

## Inoculation Effect of Nitrogen-fixing and Phosphate-Solubilising Bacteria on Seed Germination of Brinjal (*Solanum melongena* L.)

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### Abstract

Twenty rhizobacteria were isolated from rhizospheric soils of Uttarakhand Tarai region. Out of 20 isolates, 3 isolates (PB2, PB12, and PB19) were selected on the basis of morphological, biochemical, physiologically and different PGP traits. The isolates were Gram negative, catalase positive, oxidase positive, starch and gelatine hydrolysis positive. The bacterial cultures were positive for indole acetic acid production, phosphate solubilisation, ammonia production, siderophore production, and nitrogen fixing activity. Isolate PB19 was the best IAA producer strain ( $42.50\mu\text{gml}^{-1}$ ) while isolate PB2 was lowest IAA producer ( $25.34\mu\text{gml}^{-1}$ ) comparatively. All the three isolates showed siderophore production and nitrogen fixing activity in G-NFMM media. Effect of nitrogen fixing and phosphate solubilising PGPR alone and in different combinations was studied on seed germination of brinjal plant. Treatments with different combination of bacterial isolates on brinjal seeds showed maximum germination %, with consortia D (T8) and treatment 4 (PB19) by 95.83% compared to control and other PGPR combinations after 8 days of incubation. The consortia of three isolated bacteria showed significant effects on Seedling Vigour Index (SVI), germination index, and other associated germination parameters. Thus, it was concluded that the use of combination of PGPR isolates could improve seed germination and associated parameters of brinjal.

**Keywords-** Plant Growth Promoting Traits, IAA, Phosphate Solubilisation, Germination %, SVI.

### 1. Introduction

Rhizosphere is a dynamic environment, which harbours diverse group of microorganisms. Some of the bacteria that directly or indirectly stimulate plant growth have been referred to as Plant Growth Promoting Rhizobacteria (PGPR) (Bloemberg and Lugtenberg, 2011). PGPR includes *Azotobacter*, *Azospirillum*, *Rhizobium*, VAM other bacterial genera e.g. *Pseudomonas*, *Bacillus*, *Serratia* etc. Many Phosphate Solubilizing Bacteria (PSB) belonging to *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Serratia*, *Enterobacter*, *Rhodococcus* and *Arthrobacter* genera have been isolated from soil (Mamta et al., 2010; Karpagam and Nagalakshmi, 2014; Pereira and Castro, 2014). The direct promotion of plant growth involves mechanisms like nitrogen fixation, solubilization of phosphorous and iron from the soil, production of phytohormones like gibberellins, cytokinins and indole-3-acetic acid (IAA) which accelerates root growth (Bhattacharya and Jha, 2012). Bacterial Chitinase, Siderophores, HCN etc. produced in the rhizosphere can indirectly support plant growth by

suppressing hazardous effects of biotic stresses (Aeron et al., 2011; Ribeiro and Cardoso, 2012) and inhibition of phytopathogens (Bashan and Holguin, 1998).

The soil microorganisms play an important role in maintaining soil fertility and promoting plant health (Gosal et al., 2011). Conventional agriculture plays a significant role in meeting the food demands of a growing human population, which has also led to an increasing dependence on chemical fertilizers and pesticides (Santos et al., 2012). Chemical fertilizers are industrially manipulated, substances composed of known quantities of nitrogen, phosphorus and potassium, and their exploitation causes air and ground water pollution by eutrophication of water bodies (Youssef and Eissa, 2014). In this regard, recent efforts have been channelized more towards the production of 'nutrient rich high quality food' in sustainable compartment to ensure bio-safety. The innovative view of farm production attracts the growing demand of biological based organic fertilizers exclusive of alternative to agro-chemicals (Raja, 2013).

The maximum use of PGPR creates an alternative way to replace chemical fertilizer, herbicides and other chemical supplements. Most of the isolates result in a significant increase in height of plant shoot and root length and dry matter production of shoot and root of plants. PGPR as a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Selected strains of beneficial PGPR trigger a plant mediated Induced Systemic Resistance (ISR) response that is effective against a broad spectrum of plant pathogens (Mandal and Kotasthane, 2014). The present study describes rhizospheric bacterial diversity of Tarai region of Uttarakhand, India (Sharma et al., 2016). The rhizospheric isolates were evaluated for its potential of different PGP properties and inoculation effect of most efficient isolates on germination of brinjal (*Solanum melongena* L.).

