



Research Article (DOI: <http://dx.doi.org/10.15580/GJAS.2014.4.010314003>)

# Impact of Pesticides on PGPR Activity of *Azotobacter* sp. Isolated from Pesticide Flooded Paddy Soils

Chennappa G.<sup>1</sup>, Adkar-Purushothama C.R.<sup>2#</sup>, Naik M.K.<sup>3</sup>,  
Suraj U.<sup>4</sup>, Sreenivasa M.Y.<sup>1\*</sup>

<sup>1</sup>Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysore- 570 006, Karnataka, India.

<sup>2</sup>Laboratory of Plant Pathology, Faculty of Agriculture and Life Science Hirosaki University, Bunkyo-cho 3, Hirosaki 036-8561, Aomori Ken, Japan #Present address: Pavilion for Applied Cancer Research, Department of Biochemistry

Faculty of Medicine and Health sciences, Sherbrooke University 3201, Rue Jean-Mignault, Sherbrooke, Quebec J1E 4K8, Canada.

<sup>3</sup>Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Raichur – 584104, Karnataka, India.

<sup>4</sup>NRC on DNA finger printing, NBPGR, PUSA Campus, New Delhi - 110012 India.

## ARTICLE INFO

Article No.: 010314003

DOI: 10.15580/GJAS.2014.4.010314003

Submitted: 03/01/2014

Accepted: 28/04/2014

Published: 07/05/2014

**\*Corresponding Author**

Sreenivasa M. Y.

E-mail: [sreenivasamy@gmail.com](mailto:sreenivasamy@gmail.com)

Phone: +91-821-2411208;  
+91-821-2419733

## ABSTRACT

*Azotobacter* strains were isolated from paddy soils by serial dilution agar plate method. The colonies were glistening; smooth, slimy, brown to black in morphology on Jensen's N-free agar plates and the cells were Gram-negative bacteria. Biochemically, they were positive for indole production, citrate, catalase and Voges Proskauer test. Further, *Nif* gene sequence results revealed the presence of seven *Azotobacter* species. The effect of 1, 3 and 5% pesticides concentration viz., pendimethalin, chloropyrifos, glyphosate and phorate on nitrogen fixation, indole acetic acid, gibberllic acid, phosphate solubilization and bioassay of *Azotobacter* sp. were studied. Among all the species, GVT-1 strain was found to fix nitrogen at a maximum of  $30\mu\text{gN}_2\text{ ml}^{-1}\text{ day}^{-1}$ , produced highest quantity gibberllic acid ( $10\mu\text{g25ml}^{-1}$ ) and able to solubilize the phosphate at the rate of 9.8cm by forming the halo zone which was supplemented with 5% phorate. Similarly, GVT-1 strain produced a highest amount of indole acetic acid ( $31.8\mu\text{gml}^{-1}$ ) in 5% pendimethalin. Further bioassay activities of GVT-1 strain found efficient in increasing the root, shoot length and vigor of the plant. From these results it is clear that the *Azotobacter* sp. not only produces plant growth promoting substances but is also resistant to different pesticides and is not affected by the bacterial activity.

## Keywords:

*Azotobacter*; *Nif* gene; PGP activity; Bioassay; Pesticides

## INTRODUCTION

Rice is one of the major agricultural crops and cultivation is found throughout India under various cultivation practices. In the last two decades the production and utilization of rice was increased with the population to maintain food security. To improve the production of rice, several cultivation practices have been adopted. Among all the practices, chemical pesticides are in the race and used excessively for the control of plant diseases and pests. Some of the pesticides have direct effect on soil microbiological aspects, environmental pollution and health hazards (Martin et al., 2011). These pesticides may cause everlasting changes in the soil microflora (Aleem et al., 2003), adverse effect on soil fertility and crop productivity, inhibition of nitrogen ( $N_2$ ) fixing bacteria, (Sachin, 2009), interference with ammonification (Reinhardt et al., 2008). Several toxic and carcinogenic pesticides have banned in many of the countries including India. Several species are known to tolerate many of pesticides and some of them are known to degrade several pesticides in different conditions (Tejera et al., 2005). The pesticide resistant and degrading bacterial group includes many species such as *Pseudomonas*, *Flavobacteria*, *Azotobacter*, *Acetobacter*, *Arthrobacter*, *Alcaligenes*, *Bacillus*, *Enterobacter* and *Klebsiella*. Among all bacteria, plant growth promoting rhizobacteria (PGPR) have more influence on soil physiological properties. PGPR can helps to replace chemical fertilizer nitrogen for the sustainable cultivation by fixing the atmospheric  $N_2$  and producing growth promoting substances (Ahmad et al. 2005). Many species of *Pseudomonas*, *Bacillus* and *Azotobacter* can grow and survive at extreme environmental conditions (Jimenez et al. 2011). Among them, the genus *Azotobacter* are common nitrogen fixing bacterium found in agricultural soils playing different beneficial roles by secreting vitamins, amino acids, siderophores and auxins (Aquilanti et al., 2004; Sharma et al., 2009). *Azotobacter* produces indole acetic acid (IAA), gibberellic acid (GA) which are important plant growth hormones and these hormones will helps in seed germination and plant growth considerably (Asma et al., 2012). Several species of *Azotobacter* are isolated and identified in all the soil conditions and well known species are *A. vinelandii* and *A. chroococcum* (Page and Shivprasad, 1991). Several pesticides are being used for the cultivation of rice viz., pendimethalin, phorate, glyphosate, simazine, malathion, endosulfan etc, which are completely degraded by *A. chroococcum* and are greatly studied since last two decades because of its PGPR activity for sustainable agriculture (Moneke et al., 2010). *Azotobacter* species can tolerate and survive in extreme environmental condition by producing cysts. Pesticide tolerant *Azotobacter* species was isolated from paddy soils and are known to produce IAA in media supplemented with 5% pesticides (Chennappa et al., 2013). Similarly neutral effect of endosulfan ( $2\text{mgL}^{-1}$ ) was also reported on IAA production and nitrogen

fixation activity of *A. chroococcum* respectively and which is used as a sulphur source (Castillo et al. 2011). Hexachlorohexane, phorate and carbofuran have increased the bacterial population under invitro conditions and no inhibitory effects was recorded (Das and Mukharjee, 2000). Special interest was taken in the present study because in Northern parts of Karnataka, for cultivation of paddy high level of pesticide are being used to control the pests and diseases of paddy. Present investigation reveals that the *Azotobacter* species can fix  $N_2$ , produces IAA, GA and phosphate solubilization (PS) in the media containing different types of pesticides concentration and the investigation was carried with the following objectives; 1) Isolation of efficient PGPR *Azotobacter* species from paddy soils, 2) Molecular characterization of  $N_2$  fixing *Azotobacter* species, 3) Effect of pesticides on  $N_2$  fixation, IAA, GA production, PS and bioassay activities.

