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Andhare Aishwarya A
Department of Microbiology and Biotechnology, Dayanand Science College, Latur, Maharashtra, India

Poudel Anju S
Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Deshmukh Amol J
Department of Plant Pathology, College of Agriculture, Navsari Agricultural University, Waghai, Gujarat, India

Dargad Jaiprakash S
Principal, Department of Physics, Dayanand Science College, Latur, Maharashtra, India

Correspondence
Andhare Aishwarya A
Department of Microbiology and Biotechnology, Dayanand Science College, Latur, Maharashtra, India

Isolation of *Azotobacter* and study of its effect as a liquid formulation on seed germination and growth parameters of green gram (*Vigna radiata* L.)

Andhare Aishwarya A, Poudel Anju S, Deshmukh Amol J and Dargad Jaiprakash S

Abstract
An experiment was conducted to isolate and identify the Azotobacter spp. from different soil habitats of Latur district Maharashta and to study its effect on seed germination and growth patterns in green gram crop and it was found that, *Azotobacter* sp. was a free living bacterium in natural soil habitats and can be isolated easily on *Azotobacter* specific Ashby's Agar media. In all three isolates of *Azotobacter vinelandii* were isolated and identified from the soil collected of different habitats. A pot experiment was formulated to study effect of *Azotobacter* seed treatment on seed germination and growth parameters of green gram. The results revealed that the seed bacterization with all the three isolates of *Azotobacter vinelandii* was responsible to increase seed germination, speed of seed emergence, seedling vigour index, plant height and numbers of branches per plant in green gram crop.

Keywords: liquid formulation, seed germination, *Vigna radiata*

Introduction
Mungbean [*Vigna radiata* (L.) Wilczek] is a tropical grain legume. Until 2009 annual production of this crop was approximately 35,000 tons generating over $25 million in exports to Japan, India, USA and Philippines. Based on the current and projected demand of this crop, there is potential to increase the size of the mungbean industry to 1,00,000 tons per annum. Historically, poor famer perception of mungbean was as a low-yielding high-risk crop after a winter cereal (Lawn and Russell 1978) [9] has also limited the expansion of the crop into new areas and planting windows (Robertson, Carberry *et al.* 2000) [14]. Recently released mungbean cultivars has about 20% yield advantage as compared to earlier released cultivars over the globe. The cultivation of recent cultivars was observed to be resulting in higher profitability along with boosting growers' confidence and improvement in the perception about the crop. However, its yield potential is still less than that of other legumes such as yield of up to 5 t/ha of newly released peanut and soybean cultivars in good rainfall years. This suggests that realization of high commercial yield from legumes is possible. In soybean and peanut, selection for a high partitioning trait has apparently contributed to the development of higher yield potential (Cui and Yu 2005; Duncan, McLoud *et al.* 1978; Gifford, Thorne *et al.* 1984; Lal, Hariprasanna *et al.* 2006) [3, 4, 5]. The possibility of improving partitioning traits has, however, not received similar attention in mungbean.

'Biofertilizer' is a substance which contains living microorganism which when applied to seeds, plant surfaces or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Chemical fertilizers directly increase soil fertility by adding nutrients. However biofertilizer add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth promoting substances. Liquid biofertilizer technology is an alternative solution to carrier based biofertilizers. It comprises aids to preserving organism, to delivering them to their targets and improves their activities. Unlike the lignite based biofertilizers, liquid biofertilizers have a longer shelf life (Rao, 2007) [13]. By applying an appropriate liquid biofertilizer, the overall cost of production will be much lower as compared to traditional chemical fertilizers (Chin, 2010) [2]. *Azotobacter* is commonly found in rhizosphere and phyllosphere of plants and is very effective for the improvement of soil fertility and crop productivity. It can fix nitrogen directly from the atmosphere that helps plants in better grain production. Besides, nitrogen fixation, *Azotobacter*

