In-vitro study of endophytic bacteria: Screening of Indole-3-acetic acid production and phosphate solubility

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Abstract. Plant growth promoting bacteria (PGPB) are found in the rhizosphere of plants as a heterogeneous group. Among its characteristics are the genetic capacity to produce indole-3-acetic acid from L-tryptophan and the production of organic acids and phosphate solubilizing enzymes. The production of indole-3-acetic acid of each bacteria culture was measured using the Salkowski reagent applying the liquid spectrophotometry technique. On the other hand, to evaluate the potential of organic acid production of phosphorus solubility, the National Botanical Research Institute, Pune (NBRIP) culture medium was used applying the liquid spectrophotometry technique. All evaluated bacteria showed the ability to produce indole-3-acetic acid and organic acids of phosphorus solubility. The strains of Pseudomonas sp. examined in this study were the most efficient in the production of AAI and the strains of Bacillus sp. were the most efficient in the solubility of phosphates.

Key words: Indole-3-acetic acid pathways, phosphate-solubilizing microorganisms, plant growth-promoting bacteria.

Resumen. Las bacterias promotoras del crecimiento vegetal (PGPB) se encuentran en la rizósfera de las plantas como un grupo heterogéneo. Entre sus características figuran la capacidad genética de producir ácido indol-3-acético a partir de L-triptófano y la producción de ácidos orgánicos y enzimas solubilizantes de fosfato. Se midió la producción de ácido indol-3-acético de cada bacteria utilizando el reactivo de Salkowski aplicando la técnica espectrofotometría de líquidos. Por otro lado, para evaluar el potencial de producción de ácido orgánicos de solubilidad de fósforo se utilizó el medio de cultivo National Botanical Research Institute, Pune (NBRIP) aplicando la técnica de espectrofotometría de líquidos. Todas las bacterias evaluadas mostraron la capacidad de producir ácido indol-3-acético y ácidos orgánicos de solubilidad de fósforo. Las cepas de *Pseudomonas* sp. examinadas en este estudio fueron las más eficientes en la producción de AAI y las cepas de *Bacillus* sp. fueron las más eficientes en la solubilidad de los fosfatos.

Palabras clave: Bacterias promotoras del crecimiento vegetal, microorganismos solubilizadores de fosfatos, vías del ácido indolacético.

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1. INTRODUCTION

To feed a growing population, the world needs to start increasing agricultural productivity considerably, but in a sustainable and environmentally friendly way. This involves examining many of the existing approaches to agriculture that include the use of chemical fertilizers, herbicides, fungicides, and insecticides (Glick, 2012). Plant growth promoting bacteria (PGPB) are a heterogeneous group of bacteria that can be found in the rhizosphere, root surface, and in association with roots (Kloepper, Lifshitz, & Zablotowicz, 1989). The direct promotion by PGPB involves providing the plant with substances that promote plant growth. Many plant-associated microbes synthesize phytohormones such as gibberellins, cytokinins, jasmonic acid, absicic acid, ethylene, and indole-3-acetic acid.

One of the most physiologically active auxins is indole-3-acetic acid (IAA). The IAA is a common product of L-tryptophan metabolism, produced by various microorganisms including plant growth promoting bacteria. The IAA aids in the production of longer roots with a greater number of root hairs and root laterals that are involved in nutrient absorption, this produces a direct effect on plant-disease resistance (Mohite, 2013). Microbial production of IAA is special phylogenetic, this is derived from an evolution acquired by the ancient symbiosis that these organisms have sustained in soil-plant. This ability can stimulate the production of plant biomass, and allows the bacterium to generate metabolites for the synthesis of its own energy (Hoffman, Gunatilaka, Wijeratne, Gunatilaka, & Arnold, 2013). On the other hand, one of the problem is that the good results obtained in vitro cannot always be reliably reproduced in the field (Keister & Cregan, 1991).

Furthermore, to the production of IAA phytohormone, PGPBs allow solubilization of insoluble phosphates. There are two components of phosphorous in the soil, soluble and insoluble phosphates. A large proportion is present in insoluble forms and is therefore not available for plant nutrition. The most practical way for the availability of phosphates in the soil is often due to artificial applications (Richardson, 1994). To convert insoluble phosphates into a form accessible to plants, is an important trait for a PGPB to increase plant yields (Igual, Valverde, Cervantes, & Velázquez, 2001). On the other hand, phosphate solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the crop (Reyes, Bernier, Simard, & Antoun, 1999). Organic acid as 2-ketogluconic acid production, is the key point for phosphate solubilization processes by PGPB (Halder, Mishra, Bhattacharyya, & Chakrabartty, 1990).

On the other hand, it has been shown that the beneficial effects of endophytic bacteria on their host appear through several mechanisms. Such as antibiosis, growth stimulant, defense induction, parasitism, competition and interference signals (Eljounaidi, Lee, & Bae, 2016). Consequently, growth-promoting rhizobacteria and their interactions with plants are commercially exploited and have scientific applications for sustainable agriculture (Gouda et al., 2018). There is a need for effective biological treatments to cure plants infected by vascular wilt pathogens. Furthermore, most of the pathogens that cause this disease are transmitted through the soil. Chemical control methods can be expensive and inefficient and have a negative impact on ecosystem and human health (Eljounaidi et al., 2016).

Phosphorus and potassium are also known to be the most important essential minerals for plant growth and development. These elements help the synthesis of cells, enzymes, proteins, starch, cellulose and vitamins (Nath, Maurya, & Meena, 2017). The bacteria of the rhizosphere contribute to the solubility of the elements in mineral state, arranging them so that they can be assimilated by the plant (Mehta & Nautiyal, 2001). In previous research, a greater potential in phosphorus solubility has been detected in Bacillus strains (Ahmad, Ahmad, & Khan, 2008). The *Bacillus licheniformis* species produces significant amounts of 3-indolacetic acid (~ 23 µg/mL) (Saha, Maurya, Meena, Bahadur, & Kumar, 2016). These studies arise from the problem of contamination in the environment with metals and organic compounds from applications of fertilizers and pesticides in crops (Glick, 2012). The objectives of this study were:

- Determine the potential for the production of indolacetic acid IAA by the endophytic bacteria studied.

- Evaluate the ability of the bacteria studied to solubilize phosphates and make it assimilable to plants.

2. MATERIALS AND METHODS

Selection of isolates

The bacteria used are part of the biostimulate bacteria collection from the laboratory of Plants and Pathogens at the Haute École Du Paysage, D'ingénierie Et D'architecture De Genève HEPIA. The collection has two origins: one part collected by (Le Guen, 2018)) and other part by (Giroud, 2018) (Table 1). The bacteria were multiplied in Tryptic Soy Agar (TSA) or Lysogeny Agar (LBA) culture media as appropriate to the species. The incubation was carried out at a temperature of 25 °C.

Table 1. Collection of bacteria strains used for this study.

