Malaya Journal of Biosciences 2017, 4(2):88-96
ISSN 2348-6236 print /2348-3075 online

Malaya Journal of Biosciences

www.malayabiosciences.com

RESEARCH ARTICLE

Open Access Full Text Article

Isolation and characterization of Indole acetic acid producing strain from rhizospheric forest soil of Visakhapatnam District, Andhra Pradesh, India

Devi Soundarya S and Padal S B

Department of Botany, Andhra University, Visakhapatnam - 530003, A.P, India.
For Correspondence E-mail: devisoundarya32@gmail.com

Article Info: Received 27 Nov 2017; Revised: 26 Dec 2017; Accepted 28 Dec 2017.

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are of great importance in agriculture for better crop yield without pathological stress to plants and soil as well. Indole Acetic Acid (IAA), one of the plant growth promoting components, is an important property of rhizosphere bacteria which facilitates adequate crop yield. It is already reported that IAA producing bacteria is an efficient biofertilizer in agriculture to promote plant growth. Hence, isolation of such bacteria is very crucial for agriculture fields for better nutrition. The present study is aimed to isolate IAA producing bacteria from different forest soils (Salugu, Vanthalamamidi, Panneddu mailu, Dallapalle and Gurragaruvu village) of Paderu mandal, Vishakhapatnam district, India. Organisms isolated from these rhizosphere soils were characterized and screened for IAA production. Among the 55 isolated bacteria, 6 isolates (from Gurragaruvu village) showed IAA producing activity and out of these 6 isolates one isolate showed high IAA production. Then 16S rRNA gene sequencing was performed for this promising isolate and based on the sequence homology, the bacterial isolate is identified as *Klebsiella Pneumonia*.

Keywords: Klebsiella pneumonia, IAA, PGPR, In vitro and 16S rRNA

1. INTRODUCTION

Microorganism associated with the rhizosphere promotes the exchange of plant nutrients, minimizes the chemical fertilizers usage and promotes plant growth as well as soil fertility [1]. Application of such beneficial microbes sustains the soil physiology [2]. Plant growth promoting bacteria are the natural potential resource which colonize roots of plants and stimulate growth and yield directly and indirectly [3]. The rhizosphere bacteria appear to have a greater potential to synthesize and release IAA as secondary metabolites than normal soil microbiota because of - the relatively rich supply of nutrients from the root exudates in the rhizosphere [1, 3]. Many rhizosphere bacteria have the capacity to synthesize Iodole Acetic acid (IAA) that has pronounced effect on plant growth and development [1, 2]. Production of IAA by microbial isolates varies greatly among different species or strains that depend on the availability of substrates.

Rhizosphere, a narrow region of soil that is directly influenced by root secretions and associated microorganisms with high microbial diversity [4].

Phytohormone, especially indole-3-acetic acid plays an important role in the plant growth regulation like cell enlargement, cell division, cell elongation and root initiation by tryptophan dependent and independent pathways [5]. Rhizospheric colonization of cereal crop plants and plant growth promoting activity involving indole-3-acetic acid (IAA) production by Klebsiella sp. and Bacillus sp. has been well documented [4, 6, 7]. Klebsiella species have been isolated and studied for IAA production from rhizosphere of sugarcane [8], soya bean [9], rice [6], pearl millet [10] and Kentucky blue grass [9].

Iodole acetic acid (IAA), a phytohormone, is produced in young leaves and stems from transamination and decarboxylation reactions of tryptophan [11]. Effect of IAA on plants are significant and some of them are promotes cambial activity, delays in abscission of leaves and induces flowering and fruits production [12]. Therefore, IAA has much importance in plant growth promotion as well as soil fertility and here the present study is conducted to isolate the potential IAA producing bacterial strain from forest soil samples around different places of Paderu Mandal, Visakhapatnam District, India. The potent IAA producing bacteria is further characterized by 16S rRNA sequence.

