Screening of IAA producing bacterial strains and influence of Its colonization on pulses seed germination

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***Abstract*—Bacteria colonizing plant roots are in many cases beneficial for plant growth, development, and productivity. The synthesis of phytohormone by such associated microorganisms is believed to be one of the major forms of host plant–microbial interactions. In the present study, bacteria were isolated from the rhizosphere soil of groundnut, cowpea and cotton field crops. Thirty-five bacterial strains positive for IAA production were isolated from the collected soil samples. Nutrient media and tryptophan concentrations were standardized and IAA content was analyzed by Salkowaski's reagent for the selected five bacterial strains (A10, A15, B3, C4 and C9). Maximum IAA producing two bacterial strains (A15 and B3) were screened for further studies. Selected pulses seeds were inoculated with two bacterial strains and kept for germination. Uninoculated seeds were considered as control. Seed germination ratio, length of roots, fresh weight and length of seedling was observed. Both selected strains showed Increase in length of seedling in pulses seeds compared to control. Effect of selected two strains on the pulses seeds showed positive response on seedling elongation, fresh weight & amylase activity after germination.**

***Index Terms*—IAA, PGPR, Amylase, Seedling growth**

I. INTRODUCTION

Soil is a natural habitat of agriculturally beneficial microorganisms. Rhizosphere is the layer of the soil influenced by the root, much richer in bacteria than the surrounding bulk soil. Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanismare referred to as PGPR (Plant Growth Promoting Rhizobacteria). PGPR can reveal a variety of characteristics responsible for influencing plant growth. The common traits include production of plant hormones (like auxin, gibberellin, andethylene), siderophores, HCN and antibiotics. [1] PGPR are free-living soil-borne organisms that colonize the rhizosphere, and when applied to seeds or crops enhance the plants growth or reduce the damage from soil-borne plant pathogens and increase yield. Generally, PGPR function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the environment, and protecting the plants against diseases [2] and referred as biofertilizers or microbial inoculants. [3]

The microorganisms isolated from rhizosphere region of various crop havean ability to produce Indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake efficacy. [4] IAA stimulates cell elongation by modifying certain conditions like, increase in osmoticcontents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase incell wall synthesis. It promotes embial activity, inhibitIt promotes embial activity, inhibit or delay abscissionof leaves, induce flowering and fruiting. [5]

The enhancement of plant growth by PGPR indicates their potential as biofertilizers in the field of agriculture. It has been estimated that more than 100 million tonnes of nitrogen, and phosphate-chemical fertilizers have been used annually in order to increase plant yield. [6] The potential negative effect of chemical fertilizers on the global environment and the cost associated with production has led to research with the objective of replacing chemical fertilizers with bacterial inoculants. Bacterial inoculants, which help in plant growth, are generally considered to be of two types - a) symbiotic and b) free-living. The rhizosphere bacteria promote plant

growth either directly or indirectly and increase the yield. [7] Considering to these the first objective of this study was isolate, screening the indole acetic acid producing bacteria from different rhizospheric soil and selection and optimization of Nutrient media with different concentrations of tryptophan. The secondwas tocolonization of selected PGPR bacteria on the seeds of several Indian staple food seeds (pulses) and estimated amylase activity.

II. MATERIALS AND METHODS

Sample collection and Isolation of bacteria

Rhizosphere soil from the following three field crop plants were collected from fields near the Rajkot city, Gujarat (A) Groundnut (*Arachis hypogaea*), (B) Cowpea (*Vigna unguiculata*) (C) Cotton (*Gossypium hirsutam*). Soil solution for each sample were prepared with sterile distilled water. Samples were diluted up to 10^{-6} and spreading was carried out on N-agar plates. The plates were incubated at 37˚C for 24 h in incubator for bacterial growth. Isolated bacterial colonies were screened based on their morphological characters and production of IAA. Isolated colonies were transferred regularly on N-agar slant to maintain culture conditions for the bacteria.

