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A Comprehensive Study on IAA production by *Bradyrhizobium japonicum* and *Bacillus subtilis* and Its Effect on *Vigna radiata* Plant Growth

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ABSTRACT

Background: The use of chemical fertilizers and pesticides raises concerns about environmental pollution, health hazards and the destruction of biotic groups that support plant growth. Plant growth-promoting rhizobacteria (PGPR) thrive in the rhizosphere of plants are the auspicious alternative for these chemicals. PGPR plays a critical role in plant growth and development, along with biocontrol activities.

Methods: In this present study, two effective microbes, *Bradyrhizobium japonicum and Bacillus subtilis* were chosen and their ability to produce Indole Acetic acid (IAA) was determined. Optimization of IAA production was carried out in different cultural conditions. Further, *in-vitro* studies were carried out to analyze the effect of these bacteria on the growth of *Vigna radiata*.

Result: Our investigations showed that both organisms have the potential to produce IAA under standard conditions. IAA production is maximum when using *Bradyrhizobium japonicum* with the supplement of Carboxymethyl cellulose and yeast extract as C and N source, respectively. L-Tryptophan concentration has a positive effect on production. Further, the application of bacterial cultures has shown more significant improvement in plant growth in terms of root and shoot length and weight of crop material. The current findings recommend that *Bradyrhizobium japonicum* can be a suitable organism for application as a plant growth promoter.

Key words: *Bacillus subtilis*, *Bradyrhizobium japonicum*, Indole acetic acid, PGPR, Pot assay.

INTRODUCTION

In the modern cultivation process, excessive use of chemical fertilizers has led to extensive contamination of soil, air and water. Unwarranted utilization of these agrochemicals exerts deleterious effects on soil fertility and pollutes the environment. To prevent this problem and obtain higher plant yields, replacement of agrochemicals is need of the day. In the direction of a sustainable agronomic vision, crops produced need to be equipped with disease resistance, salt tolerance, drought tolerance, heavy metal stress tolerance and better nutritional value. The above-desired crop properties can be fulfilled by using soil microorganisms (bacteria, fungi, algae, *etc*.) that increase the nutrient uptake capacity and water use efficiency. Among these potential soil microorganisms known as plant growth promoting rhizobacteria (PGPR) are the most promising agents (Singh and Singh, 2018).

Plant rhizosphere is an ecologically assorted niche and PGPR are the common inhabitants that colonize plant roots and enhance yield by producing plant growth regulators without environmental contamination (Ansari and Ahmad, 2018). PGPR can alter the stimulation of plant growth by direct or indirect mechanisms. Indirect effects are related to the production of metabolites such as antibiotics, Hydrogen Cyanide (HCN) and siderophores. Direct effects are dependent on the creation of plant growth regulators, improvement in plant nutrient uptake, or promote systemic resistance of the plant (Mohite, 2013; Walia *et al*., 2014). Many bacterial species belonging to *Enterobacter, Azospirillum, Alcaligenes, Acinetobacter, Arthrobacter, Bacillus sp., Bradyrhizobium, Erwinia, Flavobacterium, Pseudomonas, Rhizobium and Serratia* have already reported for their plant-growth-promoting and allied activities (Shahid and khan, 2018). They influence plant growth directly or indirectly, along with improvement of soil fertility by various growth-promoting activities, hence referred to as plant growth-promoting rhizobacteria, *i.e*., PGPR (Das *et al*., 2013). The mechanism of enhancing the plant growth and crop yield possibly through (a) synthesize of growth hormones (Indole acetic acid, gibberellic acid and cytokinin's) (b) production of ACC deaminase to reduce the level of ethylene in the roots (Shaharoona *et al*., 2006); (c) asymbiotic nitrogen fixation (d) antagonism against

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phytopathogenic microorganisms and (e) solubilization of mineral phosphates and mineralization of nutrients (Walia *et al*., 2014).