Bacterium	Culture medium	Plant of origin	Collection
Bacillus subtilis C5B	LBA	*Actinidia* sp.	(Le Guen, 2018)
Pseudomonas graminis F33	TSA	*Actinidia* sp.	(Le Guen, 2018)
Enterobacter sp. *B4*	LBA	*Actinidia* sp.	(Le Guen, 2018)
Pseudomonas koreensis B5	TSA	*Actinidia* sp.	(Le Guen, 2018)
Frigoribacterium sp. *B7*	LBA	*Actinidia* sp.	(Le Guen, 2018)
Kocuria rhizophila B3	LBA	*Actinidia* sp.	(Le Guen, 2018)
Pseudomonas koreensis B11	TSA	*Actinidia* sp.	(Le Guen, 2018)
Pseudomonas fluorescens B3	TSA	*Solanum lycopersicum*	(Giroud, 2018)
Pseudomonas moraviensis B6	TSA	*Solanum lycopersicum*	(Giroud, 2018)
Pseudomonas koreensis B7	TSA	*Solanum lycopersicum*	(Giroud, 2018)
Pseudomonas palleroniana B10	TSA	*Solanum lycopersicum*	(Giroud, 2018)
Pseudomonas fluorescens B17	TSA	*Solanum lycopersicum*	(Giroud, 2018)
Bacillus simplex B19	LBA	*Solanum lycopersicum*	(Giroud, 2018)
Bacillus safensis B23	LBA	*Solanum lycopersicum*	(Giroud, 2018)
Bacillus subtilis B25	LBA	*Solanum lycopersicum*	(Giroud, 2018)
Bacillus aryabhattai B29	LBA	*Solanum lycopersicum*	(Giroud, 2018)
Pseudomonas koreensis B12	TSA	*Actinidia* sp.	(Le Guen, 2018)

Determination of indol-3-acetic acid (IAA) production

Inocula preparation. Culture medium Tryptic Soy Broth (TSB) and Lysogeny Broth (LBB) were prepared both with L-tryptophan amino acid, precursor of IAA (Szkop & Bielawski, 2013) at a concentration of 150 mg/L. Then, 5 µL of bacterial isolate were incubated in 10 mL of culture medium with tryptophan (TSB or LBB as appropriate to the strain) in 15 mL Falcon® test tubes. Finally, the incubation was maintained by stirring at 120 rpm at 22 °C using the Infors HT orbiton® shaker. Duplicates of each strain were made following the same protocol (Figure 1).

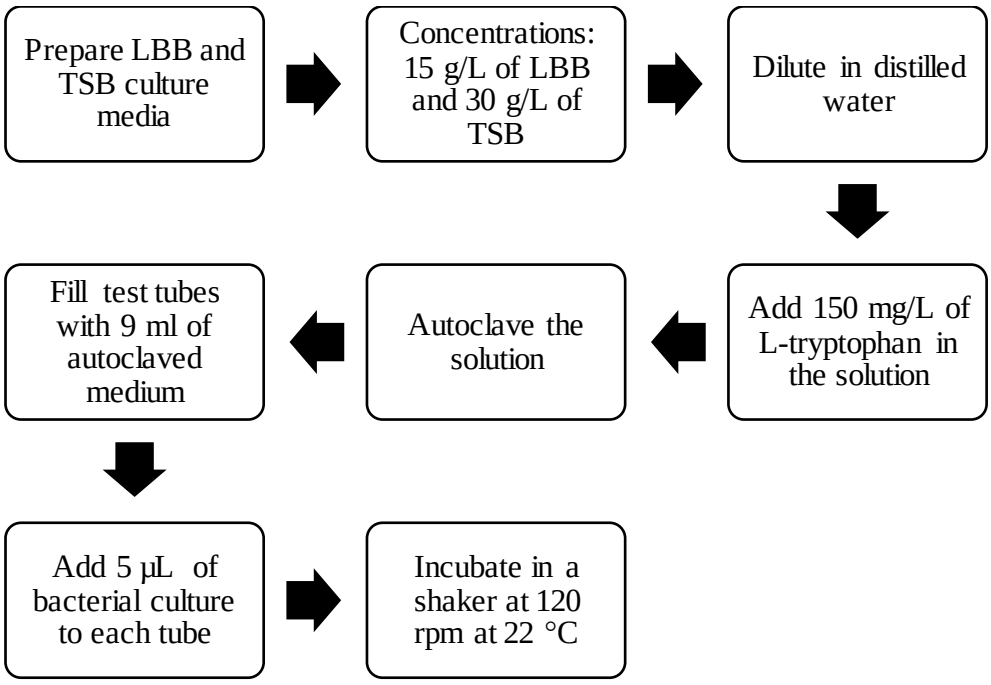

Figure 1. Bacterial inoculation protocol proposed by (Szkop & Bielawski, 2013) with modifications to perform the IAA production colorimetric test.

In vitro screening of bacterial isolates for IAA production. Bacterial culture medium with tryptophan (1.5 mL) was taken and placed in a 2 mL Eppendorf® tube. Subsequently, using a Hettich Zentrifugen Mikro® 22R centrifuge, the tubes were centrifuged at 10,000 rpm for 5 minutes at 20 °C. At the end of the centrifugation 1 mL of supernatant collected and added to 1 mL of Salkowski reagent (Glickmann & Dessaux, 1995), this reagent consist of 12 g of $FeCl_3$ per liter at H_2 SO_4 at 7.9 M. The mixture was placed in 2 mL Eppendorf® tubes and stored for 30 minutes at 21 °C in a dark place.

Finally, the previously stored mixture (2 mL) it was placed in standard cells for spectrophotometry. The IAA production it was quantified by the liquid spectrophotometry method with the use of Lambda® 35 UV/VIS spectrophotometer. The absorbance was taken at 530 nm (Glickmann and Dessaux, 1995). Measurements were made according to the incubation time of the culture medium with tryptophan, at 24 h, 48 h, 72 h, and 96 h.

IAA calibration standard curve. The standard calibration curve was made under the proposed protocol for (Gordon & Weber, 1951) with modifications proposed by Bastien Cochard. Seven samples of medium (TSB or LBB) were prepared with L-tryptophan at different concentrations of indol-3-acetic acid (200, 100, 50, 25, 12.5, 6.25, 3.125 mg/L). Each solution was placed in a liquid spectrophotometer cell; the measurement was made at 530 nm of visible spectrum. In the calibration curve of LBB medium, the data allows 95% predictability using the equation $y = 133.92x - 14.288$ (Figure 2). Moreover, in the calibration curve of TSB the data allows 99% predictability using the equation $y = 598.82x - 3.8182$ (Figure 3). The variability factor is not considered in both calibration curves, it is based on a single repetition.

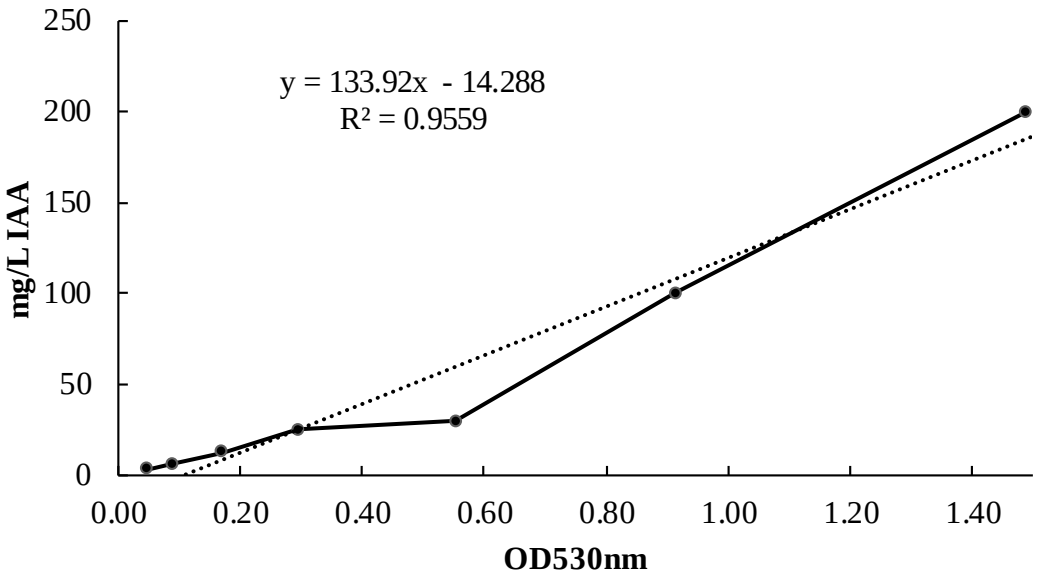

Figure 2. Concentration-absorbancy curve for indoleacetic acid in medium LBB + 150 mg/L L-tryptophane.

