronutrients (Kloepper et al., 1980; Vessey, 2003).

Plants inoculated with *Bacillus* isolates, showed an effect that was related to the degree of bacterial host-isolated specificity, which allowed better absorption of essential elements, such as N and P found in the plant. That together with the phytohormones excreted by the roots, have a physiological action, which is favorable and cause a greater development of the aerial part of the crop.

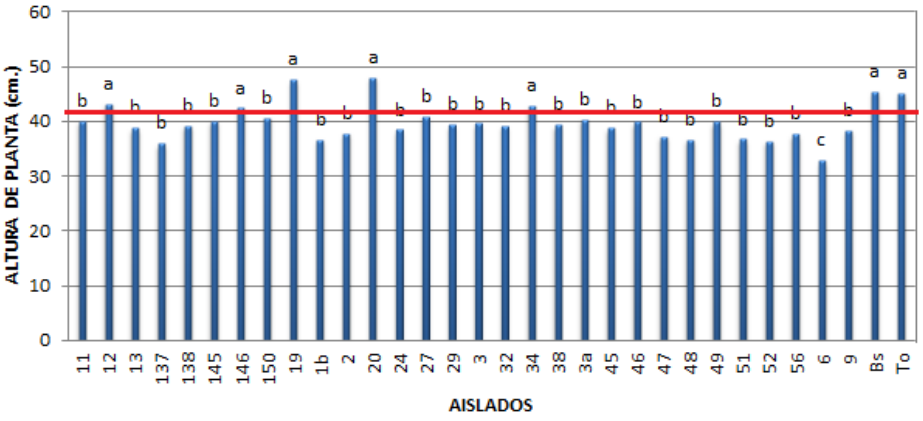

Figure 1. Plant height variation in potato plants with the different bacterial isolates.

Root length

Root length was similar with the inoculation of the isolates; however, isolate 1b favoured root length (43.5 cm), compared to other isolates including the negative (To) and the positive control with *B. subtilis* (Bs) (36.33 cm and 37.5 cm respectively). The least develop was isolate 146 (34.73 cm).

Different studies have shown that the use of *Bacillus* increases the surface area and root length, showing the potential of this microorganism to be used as a biofertilizer alternative. They also reported that certain strains of *Rhizobium* inoculated in lettuce seedlings (*Lactuca sativa* L.) increased their growth due to the production of indole acetic acid (IAA), a plant hormone that promotes radical or vegetative development (Peña and Reyes 2007).

Root weight

Root weight of the inoculated plants was similar in all the strains; however, isolate 13 favoured relatively higher root weight (37.72 gFW) compared to Bs and To isolates (25.11 and 28.32 gFW, respectively).

Success in promoting plant growth, when beneficial bacteria are introduced, depends to a large extent, on the time of its establishment and their persistence throughout the root growth season (Schippers et al., 1987). One of the most important effects is the modification of root morphology, which includes a phytostimulation of this organ and a significant increase in the formation of radical hairs (Dobbelaere et al., 1999).

Root volume

Root volume had no significant differences (p=0.005) between the isolates. But nevertheless; the isolated 32 developed relatively higher root volume (32.83 ml) compared to 26.83 ml developed with the Bs and 26.67 ml with the To. The main effects of growth promoting bacteria on grasses have been associated with emergence, root development and yield effects. *Azospirillum* favors changes in the absorption of NO3, NH4, PO4, K and Fe, which increases the accumulation of minerals in leaves and stems. It has been suggested that the increase in mineral absorption is due to a general increase in root volume and not to a more effective ion absorption mechanism (Bowen and Rovira, 1999).

Tuber number

The number of tubers developed by plants with bacterial inoculation was similar for different isolates. However, among the isolates that relatively favoured the development of the number of tubers was the isolate 34 with an average of 12.17 units, in relation to *B. subtilis* (Bs) with 7.83 and the negative control without inoculum (To) with 7.5 units On the other hand, the isolate that favoured less development was the isolate 20 with 6.17 units.

