International Journal of Advanced Life Sciences (IJALS)
ISSN 2277 – 758X
Bhargavi and Tallapragada, IJALS, Volume (9) Issue (1) February - 2016. RESEARCH ARTICLE

Bacillus sp as potential plant growth promoting rhizobacteria

V.G. Bhargavi Rayavarapu and Tallapragada Padmavathi*
Department of Microbiology, Center of PG Studies, Jain University, 18/3, 9th Main Road, 3rd Block, Jayanagar, Bangalore, Karnataka - 560011, India.
E-mail : Vam2010tpraviju@gmail.com

Corresponding Author
Tallapragada Padmavathi
Department of Microbiology, Center of PG Studies, Jain University, 18/3, 9th Main Road, 3rd Block, Jayanagar, Bangalore, Karnataka 560011, India.
Email :
Vam2010tpraviju@gmail.com

Article History
Received on 09 December, 2015; Received in revised form 26 December, 2015; Accepted 09 January, 2016

Abstract

Plant Growth Promoting Rhizobacteria (PGPR) present in the rhizosphere influences the plant growth and productivity by various mechanisms. Most efficient PGPR strains are involved in the development of plant growth by producing various regulatory hormones or chemicals in their vicinity of the rhizosphere, may fix atmospheric nitrogen, reduce toxic compounds, synthesize siderophores and suppress the growth of pathogenic microorganisms. The *Bacillus* was isolated from rhizosphere and biochemically characterized and screened *in vitro* for their PGPR traits like production of IAA, Phosphate solubilization, Siderophore, Ammonia, HCN, Catalase, Organic acid production and observed for heavy metal and salinity tolerance. *Bacillus* showed positive response towards all the PGPR traits which are responsible for plant growth. Thus, *Bacillus* can enhance the plant growth and also combat with heavy metal and salinity stress of soil. It is eco friendly. Hence, *Bacillus* can be recommended as bio fertilizers for agricultural crops instead of using as chemical fertilizers. The main objective of the present study was *Bacillus* being a potent plant growth promoting rhizobacteria (PGPR) was added as biofertilizer for increasing the crop yield instead of using chemical fertilizers.

Keywords : Indole 3 acetic acid (IAA), Gibberilic Acid (GA), Hydrogen Cyanide (HCN) and Casamino acid (CAS).

Introduction

In Rhizosphere plant-microbe interaction can be beneficial, neutral or deleterious. Free living bacteria referred as Plant Growth Promoting Rhizobacteria (PGPR), exhibit active root colonization in the rhizospheric zone. PGPR strain enhances the plant growth by direct or indirect mechanisms. PGPR improves the plant growth and yield of agricultural and horticultural crops. Strains like *Pseudomonas, Klebisella, Azotobacter, Azospirillum, Bacillus, Burkkolderia* and *Serratia* are considered as PGPR traits that enhance the plant growth (Mishra *et al.,* 2010). The direct mechanism involved in enhancing the plant growth is by producing plant hormones like Indole Acetic Acid (IAA) and Gibberellic Acid (GA), and provides nutrients to the host plant by producing siderophores, phosphate solubilization and fixes atmospheric nitrogen (Mahalakshmi and Reetha, 2009). The indirect mechanism involves the production of antibiotics, lytic enzymes, hydrogen cyanide and catalase which acts as a biological control of plant pathogens and microbes.

Interaction of beneficial microbes and plant are the primary determinants for plant health and soil fertility. Mostly 2 - 5% of rhizospheric bacteria are PGPR. Spore forming bacteria like *Bacillus* were more efficient PGPR compared with non spore forming bacteria like *Pseudomonas* because spores are most resistant and robust compared with vegetative cells. *Bacillus* is most efficient PGPR that enhances the plant growth by producing a vast variety of substances (Ajay Kumar *et al.,* 2012).

Bacillus and *Pseudomonas* sps are most predominant colonies in the rhizosphere, suppresses the plant pathogens by producing antifungal compounds. PGPR and their application to reduce the usage of chemical fertilizers and pesticides and achieve sustainable crop yield in agriculture and horticulture. PGPR also induces the Induced systemic resistance (ISR) in host plant and reduces the incidence of disease severity in host plants against pathogens. It is called as rhizobacteria mediated Induced Systemic Resistance (ISR) (Kumar *et al.,* 2011).

