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PGP potential, abiotic stress tolerance and antifungal activity of *Azotobacter* strains isolated from paddy soils

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Azotobacter strains were isolated by serial dilution method and colonies were viscous, smooth, glistening, and brown to black colour on Jenson's N-free agar. Morphological and biochemical tests showed characteristic features of *Azotobacter*. Further, molecular analyses revealed the presence of different *Azotobacter* species *viz*., *A. armeniacus*, *A. chroococcum*, *A. salinestris*, *A. tropicalis* and *A. vinelandii.* The isolates were tested for their ability of nitrogen fixation, indole acetic acid (IAA), gibberllic acid production and phosphate solubilization. Four isolates (GVT-1, GVT-2 KOP-11 and SND-4) were efficient in fixation of highest amount of N_2 (29.21 µg $NmL^{-1}day^{-1}$), produced IAA (25.50 µg mL^{-1}), gibberllic acid (17.25 µg 25 mL^{-1}) and formed larger P solubilizing zone (13.4 mm). Some of the *Azotobacter* strains were produced siderophores, hydrogen cyanide and were positive for ammonia production with respect to antifungal activity of *Azotobacter* was tested with dual culture method and *A. tropicalis* inhibited the growth of *Fusarium, Aspergillus* and *Alternaria* species. *Azotobacter* isolates were tested against salt (0-10%), temperature (4-55ºC), pH (5.0-10) and insecticide chloropyrifos (0-3%) tolerance study. Among them, *A. chroococcum* was found tolerant to a maximum of 6% NaCl with a temperature of 35-45ºC and to a pH up to 8. All the 4 strains showed effective growth against 3% chloropyrifos concentration. The studies revealed that the *Azotobacter* strains not only produced plant growth promoting substances but are also tolerant to abiotic stresses such as temperature, pH and insecticides.

Keywords: Bioremediation, Chloropyrifos, Gibberllic acid, Indole acetic acid (IAA), Nitrogen fixation, Pesticides, Stress

Plant growth promoting rhizobacteria (PGPR) are important for soil fertility and crop productivity[1-4]. PGPR is a bio-alternative for chemical fertilizer nitrogen for sustainable agriculture by fixing the atmospheric free nitrogen (N_2) and producing growth promoting substances[4-6]. Among the entire PGPR group, *Azotobacter* are ubiquitous, aerobic, free-living and N_2 fixing bacteria commonly living in soil, water and sediments[7,8]. Many species of *Pseudomonas*, *Bacillus* and *Azotobacter* can grow and survive at extreme environmental conditions *viz*., tolerant to higher salt concentration, pH values and even in dry soils with maximum temperature[5,9]. Being a major group of soil borne bacteria, *Azotobacter* play many beneficial roles[10,11] and are known to produce varieties of vitamins, amino acids, plant growth hormones, antifungal antibiotics, hydrogen cyanide (HCN) and siderophores[12,13]. These growth promoting substances such as indole acetic acid (IAA), gibberellic acid (GA), arginine, etc., have direct influence on seed germination, shoot and root length of several agriculture crops[1,4,13].

The diversity and morphological characters of these species are well studied since last two decades because of its plant growth promoting activity for sustainable agriculture *viz*., *A. beijerinckiii*, *A. chroococcum*, *A. paspali* and *A. vinelandii*[9]. Reports are also available on the factors influencing the *Azotobacter* population in soil such as pH, phosphorus (P), carbon content[14], soil aeration and moisture contents. *A. chroococcum, A. nigricans, A. salinestris, A. tropicalis* and *A. vinelandii* are most commonly found in all the soil conditions and are tolerant of the commonly used pesticides, such as endosulfan, chloropyrifos, pendimethalin, phorate and glyphosate[15]. Several reports have already demonstrated that *Azotobacter* spp. not only tolerate but also degrade heavy metals. *A. chroococcum* and *A. vinelandii* have biodegradation efficiency of endosulfan, phorate, etc.[16,17]. *Azotobacter* species produce antimicrobial compounds (2, 3 dihydroxy benzoic acid, aminochelin, azotochelin, protochelin

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and azotobactin) which are known to inhibit the growth of many common plant pathogens *viz*., *Alternaria*, *Aspergillus*, *Curvullaria*, *Fusarium* and *Rhizoctonia* species[7,18].

In the present investigation, the soil samples collected from saline paddy fields of northern parts of Karnataka region exposed to higher temperature of above 45°C being an arid region. The soil samples were also collected from different paddy fields known to receive high dosages of pesticides. The different strains of *Azotobacter* species were isolated and characterized regarding plant growth promoting activity. Further, insecticide, abiotic stress tolerance and antifungal activity of selected *Azotobacter* strains were also studied.

Materials and Methods

Sampling, isolation and Identification of *Azotobacter* species

Soil samples were collected from different locations of paddy soils of Karnataka at a depth of 0 to 15 cm. *Azotobacter* strains were isolated by serial dilution agar spread plate method on Jenson's N- free media[1,19]. The inoculated and control plates were maintained in triplicates for higher accuracy of the results. The plates were incubated at 28±2ºC for 5 days. After incubation, discrete and well-grown colonies were enumerated[20]. *Azotobacter* cultures were identified and characterized based on the morphological features and biochemical tests as described in the Bergey's Manual of Bacteriology. Pure cultures were obtained by repeated sub-culturing on the same media slants.

