

Evaluation of Bacillus and Pseudomonas Isolates from Soil as Plant Probiotics

Ekta Sehgal*

Department of Food Science and Technology CCS HAU, Hisar, India

Corresponding author: Ekta Sehgal, Department of Food Science and Technology, CCS HAU Hisar, India; Email: aku7ekta@gmail.com

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Abstract

Preparations of live microorganisms (bacteria, fungi) utilized for improving plant growth and crop productivity are generally referred to as biofertilizers or microbial inoculants Plant Growth Promoting Rhizobacteria (PGPR) are free-living, soil-borne bacteria, isolated from the rhizosphere, which, when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil-borne plant pathogens.

Keywords: Rhizobacteria; Bacillus, Bacteria, Fungi

Introduction

Pathogenic microorganisms affecting plant health are major threat to food production, and traditional methods *viz* crop rotation, breeding for resistant plant cultivars, and application of chemical pesticides, seem to be insufficient to control root diseases to important crop plants. Further, it appears inevitable that fewer pesticides will be used in future and that greater reliance will be laid on biotechnological applications including use of microorganisms as antagonists. Therefore, there arises a need to find alternatives of chemicals used for disease control and improving plant growth. *Bacillus spp.* expresses antagonistic activities by suppressing the pathogens [1-5].

Sample collection, preparation and analysis

Soil sample was collected from a village named Amlapur, lying in the vicinity of Karnal, India. Soil samples were collected in L.D.P.E. and then brought for *in vitro* works.

Isolation of bacterial strains

Bacterial strains to be isolated were pre-decided on the basis of I.A.A. producing capacity of the microorganism's i.e. *Pseudomonas fluorescens* and *Bacillus subtilis*. Isolation was achieved by serial dilution method. For serial dilution 1 gm of soil was dissolved in 10 ml distilled water. Seven test tubes each with 9 ml distilled water were prepared. One ml from stock solution was aseptically transferred to first test tube making the dilution 10⁻¹. Solution was then vortexed for proper mixing and 1 ml from this tube (10⁻¹) was transferred aseptically to the next test tube making it as 10⁻². Similar transfers were made till 10⁻⁹

dilution was achieved. From dilutions, 10⁻² to 10⁻⁹, were spreaded to sterile Medium Agar plates, The plates were then kept for incubation at 37 degrees Celsius for 48-72 hrs. Streak plate method and spread plate technique was used for the isolation of bacterial colonies. Selective medias were used to recognize the desired microorganism.

Different media used for isolation of bacteria

King's B media: It is the selective media for the isolation of *Pseudomonas fluorescens* and during pour plating it was used for the identification. After incubation, well separated individual colonies with green color pigments were marked and detected. The individual colonies were picked with sterile loop and transferred to fresh King B medium containing test tubes and the obtained pure cultures were stored in refrigeration at 4 degrees Celsius for further use.

Nutrient agar media: It was used for isolating *Bacillus* species using dilution. Rhizosphere soil sample was suspended in 99 ml of 0.85% normal saline (pH-7.0) and shaken vigorously at 150 r.p.m. at 18 degrees Celsius for 1 hr. The resulting slurry was serially diluted and then spread plating was performed on Nutrient Agar medium. The cultures obtained were incubated at 37 degrees Celsius for 2 days. For experimental use, isolates were transferred, when needed, to, Nutrient Agar medium stored at 4 degrees Celsius.

Morphological characterization of the obtained isolates: Morphological study of the isolates was done on a solid media. To observe cellular morphology cells were observed under microscope by making smear of the purified colonies selected.

Colony characteristics: Colony characteristics of strain like Colony Size, Shape, Opacity, Margin and Motility were studied on nutrient agar plate [6-12].

Gram staining: A bacterial smear was prepared and heat fixed on slide, the smear was flooded with crystal violet for 30 sec. Followed by a gentle wash with tap water. Then flooded the smear with Gram's iodine for 30 sec. And washed with tap water, kept the slide in slanting position and decolorized the stain with 90% ethyl alcohol by dropping the reagent drop-wise until the crystal violet fails to wash from the smear. Washed it with tap water and counter stain with safranin after 15 sec. And washed again with tap water and air-dried. The smear was examined under oil immersion in a light microscope. The Gram-

positive bacterial cells appeared violet while Gram-negative cells turned pink to red [13-19].

