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Invitro Evaluation of Indigenous Plant Growth Promoting Rhizobacteria Isolated from *Jatropha Curcas* Rhizosphere

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Abstract

In this study five different isolates MS1, MS2, MS3, MS4, and MS5, were isolated from the rhizosphere of *jatropha curcas* rhizosphere and these isolates were characterized and screened *in vitro* for their plant growth promoting traits like production of indoleacetic acid (IAA), ammonia (NH3), hydrogen cyanide (HCN), siderophores, phosphate solubilization, EPS production, and ACC deaminase production. The maximum production for Phosphate solubilization was in MS3 (49 µg/ml), IAA production MS1 (52 µg/ml), siderophores production MS5 (32 µg/ml), and ACC deaminase production MS3 (82 nm α-ketobutyrate mg^{-1} h^{-1}). MS1, MS3, MS5 shown HCN production positive while these all three were also good exo polysaccharide producers. The isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions. It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promote root and shoot growth as well as nodulation. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil-plant system is needed to uncover their efficacy as effective PGPR.

Keywords: PGPR (Plant growth promoting rhizobacteria)/ jatropha rhizosphere/ PGP potentials/ synergistic / IAA production / phosphate solubilization

Introduction

Plant growth promoting rhizobacteria (PGPR) are very small portion of rhizobacteria (2–5%) that promote the growth. PGPR use one or more direct mechanism of action to improve plant growth and health. In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus and production of siderophores that chelate iron and make it available to the plant root. Improvement of plant uptake by phosphate solubilization or N2 fixation and phytohormone production like indole -3-acetic acid are examples of mechanisms of direct influence on plant growth. The indirect effects can be exerted by antibiotic production, depletion of iron from the rhizosphere, induced systemic resistance, synthesis of antifungal metabolites, production of fungal cell wall lysing enzymes, competition for sites on the root, stimulation of other beneficial symbioses and degradation of xenobiotics in inhibitor-contaminated soils. The PGPR are defined by three intrinsic characteristics (Barea et al., 2005): (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities, and (iii) they must promote plant growth. The PGPR are known to participate in many important ecosystem processes. They were first used for agricultural purposes in the former Soviet Union and India and are now being tested worldwide (Lucy et al., 2004). A wide array of beneficial rhizosphere bacteria have been categorized as PGPR including mainly *diazotrophs, bacilli, pseudomonads* and *rhizobia* (Antoun and Prevost, 2006). *Jatropha curcas* L or Physic nut is a drought resistant large shrub or small tree, belonging to genus Euphorbiaceae, producing oil containing seeds. *Jatropha* (*Jatropha curcas* L.) is a hardy non-edible oil-seed plant that can sustain harsh environments, adapt well to semi-arid marginal, and wastelands and hence has been treated as a potential alternative energy source.

Materials and methods

Microbial Diversity study

Take 1gram of soil collected from the rhizosphere and add in sterile distilled water aseptically to get suspension of rhizosphere soil. Dilutions were carried out and were counted for countable colonies using Neuberg's chamber, accordingly 10^{-2} ,10^{-3} ,10^{-4} and 10^{-5} dilutions were selected. Six different media were used to study diversity of soil. Different specific medium were used to study the diversity of specific organisms. Martin's Rose Bengal agar medium is used to study the diversity of Fungi, Ashby's Mannitol agar medium is used to study the diversity of Azotobacter, Yeast Extract Mannitol medium is used for Rhizobium, Pikovskyaya's agar medium is used for the phosphate solubilizing organisms, Mac Conkey's agar medium is used for E.coli and Enterobacter, NFB medium is used for Azospirillium and Nutrient agar is used for cultivation of variety of organisms. A loopful of each dilution were then streaked on Nutrient agar plates, Mac Conkey's agar plates, Ashby's Mannitol agar plates, Yeast Extract Mannitol agar plates, Pikovskyaya's Agar plates, NFB agar plates and Martin's Rose Bengal agar plates.

Characterization for plant growth promoting potentials

Growth curve profile

Growth curve of two isolates was determined by viable cell count method. Growth profile of MS1 to MS5 was determined by inoculating early exponential phase culture in 50 ml of nutrient broth. Samples were withdrawn after every 4 h. Suitable dilutions were plated on solid medium and CFU per ml enumerated later (Pandey and Maheshwari, 2007).