Materials and methods

Isolation and identification of rhizosperic bacteria

Soil samples were collected in and around Bangalore, India. Samples were placed in plastic bags and stored at -4°C. Soil samples (10g) were taken into 250 ml conical flask, to that 90 ml of distilled water was added and kept in a rotary shaker for 15min. 1ml of soil suspension was serially diluted up to 10^{-8} dilutions. 0.1ml of sample was spread on nutrient agar plates and incubated at 37°C for 24hr. Experiment was carried out thrice to get a pure culture. The colonies were identified by performing Gram's staining and biochemical tests like indole, MR-VP, Starch, Citrate, Gelatin. The colonies were identified on the basis of Bergey's manual of systematic Bacteriology (Rani, 2012).

Characterization of rhizobacteria for PGPR trait

Detection of Phosphate solubilization

Phosphate solubilization by bacterial isolates was detected in Pikovskaya's broth (Pikovskaya, 1948). Bacterial isolates were inoculated into Pikovskaya's broth and incubated in a rotary shaker for 5days at 37°C and observed the solubilization.

Detection of organic acid production

Organic acid production was detected by inoculating test bacterial culture in minimal salt medium (MM9 broth) for 2 to 3days at 37°C. Appearance of pink color in the medium indicates organic acid formation using methyl red as an indicator (Trivedi *et al.,* 2013).

Detection of Ammonia production

Bacterial isolates were tested for ammonia production in peptone water as described by Ajay Kumar *et al.* (2012). Fresh bacterial culture was inoculated in to peptone water and incubated for 2 to 3 days at 37°C. After incubation 0.5 ml of Nessler's reagent was added and observed the development of yellow color indicates the ammonia production.

Hydrogen cyanide production (HCN)

Bacterial culture was tested for HCN production by the methodology described by Castric (1975). Test culture was inoculated in nutrient broth embedded with 4.4g of glycine and whattmann filter paper soaked in 2% sodium carbonate and 0.5ml of picric acid solution and kept at the top of the test tube. Tubes were incubated for 2 to 3 days at 37°C and observed the formation of orange to red color indicating the HCN production.

Catalase activity

48 hr old test bacterial culture was placed on a clean glass slide and 3% of H_2O_2 was dropped and mixed with tooth pick. Observation of bubble formation indicates the positive test for catalase (Ajay Kumar *et al.,* 2012).

Heavy metal tolerance

Heavy metal tolerance by bacterial strains was detected by agar dilution method. Freshly prepared agar plates embedded with different heavy metals like Co, Zn and lead of 100µg/ml were inoculated with overnight grown cultures. Tolerance was observed by the appearance of the bacterial growth on the plates after the incubation at 37°C for 24 to 48 hrs (Yogendra *et al.,* 2013).

DO NOT COPY

Detection of Siderophore production

Siderophore production by bacterial isolates was detected by inoculating into succinate medium embedded with 1% glucose (Trivedi *et al.,* 2013). Test culture was inoculated into succinate medium containing K_2HPO_4 6g, KH_2PO_4 3g, $MgSO_4$ 0.2g and Ammonium sulphate 1g, succinic acid 4g (per liter), pH was adjusted to 7.0 and incubated in a rotary shaker for 2 - 3 days at 37°C. After incubation the broth was centrifuged at 10000 rpm for 10min. Supernatant was collected and tested for siderophore production by using chemical assay as per Schwyn and Neilands (1987).

Quantitative estimation of siderophore was detected by Payne (1994). 0.5ml of supernatant was collected and 0.5ml CAS dye reagent was added and absorbance was read at 630nm. Uninoculated medium was taken as reference. Percentage of decolorization was calculated by the formula :

% of decolorization = Ar - As ÷ Ar x 100

Ar - Absorbance of reference

As - Absorbance of sample

Detection of IAA production

IAA produced by bacteria was detected by a method described by Loper and Scroth (1986). Loop full of bacterial culture was inoculated in the nutrient broth embedded with 0.1% of tryptophan and incubated in a rotary shaker at 37°C for 2 to 3 days. After incubation nutrient medium was centrifuged at 10000 rpm for 10min. Supernatant was collected and IAA was detected colorimetrically.