Other studies done in cassava showed increases between 20-54% in the agricultural yield, as well as in other indicators such as the number of tubers per plant (between 11-43%) and the diameter of these (between 15- 19%), proving the beneficial effect on potato cultivation. Thus, in relation to the quality of the harvested tubers, significant differences were observed in all sampled calibers with an average increase between 18-39% and 26-42% in the number and weight of fruits from inoculated plants, compared to control plants and fruit production (Dibut et al., 2004; Gravel et al., 2007).

Tuber weight

Tubers weight varied (p=0.05) with the inoculation of the different isolates. Thus, potato plants with the inoculation of isolates 12, 13, 137, 138, 145, 146, 150, 19, 1b, 27, 29, 32, 34, 38, 3a, 45, 46, 47, 48, 49, 52, 6, 9 and Bs, had a higher tuber weight. The most favoured in tuber weight was the isolated 145 with 57.26 gFW, in relation to the control (To) that showed less development of tubers with 38.77 gFW (Figure 2).

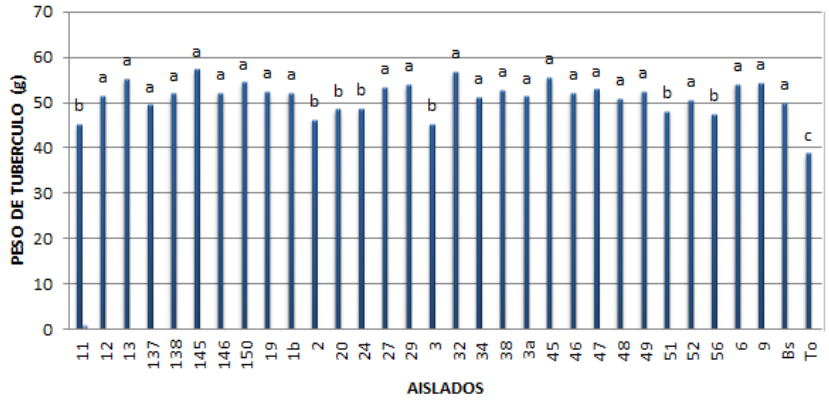

Figure 2. Tuber weight variation in potato plants with the different bacterial isolates.

Similar results were found by Soler (2012), who reported greater tuber formation (g) in plants inoculated with endophytic microorganisms, compared to control plants (uninoculated plants).

Yield

Differences (p=0.05) in yield were determined with the inoculation of the different bacterial isolates. The highest yield was observed with the inoculation of the isolates: 12, 13, 137, 138, 145, 146, 150, 19, 1b, 27, 29, 32, 34, 38, 3a, 45, 46, 47, 48 , 49, 52, 6, 9 and Bs. The plant that presented the highest yield was the one with inoculation of isolate 145 with a yield of 2.04 t/ha, in comparison to isolate To with 1.39 t/ha that had the lowest yield (Figure 3).

Similar results were obtained in potato seeds under greenhouse conditions, where the inocula were observed to promoted the growth of roots and tubers (expressed in gDW), in 22% and 80%, respectively. Microorganisms have the ability to produce auxins, which are substances with a regulatory effect on plant growth, close related to their fructification process (Srivastava and Handa, 2005). Therefore, inoculation with bacterial isolates could favor the development of potato tubers and also yield.

Cluster analysis of 32 Bacillus bacterial isolates

The 32 bacteria isolates can be grouped into 3 groups (Figure 4).

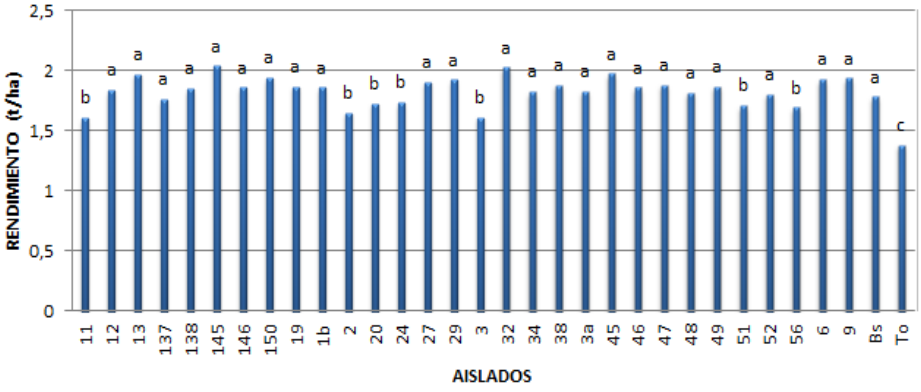

Figure 3. Yield variation in potato plants with the different bacterial isolates.