## MATERIALS AND METHODS

### Isolation and Identification of *Azotobacter* species

Soil samples were collected from 0 to 15 cm of different locations of paddy soils of Karnataka. Nitrogen fixing *Azotobacter* strains were isolated by serial dilution agar plate method and 1ml of serially diluted soil suspensions from  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions were inoculated onto Jensen's N- free media (Ahmad et al., 2008; Sharma et al., 2009). The plates were maintained in triplicates for higher accuracy of the results. The plates were incubated at  $28 \pm 2^\circ \text{C}$  for 5 days, after incubation period, the discrete and well grown colonies were enumerated (Khan et al., 2008). *Azotobacter* cultures were identified and characterized based on the morphological characters and biochemical tests as described in the Bergey's manual of bacteriology. Pure cultures were obtained by repeated sub-culturing on same media slants.

### pH tolerance

The soil pH was measured according to the protocol of Jimenez et al. (2011) and pH tolerance was carried out at different pH ranges. Different ranges of pH (5.5 to 9.5) Burk's media were prepared by adjusting the media pH (NaOH or HCL); one set with pH 7.0 were maintained as control. The test bacterial cultures were inoculated by streak plate method and incubated at  $28 \pm 2^\circ \text{C}$  for 7 days. The growths of bacterial colonies on media plates were enumerated and CFU were calculated.

### DNA Extraction and Amplification of *nif* gene

The DNA was extracted from the pure cultures of *Azotobacter* using Genei DNA isolation kit and the purified DNA was amplified with specific primer *Nif* f ( $5'$ -GGTTGTGACCCCGAAAGCTA) and *Nif* r ( $5'$ -

GCGTACATGGCCATCATCTC) for identification *nif* genes in *Azotobacter*. 25µl PCR reaction mixture contains 15.3µl of autoclaved distilled water, 2µl of purified DNA template, 2.5µl 1X Taq buffer, 2µl of MgCl<sub>2</sub>, 2µl of deoxyribonucleotide triphosphate mixture, 1µl of each primer, 0.2µl of Taq DNA polymerase (Genei). PCR amplification reaction was run in a thermocycler with following conditions (35 cycles consisting of 95° C for 1 min, 55° C for 1 min, 72° C for 1 min, and final extension at 72° C for 5 min) were done (Rajeswari and Kasthuri, 2009; Jimenez et al., 2011). The amplified were checked in 1.5% agarose gel for further confirmation.

### Cloning, sequencing and cluster analyses

PCR products were cloned into pGEM®-T Easy Vector Systems (Promega) and transformed into competent *E. coli* strain DH5α by the following manufacturer instructions. Plasmid DNA was isolated and the presence of the insert was confirmed by restriction digestion of plasmid DNA with *EcoRI* restriction enzymes (Life Sciences, Canada). The clones of interest were sequenced (Genei, Bengaluru, India) and the sequences were confirmed with NCBI BLAST database for the identity of the isolates based on previously published database sequences (Chennappa et al., 2013). Online software MEGA 5.1 was used to construct the phylogenetic tree using Maximum Likelihood NJ method.

### Effect of Pesticides on N<sub>2</sub> fixation

The *Azotobacter* strains were inoculated onto a 100ml Jenson's N-free broth in 250ml Erlenmeyer flask supplemented with 1, 3 and 5% of each pesticides viz., pendimethalin, chloropyrifos, glyphosate and phorate respectively. The flasks were incubated at 30±2° C for 10 days on a rotary shaker (120 rpm) under continuous airflow whereas, inoculated broth without any pesticides treatment maintained as control (Bano and Musarrat, 2003). The flasks were maintained in triplicates for higher accuracy of the results. The concentration of nitrogen in each liquid culture was measured by Kjeldahl method (Bremner, 1965). Nitrogen fixation was determined in terms of increase in total Kjeldahl nitrogen in comparison to control using micro Kjeldahl apparatus.

### Effect of Pesticides on IAA production

The *Azotobacter* strains were inoculated onto a 100ml Jenson's N free broth in 250ml Erlenmeyer flask supplemented with 1, 3 and 5% of each pesticides viz., pendimethalin, chloropyrifos, glyphosate and phorate respectively. The flasks were incubated at 30±2° C for 7 days on a rotary shaker (100 rpm) under continuous airflow whereas, inoculated broth without any pesticides treatment maintained as control (Bano and Musarrat, 2003). The test sets were maintained in triplicates for greater accuracy of the results. After incubation, IAA

estimation was done according to the protocol of Ahmad et al. (2008) with Solawaski's reagent. The development of pink color was read at 530nm using spectrophotometer (Genway 6506) and IAA produced was estimated by referring to a standard graph prepared with different levels of standard IAA (Beneduzi et al., 2008; Patil, 2011).

### Effect of Pesticides on GA production

*Azotobacter* isolates were inoculated onto a sterilized 100ml nitrogen free malate broth in 250ml Erlenmeyer flask supplemented with 1, 3 and 5% of each pesticides viz., pendimethalin, chloropyrifos, glyphosate and phorate respectively and incubated at 30±2° C for 7 days on a rotary shaker (100 rpm) under continuous airflow whereas inoculated broth without any pesticides treatment maintained as control (Bano and Musarrat, 2003). After incubation, the absorbance of the samples was measured at 254 nm in UV spectrophotometer. The test sets were maintained in triplicates. The GA produced by the *Azotobacter* isolates was estimated as described by Upadhyay et al. (2009).

### Effect of Pesticides on Phosphate solubilization

*Azotobacter* isolates were spot inoculated onto a Pikovskaya agar plates containing tri calcium phosphate (TCP) as a major phosphate source supplemented with 1, 3 and 5% of each pesticides viz., pendimethalin, chloropyrifos, glyphosate and phorate respectively. The plates were maintained in triplicates for higher accuracy of the results and incubated at 30±2° C for 7 days whereas inoculated plate without pesticides maintained as control. (Beneduzi et al., 2008; Sachin, 2009) and observed for the halo zone formation around the discrete colonies.

### Effect of Pesticides on Bioassay of Potential Isolates

Bioassay was carried out for the potential *Azotobacter* isolates at different concentrations of culture filtrate (100, 500, 1000 and 2000 ppm) and pesticide concentration on paddy seed germination, root length, shoot length and vigor index test. The paddy seeds were soaked for an overnight in all the concentration of culture filtrate supplemented with 1, 3 and 5% of each pesticides viz., pendimethalin, chloropyrifos, glyphosate and phorate respectively. Whereas, one set untreated (without pesticides) seeds maintained as control. The seeds were spread on blotter paper and incubated at 32±2° C for 10 days in a growth chamber. After 10 days of incubation, the effects of different pesticide concentrations on seed germination, root, shoot length and vigor index were recorded over the control (Sachin, 2009). The experimental set up was maintained in triplicates for higher statistical accuracy of the results.

### Nucleotide sequence accession number:

All the *Azotobacter* sequences were deposited in NCBI genbank along with location of the isolates and the allotted accession numbers are: KF470798, KF470799, KF470800, KF470801, KF470802, KF470803, KF470804, KF470805, KF470806, KF470807, KF470808 and KF470809.