Indole Acetic Acid (IAA) is one of the most physiologically active auxins and acts as an essential signal molecule. It is a typical product of L-tryptophan metabolism, translocate carbohydrates during its synthesis, which can be produced by several microorganisms, including PGPR, in which it acts as a factor that promotes microbial growth. In its natural form, IAA, well known for its ability to regulate critical physiological processes such as cell growth, cell enlargement, cell division, root initiation, increased growth rate, phototropism, geotropism, apical dominance (Frankenberger and Arshad 1995) tissue differentiation and tactic responses (Etesami and Beattie, 2018). Recently, it's also been reported that the IAA has the potential to stimulate cell elongation, flowering, fruiting by increased osmosis and enhanced protein synthesis; instead, it delays the abscission (Raval and Saraf, 2020). The present study was undertaken for production, partial purification and detection of plant growth hormone using *Bradyrhizobium japonicum* and *Bacillus subtilis.* Further, optimization of various chemical factors was carried out for maximum production of IAA and the effect of these bacteria on the plant growth of green gram plant (*Vigna radiata*) was evaluated using pot assay.

MATERIALS AND METHODS

The cultures of *Bradyrhizobium japonicum* MTCC120 and *Bacillus subtilis* was procured in November 2017 from the Centre of Bioscience and Nanoscience Research (CBNR), Echanari and maintained on Yeast Extract Mannitol (YEM) agar medium stored at 4°C. The laboratory experiments were conducted at the PG and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore, India. Chemicals and media used in the present study were procured from Hi-media Laboratories, Mumbai, India.

IAA production and determination

The bacterial cultures were inoculated with 100 ml of sterilized YEM medium and covered to avoid loss of IAA secreted. The flasks were incubated at 28°C on a rotary shaker at 120 rpm for a period of 96 h. The production of IAA was determined using Salkowaski's method, as described by Gordon and Weber (1951). After incubation time, 5 ml of culture suspension was collected and centrifuged at 1000 rpm for 20 min. Then IAA was determined by adding 1 ml Salkowaski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) in the culture supernatant. The contents were mixed well and allowed to stand in the dark at room temperature for 30 min. An uninoculated liquid medium served as control. Spectroscopic absorbance measurements did quantitative assays of IAA at a wavelength of 536 nm.

Extraction and purification of IAA

Bacterial cultures cultivated in the YEM medium and the bacterial cells were separated by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5–3.0 with 1N HCl and extracted twice with double volumes of ethyl acetate. Ethyl acetate fraction was dried and evaporated in a rotary evaporator at 40°C (Patel and Saraf, 2017). The procedure repeated several times to ensure proper purification. The supernatant was dialyzed against distilled water for 24 hours and used for further studies.

Paper and thin layer chromatography of IAA

The extracted sample and the standard IAA applied to Thin Layer Chromatography (TLC) plates (Silica gel G plate, thickness 0.25 mm) and run by using a solvent system of 1-Propanol: water in the ratio of 8:2. The appearance of pink spots by spraying Salkowaski's reagent was observed and R*f* values compared with standard IAA (100 µg/ml) as a positive control.

Optimization of media and physical conditions for IAA production

Optimization of the carbon source, nitrogen source, tryptophan concentration was performed to achieve an enhanced yield of IAA by one factor at a time analysis. Glucose (1%) in YEM media was replaced with other carbon sources like sucrose, maltose and carboxymethylcellulose. Likewise, experiments were carried out by using beef extract, Ammonium sulfate and Yeast extract as a nitrogen source. The effect of L- tryptophan concentration was determined by supplying with different levels of L-Tryptophan (100, 200, 300, 400, 500 µg/L).

Assessment of plant growth-promoting activities

HCN production

Production of hydrogen cyanide (HCN) by the bacterial cultures was detected with the method used by Lorck (1948). Discoloration of the filter paper from orange to brown after incubation of 4-5 days indicated the production of HCN.

Ammonia production

The bacterial cultures were inoculated in peptone broth and incubated for three days at 28°C. Upon addition of 1 ml of Nessler's reagent, the development of yellow to the brownish-orange color indicated the positive test (Cappucino and Sherman, 1992).