## **2. Materials and Methods**

### **2.1 Isolation, Purification and Identification of Isolates**

Rhizospheric soil of 2 months old brinjal fields in different areas of Kashipur region (Tarai Region of Uttarakhand) in India was used for isolation of PGPR organisms. Rhizospheric soil samples were collected and transported to laboratory under sterile conditions. Soil suspension was prepared by suspending approximately 1 gm of soil in sterile distilled water and vortexed. The suspension was serially diluted up to  $10^8$  and 100  $\mu$ l of inoculum was plated in triplicate on nutrient agar and Jensen agar medium. Plates were incubated at 28°C for 3 days. Well-isolated colonies were selected based on morpho-phenotypic characteristics (John et al., 1994; Cheesbrough, 2006), purified and maintained on nutrient agar, Jensen's agar media at 4°C. Nitrogen fixing and phosphate solubilising isolates were selected for their effects on brinjal seed germination (Sharma and Rai, 2015).

## **2.2 Evaluation of Plant Growth Promoting Traits of Isolated Rhizospheric Flora**

The isolated soil bacteria were screened for the production of indole acetic acid, phosphate solubilisation, ammonia, siderophore production and nitrogen fixing activity.

### **2.3 Indole Acetic Acid Production**

To observe Indole acetic acid production, exponentially grown cultures ( $10^8$  cells  $\text{ml}^{-1}$ ) of the rhizospheric bacterial isolates were incubated in Luria-Bertani broth (pH 7.0) amended with L-tryptophan (0.1%) and incubated at 28-30°C under shaking at 120 rpm for 72 h. Supernatant of the isolates were collected by centrifugation at 8,000 rpm for 12 min at 4°C. 2 ml supernatant of each isolates was transferred separately into fresh tube to which 2-3 drops (100  $\mu\text{l}$ ) of 10 mM O-phosphoric acid and 4 ml of Salkowski reagent in the ratio 1:2 was added (Gorden and Weber, 1951). The mixture was then incubated in dark for 30 min for the development of pink colour (IAA production) and absorbance was measured at 535 nm using spectrophotometer (Sharma and Rai, 2015).

### **2.4 Phosphate (P) Solubilisation**

Phosphate solubilisation ability of isolates was checked by spotting them on Pikovaskaya's agar (Pikovaskaya, 1948) plates based on clearing zone around the bacterial growth (due to the solubilisation of inorganic phosphate by bacteria) after incubation at  $28 \pm 1^\circ\text{C}$  for 3-4 days (Sharma and Rai, 2015).

### **2.5 Ammonia Production**

Qualitative detection of ammonia production was done using the method given by Cappuccino and Sherman (1992). Bacterial isolates were grown in peptone water for 2-3 days at optimum growth temperature. After incubation, 1ml of Nessler's reagent was added in each tube. Tubes showing faint yellow colour indicated small amount of ammonia, and deep yellow to brownish colour indicated maximum amount of ammonia (Dey et al., 2004).

### **2.6 Siderophore Production**

Siderophore production was tested qualitatively using chrome azural (CAS) agar and Universal Chemical Assay (CAS) (Schwyn and Neilands, 1987). The bacterial isolate was streaked on the CAS agar plates and incubated at  $28 \pm 2^\circ\text{C}$  for 3-4 days (Sharma et al., 2016).

### **2.7 Screening of Nitrogen-Fixing Activity**

Single colony grown on nitrogen free medium was taken and inoculated into G-NFMM containing 0.0025% (w/v) BTB solution (bromothymol blue solution). It was observed after one week incubation for the appearance of blue green colour that confirms nitrogen fixing activity (Sharma and Rai, 2015).

## 2.8 Evaluation of Soil Isolates/PGPR Isolates as Biofertilizer *in Vitro* Conditions

Finally three bacterial strains were selected on the basis of morphological, biochemical and PGP (Plant Growth Promoting) traits. Seeds of brinjal (*Solanum melongena* L.) var. Pant Rituraj were taken from G. B. Pant University of agriculture and technology, Pantnagar, Uttarakhand, India.