Nif gene sequences of different *Azotobacter* species deposited in NCBI genbank isolated from paddy soil sample and the accession numbers are KF881088, KF881089, KF881090, KF881091, KF881092, KF881093, KF881094, KF881095, KF881096, KF881097, KF881098, KF881099, KF881100, KF881101, KF881102, KF881103, KF881104, KF881105, KF881106, KF881107, KF881108, KF881109, KF881110, KF881111, KF881112, KF881113, KF881114, KF881115, KF881116, KF881117, KF881118, KF881119, KF881120 and KF881121.

All the *Azotobacter* strains sequences were deposited in NCBI genbank along with location of the isolates. Accession numbers are: JX262171, JX262167, JX262163, JX262175, JX262166, JX262173, JX262172, JX262174, JX262168, JX262165, JX262169, JX262170, JX262164 and JX262176 (Chennappa et al., 2013).

## RESULTS AND DISCUSSION

### Isolation and identification of *Azotobacter*

A total of 35 *Azotobacter* strains were isolated from saline paddy soils of Karnataka by serial dilution spread

plate method on Jenson's N- free media. All the isolates produced melanin like water- soluble brown, light brown, pale yellow, milky white to black pigments and formed sticky colonies. These isolates were Gram -ve, some of them were produced cysts and they were positive for indole production, citrate utilization catalase production and negative for methyl red and starch hydrolysis (Table 1). The production of sticky pigments and cyst formation is one of the typical identification characters of the genus *Azotobacter*. Similarly Aquilanti et al. (2004) reported three different isolation methods for rhizosphere soils of *Azotobacter* and identified viz., *A. chroococcum*, *A. vinelandii*, *A. paspali*, *A. beijerinckii*, *A. salinestris*, *A. armeniacus*, *A. brasilense*, *A. insignis*, *A. agilis* and *A. nigricans* were genetically differ from each other but not with morphological characters. In reference to this study, *Azotobacter* species are aerobes and the activity will be more in non-water logged soils. Akhter et al. (2012) reported that the soil salinity and pH was increased because of the high inputs of the pesticides for the cultivation of paddy. Zhan and Sun (2010) reported the diversity of free living nitrogen fixing microorganisms from soils of copper mining waste lands and found different uncultured microorganisms which are efficient nitrogen fixing and are closely related to diazotrophic group. Tejera et al. (2005) reported the similar kind of morphological and biochemical characteristic features of *Azotobacter* species isolated from sugarcane rhizosphere soils. All these reports clearly indicate the distribution of *Azotobacter* species and their activities in different soil ecosystem.

**Table 1: PGP Potential *Azotobacter* strains isolated from northern parts of Karnataka region and their genbank accession number.**

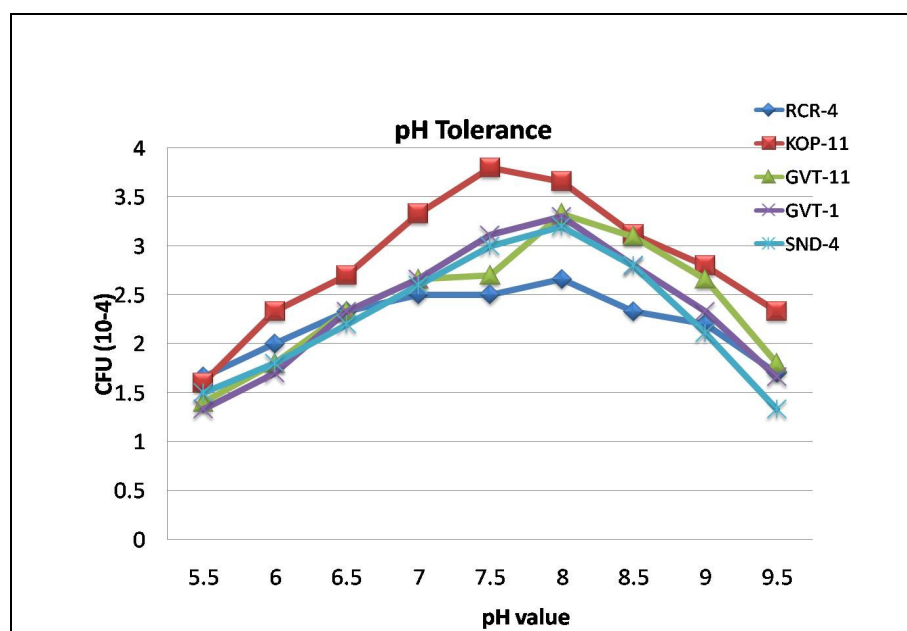
Location	Description	Pigments	Soil pH	* Accession number	* Nif gene accession number
GVT-1	<i>Azotobacter salinestris</i>	Brown	8.8	KF470807	KF881089
GVT-11	<i>Azotobacter armeniacus</i>	Yellow brown	7.9	KF470809	KF881097
SND-4	<i>Azotobacter chroococcum</i>	Brownish black	7.8	KF470801	KF881100
KOP-11	<i>Azotobacter tropicalis</i>	Dark brown	8.5	KF470808	KF881108
RCR-4	<i>Azotobacter vinelandii</i>	Pale brown	8.5	KF470802	KF881116
DVD-7	<i>Azotobacter sp.</i>	Light brown	7.5	KF470804	KF881104
YG-7	<i>Azotobacter nigricans</i>	Pale white	7.8	JX262168	KF881107

\* Representative

### pH tolerance

The pH of collected soil samples were in the range of 6.0 to 9.2 and pH 6.5 to 7.5 ranges is suitable for the growth of *Azotobacter* species. Among all the strains of *Azotobacter* KOP-5 strain was survived at a pH of 9.5 and did not observed any inhibition of growth at higher pH range. DVD-7 strain was sensitive to the pH of 8.2 and no growth was observed above 8.2 pH value (Fig 1.). The genus *Azotobacter* is ubiquitous in nature and they can grow with pH ranging from 6.0 to 9.0 of different

climatic temperatures (Jimenez et al., 2011). Paddy soil salinity was increased because of the maximum use of pesticides and chemical fertilizers for the control of pests and diseases of paddy under irrigated conditions. Similarly Chennappa et al. (2013) reported the species of *Azotobacter* are tolerant to pesticides of 1 to 5% concentration and were isolated from saline paddy soils. This result clearly indicates the species of *Azotobacter* are resistant to higher pH values and can tolerate up to pH value of 9.5 in the presence of different pesticide concentration.



