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The root endophytic fungus *Curvularia geniculata* from *Parthenium hysterophorus* roots improves plant growth through phosphate solubilization and phytohormone production

Perumalsamy Priyadharsini, Thangavelu Muthukumar*

Root and Soil Biology Laboratory, Department of Botany, Bharathiar University, Coimbatore 641046, Tamilnadu, India

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ABSTRACT

The plant growth promoting ability of *Curvularia geniculata* (Tracy & Earle) Boedijn, a dark septate endophyte (DSE) fungus isolated from *Parthenium hysterophorus* L., was examined under *in-vitro* conditions for the first time. The abilities of the fungus to solubilize different sources of phosphorus (P) [$AlPO_4$, $FePO_4$ and $Ca_3(PO_4)_2$] and to produce indole acetic acid (IAA) were also determined. *C. geniculata*-inoculated pigeon pea (*Cajanus cajan*) plants exhibited superior growth over uninoculated control plants and the fungus solubilized different sources of P in the order of $FePO_4$>$AlPO_4$>$Ca_3(PO_4)_2$. The pH of the culture medium, fungal biomass production and acid phosphatase activity was significantly influenced by phosphate source and incubation period. IAA production, confirmed by chromatographic analysis, increased with increasing concentrations of tryptophan in a non-linear manner. It can be concluded from the results that *C. geniculata* mediates plant growth through phosphate solubilization and phytohormone production. This would enable the use of this fungus as bioinoculant in plant production systems.

1. Introduction

Endophytic fungi reside within tissues of all plant parts without exhibiting any apparent symptoms. Some of these fungi enhance plant growth by various mechanisms including the solubilization of bound soil nutrients and the production of phytohormones (Radhakrishnan et al., 2015). The metabolites produced by endophytic fungi also possess antimicrobial, anticancer and insecticidal properties (Aly et al., 2010). Among the endophytic fungi, sterile or conidial forms that have melanized, regularly septate hyphae and form microsclerotia within host cells are termed dark septate endophytes (DSE; Jumpponen and Trappe, 1998). These fungi improve plant growth and health under adverse environmental conditions (Khan et al., 2015). Although several studies have examined the production of hydrolytic enzymes that are involved in organic matter decomposition by these fungi (see Mandyam et al., 2010 and references therein), studies on their role in solubilizing insoluble nutrients in soil and making them available to plants are scarce.

Phosphorus (P) is one of the important macronutrients for plant growth and development. However, P availability in tropical soils is very low (Mahamuni et al., 2012). The negatively charged inorganic phosphate ion reacts readily with cations such as aluminium (Al^{3+}), iron (Fe^{3+}) and calcium (Ca^{2+}) to form insoluble complexes. Subsequently, phosphate usually exists as tri-calcium phosphate [$Ca_3(PO_4)_2$] in alkaline soils and as $FePO_4$ and $AlPO_4$ in acidic soils (Kapri and Tewari, 2010). These forms of phosphate are not easily available to plants. Thus, the application of synthetic P fertilizers is necessary in such soils to optimize crop production. However, the use of synthetic fertilizers has several undesired effects on both biotic and abiotic components of ecosystems. An alternative strategy to reduce the use of synthetic fertilizers is to exploit resident micro-organisms, especially those that are capable of solubilizing insoluble nutrients in the soil. Previous studies have affirmed that fungi are more efficient in solubilizing bound nutrients than other soil micro-organisms (e.g., Pradhan and Shukla, 2005; Gupta et al., 2007). For example, Gupta et al. (2007) showed that fungal isolates solubilized $Ca_3(PO_4)_2$ and rock phosphate to a greater extent than bacteria.

Phytohormones are signaling molecules that act as chemical messengers in controlling plant growth. These molecules regulate the metabolic activity of plants even at very low concentrations (Davies, 2010) and have a wide range of applications in agriculture

* Corresponding author.
E-mail address: tmkum@yahoo.com (T. Muthukumar).

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(Ali and Vora, 2014). The major phytohormone indole acetic acid (IAA) regulates the elongation, division and enlargement of cells (Ishida et al., 2013; Khan et al., 2015). In spite of its plant origin, IAA is also produced by soil and endophytic micro-organisms and is known to improve plant growth (Hasan, 2002). Recently, endophytic fungi such as *Aspergillus*, *Chaetomium*, *Exophiala*, *Fusarium*, *Paecilomyces*, *Penicillium* and *Phoma* colonizing the tissues of aerial plant parts have been shown to produce IAA (Khan et al., 2008, 2011a,b; 2012a,b; 2014; Khan and Lee, 2013; Mishra et al., 2014). IAA produced by these fungi has been shown to improve root proliferation and plant biomass (Hoffman et al., 2013).