Molecular characterization

Genomic DNA was extracted from *Azotobacter* strains using DNA extraction kit as per the manufacturer's protocol and purified with PCR purification kit (Hi-Media). Purified DNA was amplified with 27f (AGAGTTTGATCMTGGCTCAG) and 519r (GWATTACCGCGGCKGCTG) primer set. The 25 µL PCR reaction mixtures contains DNA template 50 ng, 1X *Taq* buffer, 0.2 mM of each of deoxyribonucleotide triphosphate mixture, 1 µM of each primer, 1.5 mM $MgCl_2$, 2U of *Taq* DNA polymerase (Bangalore Genei). PCR amplification reaction was carried out as in Chennappa *et al.*[15]. The PCR products were cloned into pGEM®-T Easy Vector Systems (Promega) and transformed into competent *E. coli* strain DH5α by following the manufacturer instructions. Plasmid DNA was isolated and the presence of insert was confirmed by restriction digestion of plasmid DNA with *Eco*RI restriction enzymes (Life Sciences, Canada). The clones of interest were sequenced (Xcelris Pvt. Ltd, Ahmadabad, India), and the sequences were confirmed with NCBI BLAST database for identity of the isolates based on previously published database sequences. Online software MEGA 5.1 was used to construct the phylogenetic tree using Maximum Likelihood NJ method (http://megasoftware.net).

PGPR activity of *Azotobacter* species

In vitro IAA production

Strains of *Azotobacter* were inoculated in Jenson's N- free broth media supplemented with 1% tryptophan to enhance the bacterial growth. One set without bacterial culture was maintained as control and both were incubated at 28±2ºC for 7 days. After incubation, the broth cultures were centrifuged at 5000 rpm for 15 min. The test sets were maintained in triplicates. The IAA estimation was done according to Ahmad *et al.*[1] with Solawaski's reagent (50 mL of 35% perchloric acid; 1mL of 0.5N $FeCl_3$). The development of pink colour was read at 530 nm using spectrophotometer (Genway 6506), and the IAA produced was estimated by referring to a standard graph prepared with different levels of standard IAA[21].

In vitro GA production

The gibberllic acid produced by *Azotobacter* isolates was estimated as described by Upadhyay *et al.*[22]. *Azotobacter* isolates were inoculated in sterilized nitrogen free malate broth medium and incubated for 7 days at 28±2ºC and uninoculated set was maintained as control. After incubation, 2 mL of zinc acetate was added to the 25 mL culture filtrate and allowed for 2 min. About 2 mL of potassium ferrocyanide was added to the mixture and centrifuged at 1000 rpm for 15 min. About 5 mL of supernatant was mixed with 5 mL 30% HCL and incubated at 20ºC for 75 min. The absorbance of the samples was measured at 254 nm in UV spectrophotometer. The test sets were maintained in triplicates. The amount of GA present in the extract was calculated from the standard curve of GA.

In vitro phosphate solubilization

Azotobacter isolates were inoculated by steak plate method onto Pikovskaya agar media plates containing tricalcium phosphate (TCP) as a phosphate source. The inoculated plates were incubated for 5-7 days at 28±2ºC[23] and observed for the halo zone formation around the discrete colonies. The plates were maintained in triplicates for higher accuracy of the results.

In vitro Nitrogen fixation

Azotobacter isolates were grown on 50 mL Jenson's N- free broth in conical flask on a rotary shaker (100 rpm) under continuous airflow at 32ºC for 7 days and uninoculated media served as control. The concentration of N_2 in each liquid culture was measured by Kjeldahl method[24]. The flasks were maintained in triplicates for higher accuracy of the results. The N_2 fixation was determined in terms of increase in total Kjeldahl nitrogen (mg/L) in comparison to control using micro Kjeldahl apparatus[25].

Ammonia production

Freshly grown cultures were inoculated in 10 mL peptone water in test tube and incubated for 48-72 h at 28°C. After incubation period, Nessler's reagent was added to each of the tube and development of colour was recorded[26].

Siderophore production

Siderophore production was determined by Chrome Azurol S (CAS) assay. CAS agar medium was prepared according to procedure given by Ponmurugan *et al*.[27] and CAS agar was prepared from 4 solutions which were sterilized separately.

HCN production

All the isolates were screened for production of HCN by adapting the Lorck method[28]. Nutrient broth was amended with 4.4 g glycine/L and 25 mL of this medium broth was poured into the sterile Petriplates and were allowed to solidify. After solidification, the strains to be tested were streaked over the medium in a zig-zag manner. A sterile Whatmann filter paper No-1 discs (9 cm) was soaked in 2% sodium carbonate in 0.5% picric acid solution and was placed on the lid of the each petriplate. These Petriplates were then sealed with parafilm without any gap and incubated at 28°C for 4 days and development of orange to red colour was observed (Ponmurugan *et al*.[27]).