**Fig 1: Effect of pH on the growth of *Azotobacter* species isolated from paddy soil samples.**

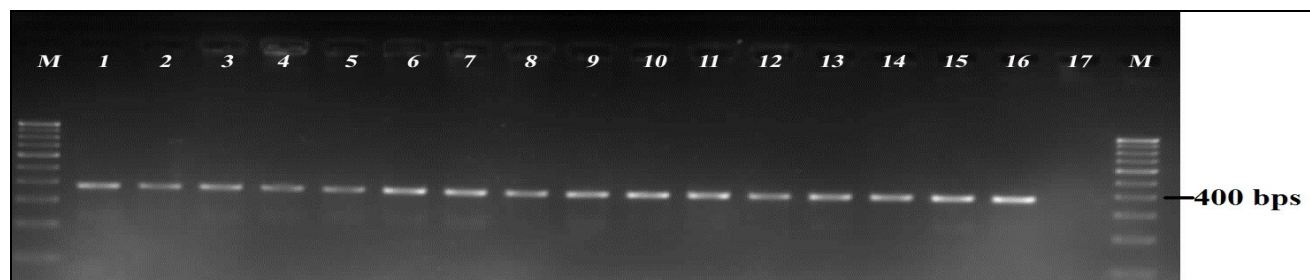
### Amplification and sequence analyses of *nif* genes

Based on preliminary morphological identification, all 35 isolates were selected for *nif* gene studies. After amplification of *nif* genes of *Azotobacter*, amplicons size was around 350bp (Fig 2). BLAST data result confirmed that they were matched 98 to 100 per cent in homology

and all the sequences were similar to published NCBI database sequences of *nif* genes *Azotobacter* species viz., *A. vinelandii* (RCR-4), *A. salinestris* (GVT-1), *A. chroococcum* (SND-4), *A. sp.* (DVD-7), *A. tropicalis* (KOP-11), *A. nigricans subsp nigricans* (YG-7) and *A. armeniacus* (GVT-11) respectively (Table 1). Similarly Zehr et al. (2003) and Bhatia et al. (2009) clearly

reported the major clusters of *nif* genes in *Azotobacter* and many of diazotrophic nitrogen fixing bacterial species isolated from cotton soil samples and found similar *nif* genes in the present study. After BLAST data result, *A. tropicalis* and *A. armeniacus* and *A. salinestrus*

were found new to the paddy soils; till now no such data is available on *A. tropicalis*, *A. armeniacus* and *A. salinestrus* species and are equally beneficial to the soil ecosystem.



**Fig 2:** Amplification of *nif* genes at 400bps of different *Azotobacter* species, Lane M is 100bp marker. Lane 1, 2 = *A. Sp.*, Lane 3, 4 = *A. vinelandii*, Lane 5, 6 = *A. chroococcum*, Lane 7, 8 = *A. salinestrus*, Lane 9, 10 = *A. tropicalis*, Lane 11, 12 13 = *A.nigricans subsp nigricans*, Lane 14= *A. armeniacus*, Lane 15 = *A. vinelandii* (JX262166), Lane 16= *A. salinestrus* (JX262173), Lane 17= -ve Control (Distilled water).

**Table 2: Effect of pesticides concentration on Nitrogen fixation and Indole acetic acid production of *Azotobacter* species isolated from paddy soils.**

Pesticides	Nitrogen Fixation ( $\mu\text{gNml}^{-1}\text{day}^{-1}$ )					IAA Production ( $\mu\text{gml}^{-1}$ )				
	RCR-4 *	KOP-11	GVT-11	GVT-1	SND-4	RCR-4	KOP-11	GVT-11	GVT-1	SND-4
<b>Pendimethalin</b>										
Control	32.2	31.2	33.7	34.5	31.4	29.4	30.2	32.5	33	31
1%	31.8	30.1	32.5	33.5	31.2	28.4	29.8	31.2	32.6	30.6
3%	28.4	29	30.8	31.4	27.5	27.3	29.1	31	32.2	30
5%	15.6	17.2	15.2	17.5	15.1	26.6	28.4	30.3	31.8	29.5
<b>Chloropyrifos</b>										
1%	27.2	26.4	27	27.8	25.76	27.3	25	27.4	28	26.45
3%	20.4	21	22	23.4	20.35	22.4	21	22	24.2	22.4
5%	15.5	16.3	17.2	17	14	15.85	15.3	15.85	19.2	17.75
<b>Glyphosate</b>										
1%	31	29.8	32.24	33.2	31	28.8	29.4	31	32.4	29.4
3%	28.24	29.2	31.2	32	30.85	27.5	27	29	30	28.6
5%	10.12	8.9	11.1	14	10.35	11	10	12	15.45	11.8
<b>Phorate</b>										
1%	31	31	32.5	33.2	30.3	27	28.4	29	30	29
3%	29.5	28.6	31	32.3	28.7	20	23	22	27	24
5%	25	26	28	30	26	13	17.3	14	22	16

\* RCR-4 *Azotobacter vinelandii*, KOP-11 *A. tropicalis*, GVT-11 *A. armeniacus*, GVT-1 *A. salinestrus*, SND-4 *A. chroococcum*

### ***Nif* gene cluster analyses**

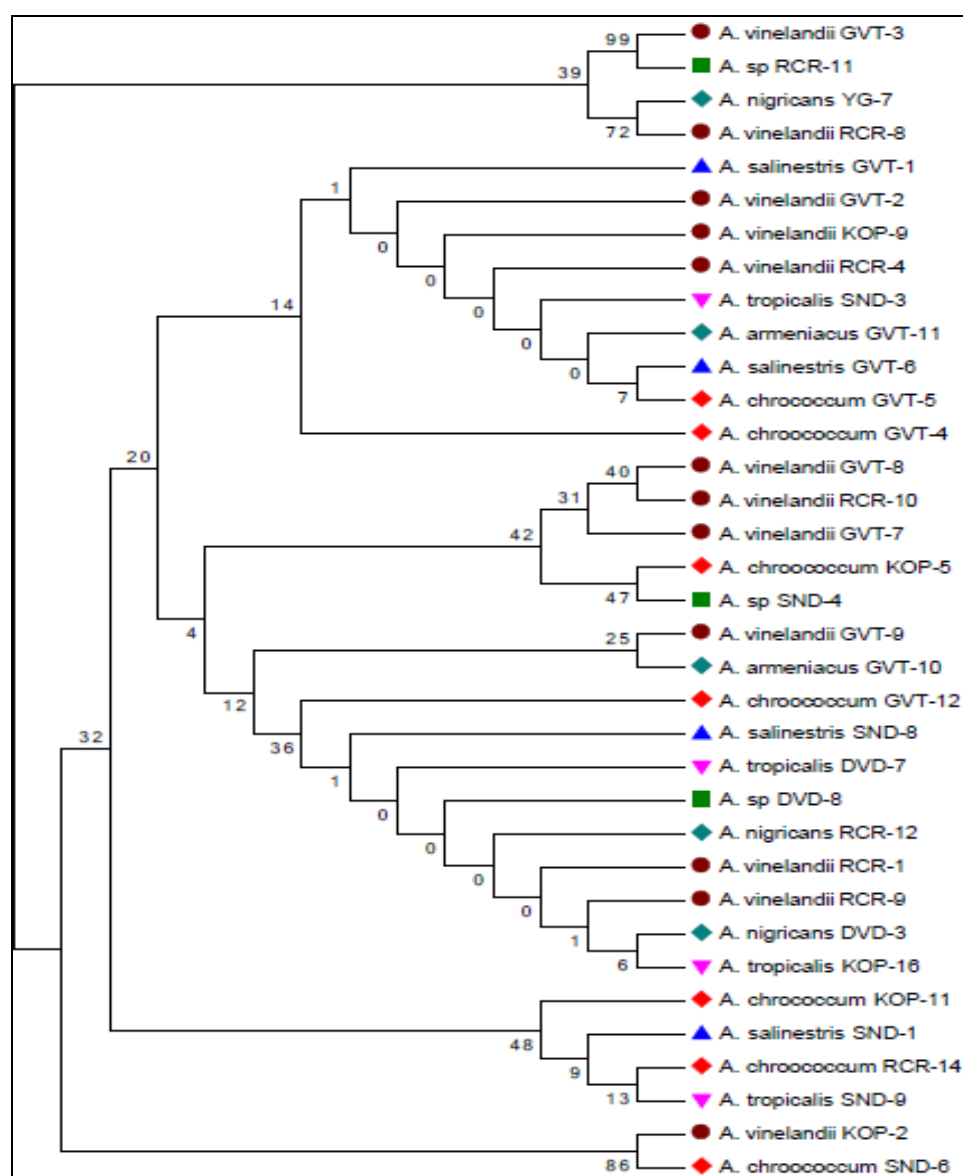
Based on the BLAST data analyses, phylogenetic tree of *Azotobacter nif* gene reveals that it formed different cluster nodes and produced individual clusters with *Azotobacter nif* gene sequences. Only GVT-3, RCR-11, YG-7 and RCR-8 Sequences produced entirely different nodes and but all the other species were identical to one

