Article History:
Received 16th February, 2014
Received in revised form 23rd February, 2014
Accepted 11th March, 2014
Published online 28th March, 2014

Key words:
PGPR, Bergey’s manual of Systematic Bacteriology, Streptomyces spp. minralini7, In vitro study.

INTRODUCTION
Plant growth promoting bacteria utilizes a group of microbes for the plant growth enhancement and aids as biofertilizers with no side effects via direct or indirect mechanisms. Direct mechanisms involve fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores, and synthesis of plant growth hormones i.e. Indole-3-acetic acid (IAA), gibberellic acid, cytokinins, and ethylene (Nelson, 2004). Indirect mechanisms involves the biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophore or through competition for nutrients and space which can further improve significantly plant health and promote growth, as evidenced by increase in seedling emergence, vigor, and yield (Khan, 2006). There are many papers related to the advantages and screening of PGPR from crop plants particularly rice, maize and sugar cane but no reports were found from medicinal plant leaves. Hence, this present study focuses on isolating beneficial PGPR endophytic actinomycetes present commonly in Neem and Tulsi leaves and determining its effect for the improvement of growth and germination on Tomato plant (Solanum lycopersicum).

MATERIALS AND METHODS
Sample collection
Healthy leaf samples of common medicinal plants, Azadirachta indica (Neem) and Ocimum sanctum (Tulsi) with high medicinal value were gathered from the garden of Nirmala College for Women, Coimbatore (11.0183° N, 76.9725° E) belonging to Western Ghats of Southern India during the period of December 2011 to January 2012. The collected plant materials were taken to the laboratory, preserved at 4°C in sealed plastic bags and subjected to isolation work within 96 hours.

Selective isolation of endophytic actinomycetes
Healthy leaf samples were cut into small pieces (2*2 cm) and washed by running tap water for 1-2 minutes to remove the soil particles completely. The resultant were subjected to a five-step surface sterilization procedure as per the method of Sheng et al., (2009): a 4 to 10-min wash in 5% NaOCl, followed by a 10-min wash in 2.5% Na2S2O3, a 5-min wash in 75% ethanol, a wash in sterile water, and a final rinse in 10% NaHCO3 for 10 min to disrupt the plant tissues and inhibit the fungal growth. At this point, the final washed solution was spread onto ISP 2 agar and incubated at 28°C for 2 to 8 weeks.

Method 1. The surface treated samples were aseptically transferred on HV (Humic acid Vitamin agar) and incubated at 28°C for 2 to 8 weeks.

Method 2. High speed centrifugation method for selective isolation of actinomycetes has been carried out with slight modifications as discussed by Kizuka et al., (1997). Five grams of the sterile checked and surface-treated leaf tissues were placed in a 50 ml centrifugation tube, containing 5 ml of sterile tap water and stirred in a mini centrifuge for 30 seconds. After allowing it to suspend at 27°C for 60 minutes, the suspension was centrifuged at 3000 rpm for 10 minutes to remove soil particles. The supernatant was then centrifuged at 10000 rpm for 10 minutes and the resulting supernatant was further centrifuged at 20000 rpm for 20 minutes. The resulting supernatant was filtered

* Corresponding author: Mrinalini J Singh
Department of Botany, Nirmala college for Women, Coimbatore – 641 018, Tamilnadu, India
through 0.22 µm pore size membrane filter and the condensed supernatant was streaked onto HV agar supplemented with nalixidic acid (50 mg/ml) and nystatin (100 mg/ml). The media was then incubated at room temperature (28 ± 2°C) for 2 to 8 weeks.

Identification of actinomycetes

The isolates picked up from HV agar plates were purified on ISP media (International Streptomyces Project) and incubated at room temperature (28 ± 2°C) for 30 days. The isolates were identified according to morphological criteria, including characteristics of colonies on plate, morphology of substrate and aerial hyphae, morphology of spores and pigment production as discussed by Williams et al., (1989) and Shirling et al., (1966). The isolates present commonly in both plants were preserved in 20% (v/v) glycerol for subsequent PGPR investigation.

Invitro screening of isolates for plant growth promotion activities

IAA Production

Indole acetic acid (IAA) production experiment was carried out as described by Brick et al., (1991). The test cultures were grown separately on nutrient broth enriched with 50 mg/ml of L-tryptophan at 30°C for 48 h. Fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution). The tubes were kept at room temperature for 20 minutes. Development of pink color indicates IAA production.