Biochemical tests

Pseudomonas fluorescens

Catalase test: A bacterial colony was picked from the plate and transferred on the glass slide in a drop of water. Few drops of H₂O₂ (dilute 30% commercial solution 1:10) was placed over the culture. Evolution of gas bubbles indicated the positive result.

Starch Hydrolysis: Nutrient agar medium was supplemented with 2% soluble starch. The isolated strains were streaked on the solidified starch agar plate and incubated at 37 degrees Celsius for 24-48 hrs. As growth appeared in the plates, iodine solution was flooded and clear zones against blue-black ground were observed [20-26].

Fluorescent pigment test: The test tubes containing sterilized Kings B medium were inoculated with the isolate of *Pseudomonas* species incubated for 5 days and observed. Yellowish green pigment observed under UV light (365 nm) indicated positive results.

Phosphate solubilisation: The plates were prepared with Pikovskaya's agar plate and phosphate solubilising activity was estimated after 4 days of incubation at 28 degrees Celsius. Phosphate solubilization activity was determined by the development of the clear zone around the bacterial colonies.

Methyl red and voges-proskauer test: Four MRVP broth test tubes were taken, three tubes (2 sets) were inoculated with P6, P7, P8 isolates and one as control respectively. All the tubes were incubated at 37 degrees Celsius for 48 hrs. And 5 drops of methyl red indicator were added into the inoculated tubes and observed for the color changes. To the control and second set of tubes added, 10 drops of VP-1 and 2-3 drops of VP-2 reagent. Observed for the color change.

Indole production: The Tryptone Broth was inoculated with bacterial strain and incubated at 28 degrees Celsius for 48 hrs. Kovac's reagent (1 ml) was added to each test tube including the control. Appearance of a cherry (deep red) coloured layer on the top of the tubes was taken positive test for the Indole production [27-32].

Biochemical tests

Bacillus subtilis

Catalase test: A bacterial colony was picked from the plate and transferred on the glass slide in a drop of water. Few drops of H₂O₂ (dilute 30% commercial solution 1:10) was placed over the culture. Evolution of gas bubbles indicated the positive result.

Starch hydrolysis: Nutrient Agar medium was supplemented with 2% soluble starch. The isolated strains were streaked on the solidified starch agar plate and incubated at 37 degrees Celsius for 24-48 hrs. As growth appeared in the plates, iodine

solution was flooded and clear zones against blue-black ground were observed [33-39].

Phosphate solubilisation: The plates were prepared with Pikovskaya's agar plate and phosphate solubilising activity was estimated after 4 days of incubation at 28 degrees Celsius. Phosphate solubilization activity was determined by the development of the clear zone around the bacterial colonies.

Methyl red and Voges-Proskauer test: Four MRVP broth test tubes were taken, three tubes (2 sets) were inoculated with P6, P7, P8 isolates and one as control respectively. All the tubes were incubated at 37 degrees Celsius for 48 hrs. And 5 drops of methyl red indicator were added into the inoculated tubes and observed for the color changes. To the control and second set of tubes added, 10 drops of VP-1 and 2-3 drops of VP-2 reagent. Observed for the color change [40-48].

Indole production: The Tryptone Broth was inoculated with bacterial strain and incubated at 28 degrees Celsius for 48 hrs. Kovac's reagent (1 ml) was added to each test tube including the control. Appearance of a cherry (deep red) coloured layer on the top of the tubes was taken positive test for the Indole production.

Result and Discussions

6 strains were isolated from soil sample from U.P and they were identified by morphological and biochemical characteristics.

Morphological and biochemical characterization of isolates

The following morphological and biochemical tests were performed for the identification of the isolates.

Gram staining: It is a method of differentiating the bacterial species into 2 large groups i.e. Gram positive (which retain the color of crystal violet) and Gram-negative (red or pink colored). Basis on the color obtained in the electron microscope, they are termed as Gram-negative or positive.