Organic acid production

Secretion of the organic acid is an essential mechanism for PGPR to dissolve inorganic phosphorous in the soil. The bacterial cultures were screened for the organic acid production by methyl red test as per the procedure given by Cappucino and Sherman, (1992).

Phosphate solubilisation

Most of the PGPR organisms can solubilize phosphorous, which can be revealed through phosphate solubilization test. Log phase growing cells of each culture (10 µL) was spotted on Pikovskaya's medium plates (Pikovskaya, 1948). A zone of phosphate solubilization was observed after an incubation of 5 days at 28°C.

Siderophore production

Siderophore production was determined by the method of Schwyn and Neilands (1987) on the Chrome-azurol S (CAS) medium. The bacterial strains were streaked on CAS medium and incubated at 28°C for 48-72 h. The formation of orange to yellow halo around the colonies confirmed the production of siderophore.

Pot assay

To study the effect of IAA producing rhizospheric isolates on plant growth, pot assay was performed. The seeds of green gram *(Vigna radiata)* were taken and surface sterilized with 0.25% $HgCl_2$ followed by distilled water for 2-3 times. Then seeds were transferred to pots that contain soil. After then, the containers were left undisturbed for 2-3 days. Then 5 ml culture filtrate of *Bacillus subtilis* and *Bradyrhizobium japonicum* was directly dispensed into the seedlings separately; Standard IAA was dispensed in another pot, which serves as a control. Pots were sprayed with sterile distilled water every day and kept in sunlight. At the end of one week, the plants were uprooted and seedlings were measured for shoot length, root length, fresh weight and dry weight.

RESULTS AND DISCUSSION

Indole acetic acid production (IAA)

The results obtained from both qualitative and quantitative assays of IAA reflected the ability of microorganisms to produce IAA compounds. In the quantitative measurements, the highest value of IAA production was obtained by *Bradyrhizobium japonicum* followed by *B. subtilis,* as they produced (41±0.35 µg/ml) and (32±0.50 µg/ml) respectively under standard assay conditions after 72 h. Also, both *Rhizobium* sp. and *Bacillus subtilis* had been extensively explored as IAA producing bacteria (Shahid and Khan, 2018) for IAA production.

Confirmation of IAA production by paper and thin-layer chromatography (TLC)

Production of IAA was confirmed by paper chromatography by the appearance of pink spots. The results were presented in Fig 1. In the case of thin-layer chromatography, the chromatograms were developed and compared against the standards. The R*f* value for standard IAA was observed to be 0.46, whereas *Bradyrhizobium japonicum* and *Bacillus subtilis* shows an R*f* value of 0.90 and 0.67, respectively. The TLC results reported by Mohite (2013) has an R*f* value of 1.00 for extracted IAA samples.

Effect of carbon source

Glucose in the production medium was replaced by different sugars such as Sucrose, Mannitol and Carboxymethyl cellulose to select the most promising carbon source. The results observed the during optimization study was presented in Fig 2 and 3. In *Bradyrhizobium japonicum*

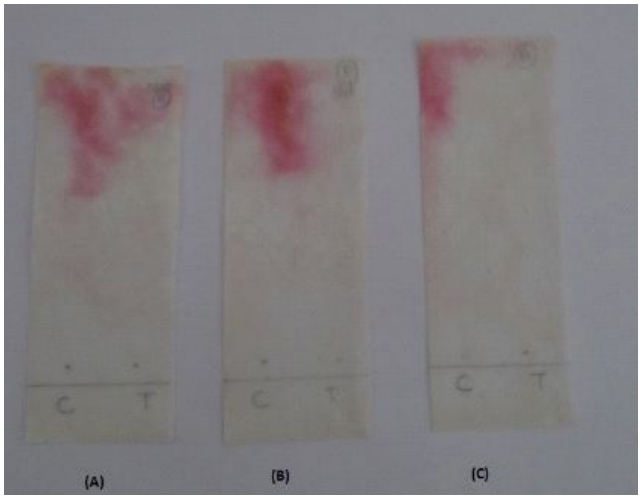

Fig 1: Confirmation of IAA production through paper chromatography. A) Standard IAA. B) *Bacillus subtilis.* C) *Bradyrhizobium japonicum.*