Colorimetric assay of IAA

1 ml of filtrate was taken and to that a few drops of orthophosphoric acid and 4ml of Salwaski's reagent was added and kept it for 30min. Appearance of pink color indicated the presence of IAA and absorbance was read at 530nm (Gordon and Weber, 1951). Estimation of IAA was calculated by using a standard curve and expressed in mg/ml over control.

Extraction of IAA

After 72 hr of incubation of bacterial isolates in nutrient medium having tryptophan was centrifuged at 10000 rpm for 10mins. The supernatant was collected and acidified with 1N HCl to bring the pH to 2.0 - 3.0. Ethyl acetate was added to the broth in 3:1 ratio for the extraction of sample. The organic phase was collected and the extraction was repeated thrice. The organic layer was allowed to dry and diluted with 0.5ml methanol or ethanol (Monita *et al.,* 2014).

Purification and detection of IAA

The diluted solution was allowed to run TLC plate coated with silica gel by using mobile phase isopropanol : ammonia : water (V/V) in the ratio 16: 3: 1 (Monita *et al.,* 2014). Standard IAA was taken in the concentration 1mg/ml. The spots were developed and observed under 256 and 360nm UV light and the Rf value was calculated. IAA in the sample was detected by using high performance liquid chromate-graphy (HPLC) with standard IAA. Amount of IAA present in the sample was calculated by using the formula :

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{dilution}}{\text{Sample amount}} \times \frac{\text{Standard amount}}{\text{dilution}}$$

Extraction and detection of Gibberellic acid (GA)

Bacterial cultures were inoculated in peptone water and incubated at 37°C for 72 hr. Culture was centrifuged at 10000 rpm for 10 min. After centri-fugation supernatant was collected and acidified with 1N HCl to bring the pH to 2.0 to 3.0. Ethyl acetate was added to the broth (V/V) in the ratio of 2 : 1. The organic layer was collected and repeated thrice for the extraction of sample and allowed it for drying. It was diluted with 0.5ml of methanol or ethanol (Bilkay *et al.,* 2010). GA was detected by Spectrophotometrically

DO NOT COPY

and HPLC using the standard GA. The estimation of GA was carried out by using the formula :

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{dilution}}{\text{Sample amount}} \times \frac{\text{Standard amount}}{\text{dilution}}$$

Salinity tolerance

To observe the salinity tolerance bacterial culture was inoculated in nutrient agar plates supplemented with different concentrations of NaCl ranging from 0.25 to 5% and incubated at 37°C for 24 to 48hr. Salinity tolerance was observed by the growth of the test culture (Damodaran *et al.,* 2013).

Results and discussion

Out of 28 colonies the most predominant single colony was selected and further biochemical and morphological characterization was carried out. Morphological characters like Gram's nature, colony characteristics and biochemical tests like indole, MR-VP, Cimmon's citrate, starch, gelatin are recorded in Table 1 and identified as *Bacillus* sp. using Bergey's manual. The *Bacillus* was further screened for PGPR traits like IAA, phosphate solubilization, siderophore, Ammonia, HCN, organic acid, catalase production and salinity and heavy metal tolerance.

Bacillus sp. showed clear solubilization in the Pikovskaya's broth. It has the ability to solubilize tri-calcium phosphate in the medium. Han *et al.* (2011) also reported that *Bacillus megaterium* exposed to nutrient limited soils (rock P) increases the nutrient availability and uptake and increases the plant growth in pepper and cucumber. Phosphorus (P) is the second most nutrient available in the soil as inorganic form. The plant absorbs inorganic form. Phosphate solubilizing bacteria (PSB) solubilize organic form into inorganic form by synthesizing phosphatases (Ahemad and Kibret, 2014).

Bacillus Sp. produce organic acids and confirmed by observing the pink color in the MM9 medium after

Table – 1. Biochemical tests for *Bacillus* sp.