2. MATERIALS AND METHODS

2.1 Collection of Samples

To isolate most efficient IAA producing PGPR, soil samples were collected from forest of Paderu, Visakhapatnam district, Andhra Pradesh, India. Here we have selected paderu area to isolate the potent bacteria because of fully vargin forest soil and it has significant variations in the soil nutrients reported by [13] through geo statistical analysis. 1 kg of each rhizospheric soil sample was brought to the laboratory in the sterile polythene bags.

2.2 Isolation and purification of bacterial isolates

Soil attached to the roots was removed followed by preparation of serial dilutions. Dilutions were plated on nutrient agar (Hi-Media) and the plates were incubated at 30oC for 48 h. Morphologically distinct colonies from agar plates were selected and purified by repeated culturing and stored in nutrient broth containing 15% glycerol at -4^{o}C temperature.

2.1.3. In Vitro Screening for IAA Production

The IAA production by the isolates was screened under *in vitro* conditions by the standard method described by Loper and Scroth [14]. Briefly, Isolates were grown in nutrient broth supplemented with tryptophan (1 mg/ml) at 30^{o}C for 72 hrs in incubator to get confluent growth. After incubation, cultures were centrifuged at 3000 rpm for 5 min. To 1 ml of the supernatant, 2 ml of Salkowski's reagent (50 ml 35% Perchloric acid; 1 ml 0.5 $FeCl_3$) were added and incubated at dark room, for 25 mins. Pink color formation confirmed the presence of IAA in the supernatant. Quantification of IAA produced by the PGPR was done spectrophotometrically read based on the absorbance of the treated supernatant at 535 nm by Hitachi U-2910 spectrophotometer. The concentration of IAA produced by the isolate in the broth was quantified by comparing the standard graph made using standard IAA procured from Himedia Laboratories Pvt Ltd, India.

3. *Identification of selected bacteria*

Selected IAA producing bacterial isolate was identified by colony morphology, microscopic examination, screening for properties study and biochemical characteristics following Bergey's Manual [15] by performing tests such as catalase, oxidase, insole, methyl red, citrate utilization, starch hydrolysis, carbohydrate fermentation i.e. glucose, sucrose, mannitol, lactose and urea utilization etc.

4.1. *Molecular Identification of selected IAA producing bacteria Using 16S rRNA Gene sequencing*

4.1.1. DNA Extraction

Bacterial Genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit – We followed the procedure according to the instructions provided by the manufacturers. Briefly, an isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. Then centrifuged at 10,000 to 12,000 rpm for 1 minute. Later, 200µl of Insta Gene matrix is added to the pellet and incubated at 56°C for 15 minutes. After that it was vortex at high speed for 10 seconds and placed the tube in 100^{0}C in heat block or boiling water bath for 8 minutes. Finally, again was vortex at high speed for 10 seconds and centrifuged at 10,000 – 12,000 rpm for 2 minutes. From the supernatant 20µl was taken for PCR reaction.

4.1.2. *PCR Protocol*

1µL of template DNA has taken in 20µl of PCR reaction solution. 27F/1492R Primers were used for bacteria, and then PCR reaction performed with

below conditions. Initial denaturation at 94℃ for 2 min and then 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec and 72℃ for 60 sec. Final Extension at 72℃ for 10 min. Included a positive control (*E. coli* genomic DNA) and a negative control in the PCR.

4.1.3. Purification of PCR products

Removed the unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

4.1.4. Sequencing protocol

Single-pass sequencing was performed on each template using below 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Bio-Systems). Using below 16S rRNA Universal primers gene fragment was amplified by MJ Research Peltier Thermal Cycler. Sequence data was aligned and analyzed for Identifying the Sample.

4.1.5. Primer Details

Primer	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

4.1.6. Bioinformatics protocol

The 16s r RNA sequence was BLAST using NCBI BLAST similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of BLAST results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [16]. The resulted aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) [17]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [18].