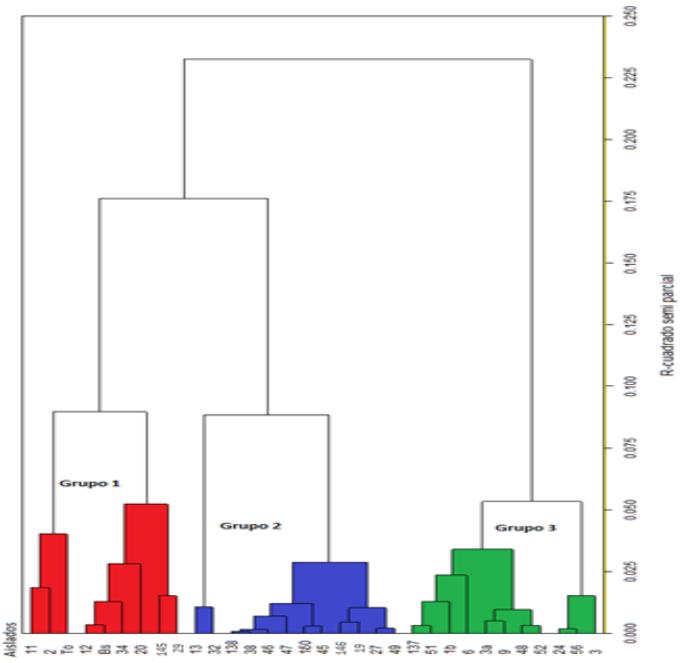

Figure 4. Clustering dendrogram of 32 bacterial isolates.

The first group is composed of the isolates: 11, 2, To, 12, Bs, 34, 20, 146 and 49, characterized mainly by greater development in plant height and not in the other variables. The second group consists of the isolates: 13, 32, 138, 38, 46, 47, 150, 45, 145, 29, 27 and 49, that have the characteristics to promote the development of root length, root weight, root volume, tuber weight and yield. The third group is integrated by the isolates: 137, 1b, 24, 3, 3a, 48, 51, 52, 56, 6 and 9, characterized for favoring the development of the number of tubers per plant.

Group 2 was selected from the 3 groups, because it favored in greater proportion the development of the phenotypic characteristics of the potato crop.

Second stage

The best isolates of the first stage (13, 19, 27, 32, 38, 45, 46, 47, 138, 146 and 150), were evaluated once more *in vitro* and greenhouse in their vegetative development. Different tests were performed in order to determined the type of bacteria inoculated.

Functional tests

Nitrogen Fixation Test

The bacterial isolates that were positive for Nitrogen Fixation were 27, 46, 47, 38, 45, 13, 32, 146 and 150. So these bacteria could be used as growth promoters in agriculture. As we obtained colony growth in the Burk medium as a positive response to this mechanism. This medium contain little amounts of nitrogen, so bacteria that develop in this medium have the ability to reduce gaseous nitrogen (N2) to produce NH4+ (ammonium), which then serves for amino acid biosynthesis (Servin and Dion, 2009). Lara et al. (2007) indicate that molecular nitrogen that exists in the atmosphere is not easily assimilated by plants because of the triple bond that binds the atoms that make the molecule difficult to break. The only form of use of this source is through the metabolic process known as Biological Nitrogen Fixation (BNF). The process that converts nitrogen into ammonia is done thanks to the activity of the enzyme complex called nitrogenase.