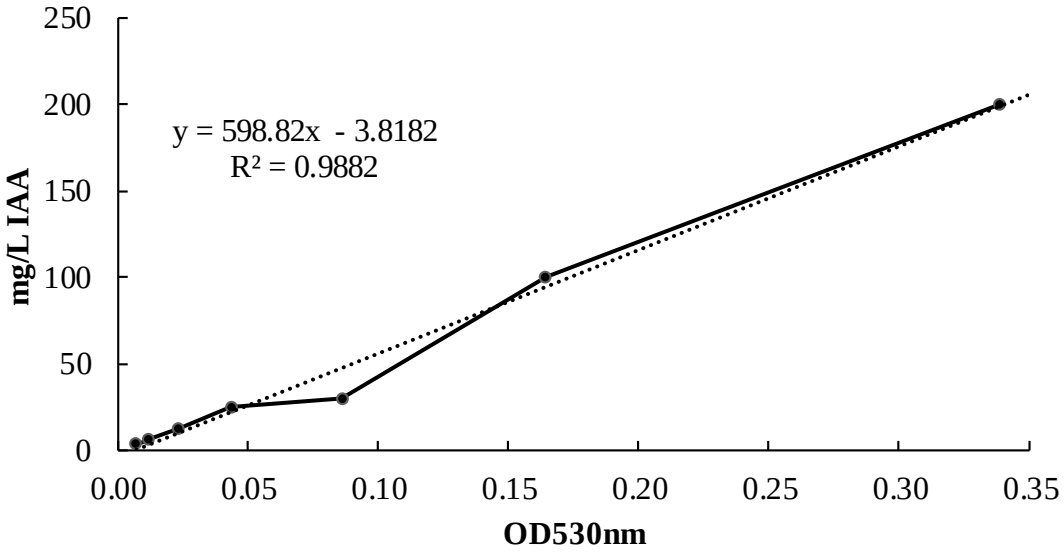

Figure 3. Concentration-absorbancy curve for indoleacetic acid in medium TSB + 150 mg/L L-tryptophane.

Determination of phosphate solubilization activity

Inocula preparation. Eight mL of liquid culture medium (TSB or LBB) were prepared in Falcon® tubes (15 mL capacity) one for each strain. An isolated colony was then taken per strain from the collection of bacteria in Petri dishes and inoculated into the tubes. The tubes were kept agitated at 120 rpm at 22 °C with the use of the Infors HT orbiton® shaker. The bacteria completed a 24-hour period at 22 °C in order to be used in the test.

Phosphate solubilization in a National Botanical Research Institute's (NBRIP) liquid culture. Phosphorus solubility activity was measured using the protocol proposed by (Liu et al., 2015) with modifications recommended by Bastien Cochard. Then 200 µL of bacteria culture of each strain (at 24 hours of incubation) were inoculated, into Falcon® tubes (15 mL capacity) containing 8 mL of NBRIP liquid medium. Finally, the tubes were kept agitated at 120 rpm at 22 °C with the use of the Infors HT orbiton® shaker. Triplicate cultures of these samples were made (Table 2).

Table 2. Composition of NBRIP + BPB (National Botanical Research Institute, Pune) liquid culture medium.

Name of the molecule	Empirical Formula	Amount (g/L)
Glucose	$C_6H_{12}O_6$	10.0000
Tricalcium phosphate	$Ca_3(PO_4)_2$	5.0000
Magnesium chloride hexahydrate	$C_{l2}Mg{\cdot}_6H_2O$	5.0000
Magnesium sulfate heptahydrate	$MgO_4S{\cdot}_7H_2O$	0.2500
Potassium chloride	KCl	0.2000
Ammonium sulfate	$(NH_4)_2SO_4$	0.1000
Bromophenol Blue	$C_{19}H_{10}Br_4O_5S$	0.0125

Source: (Mehta & Nautiyal, 2001).

Then, two milliliters of bacteria culture in NBRIP were taken and placed in Eppendorf tubes (2 mL capacity). The samples were later centrifuged at a speed of 10,000 rpm for 5 minutes at a temperature of 20 °C using a Hettich Zentrifugen Mikro® 22R centrifuge. One milliliter of supernatant was collected and placed in spectrophotometric cells, measurements of absorbance were made at an optical density of 600 nm (Liu et al., 2015) by liquid spectrophotometry method with the use of Lambda® 35 UV/VIS spectrophotometer. Measurements were made according to the incubation time of the bacteria culture medium in NBRIP at 24 h, 48 h, and 120 h (Figure 4).

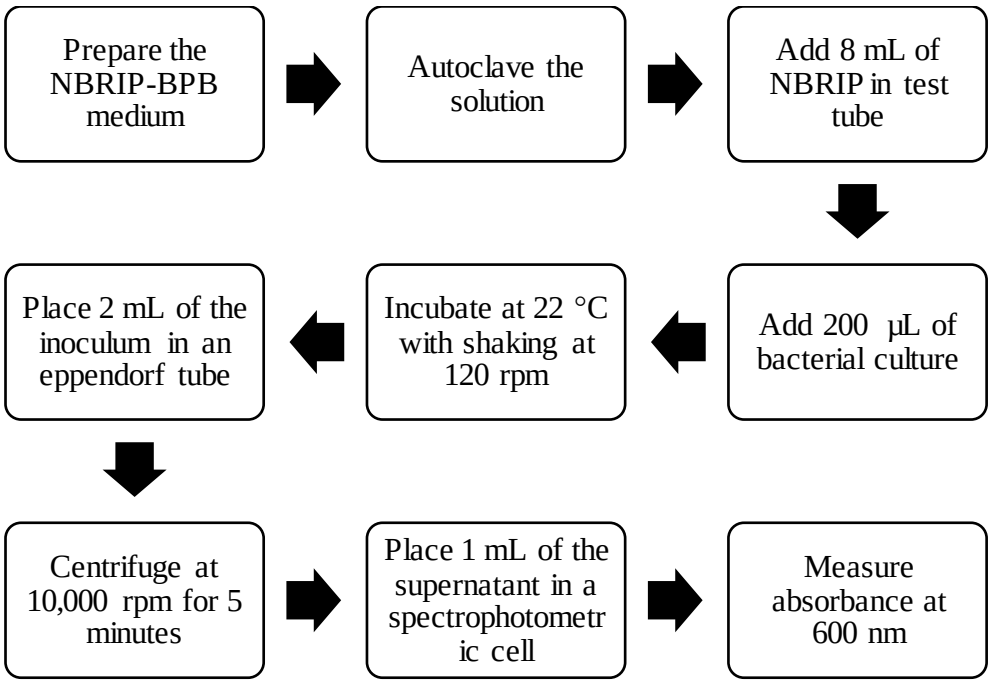

Figure 4. Screening of phosphorus solubility by PGPB protocol proposed by Liu et al., 2015 with modifications provided by Bastien Cochard.

3. RESULTS AND DISCUSSION

Screening and measure in IAA producers bacteria

All 17 isolates of *Bacillus, Enterobacter* sp., *Frigoribacterium* sp., *Kocuria* sp. and *Pseudomonas* sp. were analyzed for the production of indole-3-acetic acid, inoculated in culture medium enriched with 150 mg/L of L-tryptophan. These strains were taken from HEPIA Plants and Pathogens laboratory collection´s (Table 1). All the strains demonstrated ability to produce 3-indolacetic acid from L-tryptophan, but with different behavior based on incubation times (Figures 5, 6, 7, 8). Some bacteria produce similar amounts of IAA for this reason, the results are presented in groups of four or five bacteria, this according to their production patterns (Table 3). To understand the graphs, we must know these physiological stages: the pathways, production, latency, and degradation of IAA synthesized by bacteria. These behaviors are influenced by the environment in which the bacteria are found, each strain responds different way.