Catalase test: Catalase is an enzyme which prevents the cell from oxidative damage by reactive oxygen species like hydrogen peroxides. The presence of bubbles on reaction of bacterial species with hydrogen peroxide proves the test positive, otherwise negative [49-55].

Fluorescent pigment test: The test was done to check the fluorescence obtained under UV light study. The greenish yellow fluorescence was obtained in case of *Pseudomonas fluorescens*.

Indole test: The test was performed to determine the ability of an organism to convert tryptophan (an amino acid) into indole. The test was negative for both the strains.

MR-VP test: The test gives a good identification of microbial respiration and also confirms the differences between *E. coli* and *Enterobacter*. The positivity of test that organism is aerobic in nature [56-59].

Phosphate solubilisation: This test explains the ability of organisms to convert insoluble phosphates into soluble phosphates. More the phosphate solubilisation, more the ability of the organism towards higher yielding of growth promoting hormones for plants.

On the basis of Gram staining, 3 isolates (P1,P2,P3) were found to be Gram negative rods. Their cultural characteristics showed that the 3 isolates produced circular, convex and smooth colonies (Table 1).

The results of the biochemical tests (Table 3) performed for the identification of the isolates showed that P1,P2 and P3 isolates produced similar results with regard to gram staining (negative), indole (negative), MR (positive), VP (positive), catalase test (positive), and fluorescent pigmentation (positive). The morphological and biochemical tests confirmed the isolates to be *P. fluorescens* as reported by earlier workers [61-64].

According to Todar (2004), more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green

pigment, while the nonpathogenic saprophyte *P. fluorescens* produces fluorescent pigment that is soluble and greenish. In this study, all the three identified gram-negative *Pseudomonas* fluorescence isolates produced greenish yellow pigment on King's B media. This further confirmed the isolates to be *P. fluorescens*. All the strains showed moderate as well as fast growth [65-68].

All the isolates showed positive results in phosphate solubilization and IAA production.

The *Pseudomonas* spp. strains from rhizosphere of different crops were isolated and extensively studied by characterized *Pseudomonas fluorescens* on the basis of morphological, physiological and biochemical tests also isolated *Pseudomonas* strains from wheat rhizosphere and characterized by cultural and biochemical characteristics like production of IAA, catalase, phosphatase enzymes etc [69,70].

Table 1: Cultural characteristics of different isolates of *Pseudomonas fluorescens*.

Isolates	Cell shape	Colony type	Colony colour	Type of growth
P1	Rod	Round	Greenish yellow	Moderate
P2	Rod	Round	Greenish yellow	Fast
P3	Rod	Round	Greenish yellow	Moderate

The other 3 selected soil isolates B1,B2 and B3 were Gram positive bacilli arranged in chains. The colonies were round and creamish white showing fast to moderate growth (Table 2). The results of the biochemical tests performed for the identification of the isolates showed that B1,B2 and B3 isolates produced similar results with regard to gram staining (positive), indole

(negative), MR (positive), VP (positive), catalase test (positive), and white pigmentation [71].

Table 2: Cultural characteristics of different isolates of *Bacillus subtilis*.

Isolates	Cell shape	Colony type	Colony color	Type of growth
B1	Rod	Round	Creamish white	Fast
B2	Rod	Round	Creamish white	Moderate
B3	Rod	Round	Creamish white	moderate

Pseudomonas: *P. fluorescens* is a common gram-negative, rod-shaped bacterium. *P. fluorescens* has multiple flagella. It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. Optimal temperatures for growth of *Pseudomonas fluorescens* are 25-30 degrees Celsius.

Bacillus: *Bacillus* is a genus of gram-positive, rod-shaped, bacteria and a member of the phylum firmicutes. *Bacillus* species can be obligate aerobes, or facultative anaerobes. They will test positive for the enzyme catalase when there has been oxygen used or present.

Table 3: Biochemical characteristics of the isolates.