Phosphate solubilization

Both liquid & solid Pikovskyaya's medium was used for checking solubilization of phosphate supplied (Subbarao, 1980; Gaur, 1990). To check the amount of phosphate solubilized in the medium, 1 mL of supernatant was collected and add 9 mL of double distilled water. The whole procedure mentioned above was performed till blue colour develops and OD taken at 600 nm. Total amount of phosphate solubilized was calculated from the standard graph.

IAA production

For the detection of Indole acetic acid production Sarwer and Kremer (1995) method was used. 1 mL of supernatant and add equal amount of Salkowsky's reagent was added. Incubate for 30 min and take OD at 536 nm. Amount of IAA produced from the standard graph was estimated.

Siderophore production

Chrome azurols (CAS) assay solution was used for the detection of siderophore was followed by Schwyn and Neilands (1978). 0.5 ml supernatant was collected from each sample and 0.5 mL H2SO4 was added. Allow to cool. Add 1 mL of 1 % sulphanilic acid and 0.5 ml of 1.3 % Iodine solution. Allow to stand for 5 min. Destroy excess of iodine by adding 1 mL of 2 % sodium arsenate solution. Wait till yellow colour disappears. Add 1 mL of 0.3 % α-napthylamine solution and incubate for 30 min till pink colour develops. Take OD at 536 nm. Amount of siderophore produced from the standard graph was estimated.

HCN production

Picrate assay (Castric, 1974) was followed for the qualitative analysis of hydrocyanic acid production. Streak nutrient agar slants with isolates. After streaking a filter paper strip impregnated with 0.5% picric acid and 2.0% sodium carbonate suspended above the medium. Incubate the slants for 24 hrs. at 37 ^{0}C. Incubating heavily inoculated nutrient agar plates in an inverted position at 37 ^{0}C with picric acid indicator papers place inside the lids. After incubation observed the filter paper changes from yellow to orange brown indicates the presence of cyanide.

ACC deaminase assay

All sample measurements were carried out in triplicate. Two hundred microlitres of the toluenized cells were placed in a fresh 1.5 ml microcentrifuge tube and 20 µl of 0.5 mol l^{-1} ACC was added to the suspension, briefly vortexed and then incubated at

30^0C for 15 min. Following the addition of 1 ml of 0.56 mol l^{-1} HCl, the mixture was vortexed and centrifuged for 5 min at 12 000 g at room temperature. One millilitre of the supernatant was vortexed together with 800 µl of 0.56 mol l^{-1} HCl. Then, 300 µl of the 2, 4 dinitrophenyl hydrazine reagent was added to the glass tube, the contents vortexed and then incubated for 30 min at 30^0C. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance was measured at 540 nm (Shimadzu UV-1800, Japan) (Penrose and Glick, 2003).

Ammonia production

Quantification of Ammonia produced was determined by Vis/UV spectrophotometeric method (Dye, 1968). 1ml of supernant was taken in clear test tube. Add 1ml of Nessler's reagent. Add 9ml of distilled water and allow standing for 20 mins. Take O.D.at 540nm. (Systronic model no.166)

EPS production

Normally EPS production is studied in basal medium of all different organisms. As carbohydrate source 5% of sucrose is to be added as polysaccharide in to the medium (Modi et al., 1989). 10ml of culture suspension was collected after 5-6 days and centrifuge at 30,000 rpm for 45 mins. add thrice the volume of chilled acetone. EPS will be separated from the mixture in the form of a slimy precipitates. Precipitates were collected on a predried filter paper. Allow the precipitates to dry overnight at 50 degree C. reweigh the dried filter paper after overnight drying. Note the increase in the weight of filter paper, is the EPS produced.

Results and discussion

Total 110 isolates from GS1, 98 from GS2, 35 from GS3, 76 from GS4, and 78 from GS5 were screened finally. Only those isolates which gave maximum growth, termed as fast growers, were selected for further studies. They were purified on their respective medium. These 397 cultures were further screened on the basis of the PGPR characteristics. Total 56 isolates from GS1, 72 isolates from GS2, 10 isolates from GS3, 28 isolates from GS4, and 28 isolates from GS5 were screened on the basis of PGPR potential. Their diversity indices were studied (Table 1) for determining the species richness and evenness of PGPR in these different sites (Jha et al., 2010). The value of the Shannon index obtained from empirical data usually falls between 1.5 and 3.5 and rarely surpasses 4. It is only when there are huge numbers of species in the sample that high values are produced. Simpson index is one of the most meaningful and robust diversity measures available. In essence it captures the variance of the species abundance distribution. Thus when expressed as the complement (1-D) or reciprocal (1/D) of D, the value of the measure will rise as the assemblage becomes more even, although the reciprocal (1/D) is the most widely used form of the Simpson index. Characteristic growth shown by the culture MS1-MS (Fig. 1). The population of MS1 and MS3 was recorded as 3.6×10^7 and 3.0×10^7 CFU (colony forming unit) g–1 by dilution plate technique.