Table 3. Criteria for the establishment of groups to analyze the results according to production behavior.

Group	Selection features
A	Grown in LBB culture medium, one species of slow degradation, three species of sudden degradation.
B	Grown in LBB culture medium, one species of slow degradation, one latent species and two of sudden degradation.
C	Cultured in TSB culture medium, all of progressive production.
D	Cultured in TSB culture medium, all *Pseudomonas* sp. of progressive and latent production.

As a consequence of IAA pathways from L-tryptophan, five are known: indole-3-pyruvate (IPya), indole-3-acetamide (IAM), tryptamine (TAM), indole-3-acetonitrile (IAN) and the Trp side chain oxidase pathways (Li et al., 2018). For example, of the strains evaluated in this study; the species *Enterobacter* and *Bacillus* mostly use the IPya pathway and *Pseudomona fluorescens* uses the Trp side chain oxidase pathway (Patten, Blakney, & Coulson, 2013). Some bacteria started producing IAA at 48 hours, this is due to the time when bacteria make IAA available depends on the route the bacteria use (Goswami, Pithwa, Dhandhukia, & Thakker, 2014). In the Salkowski reagent medium there are various indole molecules, to measure only the IAA the visible spectrum of 530 nm was used (Glickmann & Dessaux, 1995).

In this study, the *Bacillus subtilis C5B* species shows a fast and high production, at 72 hours it registered a production of 28.37 µg/mL, its production decrease was slight at 96 hours. In addition, *Bacillus subtilis* has been determined by previous studies as a potential IAA producer (Idris, Bochow, Ross, & Borriss, 2004). On the other hand, the strains *Frigoribacterium* sp. *B7* and *Bacillus aryabhatai B29* started to produce similar amounts of IAA at 48 hours 12.21 µg/mL and 12.23 µg/mL successively. This production is low compared to the other strains in this group, and their production was latent going to zero at 96 hours. The *Enterobacter* sp. *B4* showed slight

differences in production over time 13.98 µg/mL 14.81 µg/mL and 15.87 µg/mL at 24 h, 48 h and 72 h successively, with its production at zero at 96 hours (Figure 5).

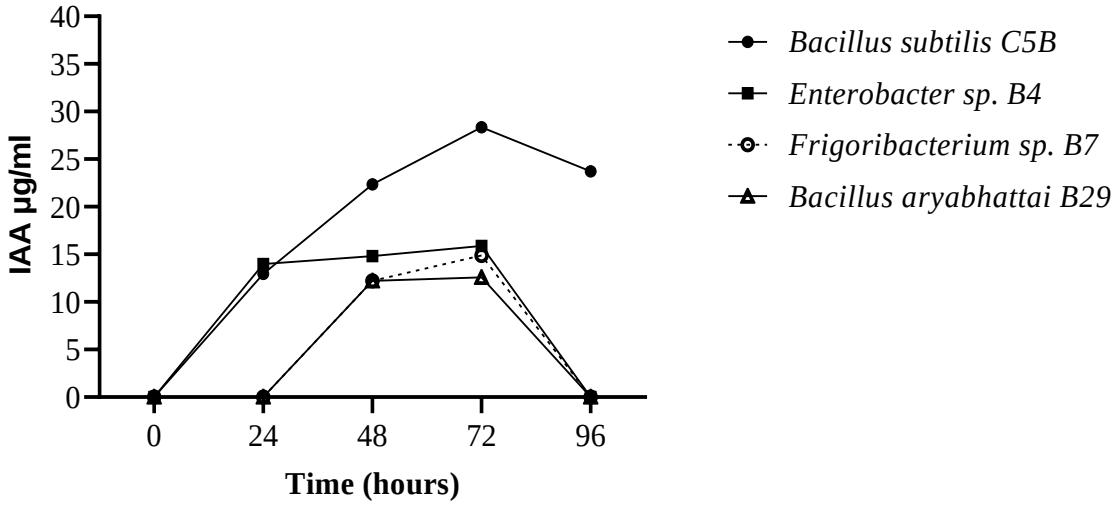

Figure 5. Bacterial behavior of IAA production over time of the strains (group A).

On the other hand, when there is no variation of the measurements over time (i.e. latency is shown in the graphs), it is because there is no degradation or production of the IAA (Gravel, Antoun, & Tweddell, 2007). This means that there is degradation of the molecule IAA in the medium, generated by the bacterial strains. Bacteria have the ability to destroy chemicals with hormonal activity (García-Gómez, González-Pedrajo, & Camacho-Arroyo, 2013). Depending on the environment, the bacteria can inactivate or mineralize IAA (Faure, Vereecke, & Leveau, 2009). For example, *Escherichia coli* hydrolyses IAA to produce indoxyl (tautomeric with 3-oxindole), which in the oxygen process dimerizes it as indigo. In most cases of the IAA degradation pathway, the final product is 2-hydroxy-indole-3-acetic acid (2-OH-IAA), OxIAA tautomer and important product in IAA degradation in plants (Scott, Greenhut, & Leveau, 2013).

In the next group is the *Bacillus subtilis B25* the most efficient strain, its maximum production was 34.64 µg/mL at 72 hours. With this result, the similarity in the production behavior between the *Bacillus subtilis* species is seen, also demonstrating their efficient production. The *Bacillus safensis B23* strain demonstrates a constant but prolonged production with a mean production in time of 12.94 µg/mL. The *Kocuria rhizophila B23* and *Bacillus simplex B19* strains had a late production at 48 h 12.07 µg/mL and 11.95 µg/mL in turn, also had a very short production period (24 hours). The production of these is relatively low compared to *Bacillus subtilis* (Figure 6).

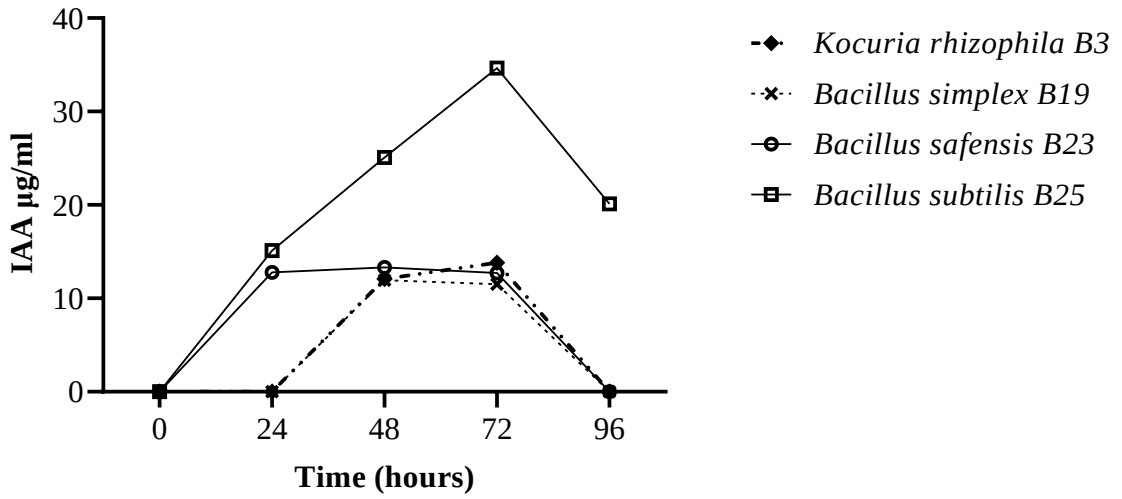

Figure 6. Bacterial behavior of IAA production over time of the strains (group B).