Screening of bacteria for IAA production

Isolated bacterial colonies were allowed to grow in Ashby's manitol broth containing L-tryptophan (5mM) for 3 days at 37˚C. After 3 days, IAA production was analyzed by Salkowaski's reagent (2ml of 0.5M $FeCl_3$ + 98ml 35% $HClO_4$) method. The broth was centrifuged at 3000 rpm for 20 min and the cell free supernatant was collected for estimation of IAA production. One ml supernatant was mixed with two ml of Salkowaski's reagent and tubes were incubated in dark. The intensity of red color developed within 30 min was measured at 530nm. Uninoculated growth medium was used as control.

Optimization of tryptophan concentration for selected media

Three different concentrations 5mM, 8mM, and 10mM of tryptophan were used for maximum of IAA production. This assay was carried out for to determine optimum concentration of tryptophan for higher IAA production by selecting bacteria. Non-inoculated growth medium was used as a control.

Media optimization

Different nutrient medium compositions Nutrient – broth, Luria-Bertani broth, Yeast Extract Mannitol broth, Ashby's Mannitol broth, Actinomycetes broth, King's-B broth were used to find out better medium for IAA production by selecting test organisms. Following six media containing different concentration of L- tryptophan (5 mM, 8 mM and 10mM) were inoculated with selected five bacterial strains.

Each isolated colony was inoculated in all above media and incubated at 37ºC for 10 days. IAA content estimated at interval of three days by Salkowski's reagent. Non-inoculated growth medium was used as a control.

Identification of organisms

Preliminary organisms were identified by gram staining. Further biochemical tests were performed to identify bacterial genus and species (HiAssorted™ Biochemical Test Kit, Himedia).

Treatment of organism on dicotyledonous seed germination

Different dicotyledonous seeds were selected Chickpea (*Cicer arietinum*), Green gram *(Vigna radiata*), Moth bean (*Vigna aconitifolia*) and Cowpea (*Vigna unguiculata*). All selected seeds were surface sterilized with 70% ethanol for 2-3 min and 3% hydrogen peroxide for 3 min followed by twice wash with sterile distilled water. Seeds were shocked for 5-6 hrs in sterile distilled water. Two selected bacterial strains (A15 and B3) producing maximum IAA level were allowed to colonize on seeds. Seeds were transferred on plates and incubated at room temperature for germination. Seed germination ratio, length of roots, length epicotyls/hypocotyls and fresh weight of seedlings was estimated after four days of germination. Further observation was taken up to ten days. Non-treated seeds were considered as control.

Estimation and extraction of Amylase enzyme

Amylase activity was measured from growing seeds. Five seedlings of each plant material were washed 2-3 times with distilled water until no foreign material remained**.** The seedlings were crushed in 0.1 N phosphate buffer, pH 6.0. The material was centrifuged at 10, 000 rpm, for 10 min at 4ºC. The supernatant of each plant was collected as an enzyme source. Crude extract of enzyme was used and preserved at 4ºC for further analyses.

Amylase assay

Amylase assay with modification of Odhav et al. [8] was followed. For the assay, 2.5ml (1% w/v) of starch solution, 2.5ml of 0.1N phosphate buffer pH 6.0, 1ml (1%w/v) of NaCl were mixed and incubated at 37ºC for 10 min. Two ml of enzyme extract and 1.0ml of distilled water was added to each tube. Tubes were incubated at 37ºC for 15 min. The reaction was terminated by adding 0.5ml of 2N NaOH and 0.5ml of DNSA reagent (12.0 g of sodium potassium tartrate, tetrahydrate in 8 ml of 2M NaOH and 96 mM 3, 5- dinitro salicylic acid solution). The tubes were kept in boiling water bath for 5 min and absorbance was measured at 540 nm after cooling the tubes.

III. RESULTS AND DISCUSSION

Plant growth hormones, which are synthesized in minute amounts, affect many activities in plant growth and development. Phytohormones are involved in the control of growth and almost every important developmental process in plants [9] (Weyers and Paterson, 2001). There are numerous reports that certain microorganisms affect plant growth through their ability to produce phytohormones. [10] Soil microorganisms have ability to release growth-promoting substances, which are useful for increasing the seed germination, plant growth and ultimately the yield. In these study thirty-five bacterial strains, isolated from the rhizosphere soil of three crops (groundnut, cowpea, cotton) showed positive test for IAA production. Varying level of IAA was produced by each strain. On the bases of maximum IAA production five isolates A10, A15 (from the groundnut rhizosphere), B3 (from cowpea rhizosphere) and C4, C9 (from cotton rhizosphere) were selected for further study.