Phosphate solubilization test

The positive isolates for phosphate solubilization were 27, 46, 47, 146 and 150 out of 11 isolates. The solubilization of phosphates is given by the secretion of organic acids such as lactic, oxalic and citric acid by microorganisms. In the test used to establish the in-vitro solubilizing phosphate activity, clear areas were developed around the microbial colonies, which indicated a change in the pH of the medium due to the synthesis of the acids mentioned above (Mehta and Nautiyal, 2001) .

Determination of bacterial isolates producing indole acetic acid (IAA)

In the indole acetic acid production test, there is a change in color in the medium from pinkish to reddish tones indicates the production of IAA from the isolates. Our results showed that isolates 27, 45 and 146 were IAA producers.

Plant growth regulators (PGRs) are organic substances that influence the physiology and development of the plant at very low concentrations (García et al., 2005). Bacteria that live in the rhizosphere can influence the growth of plants by contributing to the endogenous pool of PGRs in these, such as auxins, which include IAA (Patten and Glick, 2002). The bacterial IAA produce, would stimulate the development of the radical system and the general growth of the host plant. At the same time, it would increase the production of plant metabolites, used by bacteria for their own growth, which show a reciprocal benefit in the plant-bacteria relationship.

Biochemical tests

Catalase, Potassium Hydroxide, Amylase and Gram Stain, were the different biochemical tests used in the same bacterial isolates evaluated in the functional tests.

Catalase Test

Catalase is an enzyme that breaks down hydrogen peroxide (H2O2) into water and oxygen. Hydrogen peroxide is formed as one of the final products of carbohydrates aerobic metabolism, which, if allowed to accumulate it can be lethal to the bacterial cell. In the *Bacillus* genus, there are facultative anaerobic and aerobic bacteria, which most are catalase positive (Prescott, 2004). In the present investigation, the 11 selected isolates were positive, which confirms a characteristic of the genus *Bacillus*.

Potassium hydroxide (KOH) test

The formation of an extremely viscous suspension was observed, which when lifted with a handle, formed a thread of mucilaginous appearance. This test was done prior to the Gram staining test, since it can be deduced from the results which bacterial isolates are Gram positive or Gram negative (Prescott, 2004). In the present investigation, isolate 13 tested positive for a KOH test out of the 11 bacterial isolates evaluated, showing that it belongs to the Gram positive group; result confirmed in the Gram stain test.

Amylase test

The presence of a transparent halo around the colony indicated that the test is positive, i.e. that the bacterial isolate has hydrolyzed starch through amylase. The 11 isolates resulted positive in this test.

The production of amylases in the genus *Bacillus* is related to the complexity, nature and concentration of the nitrogen source, regulating it in a positive or negative way. The most studied complex nitrogen sources for the production of amylases in *Bacillus* species are yeast and peptone extract, although the use of amino acids and Ca+2 in the culture medium can increase production (Premila and Dhandayuthapani 2013; Thippeswamy et al ., 2006).

Gram staining test

Gram positive and Gram negative bacteria presented a coconuts or bacilli morphology, however, with the Gram staining we can identify their taxonomic group, as the Gram

positive stains violet, while the Gram negative stains pink.

Gram positive bacteria contain a thick wall of peptidoglycan and a large amount of theic acids that allow you to quickly fix and retain the initial dye which is the violet crystal, and they are also resistant to fading. In gram negatives, the opposite occurs, i.e. have a thin wall with less amount of peptidoglycan and lipids, which requires a counter staining dye such as safranine or fuchsin, in this case, the initial dye (crystal violet) is easily removed in the discoloration with alcohol, because its thin cell wall does not retain it.

Wang et al. (2007) mentions that the entire *Bacillus* genus belongs to the Gram positive group, confirming our theory that the 11 bacterial isolates studied gave a Gram positive result, which is characteristic of the genus.

Greenhouse results

The best bacterial isolates of the first phase were inoculated in potato tubers in the greenhouse, to confirm their efficiency in vegetative development.

Plant height

Plant height differences were estimated between bacterial isolates (Pr = 0.05), where the height of the potato plant increased over time.