Studies show that *Kocuria rhizophila* is efficient in the production of IAA (Goswami et al., 2014), however from a descriptive analysis *Bacillus subtilis B25* showed better results. Withal, it has been shown that *K. rizophila* is capable of producing IAA but, because it is late, it enters dormancy and degrades it very quickly. On the other hand, it is important to mention that in-plant research this strain has presented good results in the regulation of phytohormone levels in maize. In addition, a significant contribution to enhance the absorption and assimilation of nutrients in maize (Li, Sun, Zhang, Jin, & Guan, 2020).

The *P. graminis F33* strain shows increasing evolutionary production over time, with an IAA production record of 224.54 µg/mL at 96 hours of incubation. *Pseudomonas fluorescens B3* is the next strain of high production in this group, with a maximum production record of 100.41 µg/mL at 48 h. The *Pseudomonas koreensis B7* strain at 48 h showed a strange compartment, lowering its production but at 72 h, it increased and then remained constant. The *Pseudomonas koreensis B5* strain shows a drop in production after 72 h and then remains dormant. *Pseudomonas fluosrescens B17* presented a production of 134.21 µg/mL at 96 h, its avaration after 72 h was slight. *Pseudomonas fluorescens B3* showed a slight decrease in production after 48 h (Figure 7).

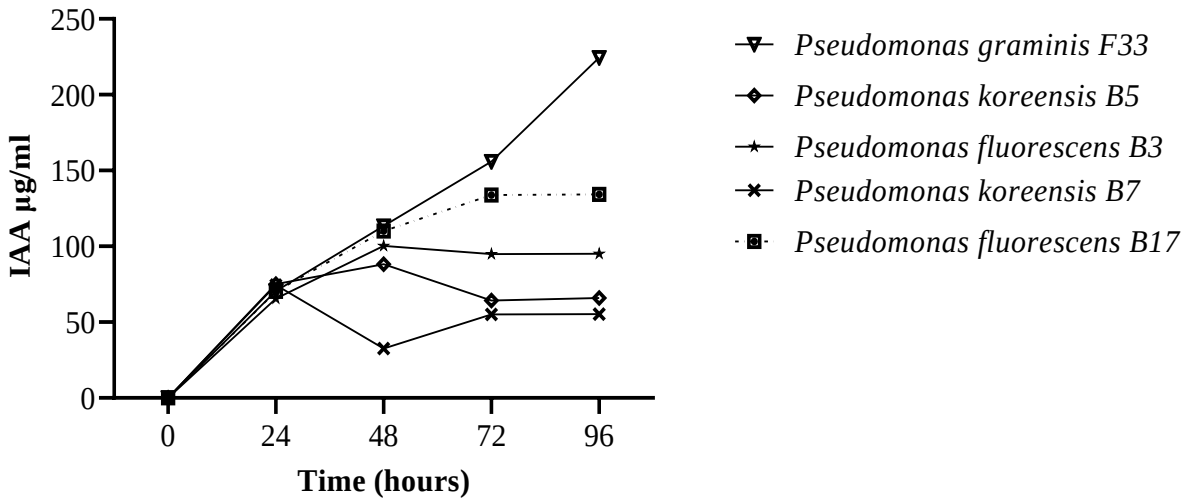

Figure 7. Bacterial behavior of IAA production over time of the strains (group C).

Studies in *Helianthus Annuus* shows that *Pseudomonas koreensis* has the ability to produce IAA in high quantities and is also an important factor in the ability it gives plants to withstand periods of drought (Macleod, Rumbold, & Padayachee, 2015). Although *Pseudomonas graminis* is known to have antagonistic ability against pathogenic bacteria, in this study it was also shown to be potential in the production of IAA (Alegre et al., 2013). The *P. fluorescens* species extracted from the rhizosphere of *Triticum* sp. have shown great IAA production ability (Meliani, Bensoltane, Benidire, & Oufdou, 2017).

In the next group evaluated, the most efficient strains were *Pseudomonas koreensis B11* with a recorded maximum of 264.81 µg/mL at 96 h and *Pseudomonas koreensis B11* with an expressed maximum of 229.57 µg/mL at 96 h. The *Pseudomonas moraviensis B6* strain showed a production of 73.67 µg/mL at 24 h, a decrease at 48 h and then and then began a slight rise. The strain *Pseudomonas palleroniana B10* presented its peak with a production of 105.41 µg/mL at 48 h and a slight decline at 72 h and a slight rise at 96 h (Figure 8).

Pseudomonas moraviensis B6 compared with the rest of *Pseudomonas* sp. used in this study, is the one with the lowest potential in IAA production. Nonetheless, its high capacity to reduce saline stress has been determined in *Triticum aestivum* where its ability to produce IAA is part of that function (Ul Hassan & Bano, 2015). *Pseudomonas palleroniana* has been used for research on drought tolerance in *Eleusine coracana* showing positive results (Chandra, Srivastava, Glick, & Sharma, 2018). Drought resistance is related to the production of phenols, when this happens the degradation of IAA begins, for this reason points in the graphs are observed where *P. palleroniana* and *P. moraviensis* reduce IAA production.

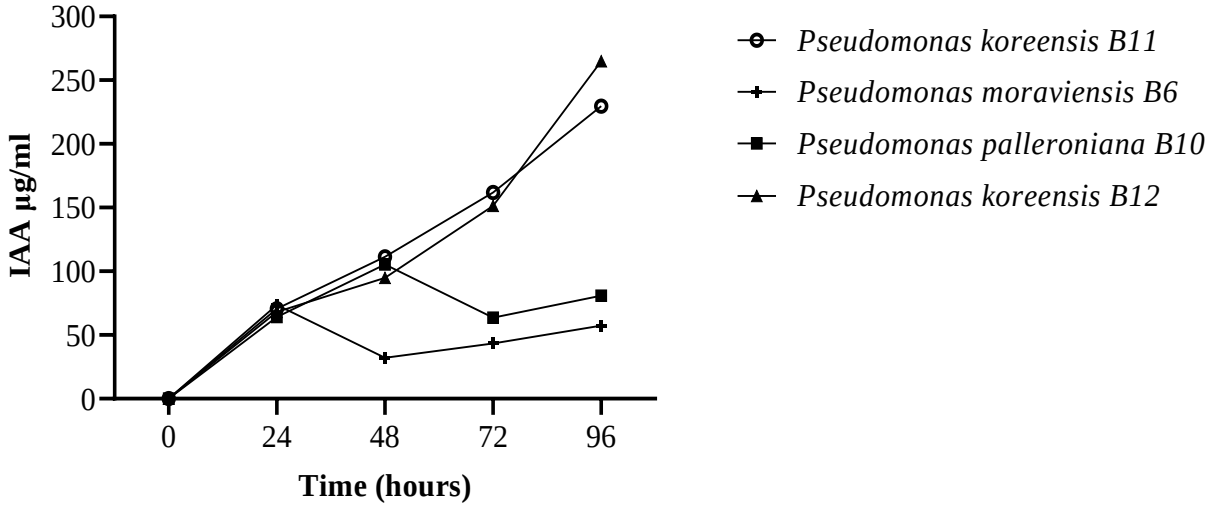

Figure 8. Bacterial behavior of IAA production over time of the strains (group D).

Screening phosphate solubility in plant growth promoting bacteria

All 14 isolates from *Bacillus, Enterobacter, Frigoribacterium, Kocuria* and *Pseudomonas* were analyzed for their potential to produce acids to solubilize insoluble phosphorus, the bacteria was inoculated into the NBRIP-BPB culture medium (Mehta & Nautiyal, 2001). The *Pseudomonas graminis F33, Pseudomonas koreensis B11* and *Pseudomonas koreensis B12* strains do not tolerate the NBRIP-BPB medium, consequently there was no growth of the strains. All the strains screened showed positive results and were classified as Minerals Phosphate Solubilizers (MPS).