also produces growth hormones *viz;* thiamine, riboflavin, nicotine, indoleacetic acid and gibberellins (Mishustin and Naumova, 1962., Shende *et al.* 1986, Jadhav and Patil, 1985) [11, 17, 7]. *Azotobacter* sp. is a common Gram-negative soil bacterium that can fix atmospheric nitrogen under aerobic conditions. This characteristic differentiates it from many other nitrogen-fixing bacteria that require anaerobic or micro aerobic conditions to protect the oxygen-sensitive nitrogenase (Setubal *et al.*, 2009) [15]. This feature also makes it an ideal candidate strain for potential co-culture with oxygen-producing phototrophs such as microalgae (Ortiz-Marquez *et al*., 2012) [18]. Additionally, *Azotobacter* sp. is considered as an ideal strain in potential biotechnology applications for the production of higher value bioproducts such as poly hydroxyl alkanoates, which could serve as potential bioplastics (Setubal *et al*., 2009) [15]. *Azotobacter* has also been reported to excrete a range of additional nitrogen compounds to serve various functions, including proteins involved in the production of alginate (Gimmestad *et al.*, 2006) [6]. In view of this background information, the present experiment was undertaken to isolate, identify and study the effect of liquid formulation of *Azotobcter* spp. inoculation on germination and growth parameters of *Vigna radiata* (Mung Beans).

Materials and Methods

Soil Sample collection

The soil samples were collected from different habitat of Latur District, Maharashtra, India on 19 July 2018 viz., Murud, Renapoor and Sawe wadi. The samples were taken from a rhizospheric soil of 15 cm depth and collected in sterile polyethylene bags, which were transported to the laboratory and stored at 4 °C until use.

Isolation of *Azotobacter* spp.

Serial dilution technique was followed to isolate *Azotobcter* spp. by preparing 10^4 to 10^7 dilution of each collected soil sample. One millilitre of each solution was pipetted out on to azotobacter specific media (Ashby's agar) plates aseptically in laminar air flow cabinet and incubated at 28 ^{0}C for 3 days in incubator. The culture plates were examined daily and each colony that appeared was considered to be one colony forming unit (cfu). After enumeration of cfu/ml, individual uncommon colony wasre cultured on fresh Ashby's agar plates for 3 days. The individual colonies were transferred further to test tubes and pure cultures thus obtained were stored at 4 ^{0} C.

Characterization and identification of *Azotobacter* spp. isolated from different habitats

Isolated *Azotobacter* spp. were identified on the basis of morphological, cultural and biochemical characteristics such as cell morphology and grams staining. Two techniques, visual observation of individual colony developed on petri dishes and micro-morphological studies in slide culture, were adopted for identification of *Azotobacter* spp. For visual observation, the isolates were grown on Ashby's agar media and incubated at 28 ^{0}C for 3 days, colonies thus developed were observed for the different cultural characters such as Color, shape, pigment formation etc., while the culture colonies were stained following Gram's staining and were critically observed at higher magnification (1000x)for micro-morphological studies moreover the colonies were also subjected to study biochemical characteristics, such as acid production through the fermentation of different sugars *viz*., Sucrose, Lactose, Glucose, Mannitol and starch by adopting standard biochemical methods as given in the Bergey's manual of systematic bacteriology (2001). Acid production through the fermentation of different sugars by different isolates of *Azotobacter* spp. was tested by using10 ml well sterilized media broth in test tube + addition of 0.1 ml individual sugar solution inoculation of + 0.1 ml individual colony followed by 3-7 days incubation for at 30^{0}C for with constant watch. Bromo cresol purple was used as pH indicator in test tubes. Change in the colour of the medium confirmed the acid production. Further the temperature tolerance test was also carried out by standard laboratory protocol. The results thus obtained in cultural, morphological, biochemical and temperature tolerance was compared with the literature of Bergy's manual and the *Azotobacter* spp. thus isolated were identified and conformed to species level.

Set up of a pot experiment

Sterilization of soil

Around 30 to 40 kg of soil was sterilized in a hot air oven at 180^0 C for 20 minutes and was used to pour in pot. 20 well sterilized earthen pots of 25 cm diameters were poured with sterilized soil and used in further study.

Preparation of liquid formulation

Liquid Formulations of individual test isolates of *Azotobacter* spp. was prepared separately using 24 hrs old cultures grown on Ashby's agar medium. Culture plates of *Azotobacter* spp. were flooded with 10 ml well sterilized distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth. After filtering the suspensions, the *Azotobacter* spp. concentration was determined using a double ruled Nabuer's haemocytometer. The spore concentration of the filtrate was adjusted to 10^8 cells/ ml using sterilized distilled water. Three individual liquid formulations *viz*., LF_1= *Azotobacter*1, LF_2= *Azotobacter*2, LF_3= *Azotobacter*3 were prepared for seed treatment.