5. RESULTS

Rhizosphere soil samples from five different villages around Paderu mandal were collected to identify the potential IAA producing strain. The geographical locations of the collected sites (except Panneddu mailu) were presented in Figure 1. R5H isolate shown in Figure 2. A total of 55 bacterial isolates were identified from the collected soil samples based on morphological characters and screened for *In vitro* IAA production. Among 55 bacterial isolates, 6 isolates (R5 series collected from Gurragaruvu village) were found to produce pink color with Salkowski's reagent, which revealed that these organisms produce IAA in the presence of tryptophan (1mg/ml) (Fig. 3). IAA production from isolates of R5 series are mentioned in Table 1. In this R5 series, R5H isolate has shown high concentration of IAA (75.61 ± 4.6) in comparison to other strains.R5 Series, plant growth promoting activities shown in (Table 2) Hence, R5H is focused to study further biochemical parameters (Table 3). To confirm species level, the present potent R5H isolate was characterized by molecular 16S rRNA gene sequencing (Figure 4). The resulted sequence was checked with the BLAST alignment for similarity matches and the phylogenetic relationship is presented in Figure 5.

6. DISCUSSION

The most widely studied group of PGPR inhabiting at root surfaces of plants and the closely adherent soil surface, the rhizosphere [19]. The plant rhizosphere is a useful and essential parameter for microbes-plants interactions for better nutrition to promote plant growth [20]. Previously reported that the PGPR inoculants supports the plant growth parameters like germination rate, seedling emergence, sustains to external stress factors and immunity towards diseases [21]. According to the previous studies rhizosphere isolates are more efficient auxin producers then the bulk soil [22]. The present R5H isolate is gram negative organism which supports the earlier studies about most IAA producing organisms are Gram negative [23 and 24] and few bacillus strains, gram positive, known to produce IAA [25]. Mirza et al., [26] reported that IAA production levels are varies from species to species and also depends on other

