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Effect of Nitrogen Fixing Bacteria for Plant Growth Promotion of *plantago ovata*(Isabgol)

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Abstract

Plantago ovata is very important medicinal herb mainly used in intestinal disorders. Nitrogen Fixing Bacteria(NFB) have ability to fix atmospheric dinitrogen in to ammonia. Present study was carried out to isolate nitrogen fixing bacteria from soil and their application on *Plantagoovata* (Isabgol) plant. NFB were isolated on different nitrogen free media such as Ashby's Mannitol Agar, Nitrogen Free Bromothymol blue medium, Rc medium etc,. Total twenty five strains were isolated and their morphological and biochemical characteristics were studied. Isolates AM1, AM2, MM5,MB2 and MB9 were selected on the basis of nitrate reduction activity. All isolates showed NH_3 production. Maximum IAA production (9.0 ìg/ ml) was showed in MM5 culture in 500 ìg/ ml of tryptophan concentration.Zone of phosphate solubilization showed in MM5 which was3mm in diameter. Pot experiment was done to study the effect NFB on isabgolplant. The treatment T3 was showed the best growth followed by T2, T1, T4, and T5 as compared to control. From the all treatments the highest length of root and shoot showed in T3 (3.2, 7.1 cm) as compared to control plantand maximum Chlorophyll a, b content showed in T3 (2.6 mg/ ml and

2.9 mg/ ml) as compared to control plant at 30 days after sowing. Total nitrogen was estimated by Kjeldahl method. The value of total nitrogen was measured recorded as follows in control (0.28%), T1 (0.41%), T2 (0.41%) at 30 days of plant growth. yield of isabgol plant can be improved by using MM5 and AM2 culture.

Keywords: *Plantago ovate,* Nitrogen Fixing Bacteria (NFB), PGP activity, Kjeldahl method.

Introduction

Isabgol*(Plantagoovata*Forsk.), an annual stemless medicinal herb that has been used in health care for many centuries in South Asia, whereas it is now widely used for its medicinal properties all over the world (MajidPouryousef et al., 2007). Isabgol has been used in medicine since ancient times, but it hasbeen cultivated as a medicinal plant only in recent decades (Gupta, 1987; Wolver et al., 1994; Handa and Kaul, 1999; Lal etal., 1999; R.Omidbaigi and M.Mohebby, 2002). The seed of Isabgol contains mucilage, fatty oil, large quantities of albuminous compound, a pharmacological inactive glycoside, namely Aucubin ($C_{13}H_{19}O_8H_2O$) and pentose sugar (R.Omidbaigi and M.Mohebby, 2002; Zahooe et al., 2004; MajidPouryousef et al., 2007). Isabgol is grown as a cash crop in parts of Rajasthan and Gujarat. The husk is used as a medicine for correcting the intestinal disorders, particularly in habitual constipation, chronic diarrhoea and dysentery (Godawat, 1999). It is alleviates kidney, diuretic and bladder complaints, gonorrhoea, arthritis and haemorrhoids (MajidPouryousef et al., 2007).

Eighty persent (80%) of the atmosphere is nitrogen (N_2). Unfortunately N_2 is unusable by most living organisms. Plant, animals and microorganisms can die of nitrogen deficiently, if surrounded by N_2 they cannot use. But it becomes available through the biological nitrogen fixation process that only prokaryotic cells have developed (Rovira 1991). Nitrogen is generally considered one of the major limiting nutrients in plant growth. The biological process responsible for reduction of molecular nitrogen into ammonia is referred to as nitrogen fixation (ClaudineFranche et al., 2008). Biological Nitrogen Fixation (BNF) is a natural process whereby the atmospheric nitrogen is converted to ammonia by a specific enzyme known as nitrogenase. Diazotrophic bacteria sach as *Rhizobium, Azotobacter, Azospirillum, Cyanobacteria* have an ability to fix atmospheric N_2. They can be classified as symbiotic and non symbiotic or free-living forms. At least 90 genera of

specialized microorganisms are known to have the enzyme nitrogenase and can fix atmospheric nitrogen in to ammonia (Murray and Jeff, 2008).

