Isolation, screening, and molecular characterization of plant growth promoting rhizobacteria isolates of *Azotobacter* and *Trichoderma* and their beneficial activities

Parameswari Kasa, Hemalatha Modugapalem, Kishori Battini

Department of Biotechnology, Sri Padmavati Mahila Visva Vidyalayam (Women’s University), Tirupati, Andhra Pradesh, India

Address for correspondence:
Dr. Kishori Battini, Department of Biotechnology, Sri Padmavati Mahila Visva Vidyalayam (Women’s University), Tirupati - 517 502, Andhra Pradesh, India. E-mail: kishori.b15@gmail.com

Abstract

**Objectives:** The present study was conducted for isolation, screening, and identification of *Azotobacter* and *Trichoderma* from different soil samples. **Methods:** A total of 10 isolates of *Azotobacter* and *Trichoderma* were isolated from rhizospheric soils. The test isolates were biochemically characterized and screened in *in-vitro* conditions for their plant growth promoting properties. DNA polymorphism of isolates was studied using randomly amplified polymorphic DNA analysis. **Results:** A total of 41 bands were scored, out of which 35 bands were found to be 85.59% polymorphic in *Azotobacter* and in *Trichoderma* among total 37 bands scored of which 29 were found to be 78.37% polymorphic. The influence of isolated plant growth promoting rhizobacteria (PGPR) strains on plant growth was studied using different parameters such as height of the plant, number of leaves, and number of branches, and bio-control activity was studied. **Conclusion:** The present results concluded that the multiple beneficial activities of PGPR traits increase the plant growth and bio-control activity.

**Key words:** Azotobacter, bio-control activity, plant growth, plant growth promoting rhizobacteria, polymerase chain reaction-randomly amplified polymorphic DNA, trichoderma

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are soil inhabitants that are able to colonize plant roots, stimulate plant growth, and increase crop yields.[1] The bacteria from soil aggressively colonize the root zone and promote plant growth are generally termed as PGPR.[2] PGPR directly enhance plant growth by a variety of mechanisms like atmospheric nitrogen fixation, siderophores production that chelates iron and make it available to the plant roots, solubilization of minerals such as phosphorus, increased uptake of nutrients such as nitrogen, phosphorus, potassium, and synthesis of phytohormones, indole acetic acid, and gibberlic acid and antifungal activity. PGPR are diverse, complex, and important assemblages in the biosphere,[3] they are considered as a group of beneficial free-living soil bacteria for sustainable agriculture and environment.[4] Along with this, they are also involved suppressing the root pathogenicity.[5] Several studies were reported the inoculation of bacteria to the plants enhances plant growth.[6-8] Different strains of PGPR genera exhibiting plant growth promoting activity are *Azooarcus, Pseudomonas, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Burkholdaria, Enterobacter, Gluconacetobacter, Rhizobiunm, Erwinia, Mycobacterium, Mesorhizobium, Flavobacterium*, etc. have been reported.[1,9-12] Rhizosphere bacteria *Azotobacter* and fungus *Trichoderma* are the well-known predominant microorganisms having novel features in improving plant growth and are the most extensively studied. *Azotobacter* is a free-living aerobic, nonsymbiotic nitrogen fixer and acts as PGPR helps in root expansion, improve uptake of plant
nutrients, protects plants from root diseases and most important improves biomass production in the rhizosphere of almost all crops. *Trichoderma* spp. is one of the efficient bio-control agents against to several plant and soil pathogenic fungi, used for plant disease control.[15-17] Inoculation of these bacteria competitively colonizes the roots of the plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both. In view of this, the present study was undertaken to investigate the PGPR activity of strains *Azotobacter* and *Trichoderma*, isolated from different crop soils and used as biofertilizers for stimulation of plant growth and bio-control without damaging the environment.

**MATERIALS AND METHODS**

**Collection of soil samples**

A total of 10 isolates were isolated from rhizospheric soils. Plant was gently and carefully uprooted, soil tightly adhering the root was collected, randomly selected, mixed and ¼ parts was used as composite rhizospheric soil sample of the crops. The pH of soil was determined in 1:2 (soil:Water) ratio, keeping 30 min as equilibration time.