another with respect to whole genome sequences data (Fig 3). Microorganisms catalyze biological nitrogen fixation with the enzyme nitrogenase, which has been highly conserved through evolution (Zehr et al., 2003). Steenhoudt and Vanderleyden (2000) reported and concluded that nitrogen fixing ability of *A. vinelandii* and *nif* HDK genes clusters. Nitrogen fixation in *Azotobacter* is complicated by the presence of three biochemically



and genetically distinct nitrogenase enzymes synthesized under different conditions of metal supply. All the amplified *nif* genes were similar to the reference *nif* gene of *Azotobacter* and *Pseudomonas*. Martensson et al. (2009) concluded that *nif* gene can be used as a molecular marker for the diazotrophic diversity study. Wu et al. (2011) isolated the *nif* gene from different diazotrophic bacteria from surface sea sediments. Soni and Goel (2011) reported the *nif* genes isolated directly from saline soil samples and these results are similar to the present results of *nif* gene of *Azotobacter* species. Upadhyay et al. (2009) isolated nitrogen-fixing bacteria from wheat rhizosphere and concluded that *nifH* gene

was responsible for nitrogen fixation. High levels of saline concentration were not hampered by the nitrogen fixing activity and on *nif* gene. This reports indicates that the divergence of *nif* genes in the ecosystem with respect to different physiological conditions. Till now no reports were found on *nif* gene of *A. salinestris*, *A. tropicalis*, and *armeniacus* species, and majority of the work was done on *A. vinelandii* and *A. chroococcum* *nif* gene clusters. Using different 16S rDNA methods for microbial diversity analyses and based on 16S rDNA analyses have given more accurate and confirmative results as compared but not with traditional methods.



**Fig 3:** *Nif* gene dendrogram based on UPGMA cluster analyses of *Azotobacter* species isolated from paddy soils and species viz., *A. sp*, *A. vinelandii*, *A. salinestris*, *A. tropicalis*, *A. nigricans subsp nigricans*, *A. chroococcum*, *A. armeniacus* and *A. salinestris*.

**Table 3: Effect of pesticides concentration on Gibberilic acid production and phosphate solubilization of *Azotobacter* species isolated from paddy soils.**

Pesticides	GA production ( $\mu\text{g25ml}^{-1}$ )					P - solubilization (cm)				
Pendimethalin	RCR-4	KOP-11	GVT-11	GVT-1	SND-4	RCR-4	KOP-11	GVT-11	GVT-1	SND-4
<b>Control</b>	15.6	17.25	15.25	17.55	15.12	13.5	12.4	13	14.2	13.4
<b>1%</b>	14.5	15.75	14	16.45	14.2	12	11.5	12	13.5	12.5
<b>3%</b>	10.8	12.5	12.5	13	11.5	8.4	9.5	9.4	12	10.3
<b>5%</b>	8	9.75	8.5	10	8.24	7	8	7.2	9.5	8.5
<b>Chloropyrifos</b>										
<b>1%</b>	13.4	14.2	12	14.5	13.8	11	11	12.2	13.5	12
<b>3%</b>	9.6	11	9	11.8	10.4	8.4	9.2	9	11	9.8
<b>5%</b>	7.8	8	7.5	9.2	8	7.1	7.8	7.5	8.8	8.5
<b>Glyphosate</b>										
<b>1%</b>	13.8	15	14	15.8	14.5	13	11	12.3	13.8	12.7
<b>3%</b>	7	7.8	8	8.8	10	11	9	11.8	13	11.8
<b>5%</b>	5	6.1	7	7.3	5.8	8	8	7.8	10	9.5
<b>Phorate</b>										
<b>1%</b>	14	15.7	13.2	16	14.2	12.2	11	11.4	13.1	11.4
<b>3%</b>	9	13.5	11.8	13.8	11	9.5	9.2	9.8	11	9
<b>5%</b>	6	9	8.7	10	9.1	7.2	6.8	7.1	9.8	6.8

### Effect of Pesticides on $\text{N}_2$ fixation

Based on *nif* gene studies, five different strains were selected and used to study the effect of pesticides on nitrogen fixation. Among 5 isolates, GVT-1 strain fixed highest amount of  $\text{N}_2$  ( $30\mu\text{gNml}^{-1}\text{day}^{-1}$ ) supplemented with 5% phorate followed by pendimethalin ( $17.5\mu\text{gNml}^{-1}\text{day}^{-1}$ ) in 100ml of Jensen's media (Table 2). The recommended dosages of the present study are, Chloropyrifos (1.75ml/l), phorate (5kg/ acre), pendimethalin (6.25ml/l) and glyphosate (5ml/l). All the 5 strains were fixed a maximum amount of  $\text{N}_2$  at 5% phorate and this concentration did not affect the nitrogen fixing activity of *Azotobacter* strains. Among all the pesticides, 5% glyphosate has negative effect on  $\text{N}_2$  fixation as compared to other concentration and fixed least amount of  $\text{N}_2$  (Azt-16). Pendimethalin 3% concentration did not affect the metabolic activity of the strain and above 3% pesticide concentration is lethal to bacterial respiration and reduced the  $\text{N}_2$  fixation. Mrkovacki (2002) stated that glyphosate not only inhibited the  $\text{N}_2$  fixation but also reduced *A. chroococcum* respiration rate by 60%. Castillo et al. (2011) reported that endosulfan concentration inhibited the nitrogenase activity of *A. chroococcum* 94 and 96 % at 2-  $10\text{mgL}^{-1}$  of endosulfan but *A. chroococcum* has

degraded endosulfan completely. Similarly in all the 3% pesticide treatments nitrogen fixation was recorded and this concentration does not have negative effect on metabolic activity. Among all the pesticides concentration, KOP-11 isolate showed maximum reduction of  $\text{N}_2$  fixation in 5% glyphosate and a maximum resistance was showed by GVT-1 isolate at 5% phorate concentration. Das and Mukharjee et al. (2000) reported, the commonly used insecticides (phorate, HCH and carbofuran) will have advantages for the growth of bacteria using as a carbon and sulphur source but few of them have showed effects on metabolic activities.