Abiotic stress tolerance studies

Salt tolerance

Jenson's N- free broth media containing different concentration of NaCl (0, 2, 4, 6, 8 and 10%) were inoculated with *Azotobacter* culture (1.0×10^4 CFU/mL) and all the flasks were incubated on a rotary shaker at 150 rpm for 10 days at 28±2ºC[22,29]. The growth rate of these strains was recorded with the optical density of the culture broth using UV/Vis spectrophotometer (Jenway 6505) at 600 nm wavelength[16]. Growth of the culture was also confirmed by plating of same broth culture onto Jenson's N- free agar plates supplemented with the same salt concentration. Total colony forming units (CFU) were also recorded for different concentration and were compared with the control plates.

Temperature tolerance

Azotobacter cultures were inoculated onto a Jenson's N- free flasks[1,19] at a pH of 7.0 and all the flasks were incubated on a rotary shaker at 150 rpm at different temperatures (4, 15, 25, 35, 45 and 55ºC) for 10 days and control flasks were incubated at 28±2ºC[12]. The growth rate of the strains was recorded with the optical density of broth turbidity using UV/Vis spectrophotometer (Jenway 6505) at 600 nm wavelength[30]. Total CFU in broth culture was also confirmed by plating onto Jenson's N- free agar plates for the temperature tolerance with the control plates. All the inoculated and control plates were incubated at same temperature ranges for 10 days[20]. All the tests were maintained in triplicates for higher accuracy of the results.

pH tolerance

The soil pH was measured and pH tolerance was carried out at different pH ranges according to the procedure as described in Jimenez *et al*.[9]. PBS buffer solution was prepared for acid tolerance studies by adjusting the pH using 1M HCl. *Azotobacter* strains were inoculated onto Jenson's N- free flasks at different pH concentrations (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0±0.5) and control flasks were maintained at pH 7.0. All the flasks were incubated on a rotary shaker at 100 rpm at 28±2ºC. The growth of the strains was recorded with the optical density of the broth using UV/Vis spectrophotometer (Jenway 6505) at 600 nm wavelength[16]. Total CFU in broth culture was also confirmed by plating onto Jenson's agar plates for the same pH tolerance along with the control plates. All the test samples were maintained in triplicates for higher accuracy of the results.

Screening of insecticide tolerant strains

The insecticide, chloropyrifos 20% EC (Swal Crop, India) has been selected for the present work, as it is widely used for the cultivation of paddy in this region. *Azotobacter* strains were inoculated to 100 mL of Jenson's N- free broth supplemented with each pesticide of 1, 2 and 3% concentration and without pesticides treatment flask inoculated with *Azotobacter* strains were maintained as control. All the flasks were incubated on a rotary shaker at 150 rpm for 10 days at

30±2ºC[12]. The growth rate of the strains was recorded with the optical density of the culture broth turbidity using UV/Vis spectrophotometer (Jenway 6505) at 600nm wavelength[16,30]. Growth of the culture was also confirmed by plating onto Jenson's N- free agar plates supplemented with the same chloropyrifos concentration and CFU was also recorded.

Antifungal activity

Antifungal activity of *Azotobacter* strains was studied using modified Jenson's media plates (with Potato Dextrose Broth). Media plates were inoculated by dual culture method with different species of common plant pathogens *viz*., species of *Alternaria alternata, Aspergillus flavus* and *Fusarium oxysporum* against *Azotobacter* strains and incubated for 5-7 days at 28±2ºC[31,32]. Observations were recorded at 24 h of intervals after 3 days of inoculation. Further, total population was also estimated and by mixing spore suspension of same fungus cultures with *Azotobacter* culture filtrate (10^3 CFU/mL) allowed for 4 h incubation. Serial dilution of the culture filtrate suspension was prepared up to 10^6 and 1 mL of serially diluted suspension was spread over Jenson's media plates and PDA plates, respectively and incubated for 5-7 days at 28±2ºC. The control plates without *Azotobacter* culture filtrate treatment were maintained in triplicates for further analyses. Total CFU were enumerated in treated plates as well as control plates.

Nucleotide sequence accession number

All the sequences of *Azotobacter* strains were deposited in NCBI gen bank along with location of the isolate using sequin online software (Sequin Application Version 12.30-2012/ info@ncbi.nlm.nih.gov). Accession numbers of the Gen Bank gen bank deposited isolates are: KF470798, KF470799, KF470800, KF470801, KF470802, KF470803, KF470804, KF470805, KF470806, KF470807, KF470808 and KF470809.

Results and Discussion

Sampling, isolation and identification of *Azotobacter* species

A total of 12 *Azotobacter* strains were isolated from paddy soil samples by serial dilution spread plate method. The isolates were sticky, water soluble, initially brown, light brown, pale yellow, milky white to black pigmented colonies which appeared on Jenson's N-free plates (Supplementary Fig. 1). The isolates were collected from water logged paddy soils and the total populations ranged between 2.4 and 5.3 ×10^4 CFUs/g of soil and were found similar to non water logged soils (Table 1). There were not much variation in population between the water logged and non-water logged soil samples. These isolates were Gram –ve, positive for indole production, citrate