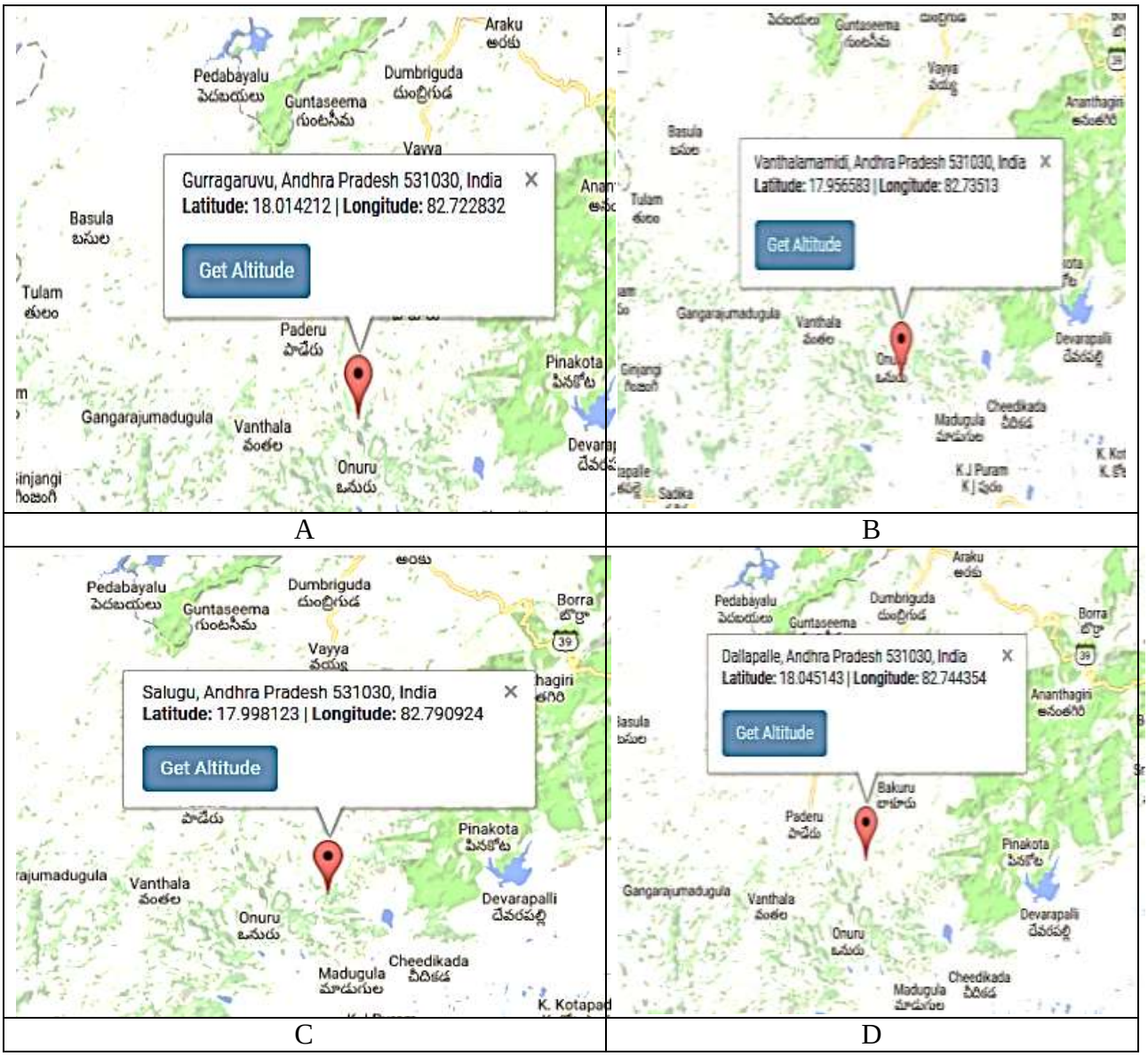

Figure 1: Geographical location of the sample collected sites. A. Gurragaruvu, B. Vanthalamamidi, C. Salugu and D. Dallapalle

Figure 2: R5 H- Isolate

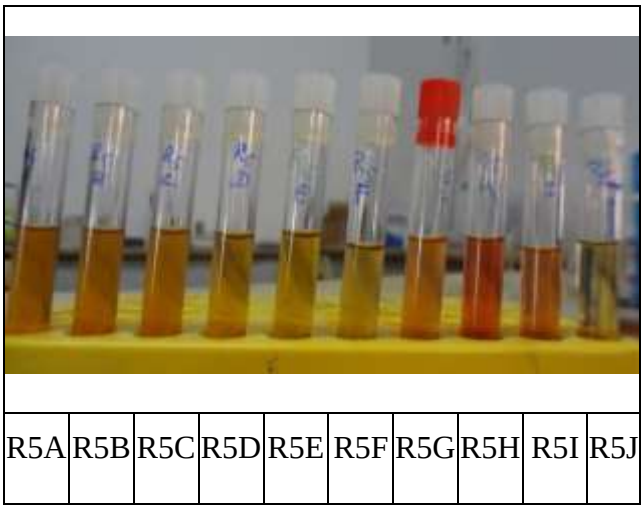

Figure 3: IAA Production by rhizosphere isolates (R5 H) showing pink color formation after addition of Salkowski's reagent

Table 1. Consolidated result showing the concentration of IAA (µg/ml) produced by the Rhizosphere Isolates under *in vitro* conditions.

S. No.	Isolate	Concentratiom Mean ± SD (µg/ml)
1	R5- A	13.43 ± 3.5
2	R5- B	15.67 ± 3.3
3	R5- C	11.33± 6.7
5	R5- G	43.33 ± 8.4
5	R5- H	75.61 ± 4.6
6	R5- I	51.12 ± 9.3

Table2. Plant growth promoting activities of Rizosphere Isolates.