Characteristics	*Bacillus*
Gram staining	+
Spore	+
Shape	Rods
Indole	-
MR	-
VP	+
Citrate	+
Starch	+
Gelatinase	+

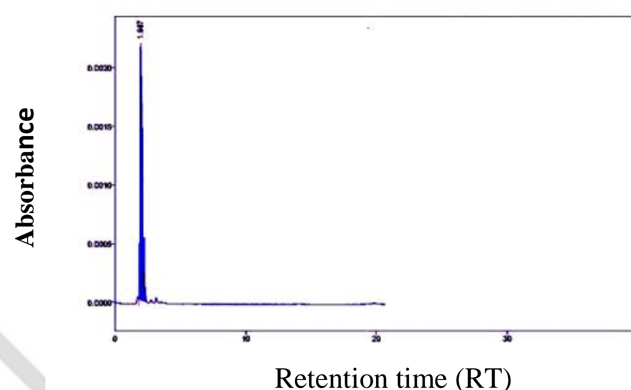

Fig.-1. HPLC Spectrum for Standard IAA

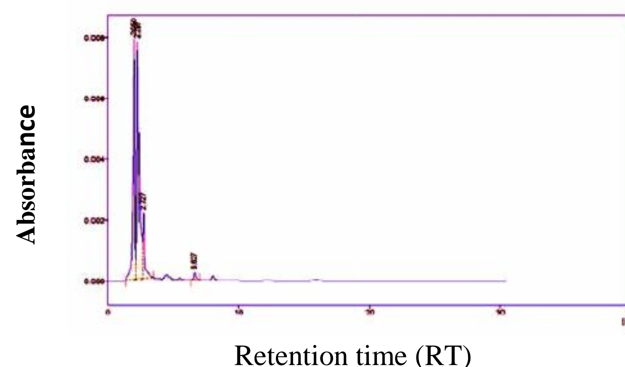

Fig.-2. HPLC Spectrum for IAA produced by *Bacillus*

addition of the methyl red indicator. The same results were found by Bharucha *et al.* (2013) by screening PGPR traits from rhizospheric soils of Alfa Alfa. Organic acids like citric acid, formic acid and oxalic acid are the elements which are involved in metabolic pathways and enhance plant growth.

DO NOT

Bacillus sp. produced ammonia was confirmed by yellow colouration by addition of Nessler's reagent. Similar results were reported by Ajay Kumar *et al.* (2012). Ammonia indirectly promotes plant growth by alleviating soil pH and inhibits growth of certain fungi (Geeta *et al.,* 2014).

Bacillus sp. produces HCN, the orange to red color in the medium confirms the presence of HCN. Kumar and Shruthi (2013) reported that *Bacillus* produces HCN from rhizospheric soils of *Lycopersicum esculentum.* HCN is a secondary volatile compound produced by bacteria which acts like a biological weed control agent (Sahran and Neha, 2011). *Bacillus* produces catalase enzyme after addition of 3% H_2O_2 the bubble formation was observed. Rhizobacteria produce catalase, which removes hydrogen peroxide (H_2O_2) from the cells.

Bacillus sp. showed heavy metal tolerance by observing the growth on the plates. Tolerance against heavy metals like Pb and Zn at 100µg/ml was observed. Yogendra singh *et al.* (2013) also reported that *Pseudomonas* exhibited heavy metal resistance and promoted plant growth.

In CAS medium *Bacillus* sp. produced orange color indicating siderophore production (Fig.-1). *Bacillus* showed 50% of decolorization. Goswami *et al.* (2015) reported that *Paenibacillus mucilaginous* plant growth rhizobacteria produces siderophores. Iron is a vital nutrient for both plants and microbes. Siderophores are the low molecular and water soluble compound which is produced by bacteria chelates Fe^{3+} into Fe^{2+} and makes available to plants.

Bacillus sp. showed positive for IAA production using tryptophan as a precursor and the development of pink color after addition of Salwaski reagent. IAA was extracted and purified on TLC plate and observed the spot under UV. Rf value for sample