Parameters	P6	P7	P8	B1	B2	B3
Gram staining	Negative	negative	negative	Positive	positive	Positive
Catalase test	+	+	+	+	+	+

Fluorescent pigment	+	+	+	-	-	-
Indole	-	-	-	-	-	-
MR-VP	+	+	+	+	+	+
Phosphate solubilization	+	+	+	+	+	+

Parameter	P6 (<i>Pseudomonas</i>)	P7 (<i>Pseudomonas</i>)	P8 (<i>Pseudomonas</i>)	B1 (<i>Bacillus</i>)	B2 (<i>Bacillus</i>)
Gram stain					
Catalase test		Positive		Positive	positive
Methyl Red		Positive	positive		positive
Indole					
Starch	negative	Negative	negative		positive

Figure 1: Biochemical characteristics of the isolates.

• The bacteria might include systematic resistance in the host plant, so it can better resist attack by a true pathogen.

• The bacteria might outcompete other (pathogenic) soil microbes, e.g., by siderophores, giving a competitive advantage at scavenging for iron.

• The bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanide.

• They produce the secondary metabolite 2,4-diacetylphloroglucinol(2,4-DAPG), the compound found to be responsible for anti-phytopathogenic and bio-control properties in these strains.

In the present investigation with PGPR such as *P. fluorescens* and *B.subtilis* and it was observed that the seed germination seedling vigor, protein and carbohydrate content increased significantly. (1999) reported that *P.fluorescens* was increasing seed germination, vigor index and field emergence (Figure 2).

Qualitative analysis of Indole-acetic-acid (IAA production by the isolates: One of the principal mechanisms of promoting plant growth is related to the capability of the microorganisms to produce plant growth promoting substances. All the 6 isolates were tested for qualitative estimation of IAA. Among 6,4 isolates were found to be positive for IAA production. The results showed that *Bacillus subtilis* strains produced more pink color on addition of salkowski reagent than *Pseudomonas fluorescens* strains.



Figure 3: Results of Qualitative production of IAA.

Effect of *Bacillus subtilis* and *Pseudomonas fluorescens* on chickpea plant growth

Among the 4 positive IAA strains were selected to study their PGPR properties. Chickpea plants (*Cicer arietinum L.*) were inoculated with the bacterial cultures. After 15 days of planting,



Figure 2: Morphological and biochemical characteristics of *P.fluorescens* and *B.subtilis*.

Fluorescent *Pseudomonas* has been recognized as biocontrol agents against certain soil-borne plant pathogens. They produce yellow-green pigments (PYOVERDINES) which fluoresce under UV light and function as siderophores. They deprive pathogens of the iron required for their growth and pathogenesis (Figure 2).

It is not clear exactly how the plant growth-promoting properties of *Pseudomonas fluorescens* are achieved; theories include:

effect of *Bacillus subtilis* and *Pseudomonas fluorescens* culture supernatants on shoot length was determined. Both sterilized and unsterilized batches were used. The sterilized batch means that the soil was sterilized so as to show the impact of only the inoculated PGPR strains (Figure 3).

Shoot length (centimeters): The table gives the details of the growth obtained when the obtained strains were used to improve the shoot length of chickpea plant. Likewise; pictures

have been attached from Day 0 to Day 15. After 15 days, it was seen that the plant where *Bacillus* was used as PGPR had reached nearly 32.3 cm whereas the plant where *Pseudomonas* was used as PGPR had growth about 15.2 cm while their control reached 3.9 and 5.8 cm, respectively, thus proving the significance and the necessity of PGPR.

Table 4: Shoot length (centimeters).