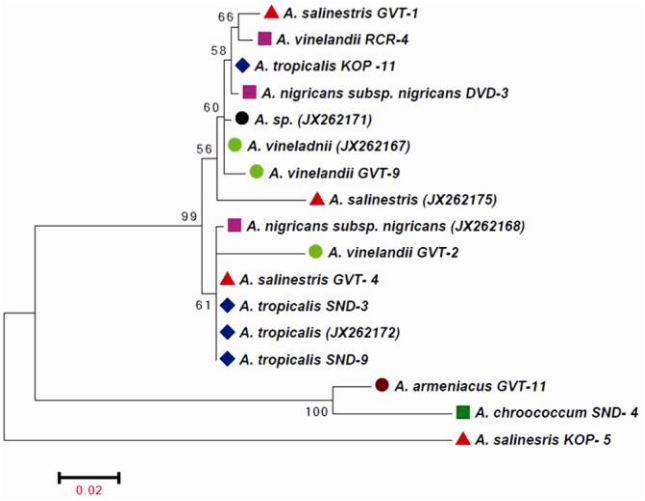

Fig. 1—Phylogenetic analyses of *Azotobacter* species based on NJ method isolated from paddy soil samples

Table 1—PGP potential *Azotobacter* strains isolated from northern parts of Karnataka region and their GenBank accession number

Location	Description	Pigments	Soil pH	Accession number
GVT-1	*Azotobacter salinestris*	Brown	8.8	KF470807
GVT-2	*Azotobacter vinelandii*	Light brown	8.1	KF470806
GVT-4	*Azotobacter salinestris*	Brown	8.4	KF470798
GVT-9	*Azotobacter vinelandii*	Pale brown	8.1	KF470805
GVT-11	*Azotobacter armeniacus*	Pale yellow brown	7.9	KF470809
SND-3	*Azotobacter tropicalis*	Dark brown	8.3	KF470799
SND-4	*Azotobacter chroococcum*	Brownish black	7.8	KF470801
SND-9	*Azotobacter tropicalis*	Brownish yellow	8.6	KF470800
KOP-5	*Azotobacter salinestris*	Brownish black	9.1	KF470803
KOP-11	*Azotobacter tropicalis*	Dark brown	8.5	KF470808
RCR-4	*Azotobacter vinelandii*	Pale brown	8.5	KF470802
DVD-7	*Azotobacter sp.*	Light brown	7.5	KF470804

utilization, catalase production and negative for methyl red and starch hydrolysis (Supplementary Table 1) features similar to the features of Bergey's manual of determinative bacteriology. Tejera *et al*.[11] reported similar kind of morphological and biochemical characters of *A. chroococcum* which were isolated from sugarcane rhizosphere soils. This indicates that *A. chroococcum* is strictly aerobic bacterium. However, *A. chroococcum* has also isolated from wet land paddy soils indicating their distribution in all soil conditions including wet land. Aquilanti *et al*.[8] reported 3 different isolation methods for *Azotobacter*. The population of *Azotobacter* in arid soil was obviously more and their activity was more conspicuous in arid soil as compared to the water logged soils observed here in this study.

All the strains were amplified with 27f and 519r primer. PCR amplicons were checked in 1.5% agarose gels, revealed all the amplicons were little above the 500 bp of DNA ladder (Supplementary Fig. 2). After amplification and cloning, the cloned fragments were sequenced and BLAST data result confirmed that all the sequences were similar to NCBI database sequences of *Azotobacter* (Table 1). Totally, six different *Azotobacter* species were identified *viz*., *A. chroococcum* (KF470801), *A. vinelandii* (KF470806), *A. salinestris* (KF470807), *A. tropicalis* (KF470799), *A. armeniacus* (KF470809) and *Azotobacter* sp. (KF470804). The phylogenetic analyses revealed that all the isolates were similar with one another in homology and formed 3 different clusters in the identity (Fig. 1). Morphologically and biochemically, all the isolates showed similar identification features but from sequence analyses confirmed the differences at species level. Similarly, Chennappa *et al*.[15] and Aquilanti *et al*.[8] reported different *Azotobacter* species *viz*., *A. agilis, A. armeniacus*, *A. beijerinckii*, *A. brasilense*, *A. chroococcum*, *A. insignis*, *A. nigricans*, *A. paspali*, *A. salinestris*, *A. vinelandii* and the cluster analyses of all the *Azotobacter* species except *A. tropicalis* and *A. nigricans* subsp. *nigricans*. The sequence detail reveals that these two species were genetically different from other species of *Azotobacter*.

PGPR activity

The highest IAA was produced by GVT-2 (25.50 µgmL^{-1}) strain supplemented with 1 mg of tryptophan (growth regulator) and SND-3 isolate produced a least amount of IAA (15.50 µgmL^{-1}). The production of IAA may vary from species to species and with the soil conditions (Fig. 2) and except SND-3 all the isolates produced equal quantity of IAA. Ahmad *et al*.[1] reported a maximum of 24.8 µgmL^{-1} of IAA produced by *Azotobacter* species when it was supplemented with 2 mg of tryptophan. Further, Patil[21] reported the highest amount (32.80 µgmL^{-1}) of IAA produced by *Azotobacter* supplemented with 5 mg of tryptophan. The variation in IAA production observed between the two experiments was due to supplementation with 2 and 5 mg of tryptophan. The GVT-2 isolate produced highest amount of IAA in 1 mg as compared to 5 mg tryptophan which was in conformity with the previous results. The addition of tryptophan has enhanced the IAA producing activity of *Azotobacter*. Similarly, Upadhyay *et al*.[22] isolated PGPR strains from different salinity soils and all the strains produced IAA in the range 3.79-119.63 µgmL^{-1}.