Seeds of brinjal were surface-sterilized with 2.4% sodium hypochlorite for 1-2 minute, then rinsed in sterile distilled water (5-6 times) and dried overnight under sterile stream of air in a laminar air flow. PGPR strains grown in NB were taken in screw-capped tubes. After sterilization, seeds were bacterized according to treatment. Carboxy Methyl Cellulose (CMC) (100mg) was added as an adhesive material. Seeds soaked in sterile nutrient broth, served as control. Known number of seeds (100) was soaked in 10 ml of rhizobacterial suspension ( $10^8$  cfu ml<sup>-1</sup>) for 12h. Then the seeds were dried under sterile stream of air. The experiment was performed in following set of treatments: (1). Sterile broth (control), (2). PB2, (3). PB12, (4). PB19, (5). Consortia A (PB2+PB12), (6). Consortia B (PB2+PB19), (7). Consortia C (PB12+PB19), (8). Consortia D (A+B+C).

The bacterized and non-bacterized seeds were transferred onto the two sheets of sterilized filter papers inside the petridishes. Eight seeds were put into each dish with three replicates. Germination data were taken till 7<sup>th</sup> days and germination % was calculated. At the end of the 15<sup>th</sup> day, potential of seed germination was assessed in terms of speed of germination, Mean Germination Time (MGT), MDG (Mean Daily Germination), PV (Peak Value) and GV (Germination Value).

The number of germinated seed was noted daily for eight days. Data were taken in triplicates. Seeds were considered as germinated when their radical showed at least 2-mm length. Seed germination percentage was calculated using the following formula (ISTA, 1999).

$$\text{Germination \%} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

Root length and shoot length of individual seedlings were measured every day from 3<sup>rd</sup> to till 15<sup>th</sup> day and the germination percentage of seeds was recorded. The vigour index of germinated seeds was calculated in different days by using the formula as described by Abdul Baki and Anderson (1973).

$$\text{Seedling Vigour index (SVI)} = \text{Seedling length (Mean root length + Mean shoot length)} \times \text{Germination \%}$$

Five seedlings were taken randomly from each rhizobacterial treatment and their fresh weight was recorded. Later, the seedlings were kept in the hot air oven for 4 days at 60°C for complete desiccation and dry weight of the seedlings was recorded.

Germination index was calculated according to the formula given by Tao and Zheng (1990).

$$\text{Germination Index (GI)} = \Sigma \text{GT/DT}$$

Where GT= Germination % & DT= Germination Days

Germination associated parameters were calculated using following formulae:

**i). Speed of Germination** Speed of germination was calculated using following formula (Czabator, 1962).

$$\text{Speed of germination} = n_1/d_1 + n_2/d_2 + n_3/d_3 + \dots$$

Where, n = number of germinated seeds, d= number of days.

**ii). Mean Germination Time (MGT)** Mean germination time was calculated using following formula (Ellis and Roberts, 1981).

$$\text{MGT} = n_1 \times d_1 + n_2 \times d_2 + n_3 \times d_3 + \dots / \text{Total number of days}$$

Where, n = number of germinated seed, d = number of days

**iii). Mean Daily Germination (MDG)** Mean daily germination was using following formula (Czabator, 1962).

$$\text{MDG} = \text{Total number of germinated seeds} / \text{Total number of days}$$

**iv). Peak Value (PV)** Peak value was calculated using following formula (Czabator, 1962).

$$\text{PV} = \text{Highest seed germinated} / \text{Number of days}$$

**v). Germination Value (GV)** Germination value was calculated using following formula (Czabator, 1962).

$$\text{GV} = \text{PV} \times \text{MDG}$$

## 2.9 Statistical Analysis

All the experiments were done in triplicates. The data obtained in the present investigations for various parameters in the experiments were analyzed statistically by using two way analysis of variance (ANOVA) on the basis of mean values to find out the significance at 1% and 5% levels, using software STPR3.

## 3. Results and Discussion

Total 20 rhizobacteria were isolated from rhizospheric soils of tarai region and were recognized as PB series 1, 2, 3 and so on. These isolates were identified by morphological and biochemical characteristics (Table 1). Among them, 3 isolates (PB2, PB12 and PB19) were selected on the basis of different plant growth promoting properties (Table 2), useful for brinjal seed germination.