It is well-known characteristic of certain phytohormones to obtain stimulatory effects on plant growth promotion. Induction of longer roots with increased number of root hairs and root length is a growth response recognized due to IAA production by rhizobacteria in the presence of tryptophan, which improves their nutrient uptake efficiency. [11] In the present study, inoculation of rhizobacteria produced IAA when L-tryptophan was added as substrate in different nutrient media. The results showed a significant difference in IAA production with the presence of different concentrations of the tryptophan (Figure 1). Amongst the five bacterial strains, A15 and B3 were producing maximum IAA with 8 mM (170.74 µg/ml) and 5 mM (388.16 ug/ml) tryptophan respectively with increasing the concentration of tryptophan from 10mM production of IAA level was declined by these five strains (Figure 1). The 5mM tryptophan concentration was producing higher amount of IAA compared to 8 mM and 10mM. These results proved that higher concentration of tryptophan was functions as inhibitor for the IAA production by selected test organisms. These showed that production of IAA by organisms depends on growth conditions and gram nature of bacteria.

From the six tested media, N-broth and Ashby's broth were best for higher IAA production with 5 mM tryptophan concentration. IAA level was estimated up to 10 days. Maximum production was observed on 8th day of inoculation. B3 produced maximum IAA (217 µg/ml) in N broth inoculated medium on 8th day (Figure 2A). A15 produced maximum IAA (310 µg/ml) in Ashby's inoculated medium on 8th day (Figure 2B). From the obtained results A15 and B3 strains were selected as a better for IAA production on Ashby's and N-broth medium. Tsavkelova et al. [12] reported that a *Bacillus* strain produced the greatest IAA in a stationary growth phase.

The colony appearance of the isolates varied significantly from each other (Table 1). The selected five strains were first identified by gram straining method. These results showed that A10, C4 and C9 were gram positive while A15 and B3 were gram negative (Table 2). Two-gram negative isolates A15 and B3 were identified based on biochemical tests determined in Bergey's manual of systematic bacteriology. [13] The results of biochemical tests reveled that A15 showed positive tests for *Shigella* sp. and B3 was positive for *Serratia ficaria* (Table 3). Effect of A15 and B3 on the pulses seeds showed positive response on seedling elongation and fresh weight (Figure 3 and Figure 4). Due to bacterial treatment Root length remained double in Chickpea, Moth bean, Cowpea and Green gram seeds after three days of seed germination (Figure 4). B3 treatment showed greater length of seedling in Chickpea, Moth bean, and Green gram while A15 showed higher length in Cowpea (Figure 4). The length of epicotyls

was four times higher with B3 treatment (8 cm) in Chickpea seedlings compared to control (1.3 cm) (Plate 1). The seedling length of Chickpea was 5.9 cm in control and 17. 4, 19.7 in A15 and B3 treated seeds respectively after seven days. The results on pulse seed germination by A15 and B3 treatment showed that B3 strain was effective for Green gram, Moth bean and Chickpea. A15 treatment showed higher length of Cowpea seedling compared to B3. Fresh weight was also higher in A15 and B3 treated pulses seed.

It has been reported that biological seed coating of *Bacillus subtilis* AF1 improved plant growth compared with control. [14] Chang et al. [15] reported that the strain of *B. cereus* QQ308 was producing growth enhancing compounds in Chinese cabbage. Dobbelaere et al. [16] assessed that inoculation of PGPR *Azospirillum brasilense* on spring wheat resulted in better germination, and early development. Khalid et al. [17] showed that responses of wheat growth to inoculation with rhizobacteria depend on plant genotype and PGPR strains as well as environmental conditions. Kishore et al. [18] reported that the *Phylloplane* bacterium, *Bacillus megaterium* GPS 55 produced IAA in L-tryptophan amended medium and increased groundnut plant growth in the greenhouse. Similarly, a rhizobacterial strain. Root growth was stimulated by the IAA producing strain *Azospirillumlipoferum*CRT1 in maize [19]. It has been previously observed that *Bacillus* and *Pseudomonas species* when applied as seed treatment colonize the endorhizosphere and increase the plant biomass. [20]