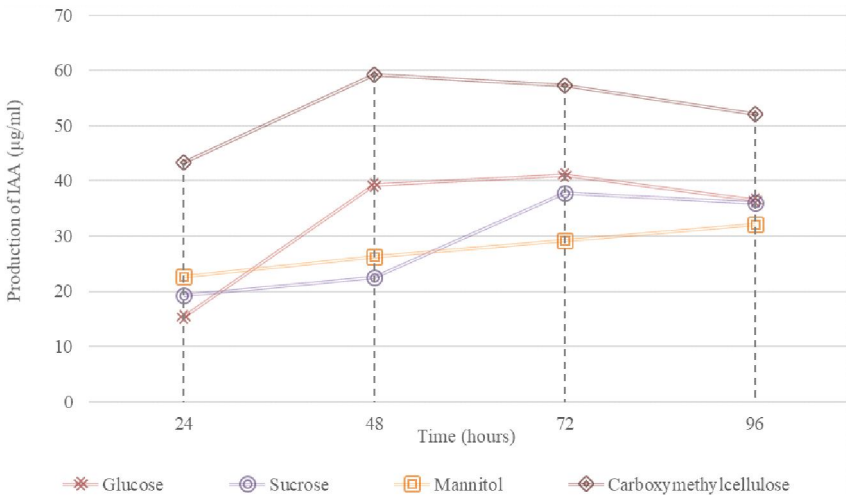

Fig 2: Effect of Carbon sources on IAA production by *Bradyrhizobium japonicum.*

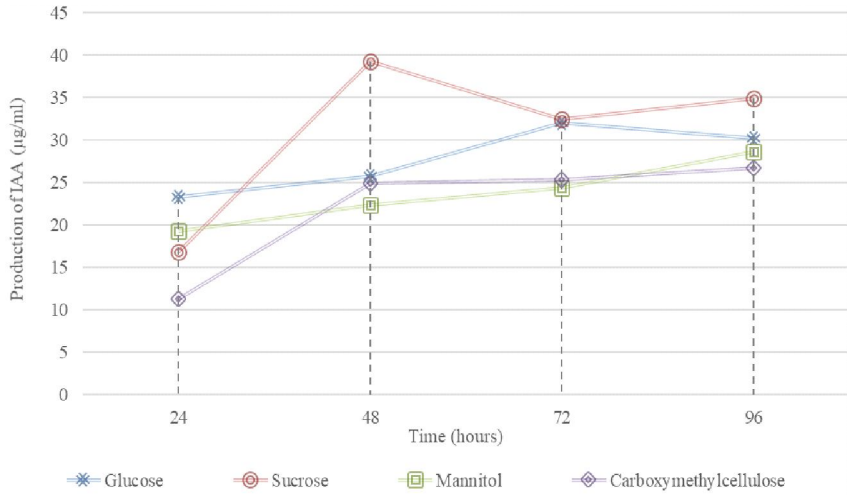

Fig 3: Effect of Carbon sources on IAA production by *Bacillus subtilis.*

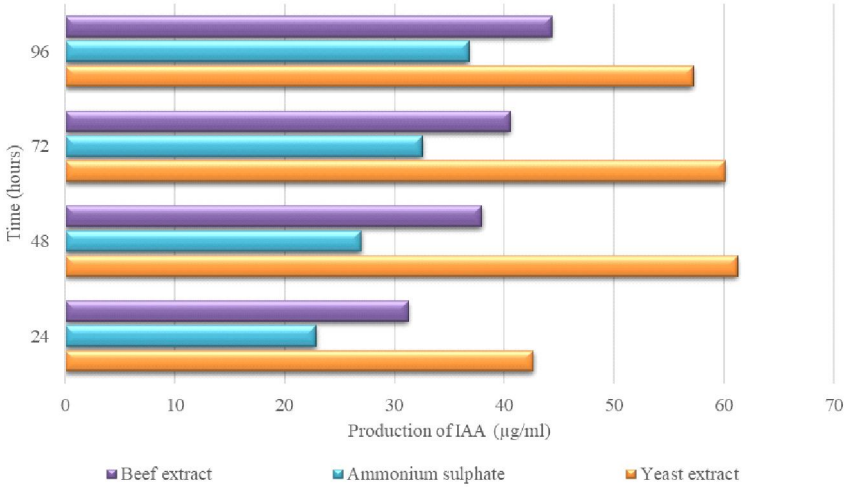

Fig 4: Effect of Nitrogen sources on IAA production by *Bradyrhizobium japonicum.*

culture production of IAA was seen to be lower in the case of Sucrose and Mannitol when compared with glucose. The most suitable carbon source was Carboxymethyl cellulose, with IAA production of 52.95 µg/ml in 48 h. Beyond 48 h of incubation, the concentration of IAA found to be reduced (Fig 2). At the same time, the results were different in the case of *Bacillus subtilis* culture. The maximum production of IAA was observed at the end of 48 h incubation with sucrose as a carbon source. However, the quantity of IAA produced was lower than the other culture. Other carbon sources showed no influence on the IAA production capacity of *Bacillus subtilis* (Fig 3). The carbon sources supplemented in broth media provide energy and improves co-factor recycling in the cells, thus contribute to the overall efficiency of IAA biosynthesis (Kumari *et al*., 2018).

Effect of nitrogen source

The impact of various nitrogen sources (yeast extract, beef extract and ammonium sulfate) was evaluated under the optimized carbon source for each organism (Fig 4 and 5). Yeast extract produced maximum IAA for both *Bradyrhizobium japonicum* (61.25 µg/ml) and *Bacillus subtilis* (48.74 µg/ml). *Bradyrhizobium japonicum* expressively utilizes yeast extract as