### Effect of Pesticides on IAA production

Among all the strains, GVT-1 strain produced maximum quantity of IAA ( $31.8\mu\text{gml}^{-1}$ ) supplemented with 5% pendimethalin over the control ( $33\mu\text{gml}^{-1}$ ). All the isolates were produced IAA in the range of 10 -  $31.8\mu\text{gml}^{-1}$  at 5% different pesticide concentrations. Among them, Azt-16 was very sensitive towards the production of IAA supplemented with 5% glyphosate and GVT-1 was resistant to 5% concentration of all the pesticides. Among all pesticides, pendimethalin did not affect the IAA production of *Azotobacter* and this strain is



resistant to 5% pendimethalin as compared to the other strains but upto 3% pesticide concentration did not affected the metabolic activity. Castillo et al. (2011) concluded that no endosulfan effect was observed on IAA production of *A. chroococcum* and very negligible variations were noticed on IAA production. Similarly Asma et al. (2012) reported the effect of endosulfan on IAA production of *Azotobacter* and found that even 50ppm of endosulfan concentration inhibited the IAA production. In the present study above 3% pesticide concentration slightly reduced the IAA production and lethal to bacterial respiration with reference to IAA production (Table 2). The production of IAA and activity vary from species to species and the environmental conditions. Similarly, Mrkovacki et al. (2002) reported that pendimethalin was efficiently degraded by *A. chroococcum* up to 55% in 20 days and did not shown any negative effect on IAA production. All the strains were isolated from paddy soils and proved the PGP potential with respect to *A. chroococcum*. Ahmad et al. (2008) reported a maximum of  $24.8\mu\text{gml}^{-1}$  of IAA produced by *Azotobacter* species which was supplemented with 2mg of tryptophan. Further Patil (2011) reported the highest amount ( $32.80\mu\text{gml}^{-1}$ ) of IAA was produced by *Azotobacter* supplemented with 5mg of tryptophan. The addition of tryptophan has increased the IAA production and influences the metabolic activity of the bacteria. Similarly Upadhyay et al. (2009) isolated PGPR strains at different salinity soil conditions and all the strains able to produce IAA at the rate 3.79 to  $119.63\mu\text{gml}^{-1}$ .

This report clearly indicates that the diazotrophic bacteria have the same type of metabolic activities in the bacterial diversity.

### Effect of Pesticides on GA production

All the *Azotobacter* isolates produced GA in the range of 5 to  $10\mu\text{g25ml}^{-1}$  supplemented with 5% pesticides concentration. GVT-1 strain produced the highest amount of GA ( $10\mu\text{g25ml}^{-1}$ ) in 5% pendimethalin and phorate as compared to the other strains (Table 3). GA is also one of the important plant growth promoting substances produced by the different species of PGPR including *Azotobacter* species. Among all the isolates RCR-4 produced least amount ( $5\mu\text{g25ml}^{-1}$ ) of GA which was supplemented with 5% glyphosate and showed sensitive towards the GA production. Similarly Asma et al. (2012) reported the effect of endosulfan on GA production of *Azotobacter* and 50ppm of endosulfan concentration inhibited the GA production. All the isolates were resistant to 3% pendimethalin concentration as compared to the glyphosate. This result clearly indicates that above 1% glyphosate concentration has inhibited the GA production ability of the *Azotobacter* similarly to endosulfan and this concentration is lethal to the metabolic activity of the *Azotobacter*. Among all the concentrations, none of the isolates showed resistant to above 1% pesticide concentration over the control. Latifi

et al. (2012) discussed the biodegradation of chlorpyrifos by *Pseudomonas putida* and strain has degraded the chlorpyrifos in 9 days of period. Similarly Upadhyay et al. (2009) reported the production of gibberellins by *Arthrobacter* ( $130.74\mu\text{gmg}^{-1}$ ) and *Bacillus* species ( $7.67\mu\text{gmg}^{-1}$ ) isolated from wheat rhizosphere under 1 to 8% of saline conditions. The GA production variation due to NaCl concentration has influenced bacterial growth rate, and all PGPR's will not produce equal quantity of GA and it varies from species to species and geographical conditions respectively.

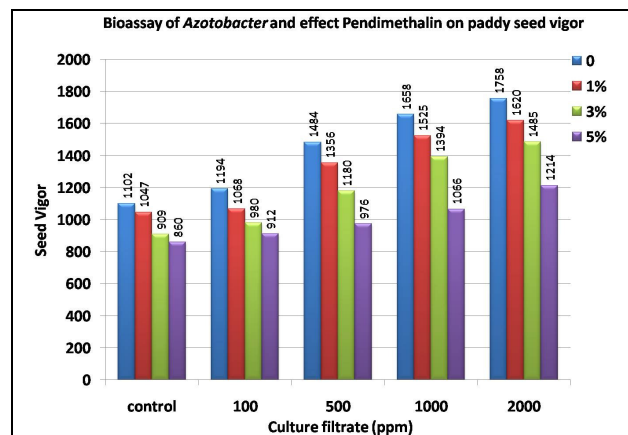
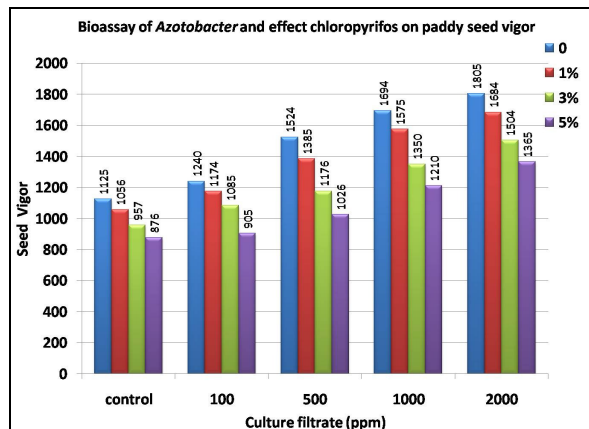
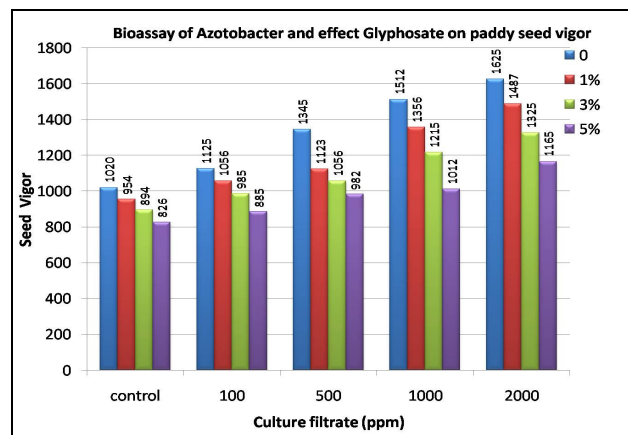
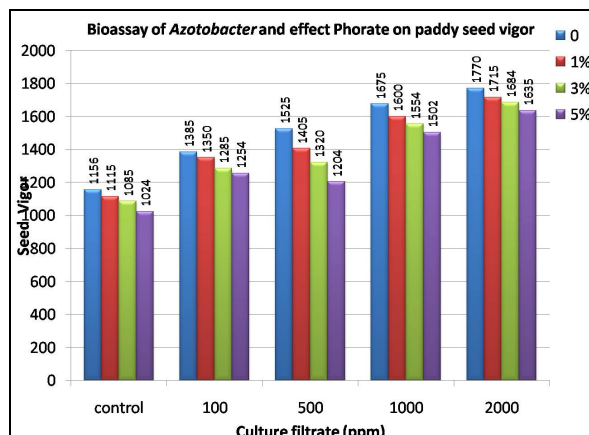
### Effect of Pesticides on P- Solubilization