Nitrogen-fixing bacteria are widely distributed in nature where they reduce atmospheric nitrogen in soil or in association with plant (Skimmer and Banfield, 2005). They have been found in a wide variety of terrestrial and aquatic habitats in both temperate and tropical regions of the word (Yooshinan, 2001). The only confirmed free living nitrogen fixing bacteria belonging to the kingdom *Eubacteria* and *Archaebacteria* are currently known to fix nitrogen (Perotto and Bonfate, 2010). Many are heterotrophic that need a supply of reduced carbon , for example: *Azotobacter spp*. and *Azospirillum species*. Others are autotrophic, that is, they reduce carbon dioxide (Graham, 2000). Nitrogen fixation generally occurs only under anaerobic or microaerophilic (i.e., low concentration of oxygen) conditions and only few strains of a particular species show such activity (Scow, 2007).

The presence of *Azotobacter sp*. in soil has beneficial effects on plants, but the abundance of these bacteria is related to many factors, soil physico-chemical (e.g.organic matter, pH, temperature, soil moisture) and microbiological properties (Kizilkaya R, 2009). *Azotobacter*are much more abundant in the rhizosphere of plants than in the surrounding soil and that this abundance depends on the crop species (Sariv Z et al.1963; Dong H et al., 2012). The species of *Azotobacter* capable of fixing an average 20 kg N/ ha/ year (Kizilkaya R, 2009). Besides, nitrogen fixation,*Azotobacter*produces, Thiomin, Riboflavin, Indole Acetic Acid Nicotin, and Giberalin (Brakel J, Hilger F, 1965). Most studied on the *Azospirillum*inoculations have suggested that nitrogen fixation was the major mechanism of plant growth (Rennie et al., 1983; Lima et ai., 1987). Up to 50 % of the N content of crop such as sugarcane, Panicum maximum and Paspalumnotatum could be supplied by associated nitrogen fixers mainly *Azospirillum*(Lima et al., 1983). Nitrogen fixing microorganism such as *Azospirillum*, directly benefits to plant for improving shoot and root development and increasing the rate of water and mineral uptake by roots (Gonzalez et al., 2005)

Materials and Methods

Sample collection

Soil sample where collected from the different isabgol farm near Unja (Mehshanadist) and Palanpur(Banaskantha dist.). They were collected in sterile polythene bags with the help of alcohol sterilized spatula.

Isolation of Nitrogen Fixing Bacteria (NFB)

Isolation of *Azotobacter*

1 g of soil sample was suspended in to 10 ml of sterile distilled water followed by serially diluted up to 10^{-4} and from that 0.1 ml of supernatant spread on Ashby's Mannitol Agar medium (it containing per liter of distilled water: 20.0 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSo_4$. $7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5.0 g $CaCO_3$, 20.0 g agar, pH 7.4±0.2). Incubacted the plates at 32 C ± 2 C temperature for 2 to 5 days(Rakeshpatel, 2011; Onyeze Rosemary et al. 2013).

Isolation of *Azospirillum*

Isolation of *Azospirillum* 1 g of soil sample was suspended in to 10 ml of sterile distilled water and from that take 0.1 ml supernatant and inoculated in to Nitrogen Free Bromothymol Blue semi-solid (Nfb) medium (containing per liter of distilled water: 5.0 g malic acid, 4.0 g KOH, 0.5 g K_2HPO_4, 0.05 g $FeSO_4$. $7H_2O$, 0.01 g $MnSO_4$. $7H_2O$, 0.01 g Mg SO_4. $7H_2O$, 0.02 g NaCL, 0.01 g $CaCl_2$, 0.002 $NaMoO_4$, 0.5% Bromothymol blue 2 ml, 0.005% Yeast extract, 3 % Agar, pH 6.8±0.2).