a primary nitrogen source and IAA quantity reaches the optimum level in 48 h of incubation time. However, nitrogen sources showed different patterns of IAA production while using the *Bacillus subtilis* organism. *Bacillus subtilis* produces maximum IAA when using yeast extract as a nitrogen source (48.74 µg/ml) with an incubation of 48 h followed by beef extract (42.88 µg/ml) and Ammonium sulfate (38 µg/ml) with an incubation of 96 h.

Effect of L-tryptophan concentration

Tryptophan acts as a precursor for IAA biosynthesis in microorganisms. Bacteria also can produce indole in the absence of tryptophan, however, in a lesser amount. Different concentrations of L-Tryptophan were used in this study to assess the induced effect on IAA production under optimized carbon and nitrogen sources. The results perceived in our study were graphically shown in Fig 6 and 7. In general, it was observed that the quantity of IAA

increased with a rise in the concentration of L-Tryptophan. *Bradyrhizobium japonicum* showed maximum and minimum production of 78.33 and 29.23 µg/ml of IAA at 500 and 100 µg/ml of tryptophan concentration, correspondingly. Whereas, *Bacillus subtilis* produced 58.88 µg/ml at 500 µg/ml of tryptophan concentration. While considering the incubation period, both organisms show a higher level of IAA after the period of 48 h incubation, beyond which slight deterioration was observed. However, the variations in the IAA production at different concentrations of L-tryptophan by the bacterial cultures indicate the intrinsic ability towards IAA production. Several studies recorded the boosted