The formation of halo zone around the colony indicates the P solubilization of the available TCP to Phosphates. After 7 days of incubation, the diameters of halo zone from edge of the colony were 7 to 9.8cm in all the 5% pesticide concentration. *A. salinestrus* (GVT-1) formed large halo zone (9.8cm) in plates containing 5% phorate concentration as compared to other strains (Table 3). In control plates the P solubilization was ranged from 13 to 14.2 cm in 7 days. All the isolates were equal in phosphate solubilization and 3% of all the pesticides showed negative effect on bacterial growth on plates. All the isolates were grown in 1% of pesticides in 4 days of incubation and 1% concentration did not affect the bacterial activity but in 3% concentration growth was inhibited by pesticides; definitely has showed bacteriostatic effect on *Azotobacter* species. Moneke et al. (2010) reported that the species of *Azotobacter* and other species viz., *Pseudomonas*, *Escherichia*, *Acetobacter* etc., can tolerate and degrade glyphosate herbicides. This report clearly indicates that all the isolates were resistant to 1, 3 and 5 % of pesticides and but the bacterial activity was inhibited as compared to the control. Previously Garg et al. (2001) reported phosphate- solubilizing activity of *Azotobacter* under *in vivo* conditions and similar results were recorded in the present study. Kumar et al. (2001) reported the P solubilizing activity of *A. chroococcum* under green house condition of wheat and P solubilization has increased the total vigor of the plant and found similar results in the present study. Upadhyay et al. (2009) isolated P solubilizing PGPR strains at 1 to 8% of NaCl conditions and all the strains able to solubilize P at the rate 4.5 to 8.0mm as compared to the present work *A. salinestrus* solubilized double with 5% glyphosate concentration. All P solubilizing species will not have same efficiency rate and GVT-1 strain found more efficient than the previous. *A. salinestrus*, *A. tropicalis*, *A. chroococcum* and *A. armeniacus* were able to solubilize equal amount of phosphate as compared to previously isolated *A. chroococcum* and *A. vinelandii* strains. These strains can be used as alternate P solubilizer as compared to the existing strains.

### Effect of Pesticides on Bioassay activity

Among 5 isolates, GVT-1 was selected for bioassay activity based on NF, IAA, GA production and PS results. Among all the culture filtrate, 2000ppm has increased the paddy seed vigor index or growth (1715, 1684 and 1635) and seed germination rate upto 95 - 98% as well as root (8.3cm) and shoot length (9.3cm) in 1, 3 and 5% phorate concentration over the control (1770) respectively. Initially in 4 days not much variation were observed on the seed germination and seedling growth but after 7 days of seed germination containing 100, 500, 1000 and 2000ppm concentration increased per cent of growth rate in 1, 3 and 5% pesticide concentration. The percent of paddy seed germination (96-100%) was increased over the control (91%) samples. The culture filtrate concentrations on paddy seeds has increased root and shoot length and vigor index at a maximum of 50% growth (Fig 4a, 4b, 4c and 4d) in 1 and 3% phorate concentration. This report clearly indicates that the isolates has capacity to increase the vigor of the plant and resistant to all the concentrations of pesticides. Sharma et al. (2009) reported *A. chroococcum* strain increased the litchi root and shoot length by 81.39% and was isolated from orchard soils. Similarly, Patil (2011) reported the

impact of *Azotobacter* culture on root and shoot length of groundnut (38.88 to 49.88) and gram (27.65 to 36.35) seeds. Sachin (2009) studied IAA production and phosphate solubilizing activities of *A. chroococcum* and concluded that the PGPR strain increased the seed germination, root and shoot length of maize respectively. Ahmad et al. (2008) reported the impact of *Azotobacter* isolates on root elongation in *Sesbania aculeate* and *Vigna radiata* plants supplemented with 1mg of tryptophan and found that all the isolates increased root length in *Sesbania aculeate* (4.7cm); *Vigna radiata* (5.5cm) over the control (3.8 and 3.5cm). *A. salinestrus* strains significantly increased seed germination, root, shoot length as well as vigor of plant as compared to the *A. chroococcum* strain respectively. This proves the potentiality of the strains with respect to the N<sub>2</sub> fixation, IAA, GA, and P solubilizing activity these isolates were equally beneficial in bioassay activities. Limited work has been done on bioassay activities of *Azotobacter* species and the effect of pesticides on NF, IAA, GA production and PS for agriculturally important crops including paddy. The present work will initiate the potentiality of the strains isolated from native soils and can be used as alternate PGP strains.

Fig. 4a: *Azotobacter salinestris* (GVT-1)Fig. 4b: *Azotobacter salinestris* (GVT-1)Fig. 4c: *Azotobacter salinestris* (GVT-1)Fig. 4d: *Azotobacter salinestris* (GVT-1)Fig 4: Bioassay of the *Azotobacter salinestris* and effect of pesticides on paddy seed vigor (growth).

## CONCLUSION

From the present study, *Azotobacter* species have been isolated and identified viz., *A. vinelandii*, *A. chroococcum*, *A. sp. A. salinestris*, *A. armeniacus* and *A. tropicalis*. All the isolates were distinct from other *Azotobacter* species in the phylogenetic diversity and were resistant to 4 different pesticides which are generally used for the paddy cultivation. Among them *A. salinestris* were fixed a maximum amount of  $N_2$ , produced IAA, GA and solubilize the P supplemented with different concentration of pesticides. The strains can withstand different pH values and the pesticide concentration under water logged soils and bioassay activity of the isolates proved the efficiency in increasing the growth of the plant respectively. Isolates were important in the field of agriculture as a PGPR to maintain the soil fertility and to improve food production with eco-friendly management.

## ACKNOWLEDGEMENT

For isolation, identification and the instrumentation facility was supported Dr. M. K. Naik, professor and Head Dept. of Plant Pathology, College of Agriculture, UAS, Raichur, Karnataka and valuable suggestions and laboratory facilities during the research work.