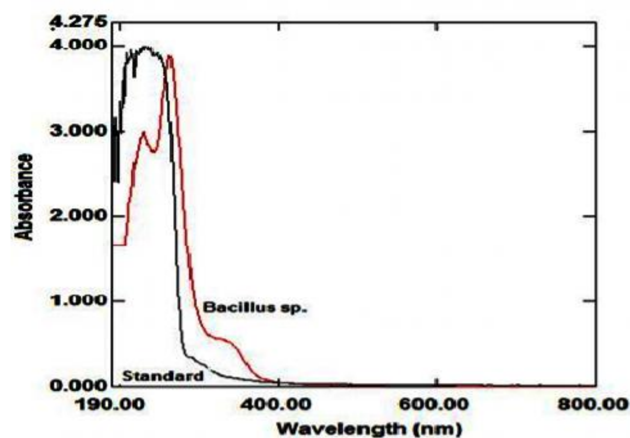

Fig.- 3. Gibberellic acid Spectrum of *Bacillus* and Standard

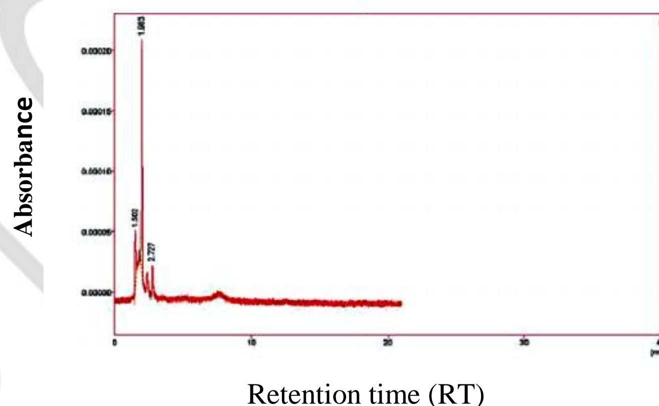

Fig.- 4. HPLC Spectrum of Gibberellic acid standard

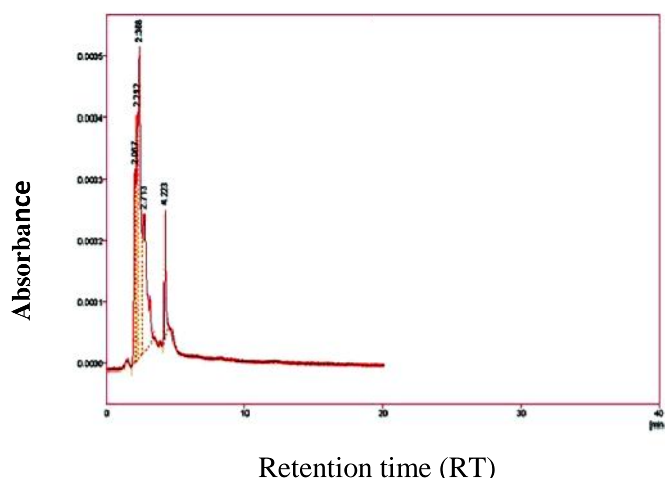

Fig.- 5. HPLC Spectrum of Gibberellic acid produced by *Bacillus*

IAA was 1.04 compared with the standard IAA Rf value of 1.04. IAA was detected by HPLC by taking standard IAA at absorption 254nm. The peak obtained for Standard IAA was 1.987 and sample IAA was 2.050 (Fig.-1 and 2). The concentration of IAA in the sample was 6.57µg/mL. IAA is a Phyto hormone and is considered as native auxin. It is a signal molecule for plant development, stimulates root growth, and modifies root architecture, increases water absorption and water holding capacity (Martinez *et al.,* 2010). IAA increases the lateral and adventitious roots and loosens the plant cell wall and releases the plant exudates that provide nutrition for rhizosperic microbes (Ahemad and Kibret, 2014). The results were supported by Swain *et al.* (2007) who reported that a positive effect of IAA produced by *B. subtillis* on edible tubercle *Dioscorea rotundata* L increases the length of root and stem, fresh weight of root and stem. Chakraborty *et al.* (2006) also reported that *B. megaterium* isolated from tea plant rhizospheric soils produces IAA and increases the plant growth. *Bacillus* produced the gibberillic acid (GA) and it was detected through spectrophoto metrically shown in Figure 3 and further confirmed with HPLC at absorption of 254 nm when compared with the standard. Through HPLC *Bacillus* produced 14.03 mg/ml of GA and the spectrum was shown in Fig.- 4 and 5. Calvo *et al.* (2014) also reported that *Bacillus* and *Pseudomonas* produce GA and helps in plant development. GA is a phytohormone which involved in cell division and cell elongation. It is essential for all plant developmental stages like stem and root initiation, seed germination and floral development.