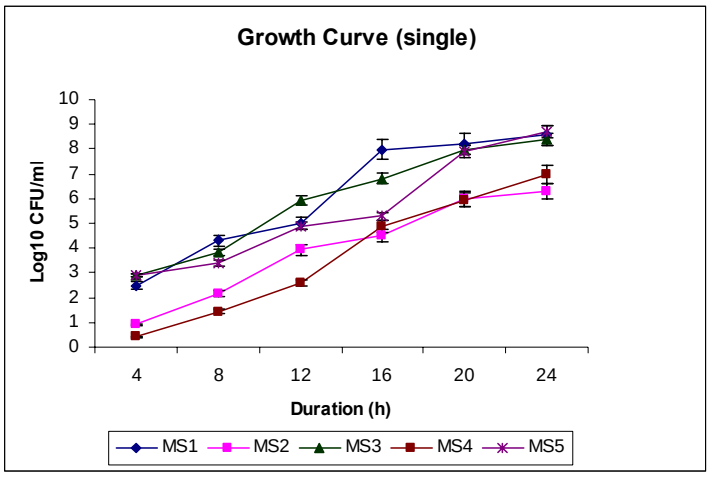

Figure1: Characteristic Growth profile shown by the isolates MS1-MS5.

Table1: Diversity indices for PGPR from different sites of *Jatropha* plantations (G1 – G5) in Gujarat.

Diversity indices	GS1	GS2	GS3	GS4	GS5
Shannon- wiener (H')	1.715	0.926	1.279	1.775	1.693
Simpson (1/D)	6.027	2.018	4.502	5.827	4.935
Simpson (1-D)	0.834	0.504	0.778	0.828	0.797

Phosphate solubilization on solid Pikovskyaya's medium after 3 d of incubation at 30 ± 2 ^{o}C, maximum zone was observed in isolate MS3 (18 mm). Significant zones were also seen in MS1 (14 mm), MS5 (12 mm), MS4 (10 mm) and MS2 (10mm). Maximum TCP solubilization in liquid medium was observed in MS3 (490 ppm) followed by MS1, MS5, MS4, MS2 in descending order of solubilization (Fig. 2). The pH of the medium also showed a decrease from 6.5 to a maximum of 4.6 after 14 d. In many isolates tested here, the final pH was same but their respective P-solubilization was different. Similar results showing no correlation between P-solubilization and pH reduction are also published by many researchers. This drop in pH may also be an attribute of glucose utilization by the isolates (Arora et al., 2008). Highest IAA production was reported in MS1 (52 µgmL^{-1}) after 96 h of incubation in dark followed by MS3 (47 µgmL^{-1}), MS4 (39 µgmL^{-1}), MS5 (32 µgmL^{-1}) and in MS2 (27 µgmL^{-1}) (Fig.3). All the isolates showed a continuous increase and decrease in the IAA production potential along with increase in incubation time. Different isolates showed different optimum incubation time for highest IAA production. Sridevi et al., (2007) showing that IAA production occurs during early stationary phase. Increase in IAA concentration occurs till 72 hrs and thereafter decrease was notice after 96 hrs of incubation.

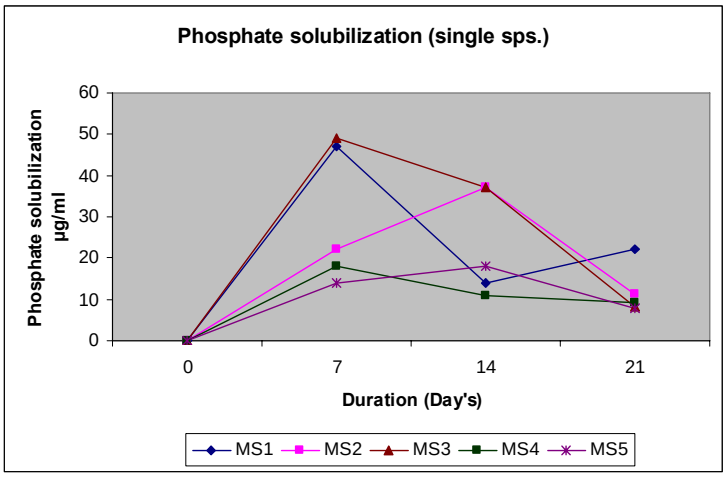

Figure1: Phosphate solubilization shown by the isolates MS1-MS5.