## REFERENCES

- Ahmad F, Ahmad I and Khan MS, (2008). Screening of free living rhizospheric bacteria for their multiple growth promoting activities. *Microbiol. Res.* 173-181.
- Akhter MS, Hossain, SJ, Hossain, SKA and Datta, RKD, (2012). Isolation and characterization of salinity tolerant *Azotobacter* sp. *Gre J Biolo Sci.* 2(3): 43-51
- Aleem A, Isar J and Malik A, (2003). Impact of long term application of industrial wastewater on the

- emergence of resistance traits of *Azotobacter vinelandii* isolated from rhizosphere soil. Biores. Tech. 86:7-13.
- Aquilanti L, Favilli F and Clementi F, (2004). Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. Soil. Biol. and Biochem. 36:1475-1483.
- Asma MT, Pallavi RB, Sonal GC, Jai SG and Prakash, DR, (2012). Effect of endosulfan on indole acid and gibberlin secretion by *Azospirillum* spp NCIM-2548 and *Azotobacter* spp NCIM-2452. Int. Res. J. Env. Sci. 1(3): 1-4.
- Bano N and Musarrat J, (2003). Isolation and characterization of phorate degrading soil bacteria of environmental and agronomic significance. Lett. Microbiol. 36:349-353
- Beneduzi A, Peres D, Vargas LK, Zanettini MHB and Passaglia LMP, (2008). Evaluation of genetic diversity and plant growth promoting activities of nitrogen fixing bacilli isolated from rice fields in south Brazil. App. Soil. Eco. 39: 311-320.
- Bhatia R, Ruppel S and Narula N, (2009). *NifH* based studies on azotobacterial diversity in cotton soils of India. Arc of Microbiol 191:807-813.
- Bremner, JM, (1965). Total nitrogen In: methods of soil analysis. Part 2, Chemical and Microbiological Properties. Agronomy 9. ASA, Madison, Wisconsin. pp. 1149-1176.
- Castillo JM, Casas J, and Romero E, (2011). Isolation of an endosulfan degrading bacterium from a coffee farm soil: Persistence and inhibitory effect on its biological functions. Sci. Total. Environ. 20-27.
- Chennappa G, Adkar-Purushothama CR, Suraj U, Tamilvandan K and Sreenivasa MY, (2013). Pesticide tolerant *Azotobacter* isolates from paddy growing areas of northern Karnataka, India. W. J. Microbiol. Biotechnol. DOI 10.1007/s11274-013-1412-3
- Das AC and Mukharjee D, (2000). Soil application of insecticides influences microorganisms and plant nutrients. App. Soil Ecol. 14: 55-62
- Garg SK, Bhatnagar A, Kalla A and Narula N, (2001). *Invitro* nitrogen fixation, phosphate solubilization, survival and nutrient release by *Azotobacter* strains in an aquatic system. Biores. Tech. 80:101-109.
- Jimenez DJ, Montana JS and Martienz MM (2011). Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable grown in Colombian soils. Braz. J. Microbiol. 42:846-858.
- Khan HR, Mohiuddin and Rahman M, (2008). Enumeration, isolation and identification of nitrogen fixing bacterial strains at seedling stage in rhizosphere of rice grown in non-calcareous grey flood plain soil of Bangladesh. J. Facul. Environ. Sci. Tech. 13:97-101.
- Kumar V, Behl RK and Narula N, (2001). Establishment of phosphate-solubilizing strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under green house conditions. Microbiol. Res. 156:87-93.
- Latifi AM, Khodi S, Mirzaei M, Miresmaeili M and Babavalian H, (2012). Isolation and characterization of five chloropyrifos degrading bacteria. Afr. J. Biotechnol. 11:3120-3146.
- Martin XM, Sumathi CS and Kannan VR, (2011). Influence of agrochemical and *Azotobacter* spp. Application on soil fertility in relation to maize growth under nursery conditions. Eurasian. J. Biosci. 5:19-28
- Martensson L, Diez B, Wartainen I, Zheng W, El-Shehawy R and Rasmussen U, (2009). Diazotrophic diversity, *nifH* gene expression and nitrogenase activity in a rice paddy field in Fujian, China. Plant soil, DOI 10.1007/s11104-009-9970-8.
- Moneke AN, Okpala GN and Anyanwu CU, (2010). Biodegradation of glyphosate herbicide *Invitro* using bacterial isolates from four rice fields. Afr J Biotechnol 9:4067-4074.
- Mrkovacki NB, Cacic NA and Milic VM, (2002). Effects of Pesticides on *Azotobacter chroococcum*. Proceedings for Natural Sciences 23- 28.
- Page WJ and Shivprasad S, (1991). *Azotobacter salinestris* spp. nov., a sodium dependent, micro aerophilic and aeroadaptive nitrogen fixing bacteria. Int. J. Syst. Bacteriol. 41:369-376.
- Patil V, (2011). Production of indole acetic acid by *Azotobacter* spp. Rec. Res. Sci. Tech 3:14-16. ISSN: 2076-5061.
- Rajeswari K and Kasthuri MG, (2009). Molecular characterization of *Azotobacter* spp. *nif H* gene isolated from marine source. Afr. J. Biotechnol. 8:6850-6855.
- Reinhardt EL, Ramos PL, Manfio GP, Barbosa HR, Pavan C and Filho CAM, (2008). Molecular characterization of nitrogen-fixing bacteria isolated from Brazilian agricultural plants at also Paulo state. Bra. J. Microbiol. 39: 414-422.
- Sachin DN, (2009). Effect of *Azotobacter chroococcum* (PGPR) on the growth of bamboo (*Bambusa bamboo*) and maize (*Zea mays*) plants. Biofrontiers 1:24-31.
- Sharma SD, Kumar P, Raj H and Bhardwaj S, (2009). Isolation of arbuscular mycorrhizal fungi and *Azotobacter chroococcum* from local litchi orchards and evaluation of their activity in the air layers system. Scientia. Horticulturae. 6:117-123.
- Soni R and Goel R, (2011). *nifH* homologs from soil metagenome. Ekologija 57:87-95
- Steenhoudt O and Vanderleyden J, (2000). *Azospirillum*, a free living, nitrogen fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24:487-506.
- Tejera N, Lluch C, Toledo MVM and Lopez GJ, (2005). Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere. Plant and soil 270: 223-232.
- Upadhyay SK, Singh DP and Saikia R, (2009). Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline

- condition. Curr. Microbiol. DOI 10.1007/s00284-009-9464-1
- Wu L, Cui Y and Chen S, (2011). Diversity of *nifH* gene sequences in the sediments of South China Sea. Afr. J. Microbiol. Res. 5(32): 5972-5977.
- Zehr JP, Jenkins BD, Short SM and Steward GF, (2003). Nitrogenase gene diversity and microbial community structure: a cross system comparison. Environ. Microbiol. 5(7): 539-554.
- Zhan J. and Sun Q. (2010). Diversity of free living nitrogen fixing microorganisms in wastelands of copper mine tailings during the process of natural ecological restoration. J. Env. Sci. 23(3): 476:487.

**Cite this Article:** Chennappa G, Adkar-Purushothama CR, Naik MK, Suraj U, Sreenivasa MY, 2014. Impact of Pesticides on PGPR Activity of *Azotobacter* sp. Isolated from Pesticide Flooded Paddy Soils. Greener Journal of Agricultural Sciences. 4(4):117-129, <http://dx.doi.org/10.15580/GJAS.2014.4.010314003>.