All the rhizospheric bacterial isolates from PB1-PB20 were grown in culture medium amended with 0.1% tryptophan, the development of pink colour with addition of Salkowski

reagent was observed into their culture filtrate. Isolates PB2, PB12 and PB19 produced 25.0  $\mu\text{g ml}^{-1}$  of IAA or more after 72 h of incubation (Table 2). The highest concentration of IAA was observed from PGPR strain PB19 (42.50  $\mu\text{g ml}^{-1}$ ) followed by PB12 (34.71  $\mu\text{g ml}^{-1}$ ) and then PB2 (25.34  $\mu\text{g ml}^{-1}$ ). It has been reported that IAA production by PGPR can vary among different species and it is also influenced by culture condition, growth stage and substrate ability (Mirza et al., 2001; Mishra et al., 2010; Kamble and Galerao, 2015).

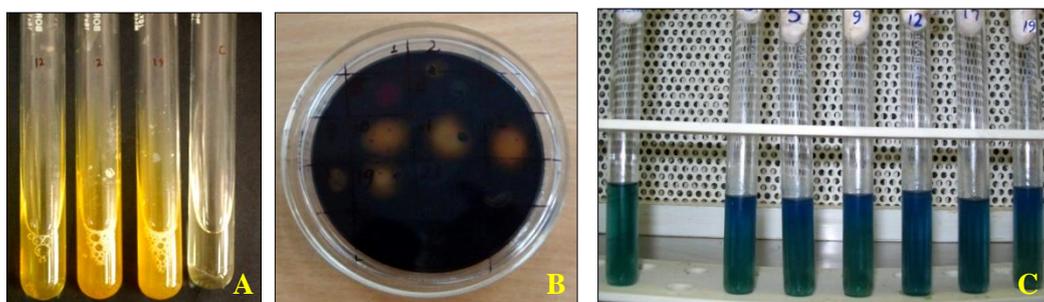
**Table 1. Biochemical characterization of selected PGPR isolates; where '+' indicates positive, '-' indicates negative**

Biochemical test	PB2	PB12	PB19
Gram Reaction	-	-	-
Catalase	+	+	+
Starch Hydrolysis	-	-	-
Gelatin Hydrolysis	+	+	+
Casein Hydrolysis	-	-	-
Methyl Red test	-	-	-
Voges-Proskauer	-	+	-
Oxidase test	+	+	+
Indole production	-	-	-
H <sub>2</sub> S production	-	-	-
Esculine hydrolysis	+	+	+

All the selected isolates were found positive for phosphate solubilisation on the pikovaskaya agar forming clear zones around streak inoculation by dissolving in tri calcium phosphate. In comparison to non-rhizospheric soil, higher concentration of phosphate solubilising bacteria is commonly found in the rhizosphere (Mishra et al., 2010). Suresh et al. (2010) indicated that most of the isolates tested in their study possessed plant growth promoting traits and that these isolates can be used as potential biofertilizers and also as biocontrol agents.

**Table 2. Plant growth promoting properties of rhizospheric isolates (+++) Strong reaction, (++) Moderate reaction, (+) Positive reaction**

Rhizospheric Isolates	Different Plant Growth Promoting Activities					
	IAA Production	PO <sub>4</sub> Solubilisation	Ammonia Production	Siderophore Production	Nitrogen Fixing Activity	Antifungal against <i>F.oxysporum</i>
PB2	+	+	+++	+	++	++
PB12	+	+	++	+	+	+
PB19	+	+	+++	+	+++	++