Incubated at 32 C ±2 C temperature for 5 to 7 days. After incubation white, thin dense pellicle was formed few mm below the surface of the medium.From that tubes pellicle was streaked on Nfb agar plates and Rojo-Congo (RC medium containing per liter of distilled water: 0.5 g K_2HPO_4, 0.2 g $MgSO_4$. $7H_2O$, 0.1 g NaCl, 0.5 g yeast extract, 0.015 g $FeCl_3$.$6H_2O$, 5 g DL-malic acid, 4.8 g KOH, 15 ml of 1:400 aqoueous solution of Congo Red(autoclave separately), added after autoclaved the medium) agar plates. All plates were incubated at 32 C ±2 temperature for 5 to 7 days (Enrique,1982; Onyeze et al., 2013; Kanimozhi and Panneerselvam, 2010).

Maintanace of bacterial isolates

Isolated Bacterial cultures were maintained on respective media such as *Azotobacter*on

AshbysMannitol Agar Medium and *Azospirillum*on Nitrogen Free Bromothymol blue medium. Preserve that slant at 4C temperature in refrigerator

Microscopic observation of isolates

Microscopic analysis was done for the study of morphological characteristics of isolated bacteria. For study Gram staining were done and observe under microscope.

Biochemical characteristics of isolates

Bacterial isolates were characterized according to Bergey'sMannualof Systemic Bacteriology. Characterization of isolates various biochemical tests were perform such as : MR-VP test, Indole test, Urea Utilization, Ammonium production, Starch Hydrolysis, GelatinLiquification test, Citrate Utilization test, Sugar fermentation, etc.

Nitrate reduction test

Inoculate PNB (peptone nitrate broth) with a loopful of culture and incubate at 32±2 C for 24 hours. After incubation add 0.5 ml of á-napthylamine reagent and 0.5 ml Sulphanilic acid in each tube. Observe the development of red colour within 30 seconds after adding test reagent indicates the presence of nitrates and positive nitrite reduction test (Rakesh J Patel, 2011).

Plant growth promoting activities

Indole -3 Acetic acid production

Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986) at different concentration of tryptophan (0, 100, 200, 300, 400 and 500 ìg/ ml).bacterial cultures were grown for 72h (*Azotobacter*) and 96h (*Azospirillum*) on LuariaBartani Broth at 32±2 C temperature. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The 2ml supernatamt mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$solution). Development of pink colour indicates IAA production. Optical Density was taken at 530 nm with the help of Spectronic 20 D^+ Spectrophotometer. From the standard graph of IAA the concentration of IAA produced by culture was measured. The standard graph was prepare IAA concentration in range of 10- 100 ìg/ml (Farah Ahmad et al.,2006).

NH_3 production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml of peptone water in each tube respectively and incubated at 30 ±2 C for 48 to72 h. After incubation 0.5 ml Nessler's reagent was added in each tube. Development of brown toyellow colour was a positive test for ammonia production (Farah Ahemad et al., 2006).

HCN production

Nutrient agar was ambeded with 4.4 g glycine / l were used for this test. The bacterial isolates were streaked on the modified agar plate. A Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plates were sealed with parafilm and incubated at 30 ±2 C for 4 days. Development of orange to red colour indicated HCN production (Farah Ahemad et al., 2006).

Phosphate solubilization

To check the phosphate solubilization activity of isolates Pikowskaya's Agar medium ambeded with bromophenol blue was used. Spot inoculation was done on Pikowskay's medium and inatcubate at 30 ±2 C for 2 to 4 Days. After incubation check the clear zone of phosphate solubilization around the colonies representing the production of organic acids as a possible mechanism of the phosphate solubilization (Guar,1990; (Farah Ahemad et al., 2006).

Pot experiment

For pot experiment sterile polythene bags were used. The garden soil was sterilized at 60 C temperature for 24 h in hot air oven. After that the approximately 500 g of soil filled in the each pot. The seeds of *plantagoovata* plant surface sterilized by 3% H_2O_2 followed by three time washed with sterile distilled water. For post study the sterilized seed were drying on sterilized Whatman No.1 filter paper. All seeds were coated with 1% CMC (Carboxy Methyl Cellulose) and allow that for 2 to 3 hr. After that the CMC coated seeds were inoculated in 2 x 10^8 cells/mlof respective culture. Allow this treated seeds to overnight for better coating around the seed. 5 to 7 seeds were sown in each pot having 20% moisture. For each treatment three such pots were maintained. Uninoculated seeds were sown in pot served as control. After 30 days of plant growth the biometric parameter was measured (Shimon Mayak et al., 2004).