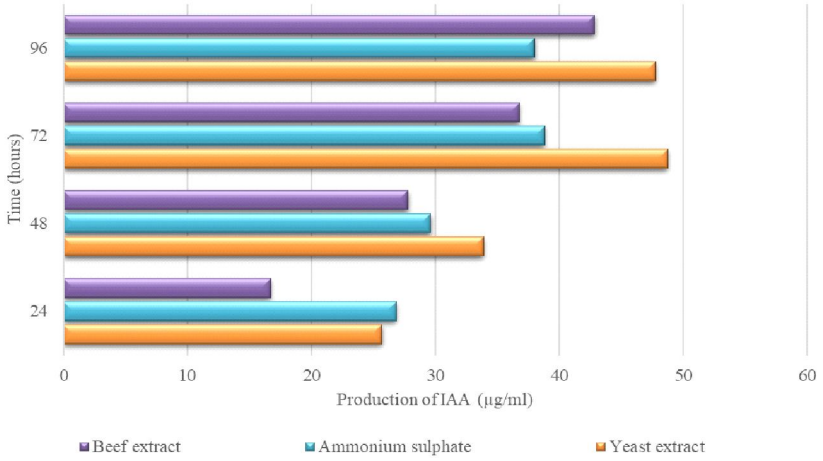

Fig 5: Effect of Nitrogen sources on IAA production by *Bacillus subtilis.*

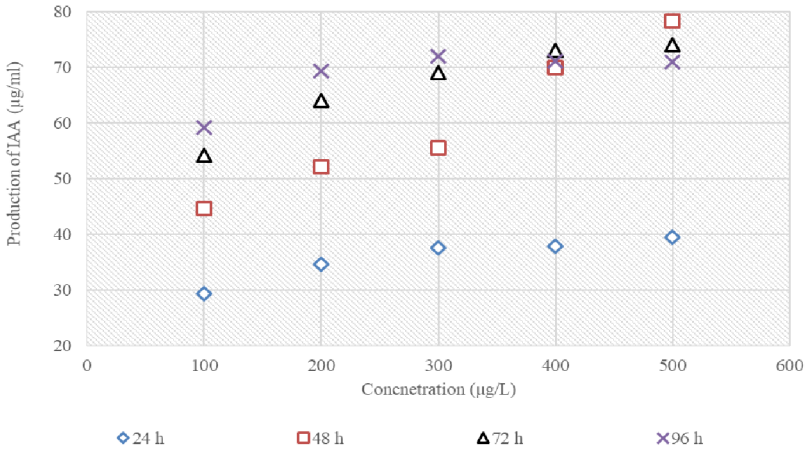

Fig 6: Effect of L-tryptophan concentration on IAA production by *Bradyrhizobium japonicum.*

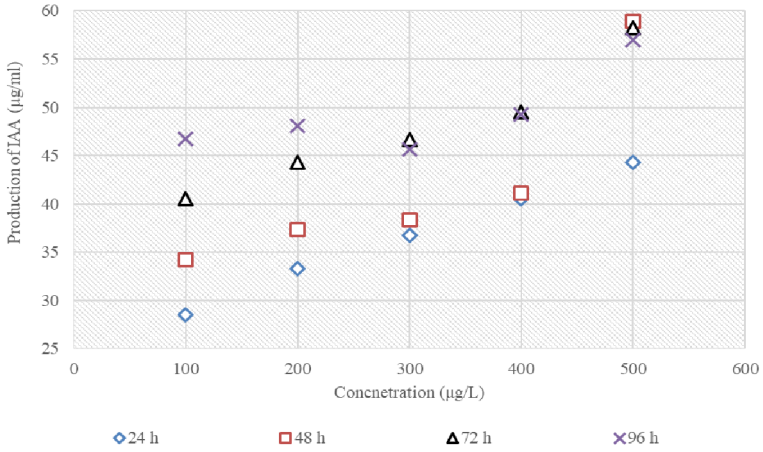

Fig 7: Effect of L-tryptophan concentration on IAA production by *Bacillus subtilis.*

Table 1: Plant growth-promoting activities of *Bradyrhizobium japonicum* and *Bacillus subtilis.*

Cultures	HCN production	Ammonia production	Organic acid production	Phosphate solubilization	Siderophore production
Bradyrhizobium japonicum	+	+	+	+	+
Bacillus subtilis	-	+	+	+	+

"-" indicates the negative result; "+" indicates the positive result.

Table 2: Effect of inoculum on growth parameters of green gram plant (*Vigna radiata*).