Bacillus sp. showed salinity tolerance was observed by growth on the plates having NaCl concentration of 0.25, 2.5 and 3.75mg/L respectively. Rezaei *et al*. (2015) also reported that the effect of PGPR on Nigella leaves under salinity stress increases the leaf area, biomass and chlorophyll content.

Conclusion

Bacillus sp. produce phyto hormones like IAA and GA, increases uptake of nutrients like phosphate and iron by siderophore production and produces ammonia. It protects cell from oxidative damage by producing catalase enzyme. It acts as a biocontrol agent by producing HCN. The *Bacillus* sp exhibited tolerance against heavy metal and salinity. Thus, *Bacillus* was considered as a potential PGPR trait which enhances the plant growth. *Bacillus* can be recommended as one of the potential bio fertilizers to the farmers instead of chemical fertilizers.

Acknowledgments

The authors thank the Department of Science and Technology of the Government of India, SERB, for financial support and Jain University, Bangalore, for providing laboratory facilities.

References

Ajay Kumar., Amit Kumar., Shikha Devi., Sandip Patil., Chandani Payal and Sushila Negi. 2012. Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study'. *Recent Research in Science and Technology.,* 4: 01 - 05.

Ankit Kumar., Anil Prakash. and Johri, B.N. 2011. Bacillus as PGPR in Crop Ecosystem'. *Bacteria in Agrobiology : Crop Ecosystems.*

Arash Rezaei., Babak Lotfi., Mohammad Jafari and Sadegh Bahamin. 2015. Survey of efects of PGPR and salinity on the characteristics of Nigella leaves'. *Biological Forum - An International Journal.,* 7 : 1085 - 1092.

Bharucha, U.D., Patel, K.C. and Trivedi, U.B. 2013. *In vitro* screening of isolates for its plant growth promoting activities from the rhizosphere of Alfalfa (*Medicago Sativa*)'. *J. Microbiol. Biotech. Res.,* 3: 79 - 88.

DO NOT COPY

Bilkay, I.S., Karakoc, S. and Aksoz, N. 2010. Indole-3-acetic acid and gibberellic acid production in Aspergillus niger. *Turk J Biol.,* 34: 313-318.

Castric, P.A. 1975. Hydrogen cyanide, a secondary metabolite of *Psuedomonas aeruginosa*. *Can. J. Microbiol.,* 21: 613 - 618.

Chakraborty, U., Chakraborty, B. and Basnet, M. 2006. 'Plant growth promotion Camellia sinensis by B. magaterium'. *Journal of Basic microbiology*., 46: 186-195.

Damodaran, T., Sah, V.,. Rai, R.B., Sharma, D.K., Mishra, V.K., Jha S.K. and Kannan, R. 2013. Isolation of salt tolerant endophytic and rhizo-spheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigour in tomato under sodic environment'. *African journal of microbiology research.,* 7: 5082 - 5089.

Dweipayan Goswami., Swapnsinh Parmar., Hemen-drasinh Vaghela., Pinakin Dhandhukia and Janki N. Thakker. 2015. Describing *Paenibacillus mucilaginosus* strain N_3 as an efficient plant growth promoting rhizobacteria (PGPR)'. *Cogent Food & Agriculture.,* 1: 1000714.

Geetha, K., Venkatesham, E., Hindumathi A. and Bhadraiah, B. 2014. Isolation, screening and characterization of plant growth promoting bacteria and their effect on *Vigna Radita* (L.) R. Wilczek. *Int. J. Curr. Microbiol. App. Sci.,* 3: 799 - 809.

Gordon, S.A. and Weber, R.P. 1951. Colorimetric estimation of indole acetic acid. *Plant Physiol.,* 26: 192 - 195.

Han, H.S., Supanjani. and Lee, K.D. 2006. Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. *Plant soil and Environment.,* 52: 130 - 136.

I ıl seyis bilkay, Safak karakoç, Nilüfer aksöz. 2010.' Indole-3-acetic acid and gibberellic acid production in Aspergillus niger'. *Turk. J. Biol.,* 34: 313-318.

Kumar, V., Bhatnagar, A.K., Srivastava, J.N. 2011. Antibacterial activity of crude extracts of *Spirulina platensis* and its structural elucidation of bioactive compound. *J. Med. Plants Res.,* 5 : 7043 - 7048.