Seed Treatment

The seeds of *Vigna radiata* collected from farm of Renapur of Latur District, Maharashtra, India. The treatment listed below was carried out to determine the efficacy of *Azotobacter spp.* as a seed treatment.

T1: 45 seeds were soaked in a suspension of 5 ml *Azotobacter*-1+ 100 ml distilled water

T2: 45 seeds were soaked up to complete imbibitions stage in a suspension of 5 ml *Azotobacter*-2+ 100 ml distilled water

T3: 45 seeds were soaked up to complete imbibitions stage in a suspension of 5 ml *Azotobacter*-3+ 100 ml distilled water

T4: 45 seeds were soaked up to complete imbibitions stage in sterilized distilled water.

T5: 45 untreated seeds were served as control

The treated seeds were air-dried for 1 hr under ambient conditions before sowing. The experiment was conducted for 6 weeks during the months of July-August 2018 under pot condition. 15 seeds of each treatment were sown in each pot and replicated four times. Irrigation, fertilizer application was done as per suitable standard practices. The observation on per cent germination was calculated by counting numbers of emerged seedlings at 10 DAS while the speed of seedling emergence was calculated by vigilance of first emerged seedlings and last count of emergence at about 5 DAS and 10 DAS respectively. Five seedlings from each repetition were

uprooted at 10 DAS, the data on seedling length was taken by routine scale measurement in millimeters and used to calculate seedling vigour index. The pots were maintained up to 6 weeks. Plant height (cm) and numbers of branches per plant were noted at 6 weeks after sowing. Data thus obtained in each repetition was analyzed and presented as average data with applying CRD design.

Table: The equations used to calculate different germination indices

Germination parameters	Equations	References
Germination percent (GP)	$\frac{\text{Number of germinated seeds at final count}}{\text{Total number of seeds sets for bioassay}} \text{X } 100$	Global method
Speed of emergence (SE)	$\frac{\text{Number of germinated seeds at the starting day of germiantion}}{\text{Number of germinated seeds at the final days of measurement}} \text{X } 100$	Modified from Islam *et al*. (2009)
Seedling vigour index (SVI)	$\frac{\text{Seedling length (mm) x Germination percent}}{100}$	Islam *et al*. (2009)

Results and Discussions

Isolation, structural and micro morphlogical studies

In all three different isolates of *Azotobacter* sp. were obtained in from Ashby's Agar plates of three different habitats and were named as *Azotobacter*- 1, *Azotobacter*- 2 and *Azotobacter*- 3. All the three isolates were found to be gram negative, circular in cell shape possesing non-transparent colonies with entire margins. *Azotobacter*- 1 and 3 were exhibited to have convex elevation while *Azotobacter*- 2 has flat elevation. *Azotobacter*- 2 and 3 were exhibited to have highly mucoid consistency while *Azotobacter*- 1 has mucoid consistency (Table-1 and Plate-1).

Table 1: Gram's reaction and colony characteristics of different *Azotobacter* isolates

Location	Isolates	Gram's staining	Form	Color	Elevation	Margin	Consistency	Density
Murud	*Azotobacter*- 1	-ve	Circular	Off white	Convex	Entire	Mucoid	Not transparent
Renapur	*Azotobacter*- 2	-ve	Circular	Creamy white	Flat	Entire	Highly Mucoid	Not transparent
Sawe-wadi	*Azotobacter*- 3	-ve	Circular	Creamy white	Convex	Entire	Highly Mucoid	Not transparent