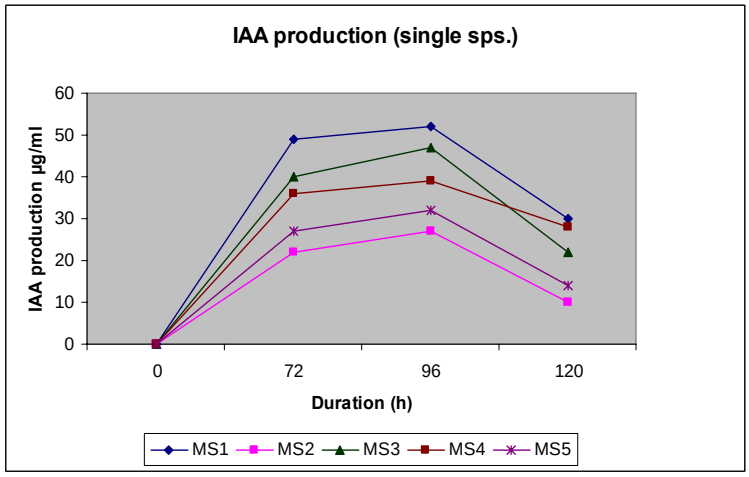

Figure3: Indole acetic acid production by the isolates MS1-MS5.

HCN production was checked in final five isolates which showed significant results in phosphate solubilization and IAA production potential. Out of these five isolates only three isolates showed HCN production after 48 and 72 h of incubation. Maximum HCN production was observed in MS1 isolate followed by MS3 and MS5 where as moderate HCN production was reported in MS2 after 48 h of incubation (Table 2). Siddique et al., (2006) observed the production of HCN by *Pseudomonas fluorescens* strain CHAO as an antagonistic factor that contributes to biocontrol of *Meloidogyne javanica* a root knot nematode, in situ and suppression of galling of tomato. Siderophore production by the isolates carried out on solid CAS blue agar showed a clear zone of decolorization representing iron chelation by the isolate in the medium. Highest zone of dye decolorization was observed in MS1 (30 mm), MS3 (22 mm), MS5 (20 mm) where as MS2 and MS4 showed a zone of 15 mm. Maximum

siderophore production in the liquid medium was shown in MS5 (32 μgmL^{-1}) after 96 h of incubation followed by MS2 (28 μgmL^{-1}), MS1 (25 μgmL^{-1}), MS4 (22 μgmL^{-1}) and in MS3 (22 μgmL^{-1}) (Table 2). All the siderophore produced was the hydroxamate type. Increase in pH was accompanied with increase in siderophore concentration. The pH increased from 6.8 to a maximum of 10 along with siderophore production. Chandra et al., (2007) reported that *Mesorhizobium loti* MP_6 isolated from root nodules of *Mimosa pudica* showed a major peak at 400nm which revealed production of hydroxymate type of siderophore. The maximum production of siderophore was 32µg/ml after 48 hrs of incubation. Study of ACC deaminase enzyme production by the final five isolates MS1, MS2, MS3, MS4 and MS5 showed that maximum ACC deaminase was produced by MS3 which was 820 $\mu Mmg^{-1}h^{-1}$ followed by MS1 (790 $\mu Mmg^{-1}h^{-1}$), MS5 (720 $\mu Mmg^{-1}h^{-1}$), MS2 (520 $\mu Mmg^{-1}h^{-1}$) and MS4 (480 $\mu Mmg^{-1}h^{-1}$) (Table 2). Almost all the rhizobacterial isolates obtained through enrichment on ACC as the sole N source showed growth-promoting activity in maize under axenic conditions but with variable efficacy. Since no efforts have been successful in isolating a bacterium capable of utilizing ACC as a precursor of ethylene (Arshad and Frankenberger, 2002) this implies that the rhizobacteria grown on ACC utilized it as a N source via deaminase trait i.e., ACC is converted into NH3 and a-keto butyric acid instead of ethylene. Thus it is highly likely that the ability of these ACC enriched rhizobacterial isolates to deaminate ACC was the responsible mechanism of action for promoted root and shoot growth because lowering of the ACC levels result in decreased endogenous ethylene production. This contention is strongly supported by the work reported by several other researchers (Glick et al., 1998).