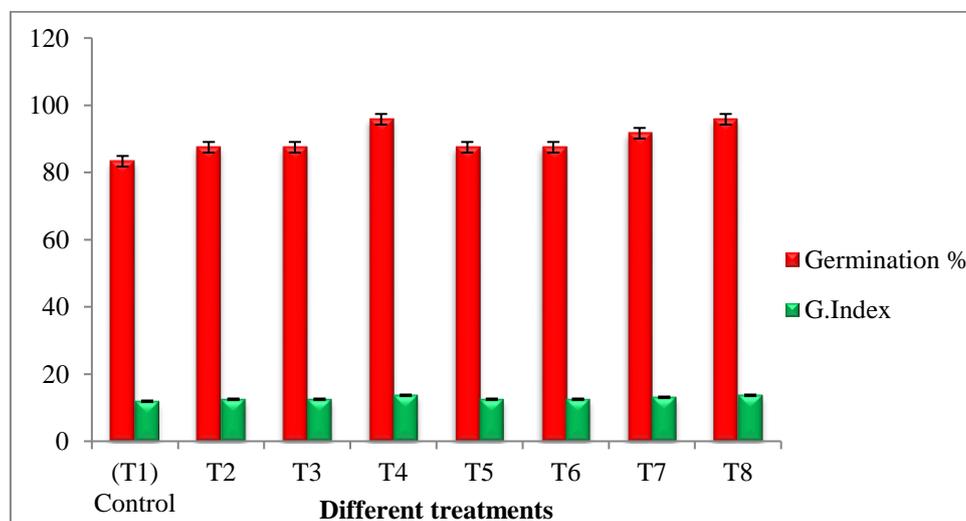


**Figure 1. Different plant growth promoting activities- A. Ammonia production; B. Siderophore assay; C. Nitrogen fixing activity**

All the bacterial isolates (PB1-PB20) were tested for ammonia production in peptone water. Among these isolates, PB2 and PB19 changed the colour of the peptone water from deep yellow to dark brown strongly indicating higher level of ammonia production but PB12 were scored as moderate (++) (Table 2 and Figure 1 A).

Isolated PGPR formed yellow coloured halo zone around the rhizobacterial colony on dark blue coloured CAS agar plates indicating the production of siderophore. The larger yellow halos varied with the isolates. The results of siderophore production indicated that the PGPR isolates PB19, PB12 and PB2 showed a higher activity (23mm, 18mm and 15mm) when compared to other isolates measured by zone of colouration surrounding the colonies on dark blue CAS medium respectively (Table 2 and Figure 1 B). Secretion of siderophore started after 12h of incubation.

All the selected rhizospheric bacterial isolates from PB1-PB20 were also screened for nitrogen fixing activity on G-NFM medium with BTB as indicator to study the release of ammonium in the culture as shown in Figure 1 C. At glucose 0.5%, PB2, PB12 and PB19 isolates were observed to fix nitrogen by changing the dark green colour to blue green colour distinctly after one week noted as positive and other recorded as negative (Table 2 and Figure 1 C).



**Figure 2. Germination per cent and germination index of brinjal plants; Values are mean  $\pm$  standard error**

The first emergence of brinjal seedling was observed on 2<sup>nd</sup> day in all treatments with control. The percent germination and days to germination varied with different bacterial consortia. The maximum germination % was observed with consortia D (T8) and treatment 4 (PB19) by 95.83%, consortia C (T7) by 91.66%, consortia A and B by 87.50% and other then by individual isolates after 8 d of incubation (Figure 2). Application of PGPR isolates significantly improves the percentage of seed germination under saline conditions (Mishra et

al., 2010). These results were similar with the findings of Dobbelaere et al. (2003) who assessed the inoculation effect of PGPR *Azospirillum brasilense* on growth of spring wheat. They observed that inoculated plants resulted in better germination, early development and flowering (Kamble and Galerao, 2015). Development of consortium of PGPR for increased and consistent effects and universal formulations to increase survival of the main bacterial strain has been suggested (Mayer, 2004).

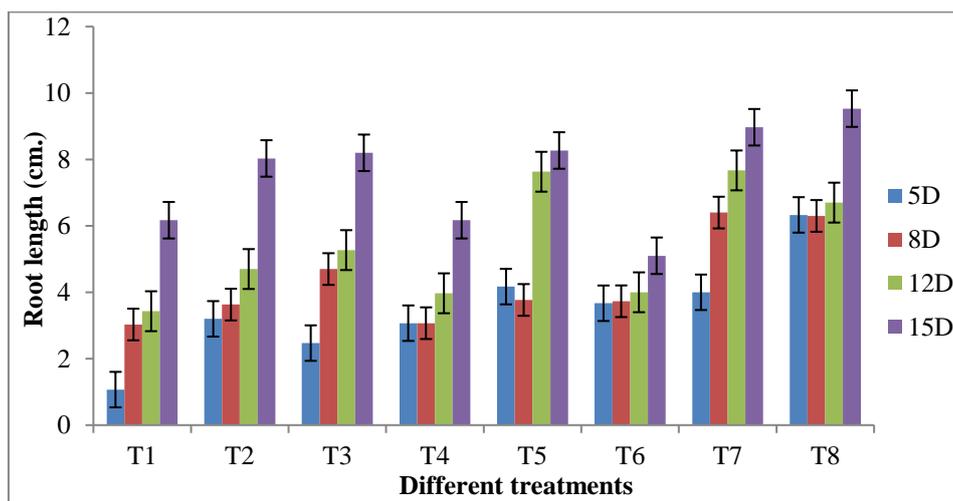


Figure 3A. Root length of brinjal in 5d, 8d, 12d, 15d in petriplate; Values are mean  $\pm$  standard error