Measurement of Biometric Parameters of Plant

After 30 days of plant growth, plants were carefully uprooted from soil (pot) and washed under running tap water to remove adhering of soil particles. The following biometric parameters of treated and untreated plant were recorded: Length of Root and Shoot (cm) , Fresh weight of Root and Shoot (g), Dry weight of Root and Shoot (g) , Number of leaves , Chlorophyll content (mg/g). Dry weight of plants was taken after drying the plants at 60 c temperature in oven for overnight (Tank and Saraf, 2008).

Determinaion of Chlorophyll content from plant tissue

Plant tissues were properly washed with distilled water to remove soil particles. Homogenise 0.5 g of fresh plant tissue in 5 ml of 80% acetone and centrifuged at 4000 rpm for 20 minute. Collect the supernatant and re-extract the residue by adding 5 ml of 80% acetone twice. After centrifugation, mix all the supernatants and make the final volume to 20 ml with 80% acetone. Mixed the tubes properly for correct results. Optical Density of different samples were measured at 645 nm and 663 nm on spectronic 20 spectrophotometer by using 80% acetone as blank (Khaleghi et al., 2012).The amount of chlorophyll a, b and total was calculated by the following formula :

Chlorophyll a (mg/g fresh weight).

= 127 x O.D. (663) 2.69 x O.D. (645) x V / 1000 x W

Chlorophyll b (mg/g fresh weight)

= 22.9 x O.D. (645) 4.68 x O.D. (663) x V / 1000 x W

Total chlorophyll (mg/g fresh weight).

= 20.2 x O.D. (645) + 8.02 x O.D. (663) x V / 1000 x W

Where as ,

W= Sample weight (g)

V= Final volume of extract (ml)

Measurement of Total Nitrogen

Total nitrogen measured by Kjeldahl method. After 30 days of plant growth soil samples were taken and brought to the laboratory for estimation of total nitrogen. The estimation of total nitrogen was carried out at gujarat laboratory, Ahmedabad.

Results

Total twenty five bacterial isolates were isolated on Ashby's Mannitol Agar plates and Nitrogen Free Bromothymole Blue Agar plates respectively from two different soil samples. From the twenty five isolates the nineteen isolates on Ashby's Agar plate and Six isolates on the NFb agar plates. The bacterial isolates were purified by sub culturing them on respective medium. The well isolated colonies were transfer on slants of the respective medium. Among the twenty five bacterial isolates five isolates were selected for further work on the basis of nitrate reduction activity. The cultural and morphological characteristics of isolates were mentioned in the table 1 respectively.

Table 1: cultural characteristics of isolates

Characters	AM1	AM2	MM5	MB2	MB9
Size	Large	Large	Big	Small	Small
Shape	Round	Round	Irregular	Round	Round
Margin	Entire	Entire	Undulate	Entire	Entire
Elevation	Convex	Convex	Raised	Flat	Flat
Texture	Smooth	Smooth	Smooth	Smooth	Smooth
Consistency	Dew drop	Dew drop	Moist	Moist	Moist
Opacity	Transparent	Transparent	Translucent	Opaque	Opaque
Pigmentation	Nil	Nil	Nil	Nil	Nil

Microscopic Observation

The morphological characteristics of all isolates were done by Gram staining. Among five selected isolates four isolates were short rod, Gram negative and one isolate was rod shape Gram positive in nature.