1-aminoacyclopropane-1-carboxylate (ACC)-deaminase activity- Germinating seed bioassay

The effect of bacterial isolates on root elongation was studied by germinating seed bioassay as per methodology (Belimov et al., 2002 and Dey et al., 2004). Seed surface of Pisum sativum were sterilized with 20% NaOCl for 3 min and washed three times with sterile de-ionized water. All the seeds were allowed to germinate at 25°C in 1% water agar plates for 48 h. Germinated seeds were individually dipped for 1 h in 20 ml bacterial cultures grown for 48 h in Nutrient Broth and transferred to wet filter paper in Petridishes taking three seeds per dish. The Petri dishes were incubated in dark at 30±0.1°C with three replications for each treatment. The seedlings treated with uninoculated nutrient broth served as control. The rootlength of seedlings was measured in cm after 5 days of incubation.

Ammonia production

The given test isolates were grown in peptone water at 30°C for 3 days in the case of bacteria and 7 days in the case of actinomycetes. One ml of Nessler’s reagent was added. Production of ammonia was detected by development of faint yellow color as described elsewhere (Bakker et al., 1987; Cappuccino et al., 1992)

Catalase test

A loop full of test culture was transferred to a sterile glass slide and a drop of 3% Hydrogen peroxide was added to the culture at once and observed for effervescence. Evolution of effervescence indicated catalase production.

Siderophore production

Thin layer of silica gel G60 slurry was prepared on glass plate and spotted with the isolated bacterium supernatant on thin layer plate, development of the chromatogram in the solvent system of methanol: chloroform (1:9). Appearance of reddish brown colour indicating the presence of siderophores discussed by Kannahi et al., (2013).

HCN production

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lork(1948). Nutrient broth was amended with 4.4 g glycine/l and the isolates were streaked on modified agar plate. A Whatman filter paper No. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2°C for 4 - 7 days. Development of orange to red color indicated HCN production.

Phosphate solubilization by bacteria

The test isolates were spot inoculated on Pikovskaya’s agar plates for phosphate solubilization under aseptic conditions and incubated for 3 days at 30°C. The appearance of clear halo zone on Pikovskaya’s agar plates showed positive phosphate solubilization ability as described by Gaur (1990).

Chitin hydrolysis

Primary screening of chitin degraders was performed by spot inoculation on colloidal chitin agar as per the standard procedure followed by Nawani et al., (2003). Clear zone of chitin hydrolysis were recorded up to 14 days at 30°C. Colloidal chitin was prepared according to the prescribed method by Berger et al (1988). Ten grams of chitin was slowly dissolved in 400 ml of concentrated Hcl with stirring at 4°C. The mixture was incubated in water bath at 37°C until viscosity decreased. To this mixture, 4.0 liters of sterile distilled water was added and left overnight at 4°C. The supernatant was slowly decanted and the precipitate was collected on a filter paper and washed extensively with sterile distilled water to attain neutral pH. The colloidal chitin was dissolved in 250 ml sterile distilled water prior to use. The colloidal chitin was washed several times with large volumes of distilled water to adjust the pH to 7.0.

Biochemical identification of selected beneficial isolates

The selected isolate which showed maximum PGPR activity were further characterized by Gram staining, motility test and biochemical tests as per methodology described by Kreig et al., (1984). The various tests performed were Oxidase, MR-VP, Indole, Citrate, Urease, Nitrate reduction and TSI test.

Molecular identification

The genomic DNA from actinomycetes used for PCR was prepared from the single colony grown on ISP 2 broth for three days. The total genomic DNA from the potent strain was isolated employing Chromous DNA isolation kit (Chromous Biotech, Bengaluru, India) according to the manufacturer protocol. The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8-27 for the forward primer and 1492-1510 for the reverse primer mentioned below.

Forward primer: 5’-AGAGTTTGATCMTGGCTCAG-3’
Reverse primer: 5’-TACGGYTACCTTGTTACGACTT-3’
A positive control (E.coli genomic DNA) and a negative control in the PCR were also included. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3500XL automated DNA sequencing system (Applied BioSystems, USA). Nucleotide sequence of 16S rRNA gene from actinomycetes strain was determined and compared for similarity level with the reference species present in genomic database bank. The NCBI BLAST program was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and the phylogenetic tree was constructed using MEGA software as discussed by Tamura et al., (2011).

In vitro plant growth study (Tomato)

In vitro plant growth studies were performed as per the methodology described with slight modifications as discussed elsewhere (Omar et al., 2013; Salem et al., 2013). Healthy seeds of Tomato (Solanum lycopersicum) were surface sterilized with 0.1% HgCl₂ for 2 min and rinsed six times with sterile distilled water. The given isolates of best PGPR activity was grown in respective broth on shaking incubator (180 rpm) at 28 ± 2°C for 24 h. The surface sterilized seeds of tomato were inoculated in respective broth culture of the test isolates for 30 min. Five seeds were sown at 5 cm depth in 250 g sterilized soil containing plastic pot. A control treatment was also maintained without inoculated seed. Pots were kept into plant growth chamber for 21 days. The experiment was setup in 3 replications. All seeds were germinated. After the initial growth, the respective plants were transferred to field treatment. After three months, shoot and root length were recorded in centimeter of each plant. Then, plants were dried in an oven at 65°C for 3 days. After this, shoot and root dry weight were recorded in gram.