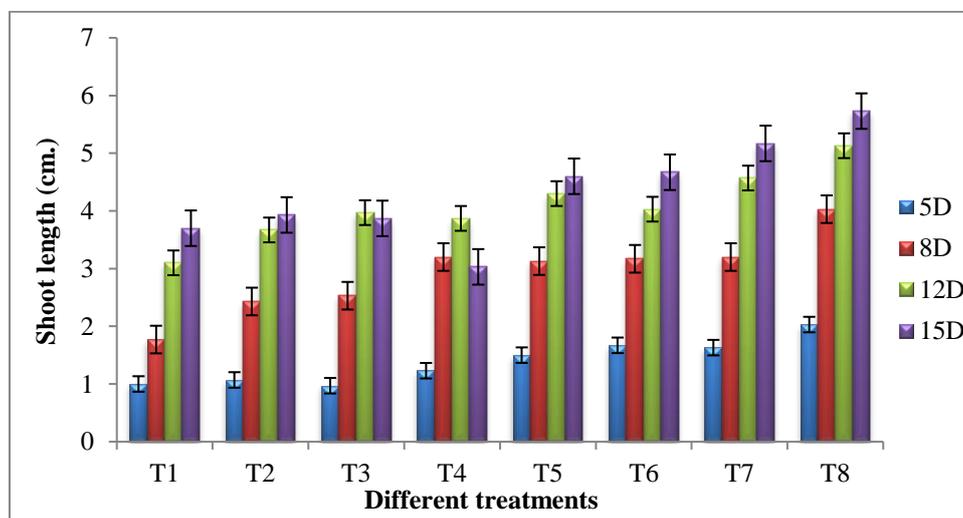


Figure 3B. Shoot length of brinjal in 5d, 8d, 12d, 15d in petriplate; Values are mean  $\pm$  standard error

Seedling growth was measured after 3<sup>rd</sup> day of seed incubation for germination in terms of root, shoot length. All treatments applied (individual bacteria and their consortia) adversely affected the seedling growth. In brinjal seedling, T8 showed increase by 52.6% and 10.33% in root length, shoot length respectively in 5d, 32.7% and 22.6% in 8d, 32.7% and 20.3% in 12d, 33.6% and 20.3% in 15d respectively comparatively to control (Figure 3 A and B). The highest root, shoot length was observed with the treatment 8 (consortia D) comparative to

other treatments in all days. Dobbelaere et al. (2003) and Çakmakçi (2005) have been reported that PGPR can increase yield and leaf area index, shoot and root weight and delay leaf senescence. Mishra et al. (2010) reported that most of isolates used in their study resulted in a significant increase of shoot length, root length and dry matter production of shoot and root of *Cicer arietinum* seedlings (Kamble and Galerao, 2015).