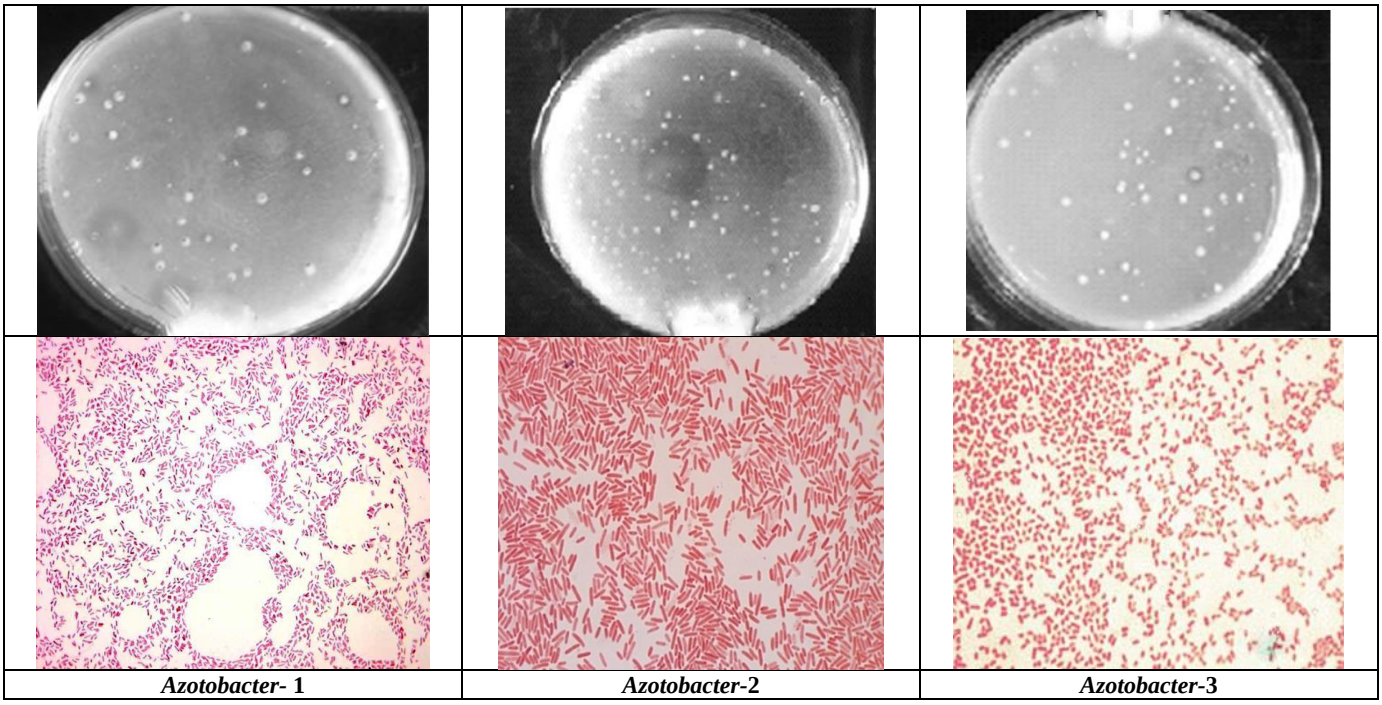

Plate 1: Gram's reaction and colony characteristics of *Azotobacter* spp.

Biochemical analysis

All the three isolates were observed to ferment and produce acids in sucrose, lactose and glucose while only two isolates viz., *Azotobacter*- 1 and *Azotobacter*-2 were observed to ferment and produce acid in mannitol. None of the isolate was observed to ferment and produce acid in starch (Table-2).

Table 2: Acid production from different sugars

Isolates	Carbohydrates				
	Sucrsoe	Lactose	Glucose	Mannitol	Starch
Azotobacter- 1	+	+	+	+	--
Azotobacter- 2	+	+	+	+	--
Azotobacter- 3	+	+	+	--	--

Screening of *Azotobacter* isolates for temperature tolerance:

The studies on screening of *Azotbacter* spp. for temperature tolerance revealed that *Azotbacter* - 3 isolate was tolerant to 45 ^{0}C followed by *Azotbacter* -1, which was found to be tolerant in 40 ^{0}C temperature. *Azotbacter* - 2 was found to be least tolerant as compared to other two isolates, which was found to be tolerant to 37 ^{0} C only (Table-3).

Table 3: Screening of *Azotobacter* isolates for temperature tolerance

Sr. No	Isolates	Temperature (0C)						
		28^{0}C	**37^{0}C**	**40^{0}C**	**45^{0}C**	**50^{0}C**	**55^{0}C**	**60^{0}C**
1	*Azotbacter* - **1**	+	+	+	--	--	--	--
2	*Azotbacter* - **2**	+	+	--	--	--	--	--
3	*Azotbacter* - **3**	+	+	+	+	--	--	--

After comparing the characters exhibited in structural, morphological, biochemical analysis and temperature tolerance tests with that of Bergy's manual, all the three isolates were identified more or less similar to that of *Azotobacter vinelandii.*

Pot trial

Highest seed germination, speed of seedling emergence, seedling vigour index, plant height and branches per plant were recorded in all the treatments over untreated control (Table-4 and Fig-1).