During the phenological development of the plants, the growth was ascending over time with the inoculation of the different isolates (Figure 5). The isolates with the highest plant height were: 19, 45, 46, 47, 138, 146, 150 and Bs, compared to the rest of the bacterial isolates: 13, 27, 32 and 38 that had the lowest plant height, including the negative control without inoculum (To).

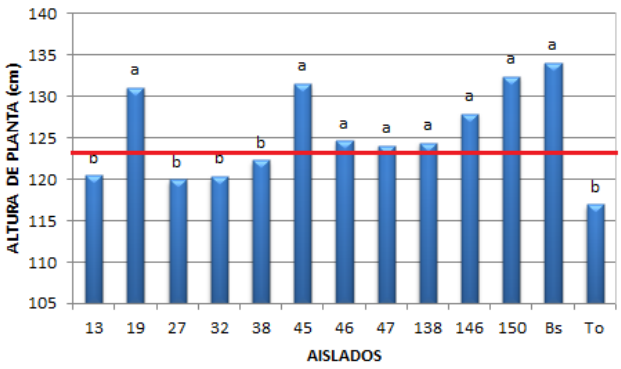

Figure 5. Plant height variation in potato with the selected bacterial isolates.

In recent years, it has been demonstrated the advantages of the association *Bacillus* in cereals, with important results in the stimulation of plant growth and nutrient gains extracted from the soil (Rodríguez, 2002). The most outstanding effects of some bacterial isolates, suggest that there is a synergism between the host and the symbionts, which allow better absorption of essential elements such as the N and P found in the plant, which probably together with the phytohormones excreted in the roots have a physiological impact, causing a greater development of the aerial part of the crop (Arshad and Frankenberger, 1998). In our study, isolated 150 (132.37 cm) and 6 other isolated, had higher plant height, which could be considered as inoculants to produce better development of the potato crop.

Root length

Significant differences were also observed between the bacterial isolates (Pr=0.0008), for root length, indicating its variation with the inoculation of the different isolates.

The potato plants that developed greater root length were those with the inoculation of isolates 13, 19, 27, 32, 45, 46, 47, 146, 150

and Bs (Figure 6), however, bacterial isolate 13 (26.39 cm) is the one that had the best root length in the plant, being able to increase the absorption surface and thus better nutrition, compared to isolates 38 and 138 with 21 cm and 18 cm, respectively. On the other hand, the negative control (To) also presented longer root length.

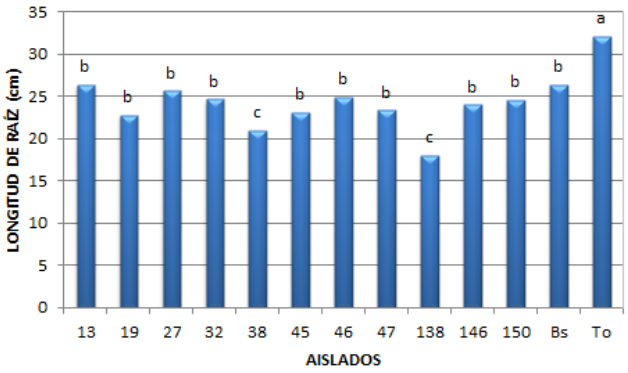

Figure 6. Root length variation in potato plants with the selected bacterial isolates.

The development of the roots is favored by the effect of inoculation of the isolates, which manifested directly in greater growth of the root of the crop. These results are consistent with those reported by Pereira et al., (1988) and Kloepper et al. (1991), who mentioned that growth promoting bacteria are characterized by increasing radical development, which has a direct impact on crop yield.

Since the 1970s, research has been carried out on the role of those rhizobacteria so called PGPR bacteria of the rhizosphere residents, beneficial for stimulating plant growth or rooting, as well as disease resistance. The bacteria may also exert neutral or harmful effects, depending on the concentration they reach in the host tissues (Bashan and Holguin, 1998). Arshad and Frankerberger, (1998) and Díaz et al. (2009), showed a negative effect on inoculation, since the negative control was able to stand out as the best treatment, which confirmed what was pointed out by Schipper et al (1987), that inoculation may have a deleterious influence.