**Isolation and identification of isolates**

Collected soil samples were air-dried for 4 h, and isolation was done by serial dilution technique.[18] Selective N-free mannitol agar for *Azotobacter* and *Trichoderma* selective medium for *Trichoderma* were used for isolation of the strains.[19] One ml of soil suspension was taken with the help of sterilized pipette and poured on the petri plate seeded with selective mediums. The plates were incubated at 37°C ± 1°C for *Azotobacter* and 28°C ± 1°C for *Trichoderma* for 5 days. Appearance of colonies was recorded, and individual colonies selected and maintained as a pure culture for further study.

The isolated colonies were sub-cultured and identified by using morphological and biochemical tests. The various tests were performed for *Azotobacter* like, catalase activity, motility test, oxidase activity, citrate test, indole test, methyl red test, Voges-Proskaer test, triple sugar ion test, nitrate reduction test.[20] Production of chlamydospores, conidial diameter, hydrolysis of gelatin, growth on glucose, nutrient agar, citric acid, lactic acid, ammonium oxalate, 4°C, 37°C, 40°C[21] tests were conducted for *Trichoderma*.

**Extraction of DNA from isolates**

Total genomic DNA was extracted from the isolated samples using Zymo-Research fungal/bacterial DNA kit. Bacterial isolates were grown in luria broth and incubated at 33°C for overnight under shaking. About 1.5 ml of culture was taken in a microcentrifuge tube, spin for 7 min and supernatant was decanted. To the pellet, 567 µl of Tris-EDTA (TE) buffer, 3 µl of 20 mg/ml proteinase-k, 30 µl of 10% sodium dodecyl sulfate were added and incubated for 1 h at 37°C. Again 100 µl of 5M NaCl and 80 µl of cetyltrimethylammonium bromide solution were added and incubated for 10 min at 65°C. Further it was extracted with equal volume of chloroform:isoamyl alcohol and the aqueous phase was transferred to the fresh tube and to this equal volume of phenol:chloroform:isoamyl alcohol was added and subjected to centrifugation at 8000 rpm for 5 min at 4°C. It was washed with chloroform:isoamyl alcohol until the clear supernatant was obtained. Then equal volume of chilled propanol was added, mixed gently and kept at −20°C overnight for precipitation of DNA. To pellet the DNA, centrifuged at 10,000 rpm for 20 min at 4°C then, pellet was washed with 70% ethanol and air-dried and DNA was dissolved in TE buffer.

**Polymerase chain reaction amplification**

Polymerase chain reaction (PCR) was performed using 16s recombinant DNA (rDNA) primers forward primer (5’-AGA GTT TGA TCC TGG CTC AG-3’) and reverse primer (5’TGA CTG ACT GAG GCT ACC TG-3’). PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2 mM deoxynucleotide triphosphates each, 2.5 µl of 10X Taq buffer, 0.36 µl 1 unit of Taq DNA polymerase, 3 µl of 10 pico mole primer. Amplifications were achieved in eppendorf thermocycler with the program consisting initial denaturation of 94°C for 3 min followed by 45 cycles each consisting denaturation at 94°C for 1 min, primer annealing temperature at 37°C for 1 min, primer extension at 72°C for 3 min, and a final extension of 72°C for 10 min. These reactions were repeated to check the reproducibility of the amplification. The banding pattern was visualized on ultraviolet transilluminator and documented by Alpha image analyzer.

**Effect of isolated strains on selected plants**

The isolated 10 samples were inoculated to *Amaranthus spinosus* and *Stevia rebaudiana* plants at 1:4 ratios. The plant growth parameters such as plant height, number of leaves, and number of branches were recorded at 15 days interval till the time of harvest.

**Bio-control activity**

Bio-control activity was performed using dual plate technique. Potato dextrose agar plates were inoculated with 5-day-old cultures of the *Fusarium*. After 2 days, a 5 mm disc of the *Trichoderma* culture is placed in the same plate at a distance of 55 mm from the phytopathogen (*Fusarium*) disc.