Days	Bacillus control	Bacillus	Pseudomonas control	Pseudomonas
Nov 19, 2014 (day 1)	0 cm	1.7 cm	1.0 cm	2.5 cm
Nov 20, 2014 (day 2)	0 cm	2.8 cm	1.3 cm	3.2 cm
Nov 21, 2014 (day 3)	0.5 cm	6.5 cm	1.5 cm	4.5 cm
Nov 22, 2014 (day 4)	1.0 cm	9.0 cm	3.2 cm	4.7 cm
Nov 23, 2014 (day 5)	1.5 cm	11.0 cm	4.3 cm	7.0 cm
Nov 24, 2014 (day 6)	2.0 cm	12.1 cm	4.6 cm	7.8 cm
Nov 26, 2014 (day 7)	2.5 cm	13.0 cm	4.9 cm	9.1 cm
Nov 27, 2014 (day 8)	3.0 cm	13.2 cm	5.2 cm	10.2 cm
Nov 28, 2014 (day 9)	3.2 cm	14.8 cm	5.3 cm	10.5 cm
Nov 29, 2014 (day 10)	3.5 cm	14.9 cm	5.3 cm	10.7 cm
Nov 30, 2014 (day 11)	3.6 cm	15.7 cm	5.4 cm	10.8 cm
Dec 1, 2014 (day 12)	3.7 cm	20.7 cm	5.5 cm	10.9 cm
Dec 2, 2014 (day 13)	3.8 cm	25.7 cm	5.6 cm	12.2 cm
Dec 3, 2014 (day 14)	3.8 cm	27.2 cm	5.7 cm	13.0 cm
Dec 4, 2014 (day 15)	3.9 cm	32.3 cm	5.8 cm	15.2 cm

Pictorial representation of plant growth for 15 days. Figure 4 showing growth of chickpea plant with the help of *Bacillus* spp. as PGPR

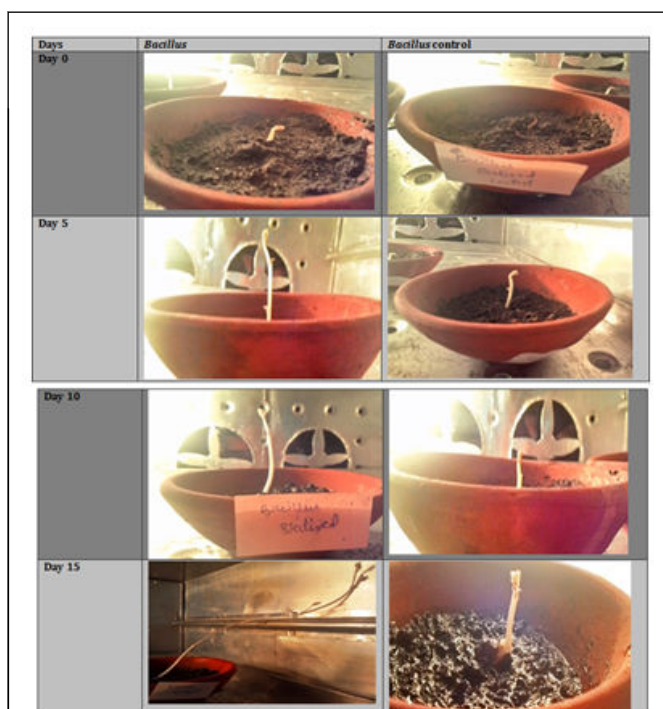


Figure 4: showing growth of chickpea plant with the help of *Bacillus* spp. as PGPR.

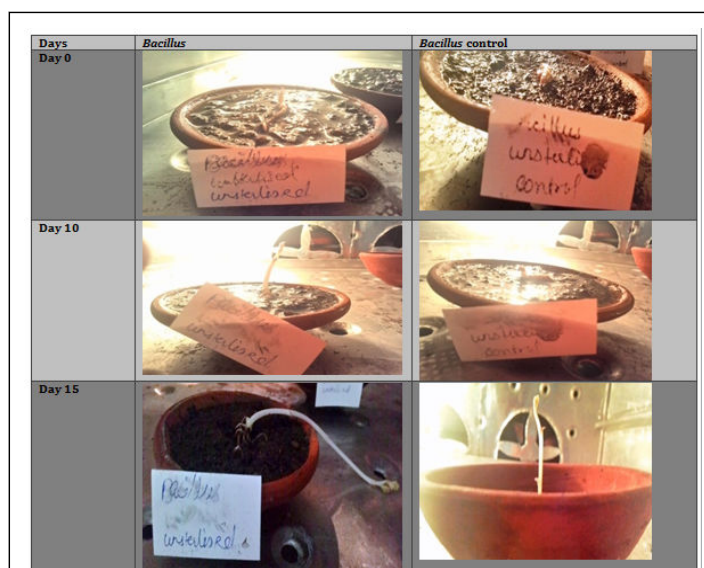
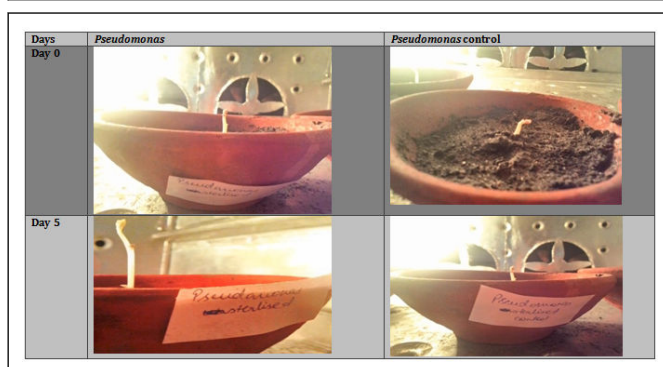