Biochemical characterization

Different biochemical test were performed to characterize the bacterial isolates. The biochemical characterization was done according to Bergey's Manual of Systemic Bacteriology vol.2. Result obtained from the different biochemical test the two isolates AM1, AM2 characterized as *Azotobacter sp.*, two isolates MB2, MB9 as *Azospirillum sp.* and one isolate MM5 as *Bacillus spp.*

Table 1: 2 Biochemical Characteristics

Tests	**AM1**	**AM2**	**MM5**	**MB2**	**MB9**
Catalase	+ve	+ve	+ve	+ve	+ve
Citrate utilization	-ve	-ve	+ve	+ve	+ve
Urease test	-ve	-ve	-ve	-ve	-ve
Phenylalanine	-ve	-ve	-ve	-ve	-ve
Starch hydrolysis	+ve	+ve	+ve	+ve	-ve
Gelatine liquification	-ve	-ve	+ve	-ve	-ve
Gelatine hydrolysis	-ve	-ve	-ve	-ve	-ve
Methyl Red	-ve	-ve	-ve	+ve	+ve
Voges-Proskauer	-ve	-ve	-ve	-ve	-ve
Carbohydrate fermentation					
Glucose	AG	AG	AG	AG	AG
Sucrose	AG	AG	A	AG	AG
Maltose	AG	AG	A	A	A
Mannitol	AG	AG	AG	AG	AG
Lactose	AG	A	A	A	A
Fructose	AG	AG	AG	AG	AG
Motility	+	+	+	+	+

Key: -ve = Negative, +ve = Positive, A= Acid production, AG = Acid and Gas production.

Nitrate reduction test Five isolates showed nitrate reduction test positive among the twenty five isolates. After addition of test reagent red colour were developed within 30 second in five isolates. Isolates were selected on the basis of nitrate reduction test.

PGPR activity

Quantitative assay of IAA production by selected isolates

Total five selected bacterial isolates AM1, AM2, MM5, MB2, MB9 were tested for the estimation of IAA in the presence of different concentration of tryptophan. Production of IAA was not observed in without addition of tryptophan.

Table 2: Indole Acetic Acid production by selected isolates

Isolates	IAA production (µg/ml) at different concentrations of tryptophan (µg/ml)					
	0	100	200	300	400	500
AM1	ND	ND	1..0	2.0	4.0	6.0
AM2	ND	1.0	3.0	4.0	5.0	7.0
MM5	ND	1.0	3.0	4.0	6.0	9.0
MB2	ND	1.0	1.0	2.0	3.0	4.0
MB9	ND	1.0	1.0	1.0	2.0	4.0

ND- not detectable.

Maximum IAA concentration ìg/ml found in T3 as follows : 9ìg/ml, 6ìg/ml, 4ìg/ml, 3ìg/ml, 1ìg/ml in 500 ìg/ml, 400 ìg/ml, 300 ìg/ml, 200 ìg/ml, 100 ìg/ml of tryptophan concentration respectively.

NH_3 production

NH_3 production checked qualitatively. All inoculated tubes were observed brown to yellow colour which was positive test for ammonia production. Table 3 showing NH_3 production.

Table 3: NH_3 production

Isolates	AM1	AM2	MM5	MB2	MB9
NH_3	+	+	+	+	+

+- represent positive

HCN production

Among all isolates MM5 showed the HCN production after 4 days of incubation. There was no HCN production was observed in other isolates except MM5.

Phosphate solubilization

Phosphate solubilization was studied using pikovskaya's medium as solid agar medium. Among the five isolates, isolate MM5, AM1, AM8 and MB9 showed zone of phosphate solubilization. Isolate MM5 showed maximum zone of phosphate solibilization and diameter of zone around colony was 3 mm after 3 days of incubation. AM2, AM1 and MB2 also showed zone of phosphate solubilization and diameter of zone as follows: 2mm, 2mm, 1.0 mm after 4 days of incubation.

Pot study

Total five treatments were given to the isabgol plant as compared to control one(without treatment). After the 30 Days the biometric parameters were studied. From the five treatments the T3 and T2 showing the best result as compared to the control plant. Root length and shoot length represent in graph1. Chlorophyll a,b also measured and mentioned in graph. Highest chlorophyll a, b content showing in T3 followed by T2, T1, T4, T5.