Root weight

Significant differences were also obtained for the variable root weight between bacterial isolates (Pr = 0.0001). So the weight of the root varied with the

Isolates that showed beneficial effects on the root weight of the plant were 13, 27, 32, 38, 46, 47 and *Bacillus subtilis* (Bs) that presented the greatest weight with 11gFW (Figure 7), subsequently isolates 27 and 38 with a weight of 8 gFW, compared to isolates 45, 138, 146, 150 and control (To) with 4.66 gFW, isolate 19 is the one that developed the lowest root weight 2.75 gFW.

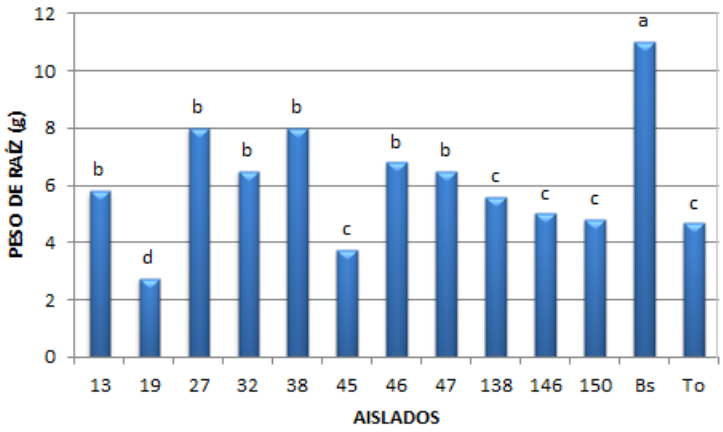

Figure 7. Root weight variation in potato plants with the selected bacterial isolates.

Several soil microorganisms common in the rhizosphere are capable of producing phytohormones, a production that has an effect on plant growth and development. Nitrogen-fixing bacteria stimulate root elongation and formation of lateral and adventitious roots, causing an increase in the radical surface (Marko e Iglesias, 2003).

Root volume

No significant differences were observed between the isolates (Pr = 0.2493), according to the analysis of variance for root volume done, however, the isolate 146 relatively favored the development of higher root volume (18.33 ml), in comparison to the other isolates that developed less than 12.33 ml including the negative control (To) and *Bacillus subtilis* (T+) (11.33 and 15.60 ml respectively). Most of the microorganism-plant associations occur at the level of the rhizosphere, a portion of the soil that is strongly influenced by the roots of the plants, through symbiotic exchanges with the plant, which stimulate the development of the root system (Aguilar, 2003).

Tuber number

Significant differences were observed, for this variable between bacterial isolates (Pr=0.0001).

The largest tuber number was observed with the inoculation of isolates 13, 19, 45, 46, 47, 138, 146, Bs (Figure 8), compared to the rest of isolates 27, 32, 38, 150 including the negative control (To), which had a smaller number of tubers.

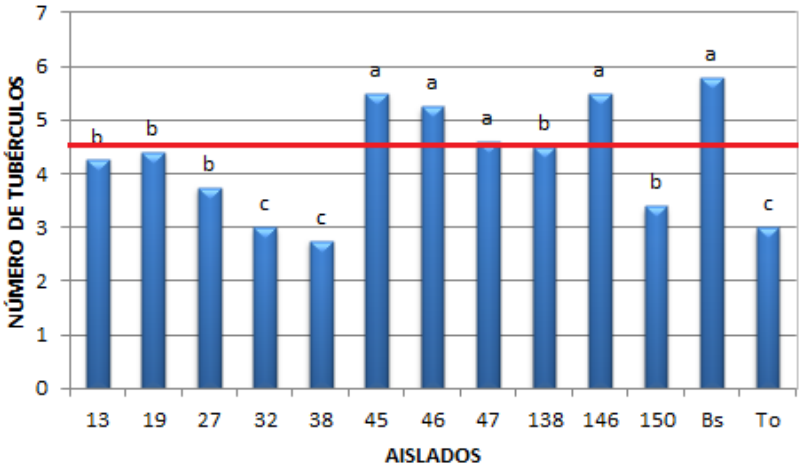

Figure 8. Variation in the number of potato tubers in plants due to the effect of bacterial isolates.