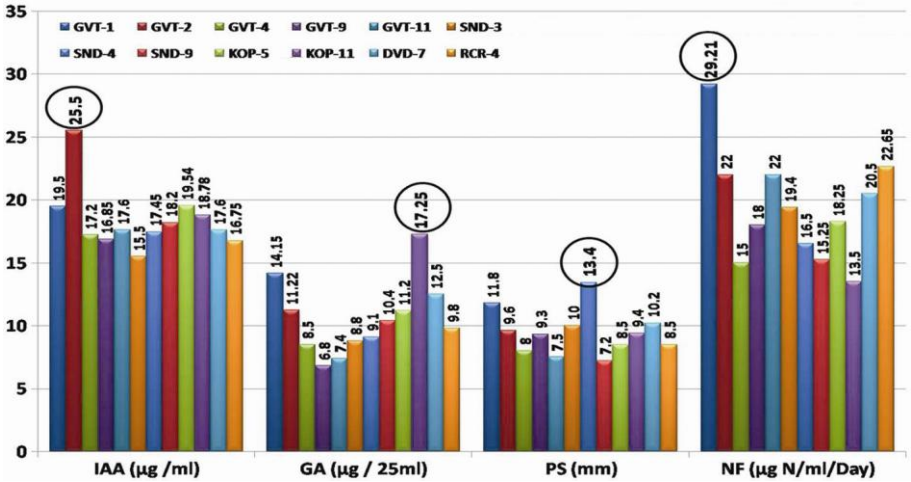

Fig. 2—PGPR activity (IAA, GA production, P solubilization and nitrogen fixation) of 12 potential *Azotobacter* strains isolated from northern parts of Karnataka region

This report clearly indicates that the diazotrophic bacteria have the same type of metabolic activities in the bacterial diversity. GVT-2 has the capacity to produce IAA at a greater rate as compared to the other isolates.

All the *Azotobacter* isolates produced GA in the range 6.8-17.25 μg25mL^{-1}. However, KOP-11 produced the highest amount of GA (17.25 μg25mL^{-1}) as compared to other species under *in vitro* conditions (Fig. 2). GA is also one of the important plant growth promoting substances produced by PGPR including *Azotobacter* species. Similarly, Upadhyay *et al*.[22] reported production of gibberellins by PGPR strain of *Arthrobacter* (130.74 μgmg^{-1}) and *Bacillus* species (7.67 μgmg^{-1}) isolated from wheat rhizosphere under 1-8% NaCl saline conditions. The variation in GA production was due to NaCl concentration that influenced bacterial growth rate. All PGPR strains will not produce equal quantity of GA and it varies from species to species. GVT-2 and KOP-11 strains proved their efficiency in IAA and GA production and thus a better PGPR strain and have greater potential of plant growth promoting efficiency with respect other strains.

The formation of clear zone indicates that the isolates were able to solubilize the available TCP to phosphates. After 7 days of incubation, clear halo zone recorded from the edge of the colony were 7.2 to 13.4 mm in diameter. Among 12 strains, *A. chroococcum* (SND-4) formed larger halo zone (13.4 mm) around the colony as compared to other strains (Fig. 2). Previously, Upadhyay *et al*.[22] isolated P solubilizing PGPR strains at 1-8% NaCl condition where in all the strains solubilized P at the rate 4.5 to 8.0 mm as compared to the present work P solubilization which found to have doubled. The NaCl concentration (1-8%) has inhibited the growth of organism and this concentration (>1%) was lethal to the bacterial growth. All P solubilizing species will not have same efficiency rate and SND-4 strain was found more efficient than the previously reported isolates. Garg *et al*.[33] reported phosphate solubilizing activity of *Azotobacter* under *in vivo* conditions and similar results were recorded in the present study. Kumar *et al*.[34] reported the P solubilizing activity of *A. chroococcum* for wheat under green house condition and P solubilization has subsequently increased the total vigour of the plant. Similar results were in the present study. *A. armeniacus, A. chroococcum, A. salinestris* and *A. tropicalis* were able to solubilize equal amount of phosphate as compared to previously isolated *A. chroococcum* and *A. vinelandii* strains and these strains can be used as a alternate P solubilizer as compared to the existing strains.

All the isolates were able to fix N_2, the highest N_2 fixation (29.21 μgNmL^{-1}day^{-1}) was recorded by *A. salinestris* (GVT-1) as compared to other strains and (Fig. 2) remaining isolates fixed almost equal amount of N_2. Among the 6 different species *A. salinestris*, *A. armeniacus* and *A. tropicalis* were found in paddy soils and were able to fix equal amount of N_2 by the known species of *A. chroococcum* and *A. vinelandii*, respectively. Page and Shivprasad[10] identified a novel *A. salinestris* species isolated from saline soils of Canada and N_2 fixation found similar to the present investigation. Kizilkaya[25] reported *A. chroococcum* that fixed 29.35 μgNmL^{-1}day^{-1} and all other species are unable to fix atmospheric free N_2 freely as compared to *Azotobacter* group. Soares *et al*.[35] reported N_2 fixation by *Azospirillum* spp. which recorded a maximum of 6.17 μgNmL^{-1}day^{-1} but as compared to GVT-1 isolate, *Azospirillum* fixed very less amount of N_2. The present results and previous data showed similar values and no such differences were recorded. Among all PGPR activity, IAA, GA and P solubilization and N_2 fixation are more important and all are related to their PGPR efficiency. One or the other way, all the strains are efficient and equally beneficial for soil fertility and for sustainable agriculture.