Germination percent (GP)

The higher seed germination per cent was recorded in treatment-3: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage which was found statistically at par with treatment-2: seeds soaked in *A. vineandi*-2 solution up to imbibitions stage and treatment-1: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage followed by treatment-4: seeds soaked in water. Lowest seed germination was recorded in control.

Speed of emergence (SE)

The higher percent speed of seed emergence was recorded in treatment-3: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage which was found statistically at par with treatment-2: seeds soaked in *A. vineandi*-2 solution up to imbibitions stage and treatment-1: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage followed by treatment-4: seeds soaked in water. The lowest percent speed of seed emergence was recorded in control.

Seedling vigour index (SVI)

The highest seedling vigour Index was recorded in treatment-3: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage which was found statistically at par with treatment-2: seeds soaked in *A. vineandi*-2 solution up to imbibitions stage and treatment-1: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage followed by treatment-4: seeds soaked in water. Least seedling vigour Index was recorded in control.

Plant height

The highest plant height at 6 weeks after sowing was recorded in treatment-3: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage which was found statistically at par with treatment-2: seeds soaked in *A. vineandi*-2 solution up to imbibitions stage followed by treatment-1: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage and treatment-4: seeds soaked in water. Lowest seed germination was recorded in control.

Branches per plant

The highest branches per plant at 6 weeks after sowing was recorded in treatment-3: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage which was found statistically at par with treatment-2: seeds soaked in *A. vineandi*-2 solution up to imbibitions stage followed by treatment-1: seeds soaked in *A. vineandi*-3 solution up to imbibitions stage and treatment-4: seeds soaked in water. Lowest seed germination was recorded in control.

Table 4: Effect of *Azotobacter*. Seed treatment on germination and growth parameters of Green gram

Treatment	10 days after sowing			6 weeks after sowing	
	Germination (%)	**Speed of emergence (%)**	**Seedling Vigour Index**	**Plant height (cm)**	**Branches/ plants (nos.)**
T_1 : seeds soaked in *A. vineandi*-1 solution up to imbibitions stage	83.33	82.05	79.98	58.00	5.25
T_2 :seeds soaked in *A. vineandi*-2 solution up to imbibitions stage	86.67	86.54	81.47	60.25	6.00
T_3 :seeds soaked in *A. vineandi*-3 solution up to imbibitions stage	88.33	86.93	84.78	65.75	6.25
T_4 :seeds soaked in water up to imbibitions stage	78.33	79.05	76.65	53.75	5.00
T_5 : untreated seeds	75.00	75.57	71.30	50.25	5.00
SEm±	2.31	2.22	2.68	1.37	0.16
CD @ 5%	6.98	6.70	8.08	4.13	0.47
CV	5.62	5.42	6.80	4.76	5.74

Fig 1: Effect of Azotobacter spp. seed treatment on seed germination and growth parameters of Green gram

The present research work carried out with an objective to study the effect liquid formulation of *A. vinelandii* inoculation on germination and growth parameters of *V. radiata.* The results obtained in present study are discussed here with in context to earlier workers. Seed inoculation of *Azotobcter* induces higher seed germination, speed of seedling emergence, seedling vigour index, plant height and branches per plant in all the treatments over untreated control. The increase in germination percentage due to inoculation of liquid bioinoculants may be due to ability to suppress the growth of antagonists present in soil and on seed coat and release of plant growth promoting substances around seed rhizosphere, as reported earlier by Jadhav and Patil (1985) [7] that *Azotobacter* as biofertilizer performed better than inorganic fertilizers in relation to increase seed germination of paddy plant. Moreover, similar types of results were also obtained by *Azotobacer* and PSB in okra. Sharma *et al.* (2007) [16] reported increase in seed germination due to inoculation with PSB in *Cicer arientinum* L. *Azotobacter* and PSB were found to be significant in increasing seed germination was also reported earlier by Pathak *et al.* (2013) [12] and Mahato *et. al.* (2009) [10].

Conclusion

Azotobacter spp. was free living bacteria in natural soil habitats and can be isolated easily isolated on *Azotobacter* specific media. In all three isolates of *Azotobacter vinelandii* were isolated and identified from the soil collected from different habitats. Seed bacterization with all the three isolates of *Azotobacter vinelandii* was responsible to increase seed germination, speed of seed emergence, seedling vigour index, plant height and numbers of branches per plant in green gram crop.

Conflict of intrest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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