RESULT AND DISCUSSION

Three actinobacterial isolates were commonly present in both Neem and Tulsi leaves. These three isolates were designated as 5,7 and 9. All isolates has shown significant PGPR activity. Especially Isolate no.7 showed positive results for all PGPR studies whereas, Isolate no. 9 showed negative response for Phosphate solubilization, ACC and Siderophore production and Isolate no. 5 showed negative response for ACC test as shown in Table no.1.

All the actinobacterial isolates showed good chitinolytic activity. Since all the three actinobacterial isolates showed good PGPR activity, both microscopy and biochemical tests were performed to study the characteristics of PGPR isolates, as shown in Table 2. The cultural characteristics of the isolate no.7 were studied since it produced better PGPR activity compared to other two isolates. Irregular, raised, grey white colonies with light yellow substrate mycelium were observed on ISP2 agar surface. Dark brown melanin pigment was produced on ISP7 agar medium and no soluble pigments were produced by the isolate. Therefore, based on actinomycetes identification key, the isolate was assigned to the genus Streptomyces (Table 3). Though it was assigned to the genus Streptomyces, the strain did not match with any of the species and therefore it may be a novel species which may be designated as mrinalini7 strain. Molecular characterization by 16S rRNA ribotyping and phylogeny analysis was carried out in order to identify Streptomyces sp. mrinalini7 up to species level.

Phylogenetic analysis and species identification

Using BLAST search in the NCBI data bank, sequences homologous to 16S rRNA gene of the isolate A5 were collected. The DNA sequences were aligned and phylogenetic tree was constructed using MEGA4 software (bootstrap method) (Fig.5). The evolutionary history was inferred using the Neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. Only values greater than 50% were shown. Comparison of 16S rRNA nucleotide gene sequence of strain mrinalini7 with corresponding Streptomyces sequences clearly showed that the organism form a distinct phyletic line with Streptomyces genera. The isolate was closely related to the type strain of Streptomyces plicatus showing a 16S rRNA gene sequence similarity of 96%. Hence the molecular results support the classification of the given isolate as a new strain which was named as Streptomyces sp. mrinalini7.
The partial 16S rRNA sequence of the isolate (840 bp) was deposited in Genebank under the accession number KF909129. Still, DNA-DNA hybridizations, phenotypic comparisons, and chemotaxonomic analysis need to be performed to confirm its novelty. Since mrinalini7 strain showed the best PGPR activity, its ability of plant growth promotion has been studied by inoculating into Tomato plant and its activity has been compared with the un inoculated control plant. Remarkable changes have been observed shown in Graph 1. The PGPR isolates significantly affected the production of tomato seedlings. Results revealed that the root length and shoot length increased in PGPR treated plants over uninoculated control. A significant increase in both fresh and dry root, shoot and fruit biomass of tomato seedling were observed in response to PGPR isolates over control.

**DISCUSSION**

In the present study, we have found that mrinalini7 strain significantly enhanced the biomass production of Tomato. Hence, it is suggested that the use of mrinalini7 strain as effective biofertilizers might be beneficial for tomato cultivation. Plant growth of tomato was enhanced due to proper root colonization of plant growth promoting rhizobacteria which provide plant hormones (IAA), phosphorus, siderophores and ammonia to plant. But the exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth.
growth promotion as discussed elsewhere (Glick, 1995; Lalande et al., 1989). Similarly PGPR can solubilize precipitated phosphates and enhanced phosphate availability to tomato plant that represents a possible mechanism of plant growth promotion under field conditions discussed elsewhere (Verma et al., 2001; Verma et al., 2010). Siderophore is one of the biocontrol mechanisms belonging to PGPR groups under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungus mentioned by Whipp (2001). In addition to siderophore, there are other mechanisms of biocontrol including antibiotics compounds, elicitation of induced systemic resistance (ISR) of plant, and lytic enzyme secretion as mentioned by Haas et al., (2005). The present study revealed that the isolated actinomycetes group seems to be the major contributor to chitin degradation.

CONCLUSION

Thus, fauna group seem to favor the predominance of isolates expressing good PGPR apart from soil bodies. Such type of study is mandatory since the efficient use of PGPR as biofertilizers replaces chemical fertilizers and pesticides for sustainable plant cultivation in India and other developing countries.

Acknowledgement

We are thankful to the authorities of Nirmala College for Women, Coimbatore, Tamil Nadu for providing necessary facilities. The first author is sincerely grateful to University Grants Commission, Government of India for providing the award of Junior Research Fellowship in Science to pursue the research work. We are also thankful to YaazhXenomics, Chennai and Chromus services, Bangalore for 16S rRNArribotyping service.

Reference