Table2: Production of siderophore, ACC deaminase and HCN by the isolates MS1-MS5.

Isolates	*Siderophore production (μgml^{-1})*	*HCN*	*ACC deaminase production ($\mu Mmg^{-1}h^{-1}$)*
MS1	25 ± 2.32	++	790 ± 8.97
MS2	28 ± 2.34	-	520 ± 4.59
MS3	22 ± 1.76	++	820 ± 8.34
MS4	22 ± 1.21	+	480 ± 2.87
MS5	32 ± 1,54	++	720 ± 4.34

Maximum concentration of ammonia production was observed in isolates MS3 and MS5 is 84µg/ml and 78µg/ml after 10 days of incubation. Other isolates showed considerable amount of ammonia production is 38µg/ml to 53µg/ml (Fig. 4). Joseph et al., (Joseph et al., 2007) reported ammonia production in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%). After incubation for 5 days maximum amount of EPS production was observed in isolate MS3 i.e. 35.6 mg/ml. MS1, MS5, MS4 and MS2 also produced

good amount of EPS in the range of 14.2 mg/ml to 24.4 mg/ml (Fig. 5). Alami et al., (2000) reported that EPS produced by root associated saprophytic bacterium (rhizobacterium) *Pantoea agglomerans* YAS34 was associated with plant growth promotion of sunflower.

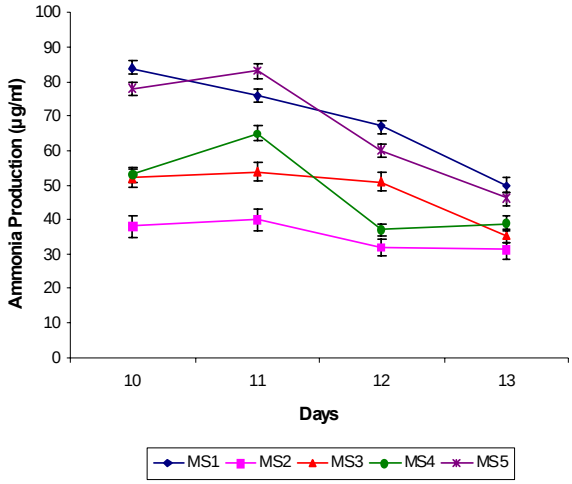

Figure4: Ammonia production by the isolates MS1-MS5.

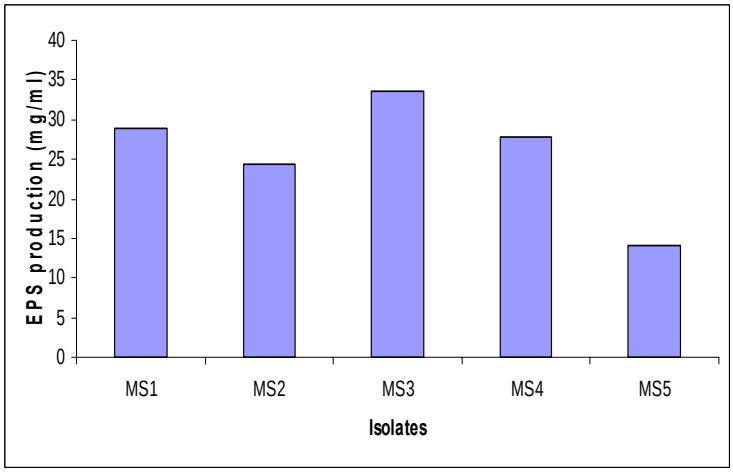

Figure5: EPS production by the isolates MS1-MS5.

Conclusion

Plant growth promoting bacteria are soil bacteria associated with plants and can facilitate plant growth and development through different ways. In short we can say that these soil bacteria can enhance plant growth in two different mechanisms. Directly they enhance plant growth by supplying the plants with required nutrients like nitrogen, phosphorus, plant hormones etc. and assisting their uptake. These bacteria also sequester metals like iron by producing siderophores thereby fulfilling

the need of micronutrients. Indirectly rhizobacteria helps plant growth by releasing biocontrol agents as well as HCN for protecting plants against phytopathogens and enzymatically lowering plant ethylene levels by producing ACC deaminase enzyme. Effective plant growth promotion also requires that a PGPR must be able to colonize the root surface of the plants as well as survive for long time in environments foreign to them. All the above mentioned studies showed that the selected soil isolates of *Jatropha curcas* rhizosphere showed all the desired PGPR traits during their *in vitro* studies and are able to perform their plant growth promoting efficiency.

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