Figure 5: showing growth of chickpea with the help of *Pseudomonas fluorescens* as PGPR

Unsterilized batch: This means that the soil was having whole lot of micro lora including the obtained strains (Figure 4).

Shoot length (cm): It proved that the soil in which *Bacillus* was added as PGPR again showed improved growth (16.4 cm) and the plant where *Pseudomonas* was added as PGPR also improved growth to nearly 6.6 cm which was applaudable on comparison with their controls i.e.2.2 cm and 5.5 cm, respectively (Figure 5).

Table 5: result of shoot length growth including other micro-organisms along with the isolated PGPR.

Days	Bacillus	Bacillus control	pseudomonas	Pseudomonas control
Nov 19, 2014 (day 1)	1.0 cm	1.0 cm	1.0 cm	1.0 cm
Nov 20, 2014 (day 2)	1.3 cm	1.0 cm	1.2 cm	1.2 cm
Nov 21, 2014 (day 3)	2.7 cm	1.0 cm	1.3 cm	1.3 cm
Nov 22, 2014 (day 4)	3.6 cm	1.0 cm	2.0 cm	1.8 cm
Nov 23, 2014 (day 5)	3.8 cm	1.1 cm	2.2 cm	2.5 cm
Nov 24, 2014 (day 6)	7.0 cm	1.2 cm	2.5 cm	3.2 cm
Nov 26, 2014 (day 7)	9.2 cm	1.3 cm	2.7 cm	4.7 cm
Nov 27, 2014 (day 8)	11.3 cm	1.4 cm	2.8 cm	4.9 cm
Nov 28, 2014 (day 9)	14.5 cm	1.4 cm	3.0 cm	5.6 cm

Nov 29, 2014 (day 10)	15.0 cm	1.4 cm	3.2 cm	5.8 cm
Nov 30, 2014 (day 11)	15.0 cm	1.5 cm	3.5 cm	6.0 cm
Dec 1, 2014 (day 12)	15.1 cm	1.6 cm	3.8 cm	6.1 cm
Dec 2, 2014 (day 13)	16.0 cm	1.8 cm	4.0 cm	6.2 cm
Dec 3, 2014 (day 14)	16.2 cm	2.0 cm	4.2 cm	6.3 cm
Dec 4, 2014 (day 15)	16.4 cm	2.2 cm	5.5 cm	6.6 cm



Figure 6: Showing growth of chickpea plant with the help of *Bacillus* spp. as PGPR along with other soil microflora.