**RESULTS AND DISCUSSION**

Isolated *Azotobacter* and *Trichoderma* colonies were sub-cultured on selective media; morphological and biochemical identification tests are tabulated [Tables 1 and 2]. Total genomic DNA was extracted, and PCR-randomly amplified
polymorphic DNA (RAPD) analyses were performed using 16s rDNA forward and reverse primers for 10 strains. A total of 41 bands were scored, out of which 35 bands were found to be 85.59% polymorphic in *Azotobacter* and in *Trichoderma* among total 37 bands scored of which 29 were found to be 78.37% polymorphic [Figure 1]. In other studies, RAPD analysis for molecular variability in *Azospirillum lipoferum* found 93.2%,[22] in *Azotobacter chroococcum* 84.4%[23] polymorphism were observed which were isolated from different agroclimatic zones of Karnataka,[24] found 55.5% and[25] found 87% intra-specific genetic variation in *Trichoderma* isolates. In the present study, the growth parameters were significantly increased in PGPR inoculated plants when compared to control plants. Increases in the biomass in the inoculated plants with 10 strains of both *Azotobacter* and *Trichoderma* were found to be significantly higher growth parameters like plant height, number of leaves, and number of branches as compared to uninoculated plant of *A. spinosus* [Figure 2] and *S. rebaudiana* [Figure 3]. Similar studies were conducted with combined inoculation of a phosphate solubilizing *Bacillus megaterium* sub sp, and a bio-control fungus *Trichoderma* spp.[26] and with the tetra-inoculants of *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* + *T. harzianum* on chickpea and observed increased germination, nutrient uptake, height of the plant, number of leaves, number of branches, pea yield, nodulation, and total biomass and less infection with pathogens.[27]

In the present study, bio-control activity of *Trichoderma* was observed against to fungal pathogen *Fusarium* using dual plate technique [Figure 4]. Several studies reported the isolates of *Trichoderma* spp. showed antifungal activity against to different fungal spp. *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium sp.*, *Fusarium graminearum*, *Fusarium oxysporum f. pisi spa* and *F. oxysporum f. Lycopersici* sp.[22,24,25,28-33] In the present study, growth of the *Stevia* and *Amaranthus* plants was observed by treatment with *Azotobacter* and *Trichoderma* inoculation by direct mechanism of PGPR by the nutrients uptake and production of phytohormone (indole-3-acetic acid) and indirect mechanism of PGPR has suppressed plant diseases like wilt disease (*Fusarium oxysporum*) by *Trichoderma*.

**Table 1: Biochemical characteristics for the identification of Azotobacter species**

| Biochemical tests | Azotobacter species |
|-------------------|---------------------|
| Gram-staining     | Positive            |
| Motility          | Motile              |
| Catalase test     | +                   |
| Nitrate reduction | +                   |
| Pigment production| +                   |
| H₂S production   | +                   |
| Urease test       | +                   |
| Citrate test      | +                   |
| Utilization of carbon source | +     |
| Glucose           | +                   |
| Fructose          | +                   |
| Maltose           | —                   |
| Indole test       | +                   |

**Table 2: Biochemical characteristics for the identification of Trichoderma species**

| Biochemical tests | Trichoderma species |
|-------------------|---------------------|
| Production of chlamydospores | Positive |
| Conidial diameter of >2 μm | +       |
| Growth on glucose  | +       |
| Growth on citric acid | +     |
| Growth on lactic acid | +     |
| Growth on ammonium oxalate | +     |
| Hydrolysis of gelatin | +     |
| Growth on nitrate agar 4°C | —      |
| Growth on nitrate agar 37°C | +     |
| Growth on nitrate agar 40°C | —      |

**Figure 1:** PCR-RAPD profiles obtained with forward and reverse primers (a) *Azotobacter*, (b) *Trichoderma* and markers on left side

**Figure 2:** (a) *Amaranthus spinosus* plants inoculated with the combination of both *Azotobacter* and *Trichoderma* showing growth after 45 days C: control, Z1–Z10, (b) Effect of isolated strains of *Azotobacter* and *Trichoderma* on growth parameters of *Amaranthus spinosus* plants

**Figure 3:** (a) *Stevia rebaudiana* plants inoculated with the combination of both *Azotobacter* and *Trichoderma* showing growth after 45 days. C: control, Z1–Z10, (b) Effect of isolated strains of *Azotobacter* and *Trichoderma* on growth parameters of *Stevia rebaudiana* plants
CONCLUSION

The results concluded that the combination of both isolated strains of *Azotobacter* and *Trichoderma* promotes plant growth and not infected with fungal pathogens. These strains may be used simultaneously as a biofertilizer and bio-control agent. These are eco-friendly in nature and cost effective, so PGPR help in improving profitability in agriculture and improve livelihoods of small and marginal farmers.

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