Organism Code	Amylase (Starch)	Protease (Gelatin)	Chitin	IAA	Catalase	Phosphate solubilizing
R5 A				+	+	
R5 B				+	+	+
R5 C			+	+	+	
R5 D					+	
R5 E	+				+	
R5 F	+	++	+		+	
R5 G			++	+	+	+
R5 H				++	+	++
R5 I				+	+	
R5 J					+	

Table 3. Morphological and biochemical characteristics of R5H isolate

Name of test	Result	Name of test	Result
Media	Nutrient Agar	Lysine	- ve
Colony morphology		Starch	+ ve
Surface	Smooth	Raff nose	+ ve
Size	Medium	Sucrose	+ ve
Color	Cloudy	Lactose	+ ve
Form	Circular	Maltose	+ ve
Texture	Mucoid	Trehalose	+ ve
Elevation	Raised	Cellobiose	+ ve
Margin	Entire	Mellibiose	+ ve
Gram stain	- ve rod shape	Ribose	+ ve
Salt 0 %	++ ve growth	Arabinose	+ ve
Salt 3 %	+++ ve growth	Xylose	+ ve
Salt 6%	+ ve growth	Ramones	+ ve
Salt 8%	+ ve growth	Mannose	+ ve
Salt 10%	No growth	Galactose	+ ve
Salt 12%	No growth	Fructose	+ ve
Catalse	+ ve	Adonitol	- ve
Oxidase	- ve	Mannitol	+ ve
Nitrate	+ ve	Sorbitol	+ ve
H2S	- ve	Dulcitol	+ ve
Urease	- ve	Inositol	+ ve
Iodole	- ve	Myoinositol	+ ve
Methyl red	- ve	Salicilin	- ve
Voges prosecure	+ ve	Asculine	+ ve
Citrate	+ ve	Chromogenic UTI agar	bluish green color growth
Gelatin	- ve	EMB	Transparent growth
Casein	- ve	MAC	Cream color growth
Ornithine	- ve	Kings	cream color growth
Arginine	- ve	TCBS	Yellow color growth

CGAAGGTTAGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA
ACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTC
AAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTG
CTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGC
AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGG
GGTGAAGTCGTAACAGGGAAACCGTAAAAAAGGAGG