Phosphorus solubilizing bacteria are capable to convert insoluble phosphorus into a bioavailable, through solubilization and mineralization processes (Behera et al., 2017). In this study, tricalcium phosphate ($Ca_3(PO_4)_2$ (Xiao, Chi, He, & Zhang, 2009) was used as a source of phosphorous in the medium. Insoluble phosphates are converted into soluble form through one of the processes of acidification, chelation, reaction exchange, and production of organic acids by phosphate solubilizing microorganisms (PSMs) (Chung et al., 2005). During the phosphate solubilization, various organic acids such as malic acid, lactic acid and acetic acid are part of the process (Behera et al., 2017).

The solubilization of insoluble phosphoric compounds is also intervened by the action of phosphatase enzymes called acid phosphatase (Coleman, 1992). Acid phosphatase is found in the cell wall of bacteria cells and in the polymeric extracellular substance surround it (Mohamed, Farag, & Youssef, 2018). The role of blue bromophenol in NBRIP-BPB medium is to change the intensity of the medium's color, as a reaction to organic acids and enzymes produced by bacteria. This means that when measuring the supernatant of the NBRIP-BPB medium at 630nm (under the technique of liquid spectrophotometry), if a reduction in the intensity of reflectance is registered, it is an indicator of organic acids and enzymes present (Mehta & Nautiyal, 2001).

Based on the results of this study, the *Bacillus subtilis C5B* strain is very efficient in the production of organic acids, reaching a reflectance reduction of 53.57% at 120 h of inoculation. All the strains

evaluated the group A (Figure 9), showed an increasing behavior in the reduction of reflectance. The *Enterobacter* sp. *B4* and *Pseudomonas koreensis B5* presented very close values in reflectance reduction at 120 h, 33.57% and 31.53% successively. The *Frigoribacterium* sp. *B7* strain presented very low yield with 6.98% reduction in reflectance at 120 h (Figure 9). In the figure, the X axis represents the percentage reduction in the reflectance of the visible spectrum OD630nm due to the presence of organic acids and enzymes in the medium. A control consisting of measuring NBRIP-BPB without inoculating bacteria in it was used as a basis.

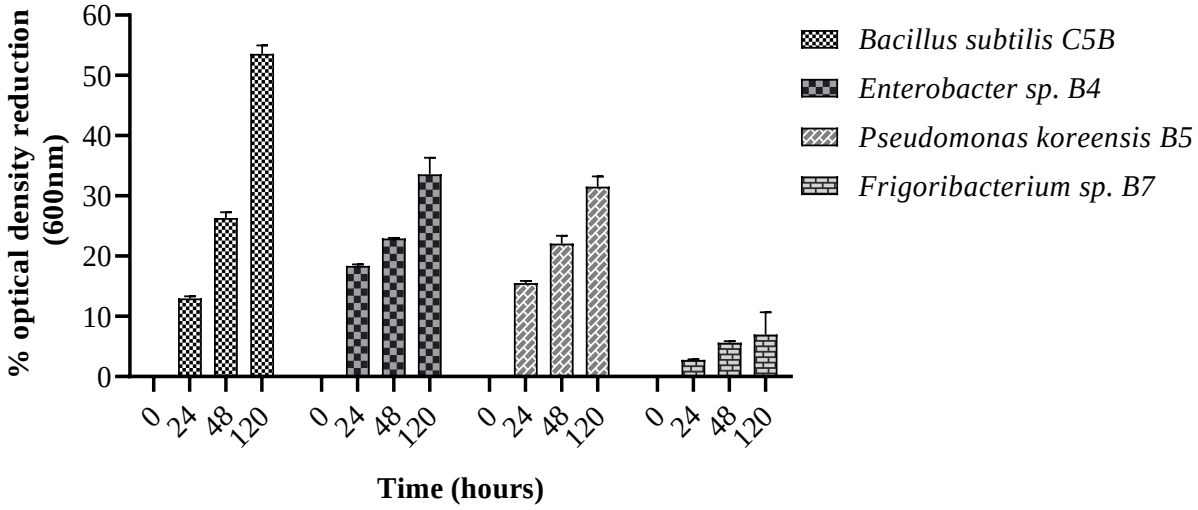

Figure 9. Evolution in time of the reduction of the optical density in the 630nm spectrum in the NBRIP culture medium (group A).

The *Bacillus subtilis* strain, coming from the rhizosphere of rice crops in the soils of Tamil Nadu, India; evidence to be capable to solubilize phosphorus and with better demonstrated ability compared to *Pseudomonas* sp. (Sivasakthi, Kanchana, Usharani, & Saranraj, 2013). The *Bacillus subtilis C5B* strain screened in this study proved efficient at solubilizing phosphorus with a percentage decrease in OD630nm of 54% measured at 120 hours of incubation. The *Enterobacter* sp. *B4* demonstrated a 34% reduction in reflectance measured at 120 hours of incubation, indicating that it is efficient in solubilizing insoluble phosphate molecules. Studies show that the *Enterobacter* species plays a very important role in the release of phosphates and iron in alkaline soils (Gyaneshwar et al., 1999).

In the group B, all the strains except *Pseudomonas koreensis B7*, showed increasing reduction in reflectance over time. The *Pseudomonas fluorescens B3* strain was the one with the best results in this group with a reflectance reduction of 30.61% at 120h. The *Pseudomonas moraviensis B6* strain registered a reflectance reduction of 25.55% at 120 h. Of this group, the *Kocuria rizophila B3* strain was the one with the lowest yield, with a reflectance reduction of 9.21% at 120h (Figure 10).

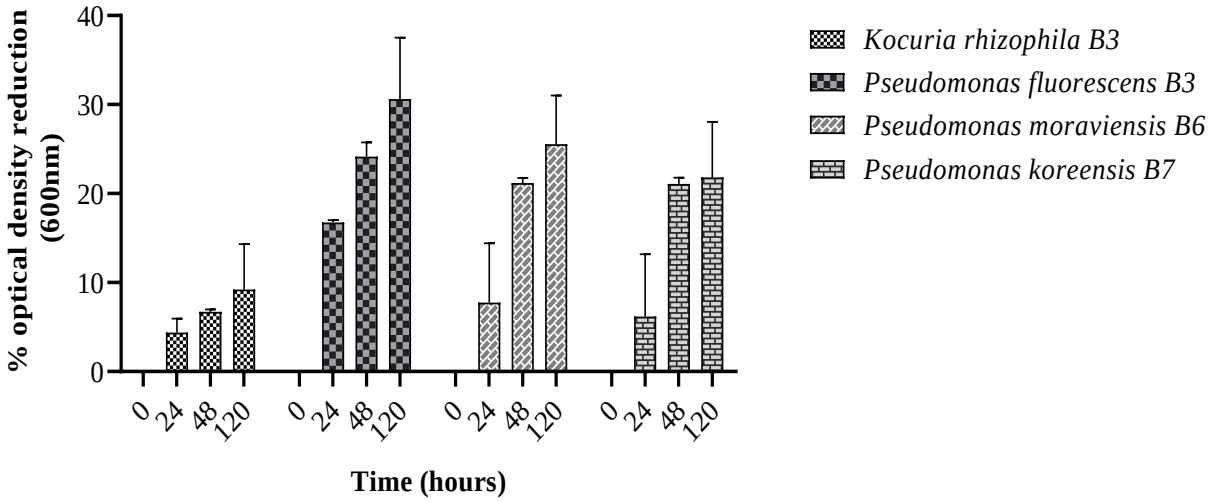

Figure 10. Evolution in time of the reduction of the optical density in the 630nm spectrum in the NBRIP culture medium (group B).