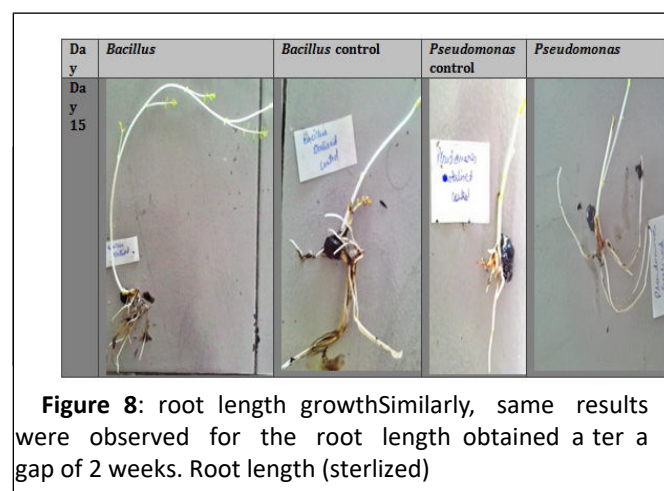


Figure 8: root length growth Similarly, same results were observed for the root length obtained a ter a gap of 2 weeks. Root length (sterilized)

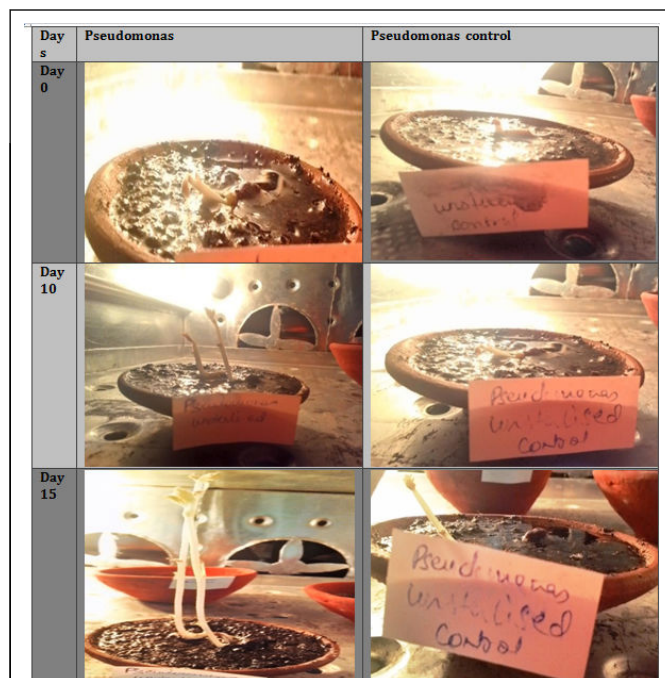


Figure 7: Showing growth of chickpea plant with the help of *Pseudomonas fluorescens* as PGPR along with other soil microflora.

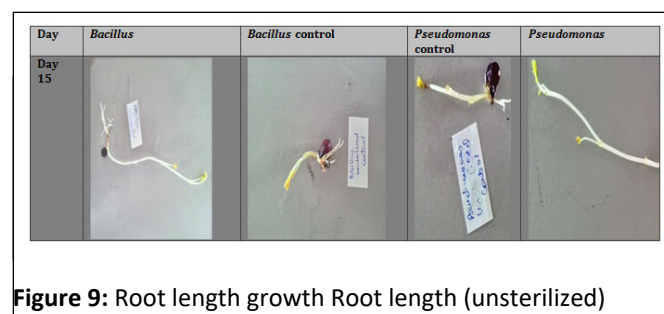


Figure 9: Root length growth Root length (unsterilized)

In all the test plant species, both the inoculants enhanced growth that was comparatively greater than control, Among the inoculants, difference was not that much statistically significant, although the highest mean shoot length for chickpea plant treated with *Bacillus* spp. was highly greater than that of *Pseudomonas fluorescens* as inferred from the obtained graph which is as follows.

Graphical representation of shoot length growth over 15 days.