Plant hormones like Auxin and gibberellins triggered the activity of specific enzymes, such as amylase that promoted early germination, which have brought an increase in availability of starch assimilation. [8] Activation of Amylase depends on storage sugar of seeds. Increase in activity was observed with seedling length. Amylase activity of treated pulses seeds was slightly higher than control after two days of seed germination. In Moth bean, and Green gram activity was higher in A15 compared to B3 while in chickpea and cowpea B3 treatment showed higher compared to A15 (Figure 5). However lower difference in amylase activity between control and PGPR treatment suggested that bacterial treatment may influenced other genes responsible for root and seedling elongation.It should be emphasized that phytohormones such as auxin do not act alone but rather may interact with one another in a variety of complex ways. [21] Increase in seed germination ratio as well as length of seedling suggest that along with IAA other metabolites are also activated to induce the growth. The other mechanism regulating the growth of pulses is needed to be study.

IV. CONCLUSIONS

As the plant develops all parameters affect three fundamental processes: Cell division, Cell expansion and Cell differentiation. All the PGPR strains produced indole compounds when the precursor L - tryptophan was added to the culture medium. The concentration of indole fluctuates with increasing concentrations of L-tryptophan. A highly significant correlation between L-tryptophan-derived auxin production by plant growth promoting rhizobacteria *in vitro* and, root length, seedling length and fresh weight of seedlings was observed in selected pulse crop seeds. In the present study, all most double increase in length of selected dicot (Chickpea, Moth bean, Cowpea and Green gram) seedling was observed. The results showed that inoculation of these selected bacterial treatments would prove stimulating effect on growth and development of selected pulses to increase the yield. Amylase activity estimated at 3rd day of incubation showed slight difference in control and bacterial treated seeds. These results suggested that instead of seed germination *Serretiaficaria*and *Shigella*sp. might be inducing cell elongation enzymes. So further study related to cell elongation parameters and its correlation with PGPR strains is needed to be study.