After incubation period, all the freshly grown cultures developed pale yellow to dark yellow colour after the addition of Nessler's reagent in the tubes. Among 12 isolates, 10 produced pale colour and the remaining two strains were dark yellow. This shows the positive results of the ammonia production (Table 2).

Siderophores are the iron chelating agents which are important for suppression of plant pathogenic organisms as well as the uptake of the iron nutrients. Out of 12 strains, 8 (GVT-1, 2, 9, SND-3, 9, DVD-7, KOP-11, RCR-4) isolates produced orange yellow (++) or golden yellow colour and two (GVT-11, KOP-5) isolates produced light orange (+) or greenish tinge around the colonies under UV light illuminator. The remaining two strains were negative (-) for siderophore production (Table 2).

Among the 12 isolates, 6 of them produced higher siderophore (>4 mm orange yellow zone), three isolates produced moderate siderophore production (2-4 mm orange zone), remaining one isolate produced less siderophore production (<2 mm orange).

Table 2—Characterization of *Azotobacter* based on NH_3, siderophore and HCN production

Isolate	NH_3 production	Siderophore production	HCN production
GVT-1	+	++	RC
GVT-2	+	++	O
GVT-4	+	-	LRC
GVT-9	+	++	RC
GVT-11	+	+	O
SND-3	+	++	O
SND-4	+	-	O
SND-9	+	++	LRC
KOP-5	+	+	LRC
KOP-11	+	++	O
RCR-4	+	++	RC
DVD-7	+	++	O

Siderophore: ++, Orange Yellow; +, Light Orange; and -, No colour. HCN Production: RC, red colour; LRC, light red colour; and O, Orange

Among all the isolates of *Azotobacter*, HCN production was identified based on the Lorck[28] method. After incubation period of 4 days, the development of orange colour was observed on the surface of the filter paper. After 7 days of incubation period, the colour intensity was high. Among the 12 isolates, 3 (GVT-1, GVT-9 and RCR-4) produced red colour, 3 other strains (SND-9, KOP-5, GVT-4) produced light red and remaining was orange colour (Table 2).

Sakthivel and Karthikeyan[36] reported the PGPR activities such as ammonia, HCN, siderophore production of the *Azotobacter*, *Pseudomonas*, *Bacillus* and *Azospirillum.* It is found that among them, 60% of *Pseudomonas* produced maximum siderophore and HCN and 15% of *Azotobacter* produced little bit low amount under *in vitro* conditions. Likewise all the isolates of *Azotobacter* are also positive for the ammonia production and similar kind of results were interpreted in the present study.

Abiotic stress tolerance studies

Based on PGP activity, only 4 efficient strains (GVT-1, GVT-2 KOP-11 and SND-4) were selected for stress tolerance study. All the four *Azotobacter* species were selected for salt tolerance studies and the growth was recorded up to 6% NaCl but *A. chroococcum*, *A. vinelandii* and *A. salinestris* showed growth up to 8% concentration. The highest CFU was recorded by *A. tropicalis* (1.55×10^4) and *A. chroococcum* (1.75×10^4) whereas the lowest was with *A. vinelandii* (CFU 1.4×10^4) at 6% NaCl (Fig. 3a).

A. chroococcum, *A. vinelandii* and *A. salinestris* were tolerant to 8% NaCl (OD 0.9) but the total CFU counts were reduced at 8% concentration. It is true that NaCl concentration has definitely negative effect on the growth and survivability of *Azotobacter* species. Upadhyay *et al*.[22] reported the NaCl tolerant *Arthrobacter* sp. from wheat rhizosphere and documented that the isolate was tolerant to 8% NaCl having same PGP activity. Akhter *et al*.[37] reported salt tolerant *Azotobacter* spp. isolated from saline soils of Bangladesh and found that the two *Azotobacter* spp. tolerated up to 10% NaCl, and few of them were sensitive to even 6%. The NaCl concentration affected the nitrogen fixation of *Azotobacter* but it fixed N continually (9.87 mgN/g of carbon) even at higher concentration.

All the 4 *Azotobacter* strains were grown at temperature 15-35ºC for 7 days of incubation at pH 7, but *A. chroococcum*, *A. vinelandii* and *A. tropicalis* were grown up to 45ºC. The highest CFU was recorded by *A. vinelandii* (1.85×10^4) incubated at 45ºC. However, all *Azotobacter* strains documented maximum growth at 35ºC and growth was reduced with increasing temperature (Fig. 3b). *A. chroococcum*, *A. vinelandii* and *A. tropicalis* were resistant to 45ºC. The pH of collected soil samples was in the range 6.0-9.2 with a pH 6.5 to 7.5 which was suitable for the growth of *Azotobacter* species at different climatic temperatures. The soil salinity has increased because of the maximum use of pesticides and chemical fertilizer in order to control the pests and diseases of crops. Pesticides persist for longer period in soils (Jimenez *et al*.[9]). The temperature in this region reaches up to 47ºC in summer. The previous reports indicated that *Azotobacter* can survive in the form of cysts up to 24 years in dry soils and also at extreme temperature and varied pH ranges[10,38].