In this study, all *Pseudomonas fluorescens* strains showed ability to solubilize phosphorus. The *Pseudomonas fluorescens B17* strain was the most effective with a reflectance reduction of 46% measured at 120 hours of incubation. Studies in Korea show that strains of *Pseudomonas fluorescens* extracted from ginseng rhizosphere are proven phosphorus facilitators for the plant (Park, Lee, & Son, 2009). Moreover, the species *Pseudomonas koreensis* has also been shown to have a high potential for phosphorus solubility, such as the results obtained in this study (Gusain, Kamal, Mehta, Singh, & Sharma, 2015).

In group C, strains *Bacillus safensis B23* was the most effective with a reflectance reduction of 50.06% at 120 h. Followed by the *Pseudomonas fluorescens B17* strain with a reflectance reduction of 46.19% at 120 h. The *Bacillus simplex B19* strain with a relatively low reflectance reduction of 25.69% at 120 h. The *Pseudomonas palleroniana B10* strain registered its highest reflectance reduction of 24.69% at 48 h and a reduction to 9.83% at 120 h. *P. Palleroniana B10* it is the only strain in this study that exhibited this strange behavior that could not be explained in this study (Figure 11).

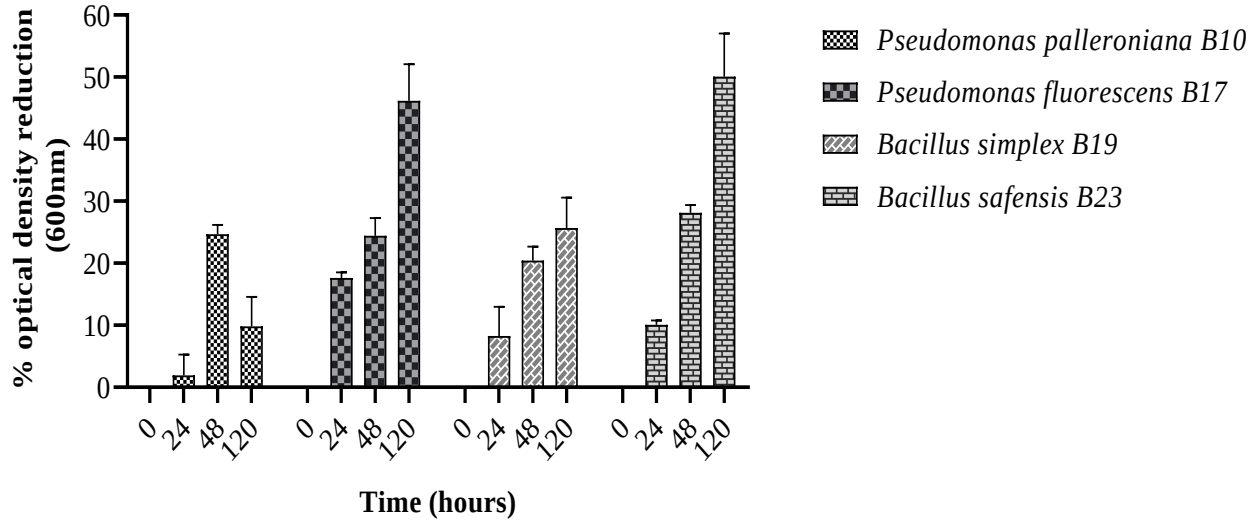

Figure 11. Evolution in time of the reduction of the optical density in the 630nm spectrum in the NBRIP culture medium (group C).

The *Bacillus safensis* species has demonstrated ability in reduction and oxidation processes in metals present in wastewater Treatment Plant (Shafique, Jawaid, & Rehman, 2017). It could not be verified on this occasion whether the organic acids and enzymes it produces are directly related to the phosphate solubility. Nonetheless, making a general analysis and knowing that the source of *Bacillus safensis* evaluated in this study is the *Solanum lycopersicum* rhizosphera; it can be deduced that it is directly related to the phosphate solubility. The *Pseudomonas palleroniana B10* strain showed a decrease in reflectance at 120 hours, the reason was not evaluated in this study. According to studies this can be an effect of the molecular change of the medium compounds (Behera et al., 2017) that cause a change in the reflectance spectrum hence the 630 nm spectrum did not detect that color change correctly.

Of all the strains evaluated in the study, the one with the best performance was *Bacillus aryabhattai B29*, with a behavior of increasing reflectance reduction and with a maximum recorded at 120 h with a 71.03% of reduction in reflectance. Then, the *Bacillus subtilis B25* species showed low efficiency, being its highest record at 120 h with a reflectance reduction of 16.34% (Figure 12).

In this study, the *Bacillus aryabhattai B29* strain proved to be the best producer of organic acids and phosphate solubilizing enzymes, compared to the rest of the strains screened in this investigation. Researchs has confirmed that this strain is an excellent promoter of plant growth (Lee, Ka, & Song, 2012). Based on these statements and knowing that the source of this strain is the *Solanum lycopersicum* rhizosphere, it can be attributed that they also have a high capacity to solubilize phosphates.

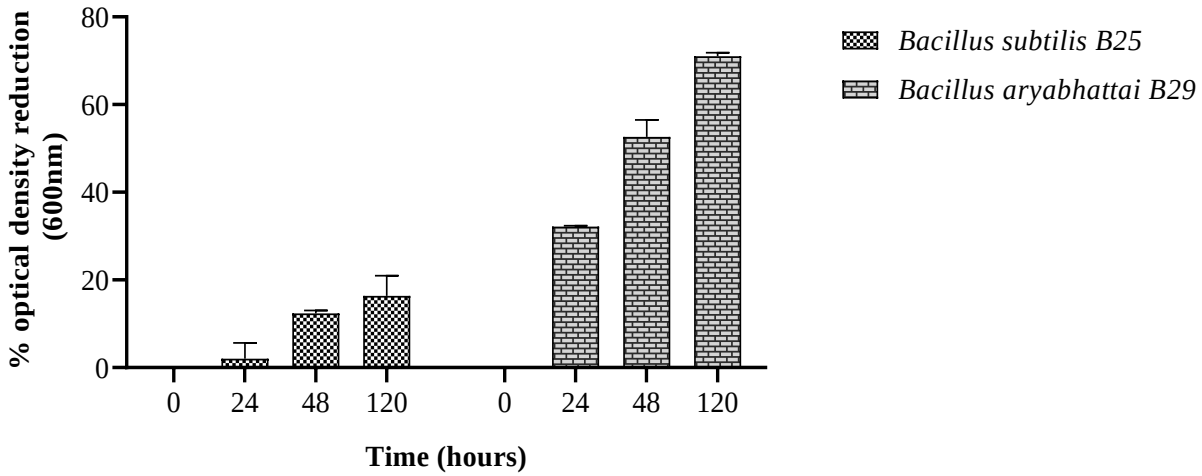

Figure 12. Evolution in time of the reduction of the optical density in the 630nm spectrum in the NBRIP culture medium (group D).

4. CONCLUSIONS

- The strains of *Pseudomonas* sp. determined to be the most efficient producers of indole-3-acetic acid, compared to the strains of *Bacillus* sp., *Enterobacter* sp., *Frigoribacterium* sp. and *Kocuria* sp. Nonetheless, all the strains screened in this study proved to be potential producers of indole-3-acetic acid.

- The strain *Bacillus* sp. proved to be the most efficient producers of organic acids and phosphate solubilizing enzymes, compared to the strains of *Enterobacter* sp., *Frigoribacterium* sp., *Kocuria* sp. and *Pseudomonas*. Notwithstanding, all the strains screened in this study proved to be potential producers of organic acids and phosphate solubilizing enzymes.