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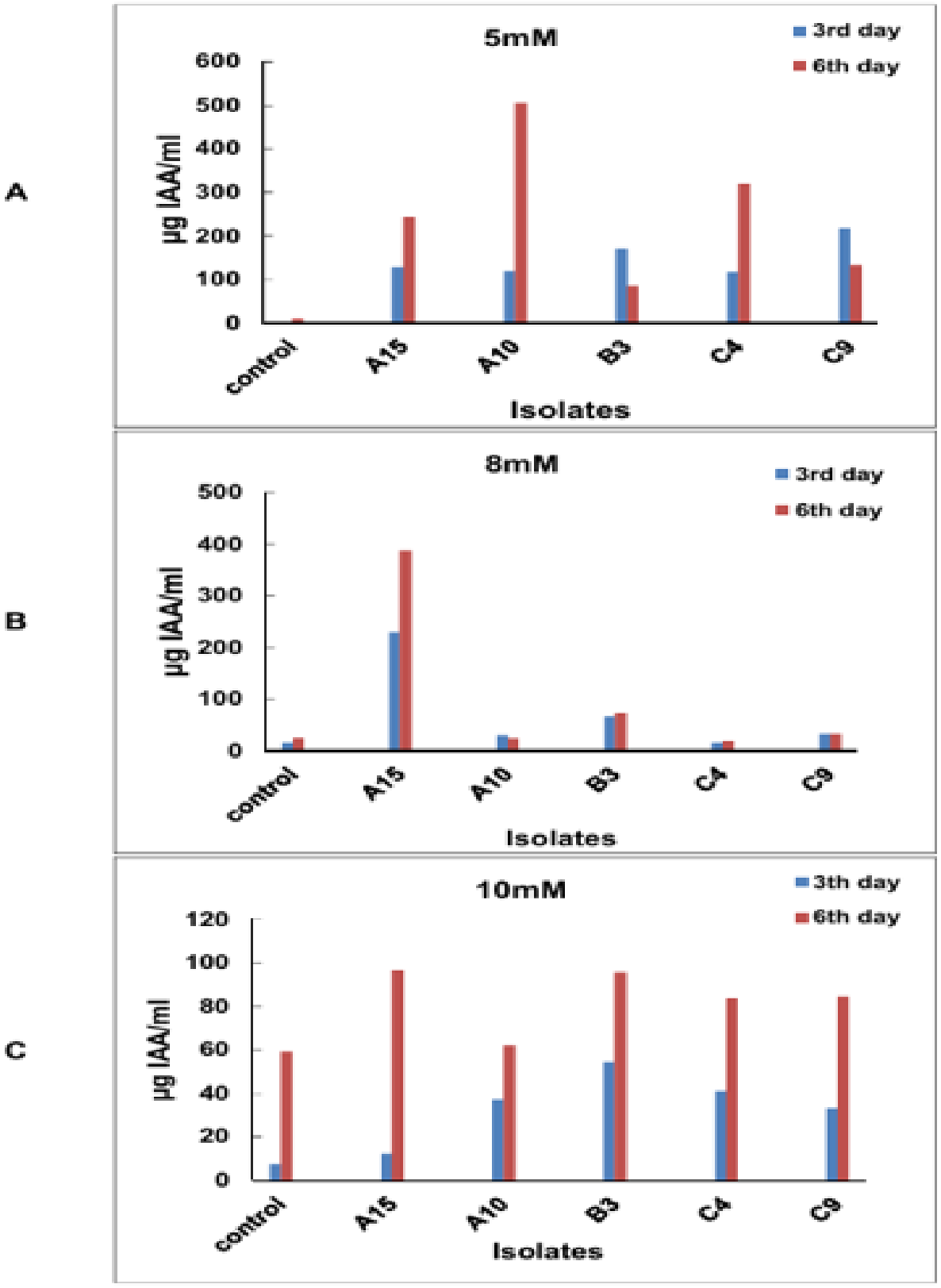

Figure 1: Changes in IAA level with varying concentration of Tryptophan (A) 5mM (B) 8mM and (C) 10 mM in N-broth