All the strains of *A. chroococcum* survived at a pH of 9.0 and there was no inhibition of growth at higher pH range. *A. salinestris* was sensitive to pH >9.0 and no growth was observed above pH of 10 (Fig. 3c). The highest CFU was recorded at a pH of 8.0 and 9.0 by *A. chroococcum*. The genus *Azotobacter* is ubiquitous in nature and as previous reports states that they can grow at a pH range 6.0-9.0 at different temperatures[9]. Similarly, Chennappa *et al*.[15] reported a species of *Azotobacter* tolerant to pesticides of 1-5% concentration and were isolated from saline paddy soils. This result clearly indicated that the species of *Azotobacter* are resistant to higher pH values and can

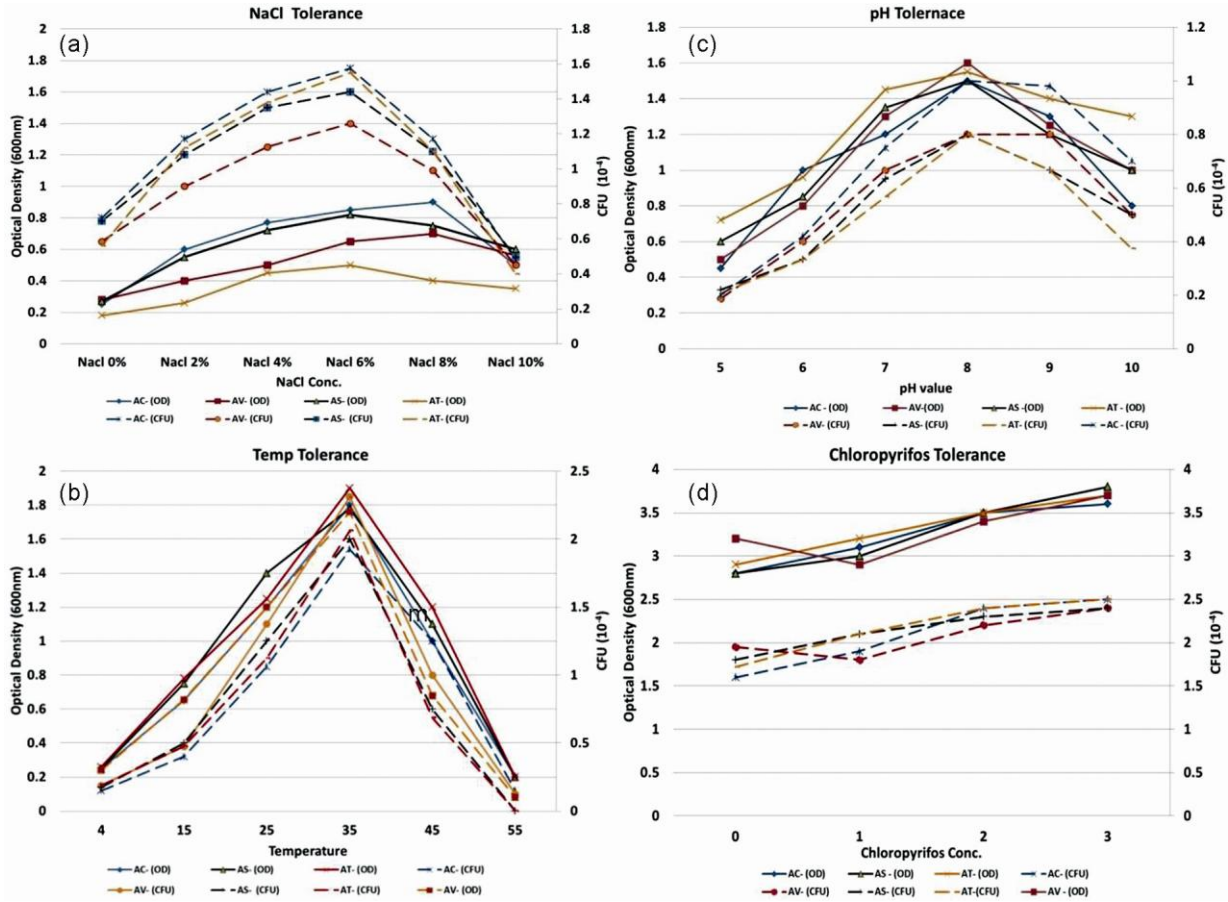

Fig. 3—Effect of (a) NaCl concentration; (b) different temperatures; (c) pH; and (d) pesticide on the growth and survivability of *Azotobacter* spp. isolated from paddy soils

tolerate up to pH value of 9.5 in the presence of different pesticide concentration.