Regarding the number of tubers, similar results were reported in other studies with

strawberry (*Fragaria versca*) (Esitken et al., 2010), raspberry (*Rubus idaeus*) (Orhan et al., 2006) and chili (*Capsicum annum*) (Datta et al., 2011), in which *Bacillus* treatments increased the number of fruits, may be due to the association that these microorganisms have with the different cultivars in which they were applied as cereals, vegetables or tubers.

Tuber weight

Tuber weight differences between bacterial isolates were also estimated (Pr=0.0001),. Weights varied with the inoculation of the different isolates.

Potato plants with the greatest weight of tubers were those with the inoculation of isolates 19, 27, 32, 38, 45, 46, 47, 146, 150, Bs and To (Figure 9), being the commercial Bs, the best isolate with 72.74 gFW, followed by isolate 45 with 71.63 gFW. The isolates that had the lowest tuber weight per plant were isolates 13 and 138 with 38.50 gFW and 36.40 gFW, respectively.

Aguilar et al. (2003), also demonstrated the benefit obtained with the inoculation of *Bacillus* bacteria in potato plants, reporting significant difference in terms of tuber mass (weight) in relation to the control.

Yield

For the variable yield, significant differences were also found among the bacterial isolates (Pr=0.0012), which indicates that the tuber yield was different with the inoculation of the different bacterial isolates.

The bacterial isolates that showed beneficial effects on plant yield were 19, 38, 45, 46, 47, 138, 146 and Bs (Figure 10), being isolate 45 the best with 3 t/ha. The isolates with less yield were isolates 13, 27, 32, 150, including the control. The isolate that developed the lowest yield was 150 with 1.48 t/ha.

In the present study, potato tuber yield showed a notable increase with the inoculation of *Bacillus*-like bacteria, presenting an increase of 68% compared to non-inoculated plants. Other studies reported the beneficial effect on potato yield with the use of *Bacillus* species, nevertheless these increases varied from 4% to 47% (Jiménez et al., 2001). In the present investigation, the increase obtained were greater to the reported in the study above, may be due to the concentration of bacteria used or the root volume.

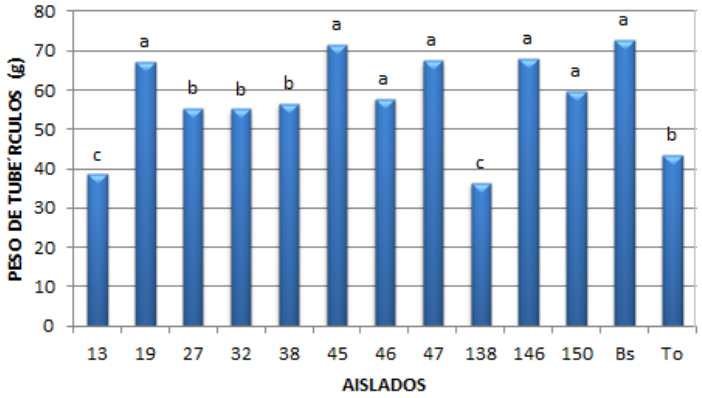

Figure 9. Tuber weight variation in potato plant with the selected bacterial isolates.

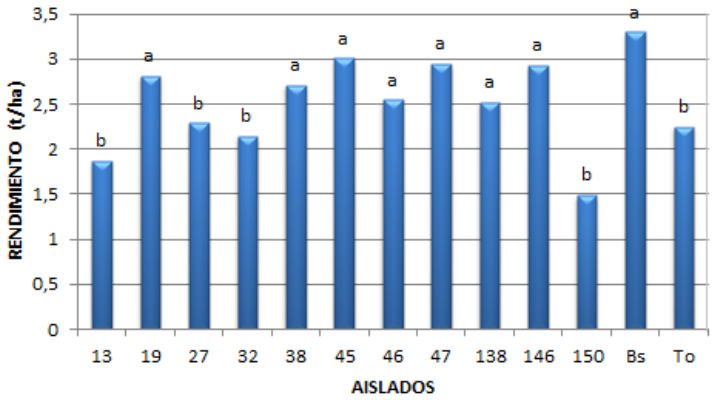

Figure 10. Yield variation in potato plant with the selected bacterial isolates.