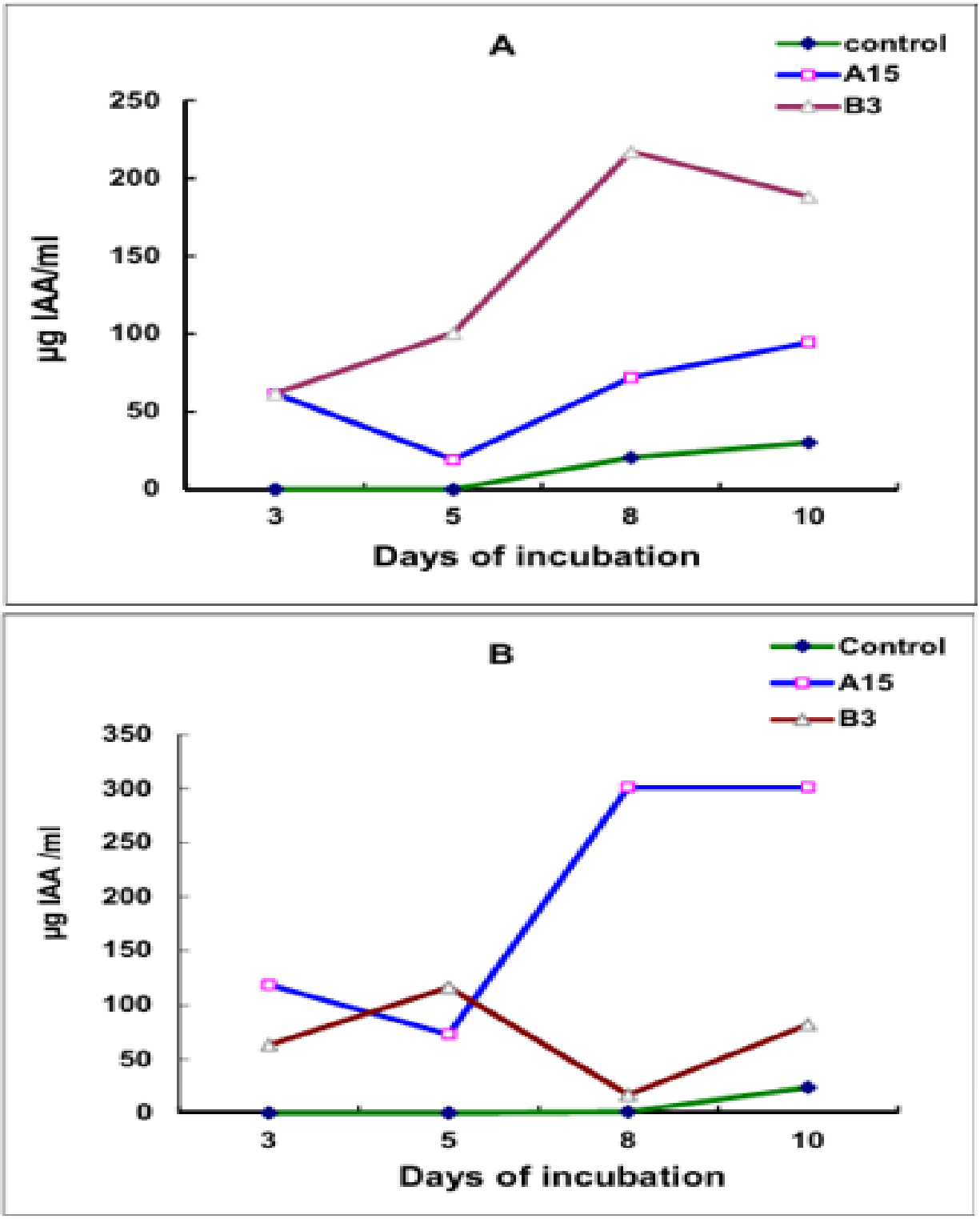

Figure 2:

A: IAA level produced with 5 mM tryptophan in N broth media

B: IAA level produced with 5 mM tryptophan in Ashby's broth media

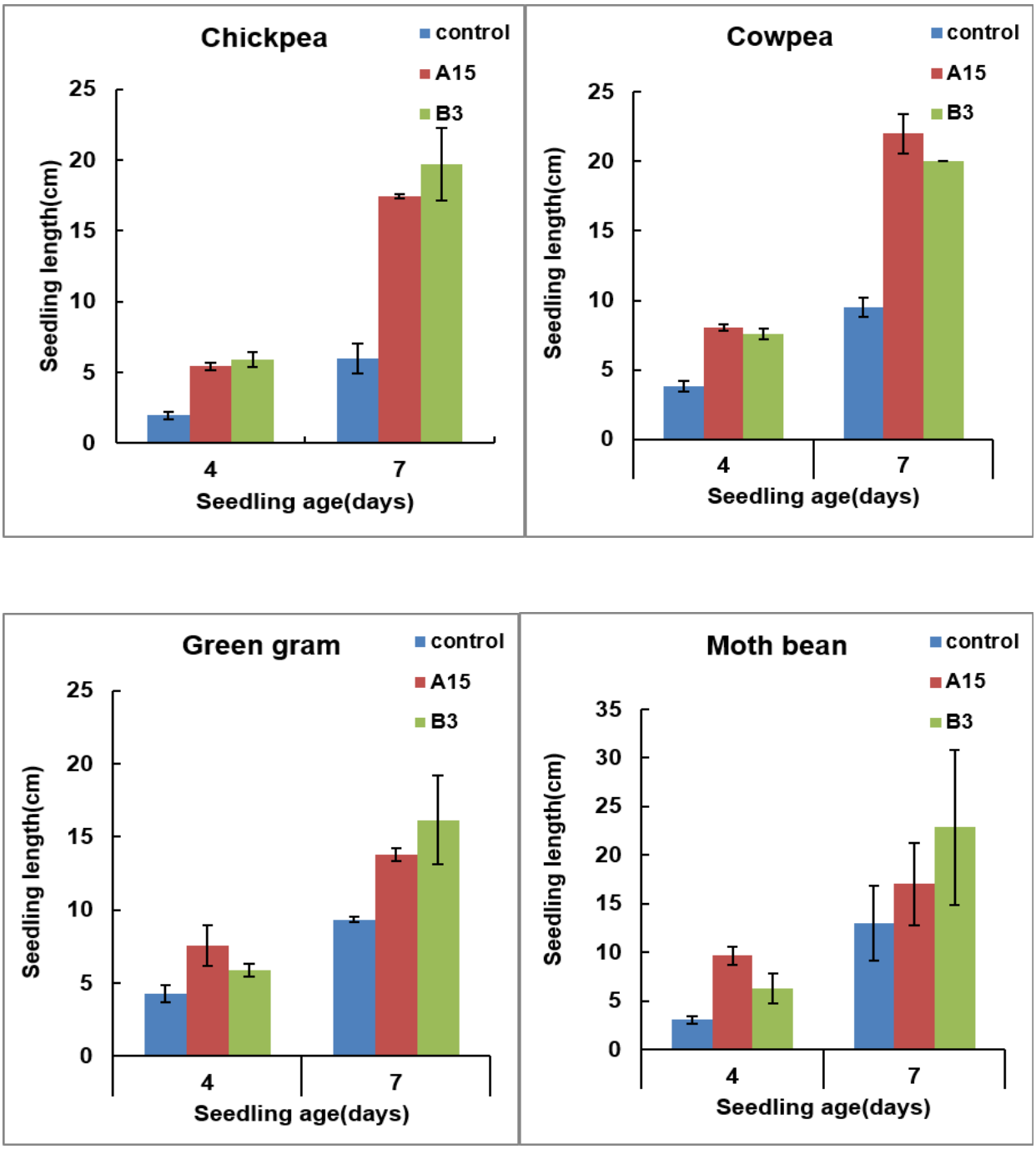

Table 1 : Colony isolated from rhizosphere

No of isolates	Colony characteristics							
	size	**shape**	**margin**	**surface texture**	**Elevation**	**opacity**	**consistency**	**pigment**
A10	Big	Round	entire	smooth	Low convex	opaque	gummy	yellow
A15	Pin point	round	entire	smooth	Low convex	opaque	moist	Yellowish orange

B3	small	round	entire	smooth	convex	transparent	moist	watery
C4	Big	Round	entire	smooth	Low convex	opaque	moist	NIL
C9	small	Round	wavy	rough	Low convex	opaque	moist	orange

Table 2: Morphological cell characteristics of five prominent isolates

Isolates	Gram staining and morphology
A10	Gram positive, purple, arrange in bunch
A15	Gram negative, pink, small rod
B3	Gram negative, pink, small rod
C4	Gram negative, purple, long filamentous
C9	Gram negative, pink, big rod

Table 3: Biochemical Characteristics of Isolates

No	Biochemical test for gram negative organisms	Original color	After incubation positive color reaction	Organism-1 *shigella* species A15	Organism-2 *Serratia ficaria* B3
1	Citrate utilization	Green	Blue	Negative	Positive
2	Lysine utilization	Light purple	Purple/dark purple	Negative	Negative
3	Ornithine	Light purple	Pink	Negative	Negative
4	Urease	Orangish yellow	Green	Negative	Negative
5	Phenyl alanine deamination	Colorless	Pinkish red	Negative	Negative
6	Nitrate reduction	colorless	Black	Positive	Positive
7	H_2S production	Oranges yellow	Yellow	Negative	Negative
8	Glucose	Pinkish red	Yellow	Positive	Negative
9	Adonitol	Pinkish red	Yellow	Negative	Negative
10	Lactose	Pinkish red	Yellow	Negative	Negative
11	Arabinose	Pinkish red	Yellow	Positive	Positive
12	Sorbitol	Pinkish red	Yellow	Positive	Positive