Figure 4: 16S rRNA sequence of R5H isolate

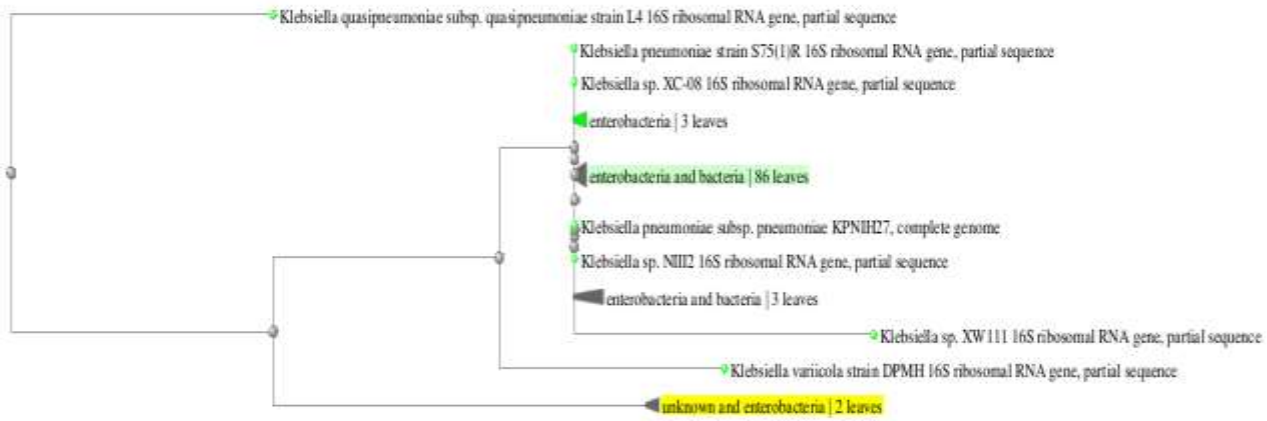

Figure.5. Phylogenetic relationship of R5H isolate on partial 16S rRNA gene sequences with 16S rDNA reference gene sequences available in NCBI. Based on the reference sequences after BLAST, the present isolate belongs to the phylum Proteobacteria, Class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae Genus *Klebsella* and species *pneumoniae*.

parameters supports the present study where among all strains, 6 isolates are IAA positive strains and the IAA production levels are varying among R5 series isolates. Van Urk Salkowski reagent is the best choice in detection of IAA production qualitative as well as quantitate studies because of its specific reaction towards IAA [27] and in the present experiment the IAA production levels are within the limits of Salkowski reagent [28]. Among all bacterial isolates, R5G and R5I strains found to be medium IAA production and R5H is the best IAA producing bacteria (75.61 ± 4.6 µg/ml) in this series as shown in Figure 2 and Table 1. Hence for further characterization R5H was selected and it was identified by 16S r RNA gene sequencing as *K. pneumoniae*. Production of IAA is considered as an effective parameter for screening the beneficial microorganisms where these bacteria have profound effect on plant growth [29 and 30]. Thus, these IAA producing *Klebsiella* strain is considered for further studies to reveal the significance on plant growth under controlled condition.

7. CONCLUSION

From this study, it is clear that rhizospheric soil can provide a rich source of IAA producing bacteria and has the ability to produce a significant amount of IAA in a tryptophan-supplemented medium. Overall six isolates were identified as IAA producing strains among which one efficient IAA producing bacteria was characterized. Among the isolates rhizospheric soil R5 H showed best IAA production. The present study need further research so that the strain could be directly applied to crop fields in different formulations for sustainable agriculture.

Acknowledgement

We convey our sincere Thanks to Department of Botany, Andhra University for permit us to carry out the study. We would like to Thank 'For U Lab' Director Mr.D.Raju for valuable support in Identification of Isolates.

Conflicts of Interest

There are no conflicts of interest.

References

1. Dastager SG, Deepa CK and Pandey A (2011). Potential plant growth promoting activity of *Serratia nematodiphila* NII-0928 on black pepper. *World Journal Microbial Biotechnology;* 27:259-265.
2. Serpil S (2012). An agricultural pollutant: chemical fertilizer. *International Journal of Environmental Science and Development*; 3 (1):77-80.
3. Afzal A and Bano A (2008). Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology*; l10:85-88.
4. Saharan BS and Nehra V (2011). Plant growth promoting rhizobacteria: A Critical Review. *Life Sciences and Medicine Research*; 2011(21):1-30.