5. RECOMMENDATIONS

- Identify the chemical compounds produced by bacteria in the NBRIP medium. This in order to know what are the organic acids and enzymes that bacteria produce to solubilize phosphates.

- Set up plant tests with inoculation of indole-3-acetic acid producing bacteria. Measure response variables in plants that determine the impact they have on plant development.

- Measure the soluble phosphorous present in the medium before inoculation and its evolution over time. Make a comparison of the amount of free phosphorous at the beginning and at the time determined as the end of the measurements.

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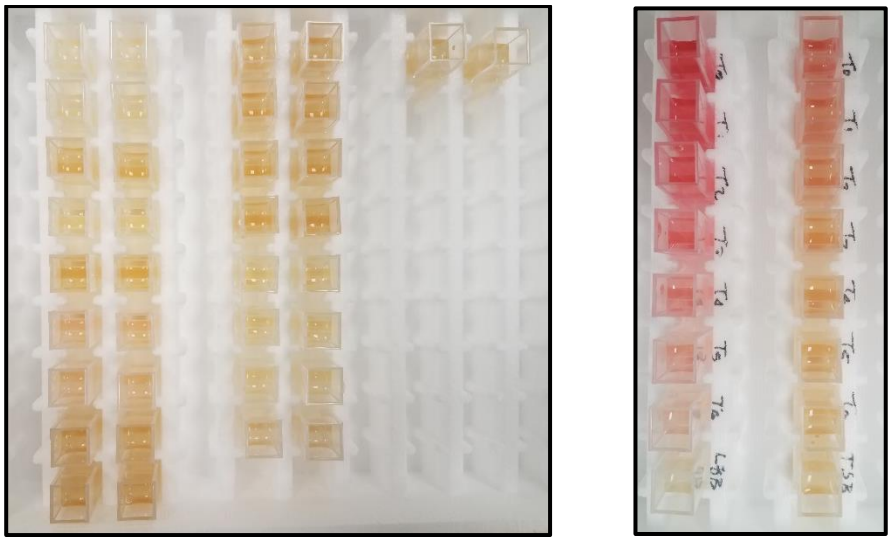
7. APPENDICES

Appendix 1. Average of OD_{530nm} measurements of duplicate bacterial cultures in medium with L-Tryptofano at different measurement times.

			Average (duplicate)			
Genus	**Specie**	**Code**	**24h**	**48h**	**72h**	**96h**
Bacillus	*Subtilis*	*C5B*	0,2034	0,2737	0,3185	0,2837
Enterobacter	sp.	*B4*	0,2111	0,2173	0,2252	0,0000
Frigoribacterium	sp.	*B7*	0,0000	0,1979	0,2179	0,0000
Kocuria	*Rhizophila*	*B3*	0,0000	0,1969	0,2099	0,0000
Bacillus	*Simplex*	*B19*	0,0000	0,1959	0,1926	0,0000
Bacillus	*Safensis*	*B23*	0,2021	0,2061	0,2017	0,0000
Bacillus	*Subtilis*	*B25*	0,2195	0,2939	0,3654	0,2570
Bacillus	*Aryabhattai*	*B29*	0,0000	0,1980	0,2007	0,0000
Pseudomonas	*Graminis*	*F33*	0,1241	0,1958	0,2668	0,3814
Pseudomonas	*Koreensis*	*B5*	0,1319	0,1537	0,1137	0,1167
Pseudomonas	*Koreensis*	*B11*	0,1243	0,1922	0,2768	0,3898
Pseudomonas	*Fluorescens*	*B3*	0,1157	0,1741	0,1647	0,1654
Pseudomonas	*Moraviensis*	*B6*	0,1294	0,0601	0,0788	0,1023
Pseudomonas	*Koreensis*	*B7*	0,1307	0,0608	0,0983	0,0989
Pseudomonas	*Palleroniana*	*B10*	0,1137	0,1824	0,1128	0,1416
Pseudomonas	*Fluorescens*	*B17*	0,1238	0,1902	0,2301	0,2305
Pseudomonas	*Koreensis*	*B12*	0,1203	0,1647	0,2593	0,4486

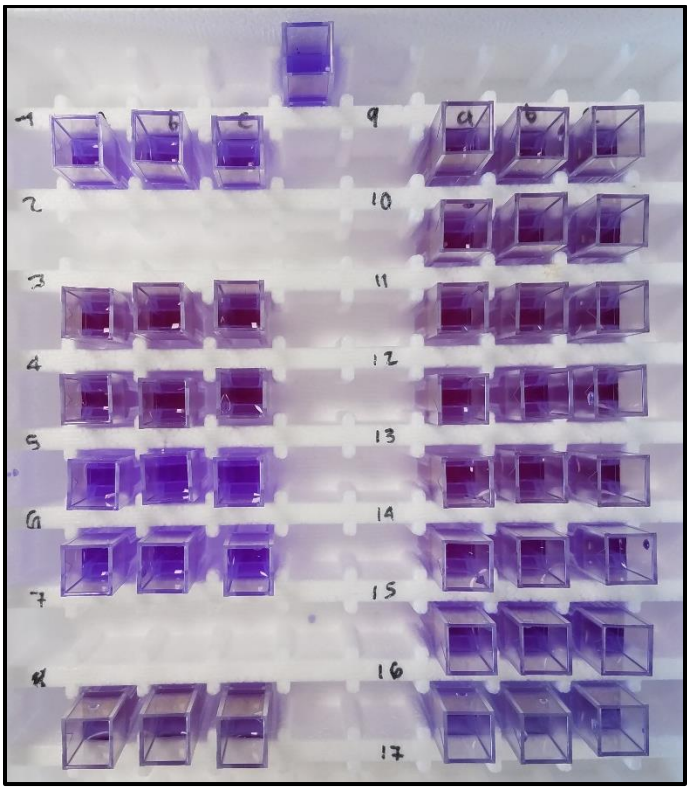
Appendix 2. Results on the right shows the difference in color gradient by the Salkowski reagent (duplicates) at 48 hours of inoculation. Results on the left shows the color gradient at different IAA concentrations used for the calibration curve. The order of the duplicates is the same as shown in the previous table in descending order.



Appendix 3. Average of OD600nm measurements of triplicates bacterial cultures in medium NBRIP-BPB at different measurement times.

	Average (triplicates)		
Bacteria	**24h**	**48h**	**120h**
Control	**2,3564**	**2,3796**	**2,2702**
Bacillus subtilis C5B	2,0508	1,7530	1,0540
Enterobacter sp. *B4*	1,9231	1,8347	1,5082
Pseudomonas koreensis B5	1,9909	1,8539	1,5543
Frigoribacterium sp. *B7*	2,2906	2,2453	2,1118
Kocuria rhizophila B3	2,2531	2,2199	2,0612
Pseudomonas fluorescens B3	1,9617	1,8052	1,5753
Pseudomonas moraviensis B6	2,1740	1,8754	1,6903
Pseudomonas koreensis B7	2,2109	1,8779	1,7749
Pseudomonas palleroniana B10	2,3966	1,7921	2,0470
Pseudomonas fluorescens B17	1,9414	1,7985	1,2215
Bacillus simplex B19	2,1616	1,8930	1,6870
Bacillus safensis B23	2,1188	1,7099	1,1337
Bacillus subtilis B25	2,3388	2,0853	1,8993
Bacillus aryabhattai B29	1,5986	1,1271	0,6577

Appendix 4. Color gradient differentiated according to the presence of organic acids and enzymes in the NBRIP-BPB medium. The order of triplicates is the same as the previous table in descending order.



Appendix 5. Collection of endophytic bacteria from the laboratory of HEPIA Plants and Pathogens cultivated and isolated in Petri.