Selection of bacterial isolates with the best characteristics such as PGRB

Through the grouping of bacterial isolates (Figure 11), the 12 isolates can be grouped into 2 groups, where the first group consisting of isolates 13, 138, 27, 32, 38 and 46 are characterized by their effect on development of small plant, smaller root size, smaller number of tubers, lower tuber weights per plant, lower root volume and lower yield; although, they promote slightly greater root development.

The second group is composed of isolates 19, 146, 45, 47, Bs and 150, which are characterized for promoting the development of taller plants with larger root size, therefore, greater root volume. Likewise, they promote the development of a greater number of tubers with a greater weight of tubers/plant and with greater yield. Although, they promote root development to a lesser extent.

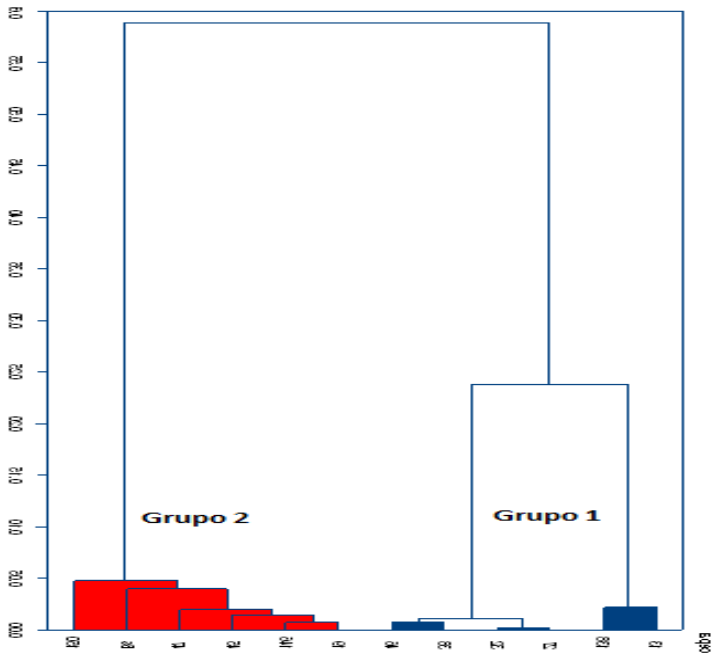

Figure 11. Clustering dendrogram of 12 bacterial isolates.

Therefore, isolates set in group 2 could be selected as growth promoters, because they promote the development of favorable characteristics such as yield.

According to the objectives set and the results obtained, together with the biochemical tests which showed that the selected isolates corresponded to the *Bacillus* genus. From a collection of 39 bacterial strains isolated from wild potato plants, 30 of those were bacterial isolates of the genus *Bacillus*. Greenhouse conditions defined that out of the 30 bacterial isolates of the genus *Bacillus* tested, 11 isolates were selected (13, 19, 27, 32, 38, 45, 46, 47, 138, 146 and 150) as growth promoters for the Huaycha variety potato crop.

In vitro conditions of the 11 isolates selected as plant growth promoters in the first stage, allows to determine that nine tested positive for nitrogen fixation (growth in the Burk medium), five showed growth in the NBRIP culture medium, and three were indole acetic acid producers. Isolates 27 and 146 had all three functions.

The 11 bacterial isolates formed 2 set, from which group 2 (isolates 19, 146, 45, 47, 150 and the commercial inoculum *B. subtilis)*, had a greater effect on the development of the plant. The isolate 45 was the one which presented greater plant height and tuber development with a yield of 3 t/ha of potatoes.

Conflict of interests

The present investigation does not present any conflict of interest with the institutions where the experiments were performed.

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