Loper, J.E. and Schroth, M.N. 1986. Influence of bacterial sources of indole-2-acetic acid on root elongation of sugar beet. *Phytopath.,* 76 : 386-389.

Mahalakshm, D. and Reetha. 2009. Assessment of plant growth promoting activities of bacterial isolates from the rhizophere of tomato (*Lycopersicon esculentum* l.)'. *Recent Research in Science and Technology.,* 1: 026 - 029.

Martínez-Viveros, O., Jorquera, M.A., Crowley, D.E., Gajardo, G. and Mora, M.L. 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizo bacteria'. *J. Soil Sci. Plant Nutr.,* 10: 293 – 319.

Meenakshi Mishra., Umesh Kumar., Pankaj Kishor Mishra. and Veeru Prakash. 2010.' Efficiency of Plant Growth Promoting Rhizobacteria for the Enhancement of *Cicer arietinum* L. Growth and Germination under Salinity'. *Advances in Biological Research.,* 4 : 92 - 96.

Mishra, R.K., Prakash, O., Alam, M. and Dikshit, A. 2010. Influence of plant growth promoting rhizobacteria (PGPR) on the productivity of *Pelargonium graveolens* L. herit. *Rec. Res. Sci. Tech.,* 2(5): 53-57

Monita, V., Patel and Rajesh K. Patel. 2014.' indole-3-acetic acid (iaa) production by endophytic bacteria isolated from saline dessert, the little runn of kutch'. *CIBTech Journal of Microbiology.,* 3: 17 - 28.

DO NOT COPY

Munees Ahemad and Mulugeta Kibret. 2013. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective'. *Journal of King Saud University.,* 26: 1 - 20.

Pavan Kumar, D., Agrawal and Shruti Agrawal. 2013. Characterization of *Bacillus* sp. strains isolated from rhizosphere of tomato plants (*Lycopersicon esculentum*) for their use as potential plant growth promoting rhizobacteria'. *International journal of agriculture technology.,* 2: 406-417.

Payne, S.M. 1994. Detection, isolation and characterization of Siderophores. *Methods in Enzymology.,* 235: 329 - 344.

Pikovskaya, R.E. 1948. Mobilization of phosphorus in soil in concentration with vital activity of some microbial species. *Microbiologiya.,*17: 362 - 370.

Rani. K. 2012. Immobilization of Vigna mungo - amylase onto NaCl and NaNO3 treated woven Bombyx mori silk Fabrics. *Asian Journal of Biological and Sciences.,* 2012, 1 (2): 96-100.

Saharan, B.S. and Nehra. V. 2011. Plant Growth Promoting Rhizobacteria: A Critical Review'. *Life Sciences and Medicine Research.,* 21.

Schwyn, B. and Neilands, J. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.,* 160 : 47 - 56.

Swain, M.R., Nasar, S.K. and Ray R.C. 2007. 'Indole-3-acetic acid production and effect on sprouting of yam (*Droscorea rotundata* L.) minisetts by B. *subtillis* isolated from culturable cow dung microflora'. *Pol .J. Microbiol.,* 56: 103 - 110.

Trivedi, M.K., Patil, S. and Tallapragada, R.M. 2013. Effect of bio field treatment on the physical and thermal characteristics of vanadium pentoxide powders. *J. Mater. Sci. Eng.,* S11: 001.

Usha Rani, M. Arundhathi and Gopal Reddy. 2012. Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. *African Journal of Biotechnology.,* 11(32) : 8085 - 8091.

Yogendra Singh, P.W. Ramteke and Pradeep, K. Shukla. 2013. Isolation and characterization of heavy metal Resistant *Pseudomonas* spp. and their plant growth promoting activities'. *Advances in Applied Science Research.,* 4 : 269 - 272.

Corresponding Author : Tallapragada Padmavathi, Department of Microbiology, Center of PG Studies, Jain University, 18/3, 9th Main Road, 3rd Block, Jayanagar, Bangalore, Karnataka 560011, India. Email : *Vam2010tpraviju@gmail.com* ***© 2016, IJALS. All Rights Reserved.***

DO NOT COPY