Samples	Root length (cm)	Shoot length (cm)	Fresh weight (g)		Dry weight (g)	
			Root	Shoot	Root	Shoot
Control	4.3±0.2	14.0±0.9	0.21	1.82	0.19	0.14
Standard IAA	6.2±0.4	18.1±1.3	0.62	3.44	0.46	0.35
Bradyrhizobium japonicum	5.9±0.3	16.9±1.0	0.48	2.96	0.41	0.29
Bacillus subtilis	5.2±0.3	15.7±1.2	0.44	2.27	0.36	0.25

production of IAA in the presence of tryptophan (Kumari *et al*., 2018). The decrease in IAA concentration after 48 h may be due to the release of IAA degrading enzymes such as IAA oxidase and IAA peroxidase, as reported by (Bhowmick and Basu, 1986).

Plant growth-promoting activities

Both cultures were further screened for other plant growth-promoting traits such as HCN production, Organic acid production, Ammonia production and Siderophore production. The results were tabulated in Table 1. The results of the current study are comparable to previous observations. The creation of hydrogen cyanide and ammonia (NH_3) are essential in plant growth-promoting activity of PGPR strains. Recently, their synergetic effect on plant growth, as well as modulation of plant metabolites, was reported by Agbodjato *et al*. (2015) and Kumar *et al.* (2016). Several researchers had reported that *Bradyrhizobium sp.* (Chaudhary and Sindhu, 2016; Ahemad and Khan, 2012; Shaharoona *et al.,* 2006) and *Bacillus sp.* (Saeid *et al*., 2018) can produce HCN, Ammonia and siderophores and also solubilize phosphate by organic acid production.

Growth augmentation in green gram plant

The effect of inoculum on growth parameters of green gram plant was shown in Table 2. Results of the pot trial study further justified the use of selected strains significantly improved the green gram plant growth and development under experimental conditions. *Bradyrhizobium japonicum* culture increased root length by about 37%, while *Bacillus subtilis* increase by 20.93% against control plants. Likewise, shoot length was found to be about 20.7% and 12.14 % higher for *Bradyrhizobium japonicum and Bacillus subtilis* isolates, respectively. However, the application of standard IAA showed a better growth rate than both the organisms. The stimulus of biological activities due to the consistent supply of nutrients may be attributed to the increase of plant height.

Inoculation of green gram with *Bradyrhizobium japonicum* and *Bacillus sp.* strains had a more significant impact on the increase in fresh and dry weight of the plant parts. Data presented in Table 2 compared the fresh and dry weight of root and shoot after treatment with bacterial culture with that of the control and standard IAA applied plants. Lowest dry weight for both root and shoot were observed in control plants. From the observations, it can be suggested that the application of Bacterial cultures can significantly increase plant growth. Approximately, about 116% of the increase in root dry weight was observed by injecting with *Bradyrhizobium japonicum* and 107% of enhancement in shoot dry weight observed when equated with control plants. Similarly, *Bacillus subtilis* enhances the dry weight of root and shoot by about 89.47% and 78.57% than the uninoculated plants. The relative water content of the root system was affected by the application of PGPR's, but there was no significant difference in shoot water content. These differences acquired in plant root systems mainly attributed to the existing soil conditions and the biological activity of the applied microbial strains. The use of PGPR strains results in higher nitrogen fixation, better acquisition of P and other nutrients through organic acid production, thereby increasing the plant growth.

CONCLUSION

Generally, chemical fertilizers and pesticides are effective and suitable in use for production and disease management of crops. Still, they are a potential hazard for the soil as well as human health. Therefore, the use of PGPR as biofertilizer and biocontrol agents is a sustainable method for agriculture due to their less toxicity and also has the capacity for crop improvement. The present study reveals that IAA produced by the individual bacterial cultures which enhance the plant growth, which was investigated through pot assay. Thus, these bacterial cultures can be used as bio-inoculants in the fields to improve crop productivity and to replace the chemical fertilizers. Further investigation is needed to know their synergistic relationship when they applied in areas as consortia.

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