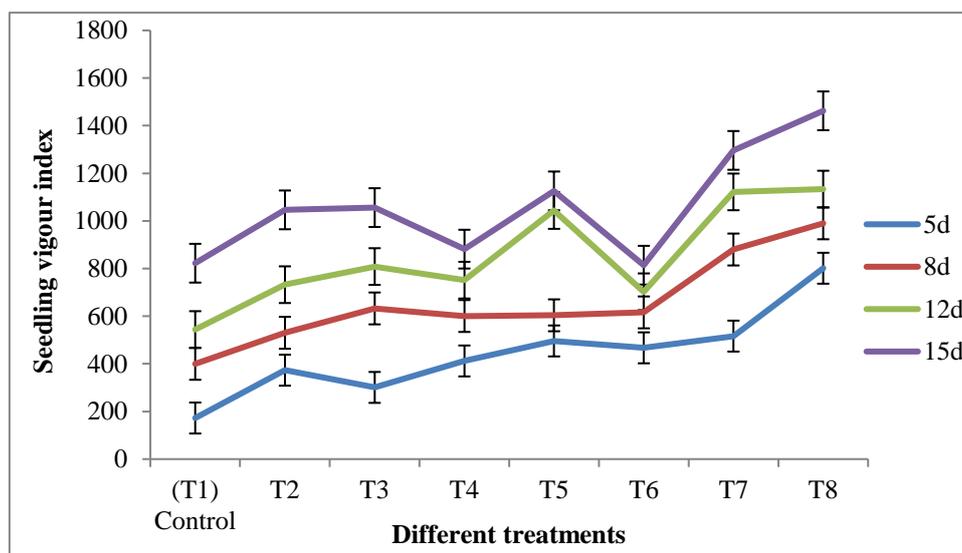


Figure 4. Seedling vigour index of brinjal in different days; Values are mean  $\pm$  standard error

The maximum seedling vigour index was found in treatment 8 (consortia D) comparative to other treatments with uninoculated control (treatment 1) (Figure 4) and maximum germination index was observed in treatment 8 and treatment 4 (13.69) comparative to other treatments (Figure 2). Trials with plant growth-promoting rhizobacteria indicated that yield and dry matter accumulation increase in wheat (De Freitas, 2000; Çakmakçi et al., 2007), maize (Ashrafi and Seiedi, 2011; Sharifi et al., 2011), sugarcane (Sundara et al., 2002) and barley (Çakmakçi et al., 2001; Şahin et al., 2004).

Likewise germination parameters like Speed of germination, Mean daily germination, Peak value and Germination value was found maximum in treatment 8 comparative to other treatments, but Mean germination time was observed maximum in treatment 4 and minimum in treatment 8 (Table 3).

As it is known that seed germination provides a suitable foundation for plant growth, development and yield. In the present experiment, application of different consortium enhanced seed potential by increasing the characteristics of seed germination. Among the treatments, application of consortium D proved best by giving the highest values for percent seed germination in different days, seedling vigour index and seed germination index. These results agree with the findings of Minorsky (2008). Mishra et al. (2009b and 2011) and Prabha et al. (2012) reported that co inoculants significantly enhanced root and shoot length

(Latha et al., 2009), Speed of germination, Mean daily germination, Peak value and Germination value. Mishra et al. (2009a), also reported that the bacterized wheat seedlings recorded higher seed germination, root, and shoot length as compared to uninoculated controls. Thus, on the basis of the roles played by consortium, we could easily visualize their direct and indirect involvement in the root and shoot growth by better improvement in seed germination characteristics.

**Table 3. Germination associated parameters of brinjal (*Solanum melongena* L.); MGT is mean germination time, MDG is mean daily germination, PV is peak value, and GV is germination value; Values are mean  $\pm$  standard error**

Treatments	Speed of Germination	MGT	MDG	PV	GV
T1 (Control)	2.16	3.42	0.95	0.93	0.92
T2 (PB2)	2.33	3.29	1.00	1.30	1.30
T3 (PB12)	2.04	3.57	1.00	0.92	0.92
T4 (PB19)	2.25	3.74	1.09	0.75	0.81
T5 (PB2+PB12)	2.22	3.47	1.00	0.83	0.81
T6 (PB2+PB19)	2.30	3.33	1.00	1.15	1.15
T7 (PB12+PB19)	2.63	3.32	1.05	1.28	1.32
T8 (PB2+PB12+19)	2.89	2.92	1.09	1.78	1.96
SEm $\pm$	0.20	0.26	0.055	0.20	0.23
CD @ 1%	0.84	1.08	0.23	0.85	0.96
CD @ 5%	0.61	0.78	0.17	0.61	0.69
CV	14.71	13.19	9.38	31.25	34.27

#### 4. Conclusion

The present study clearly indicates the potential of efficient N<sub>2</sub>-fixing, P-solubilizing, and IAA-producing bacteria on brinjal seed germination. The effect of an inoculation by a consortium was more effective than individual inoculation for all the mentioned parameters. The use of PGPR as inoculants biofertilizer could be an efficient approach to replace chemical fertilizers and pesticides for sustainable *Solanum melongena* cultivation in Tarai region of Uttarakhand in India. These isolates showed potential in field applications as PGP agents in brinjal. Further studies are required involving detailed characterization of molecular and functional properties of these PGPR for their applications in the field.

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