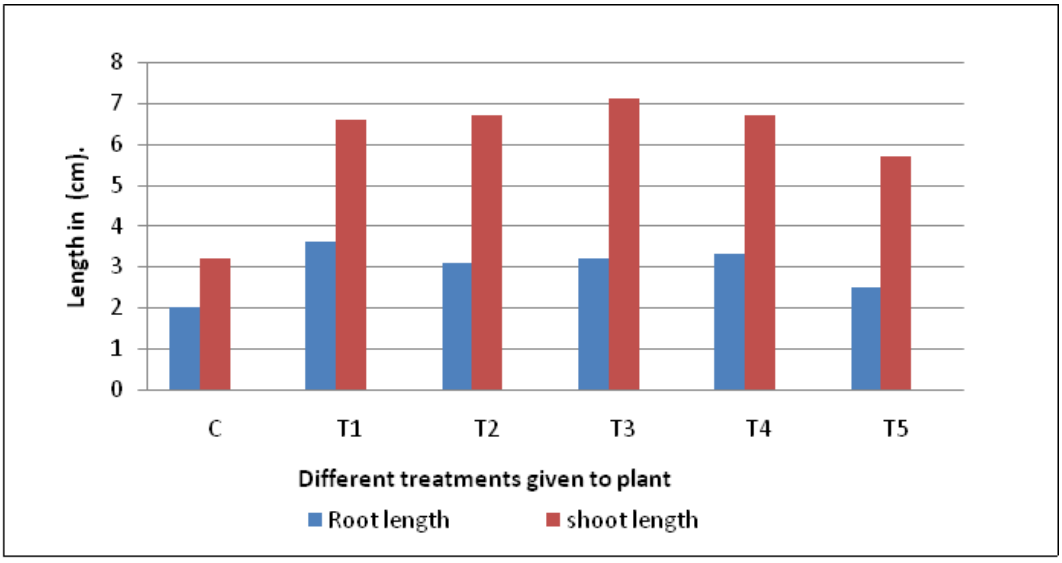

Figure: 1. Measurement of root and shoot length of plant.

From the all treatments the highest length of root and shoot showed in T3 (3.2, 7.1 cm) followed by T2 (3.1, 6.7 cm), T1 (3.6, 6.6 cm), T4 (3.31, 6.1 cm), T5 (2.5, 5.7 cm), C (2.0, 3.2 cm) at 30 days after sowing.

Numbers of leaves were counted in each treatment. 4 leaves found in T1, T2, T3, T4, T5 and 2were fond in control.Maximum wet weight of root and shoot observed in T3 (0.0014 g, 0.0378 g) followed by T2 (0.010, 0.0313) after 30 days of sowing.

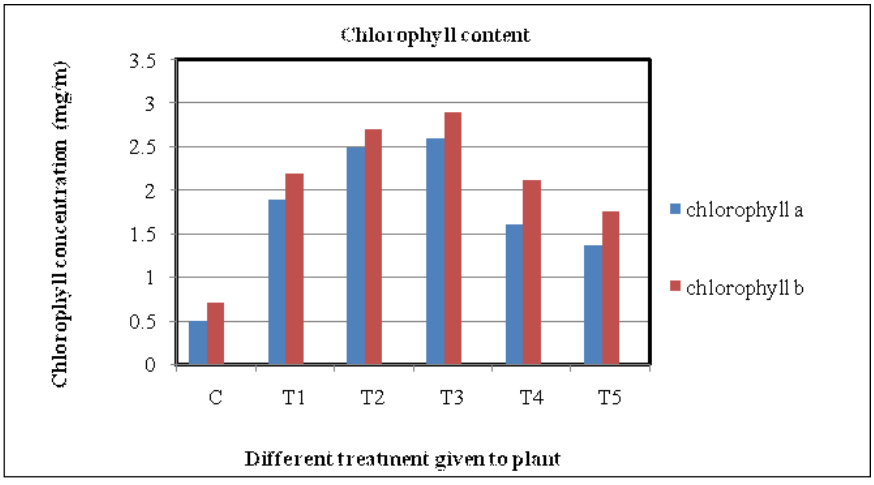

Figure: 2. Estimation of chlorophyll a, b from plant tissue.