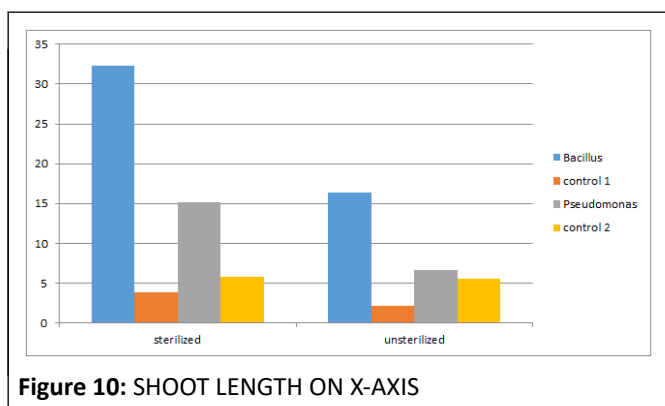


Figure 10: SHOOT LENGTH ON X-AXIS

Discussion

The objective of this project was to isolate bacterial strains of *Bacillus* species and *P. fluorescens* to show their PGPR activity on chickpea plant's shoot length and finally on the root length. The two isolated strains possessed multiple PGP traits such as catalase production, phosphate solubilization, production of IAA like compounds and also antagonism to soil borne pathogens. (Figure 7)

Coming to *Bacillus* species identification which was obtained as flat, dull, dry, creamish-white lobate colonies with optimal growth temperature range of 27-35 degrees Celsius (T1). While *P. fluorescens* was identified as round, rod-shaped, greenish yellow colonies with optimal growth temperatures ranging from 25-30 degree Celsius (T2). (Figure 8),(Figure 9)

Biochemical tests included:

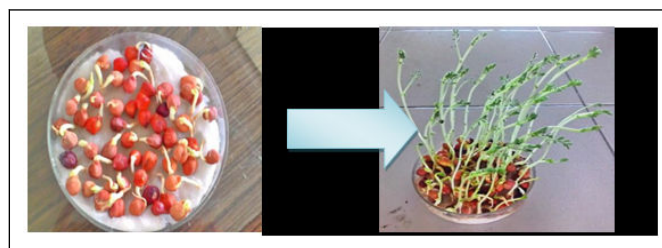
- Gram staining: out of the isolated bacteria *P. fluorescens* was gram negative and *Bacillus* was gram positive.
- Catalase test where bubble formation occurred in case of *P. fluorescens* while null was observed in case of *Bacillus* species. This indicates that *P. fluorescens* is capable of breaking down H_2O_2 into CO_2 and O_2 . This is done by it because of its property to produce catalase. Whereas *Bacillus* spp. did not produce catalase enzyme.
- *P. fluorescens* as per its name gave a greenish yellow fluorescence under UV light study.
- Indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into indole. This division is performed by a chain of a number of different intracellular enzymes, a system generally referred as "tryptophanase". Indole is generated by reductive deamination from tryptophan through the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium and energy. Pyridoxal phosphate is required as a coenzyme. The test was negative for both the strains indicating that they did not produce indole.
- MRVP was positive for both the strains proving that they are aerobic in nature. The methyl red test detects production of acid formed during metabolism using mixed acid fermentation pathway using pyruvate as a substrate. The pH indicator methyl red is added to one tube and a red color appears at pH's lower

than 4.2 indicating a positive test. The solution remaining yellow (pH=6.2 or above) indicates a negative test. The VP test uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2,3-butanediol fermentation pathway, after adding both reagents, the tube was shaken vigorously then allowed to sit for 5-10 mins. A pinkish-red color indicates a positive test meaning the 2, 3-butanediol fermentation pathway is used.

- Phosphate solubilization indicated that the organisms were able to convert insoluble phosphates into soluble phosphates (T4) gives the practical view of these *in-vitro* tests.
- The graphical representation indicates that the sterilized batch showed better shoot and root growth than the unsterilized batch. Therefore sterilized batch was taken into consideration in this *Bacillus* spp. provided growth up to 2.5 cm and *Pseudomonas* spp. up to 15.2 cm (Figure 6).

Conclusion

Our results confirm growth promotion by one representative each of both the species of bacteria (*Bacillus* spp. and *Pseudomonas fluorescens*) but only a little variation was observed in the bacterial effectiveness among the parameters (like shoot length and root length) and plant type. Considering shoot length, majorly, there were significant differences between the performances of *P. fluorescens* and *Bacillus* spp. Also, based on the results, among the representative organisms of the two genera in this study *Bacillus* is a better PGPR than *Pseudomonas*. Keeping in view, our work we recommend further comparative studies of the PGPR properties between many representatives of the two genera. (Figure 10).



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