5. Woodward AW and Bartel B (2005). Auxin: Regulation, Action, and Interaction. *Annals of Botany;* 95:707-735.
6. El-khawas H and Adachi K (1999). Identification and quantification of auxins in culture media of *Azospirillum* and *Klebsiella* and their effect on rice roots. *Biology and Fertility of Soils;* 28:377-381.
7. Mishra VK and Kumar A (2012). Plant growth promoting and Phyto stimulatory potential of *Bacillus subtilis* and *Bacillus amyloliquefaciens. Journal of Agricultural and Biological Science;* 7 (7): 509-512.
8. Govindarajan M, Kwon SW and Weon HY (2007). Isolation, molecular characterization and growth-promoting activities of entophytic sugarcane diazotroph *Klebsiella* sp. GR9. *World Journal of Microbiology and Biotechnology;* 23: 997.
9. Kuklinsky-Sobral J, Araujo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA and Azevedo JL (2004). Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology;* 6(12): l244.
10. Tiwari M, Paroda S and Dadarwal K (2003). Associative diazotroplis of pearl millet *(Pennisetum glaucum)* from semi-arid region-isolation and characterization. *Indian Journal of Experimental Biology;* 41(4) 341.
11. Lebuhn M and Hartmann A (1993). Method for the determination of indole-3-acetic acid and related compounds of L-tryptophan catabolism in soils. *Journal of Chromatography A*; 629: 255.
12. Zhao Y (2010). Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology;* 61: 49-64.
13. Desavathu RN, Nadipena AR and Peddada JR (2017). Assessment of Soil Fertility Status in Paderu Mandal, Visakhapatnam District of Andhra Pradesh through Geospatial Techniques. *The Egyptian Journal of Remote Sensing and Space Sciences;* (in press).
14. Loper JE and Schroth MN (1986). Influence of bacterial sources of indole-2-acetic acid on root elongation of sugar beet. *Phytopathology*; 76: 386-389.
15. Bergey DH (1923-1934). Bergey's Manual of Determinative Bacteriology. 1st to 4th ed., Williams & Wilkins Company, Baltimore.
16. Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*; 32(5):1792-1797.
17. Talavera G and Castresana J (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology*; 56: 564-577.
18. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, GuindonS, Lefort V, Lescot M, Claverie JM and Gascuel O (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research;* 1:36.
19. Klopper JP, Hays WR, Sharma V, Baumbusch MA, Hershman JM and Haugen BR (2004). Retinoid X receptor-gamma and peroxisome proliferator-activated receptor-gamma expression predicts thyroid carcinoma cell response to retinoid and thiazolidinedione treatment. *Molecular Cancer Therapeutics;* 3(8): 1011–1020.
20. Jeffries P, Gianinazzi S, Perotto S, Turnau K and Barea JM (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils*; 37: 1-16.
21. Egamberdiyeva. D (2007). The Effect of Plant Growth Promoting Bacteria on Growth and Nutrient Uptake of Maize in Two Different Soils. *Applied Soil Ecology;* 36 (2-3): 184-189.
22. Sarwar M and Kremer RJ (1992). Determination of bacterially derived auxins using a micro plate method. *Letters in Applied Microbiology;* 20(5): 282-285.
23. Lindow SE, Desurmont C, Elkins R, McCourt G, Clark E and Maria TB (1998). Occurrence of lndole -3- acetic acid-producing bacteria on pear trees and their association with fruit russet. Phytopothol. 88(11): 1149 -1157.
24. Datta, C., Basu, P (2000). lndole acetic acid production by a Rhizobium species from root nodules of a leguminous shrub, *Cajanus cojan*. *Microbiological Research*; 155(2): 123 – 127.
25. Wahyudi AT, Astuti RP, Widyawati A, Meryandini A and Nawangsih AA (2011). Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting Rhizobacteria. *Journal of*

Microbiology and Antimicrobials; 3(2): 34-40.

26. Mirza MS, Ahmad W, Latif F, Haurat J, Bally R, Normand P and Malik KA (2001). Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. *Plant and Soil;* 237(1):47-54.
27. Vaghasia HL, Patel GM, Chudasama RS and Bhott KR (2011). Screening of IAA from rhizosphere Micro flora of field crops. *Bioscience Discovery*; 02 (1): 94-100.
28. Ehmann A (1977). The Van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography*; 132(2): 267-276.
29. Govindarjan M, Kwon SW and Weon HY (2007). Isolatin, molecular charactertization and activities of endophytic sugarcane diazotroph *klebsiella* sp. GR9. *World Journal of Microbiology and Biotechnology;* 23(7): 997-1006.
30. Ali B and Hasnain S (2007). Potential of bacterial indoleacetic acid to induce adventitious shoots in plant culture. *Letters in Applied Microbiology;* 45(2): 128-133