Among all treatments the maximum Chlorophyll a, b content showed in T3(2.6 mg/ ml and 2.9 mg/ ml) followed by T2 (2.5 mg/ ml, 2.7 mg/ ml), T1 (1.9 mg/ ml, 2.2 mg/ ml), T4 (1.6 mg/ ml, 2.12 mg/ ml), T5 (1.36 mg/ ml, 1.75 mg/ ml), C (0.5 mg/ ml, 0.7 mg/ ml) at 30 days after sowing.

Measurement of Total Nitrogen

The total nitrogen was measured from the soil sample by Kjeldahal method. The two treatmentswere given the best result only that sample used for measurement of nitrogen. The value of total nitrogen which canmeasured recorded as follows the control (0.28%), T1 (0.41%), T2 (0.41%). The amount of total nitrogen increased in bacterial treated plant soil as compared to control plant soil.

Discussion

Nitrogen (N_2), is an essential element foe the support of all forms of life. It is found in amino acids and proteins and many other organic compounds are derived from the nitrogen fixation process (Egamberdiveva, D. and Z.Kucharova, 2008). N_2 is the most abundant gas in earth's atmosphere, it is extremely unreactive (Frank et. al., 2003). Biological nitrogen fixation is carried out only by prokaryotes, which may be free living or symbiotic in nature. Nitrogen-fixing organisms are generally active in plant root zone soil (BagaliShrimantShridhur, 2012).

In the present study total twenty five bacterial isolates were isolated from different isabgol farm soil sample. Among them 19 isolates on Ashby's Agar medium and 6 on Nitrogen Free Bromothymol blue medium respectively. characterization of selected isolates were done by studied the cultural , morphological and biochemical characteristics. From that study the isolate AM1,AM2 characterized as *Azotobacter spp*., isolate MM5 as *Bacillus spp*. and isolate MB2, MB9 as *Azospirillum spp*.

It has been assumed that inoculation with diazotrophic bacteria like Rhizobium, Azotobacter and Azospirillum enhanced the plant growth as a result of their nitrogen fixation ability. 47 isolates belonging to M. Ciceri, fluorescent Pseudomonas and Bacillus species were screened in vitro for PGP activites.(Farah Ahmad et. al., 2008).

In present study five isolates were tested for their PGP activity such as IAA production, NH_3 production, HCN production and phosphate solubilization. IAA production detected in all the five isolates such as AM1, AM2, MM5, MB2,MB9. Among five isolate the maximum production showed in MM5 which was 9.0 ìg/ml in 500 ìg/ml of tryptophan concentration. NH_3 production

also showed in all selected isolate. HCN production was not detected in all isolates only MM5 showed the little production. Phosphate solubilization was most frequently happened by *Bacillus* isolate, followed by Azotobacter andAzospirillum isolates. Highest Zone of phosphate solubilization observed in MM5 culture which was 3mm in diameter. Above five selected isolates could exhibit more than two or three PGP activity, which may promote plant growth directly or indirectly.

Saxena and Rao observed that there was significant variation in the seed yield of isabgol in different treatmentswith the maximum increase with 40 kg Nha-I.

Five treatment were given to isabgol plant and after 30 days biometric parameters were studied best growth showed in treatment T3 followed by T2 > T1 > T4> T5> C. From the all treatments the highest length of root and shoot showed in T3 (3.2, 7.1 cm) as compared to C (2.0, 3.2 cm) at 30 days after sowing. 4 leaves were found in all treatments and two leaves in untreated one. Among all treatments the maximum Chlorophyll a, b content showed in T3 (2.6 mg/ ml and 2.9 mg/ml).

Conclusion

Present study concluded that the application of isolated NFB on *Plantagoovata*wasenhanced the plant growthas compared to control plant. Moreover total nitrogen was found increased in soil after harvesting. Culture MM5 and AM2 showed the good PGP activity and enhanced plant growth, so it could be used as PGPR for improving plant growth. Among the five treatments T3 showed the best result of plant growth promotion as compared to control plant. This microbial inoculants can be use as Nitrogen biofertilizer for improving isabgol plant growth and increase soil nitrogen through nitrogen fixation.

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