Among all strains, *A. chroococcum* was tolerant and able to grow in chloropyrifos of all concentrations. However, all the strains were tolerant up to 3% chloropyrifos (Fig. 3d). The OD and CFU of all the strains increased gradually from 1.5 to 2.5×10^4 CFU at 3% chloropyrifos concentration during 24 h to 10 days of incubation. Among all, *A. chroococcum* and *A. salinestris* strains showed effective growth against 3% chloropyrifos (1.8 to 2.5×10^4 CFU and OD 2.7 to 3.7) having maximum growth during 4-5 days of incubation (Supplementary Fig. 3). Initially, *A. tropicalis* and *A. salinestris* strains showed comparatively lesser growth at 2% chloropyrifos during 4-5 days of incubation. Castillo *et al*.[16] reported the endosulfan degrading *Azotobacter* species and found that endosulfan reduced nitrogenase activity but not IAA production even at very low level (2 mg/L) and did not reduce the growth rate. Latifi *et al*.[29] isolated chloropyrifos degrading bacteria from effluent storage pools and found that bacteria utilized chloropyrifos as carbon source. These were tolerant to (2 g/L) chloropyrifos and belonged group of *Pseudomonas*.

Among all the pathogens tested, *A. tropicalis* showed a maximum zone of inhibition over ALT-2 (21.66 mm) by inhibiting the mycelial growth and sporulation (Table 3) after 5-7 days of inoculation. *A. chroococcum*, *A. vinelandii* and *A. salinestris* showed almost similar growth inhibitory action against ASP-1 isolate (13.33 and 10.00 at 5 and 7 days). *A. chroococcum* showed an effective inhibitory activity against ALT-2 (21.66 and 20.00) and ALT-3 (18.33) (Supplementary Fig. 4). Statistical analyses of antifungal activity of *Azotobacter* ranged between 44.16 and 51.94 in lower bound at 95% confidence interval in which minimum inhibition (44.16) was observed (*A. chroococcum* and *A. salinestris*) after 7 days, whereas the range in upper bound inhibition

Table 3—Antifungal activity of *Azotobacter* strains against *Aspergillus*, *Fusarium* and *Alternaria* species and zone of inhibition at different periods

Isolates / Period	*A. chroococcum*		*A. vinelandii*		*A. salinestris*		*A. tropicalis*	
	5 days	7 days	5 days	7 days	5 days	7 days	5 days	7 days
ASP-1*	13.33**	10.00	13.33	10.00	13.33	10.00	16.66	15.00
ASP-2	16.66	15.00	20.00	16.66	20.00	18.33	18.33	16.66
ASP-3	21.66	18.33	18.33	16.66	20.00	16.66	18.33	16.66
FUS-1	18.33	16.66	16.66	13.33	20.00	16.66	20.00	18.33
FUS-2	16.66	15.00	16.66	15.00	20.00	16.66	20.00	16.66
FUS-3	18.33	13.33	21.66	18.33	21.66	20.00	16.66	13.33
ALT-1	21.66	18.33	16.66	13.33	18.33	16.66	18.33	15.00
ALT-2	21.66	20.00	18.33	16.66	20.00	16.66	21.66	21.66
ALT-3	18.33	18.33	21.66	18.33	13.33	13.33	18.33	16.66

*ASP, *Aspergillus*; FUS, *Fusarium*; ALT, *Alternaria*

**Zone of inhibition (mm)

varied from 50.27 to 60.27 at 95% confidence interval in which the minimum inhibition was observed in *A. vinelandii* at 7 days. The maximum inhibitions were observed in *A. tropicalis* at 5 days with a maximum growth reduction (60.27%) as compared to other isolates (Supplementary Table 2). Similarly, Mali and Bodhankar[39] reported *A. chroococcum* that inhibited the growth of *Aspergillus*, *Alternaria*, *Fusarium* and *Azotobacter* species known to produce antimicrobial agents such as 2, 3 dihydroxy benzoic acid, aminochelin, azotochelin, protochelin and azotobactin.

Agarwal and Singh[31] reported antifungal activity of *Azotobacter* sp. against *F. oxysporum*, *R. solani* and *Aspergillus* sp. Cavaglieri *et al*.[32] reported the species of *Azotobacter* and *Arthrobacter* that inhibited root colonization of *F. verticillioides.* Fumonisin-B1 production by *Fusarium verticilliodes* was inhibited by *A. armeniacus*. A total of 0.84×10^3 CFU fungal colonies appeared in plates which were treated with *A. chroococcum* culture filtrate but in control plates the population was 2.41×10^3 CFU. All the strains of *Azotobacter* culture filtrate has reduced 76% total population of all the pathogens tested. This result indicated that *A. chroococcum* culture filtrate has antifungal properties and can control major plant pathogen by producing antifungal antibiotics. Similarly, Bhosale *et al*.[18] reported antifungal activity of *A. vinelandii* against *F. oxysporum* and found that *A. vinelandii* strain showed maximum zone of inhibition (40 mm) with lowest being recorded was 12 mm diameter.

Azotobacter species are known to be better PGPR. From the present study, it is once again proved that *Azotobacter* isolates have more influence on soil fertility and crop productivity. Molecular studies confirmed the existence of *Azotobacter* species in paddy soil ecosystem. *Azotobacter* spp. fixed maximum N_2, produced highest IAA, GA, solubilized P and showed PGP efficiency. The isolates can withstand higher salt concentration, temperature, pH, and even some insecticides also. They are effective bioagents against common plant pathogens and can control the growth of the fungus. Antagonistic and physiological properties proved their activity in improving soil fertility.

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