Although phosphate solubilization and phytohormone production have been reported for many endophytic fungi colonizing aerial plant parts, reports are scarce for endophytic fungi colonizing root systems. Therefore, knowledge of phosphate solubilization and phytohormone production by root endophytic fungi may assist crop production and yield in low fertility soils (Barrow and Osuna, 2002). Hence, the objective of the present study was to characterize and screen a DSE fungus isolated from the roots of *Parthenium hysterophorus* L. for its plant growth promotion characters, such as P solubilization and phytohormone production.

2. Materials and methods

2.1. Isolation of the fungus

Fine feeder roots of *P. hysterophorus* were collected during August 2012 from Bharathiar University campus, Coimbatore, Tamilnadu, India (11.04° N, 76.88° E; 411 m a.s.l; annual rainfall-618 mm). The collected root samples were thoroughly washed with running tap water for 5 min to remove soil and attached debris. The roots were initially surface-sterilized with 0.5% (w/v) sodium hypochlorite solution for 1 min, rinsed with sterile distilled water and then with 70% (v/v) ethanol for 2 min. The roots were then rinsed five times with sterile distilled water. The surface-sterilized roots were cut into 0.5 cm lengths using a sterile razor blade and plated into potato dextrose agar (PDA) medium in Petri dishes (90 mm diam.). The imprinting technique was used to test the effectiveness of surface sterilization (Schulz et al., 1999). The Petri dishes were incubated at 28 °C for 5 days. A pure culture of the fungus was established by transferring the hyphal tips emerging from the cut ends of the roots onto PDA in slants, which were maintained at 28 °C.

2.2. Characterization of the fungus

As the fungus failed to sporulate in culture, molecular characterization was carried out by isolating total genomic DNA from the fungal hyphae using the cetyl trimethylammonium bromide (CTAB) method (Weising et al., 1995). The polymerase chain reaction (PCR) was performed using the specific primers ITS1 and ITS4 to amplify the fungal internal transcribed spacer (ITS) region (White et al., 1990). Amplification was carried out in 20 μl PCR reaction mixtures [2 μl dNTPs (2.5 mM), 2 μl buffer (10X), $MgCl_2$ (3 mM), 2 μl ITS1 primer (10 pM), 2 μl ITS4 primer (10 pM), 1 μl template DNA (50 ng), 0.25 μl *Taq* polymerase (5U) and 10.75 μl water]. The PCR program consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation for 50 s at 94 °C, primer annealing for 30 s at 48 °C and extension for 1 min and 30 s at 72 °C, and a final extension for 6 min at 72 °C. The amplified products were sequenced at BioServe Biotechnologies Private Ltd., Hyderabad, India. The sequence similarity to database records was investigated through the National Centre for Biotechnology Information (NCBI) platform using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). The sequence has been deposited in GenBank (Accession No. KX656363).

2.3. Evaluation of plant growth promotion by the DSE fungus

Pigeon pea (*Cajanus cajan*) seeds (var CO7) were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Seeds were surface sterilized by immersing them in 2% (w/v) sodium hypochlorite solution for 1 min, followed by immersion in 70% (v/v) ethanol for 3 min and then rinsing with four changes of sterile distilled water. The effectiveness of surface sterilization was confirmed by imprinting the sterilized pigeon pea seeds on PDA medium and checking for contamination after five days. The surface sterilized seeds were germinated on wet sterile filter paper in Petri dishes under sterile conditions. Seedling growth assays were performed by transferring germinated seeds to plastic pots (85 × 50 mm, H × W) containing sterile vermiculite (155 g) at the rate of three seeds per pot. The pots were covered with a transparent polythene bag to prevent contamination. Seedlings were thinned to the single largest germinant per pot once emergence was complete. Fungal inoculation was effected by placing 1 cm diameter fungal plugs obtained from the fungal culture adjacent to the root system. Fungus-free PDA plugs served as controls. Seedlings received Hoagland's nutrient solution at four day intervals and were watered with sterile distilled water as required. Twenty-five replicates each were maintained for control and inoculated treatments. The seedlings were destructively harvested 25 days after inoculation. Of the 25 seedlings in each treatment, 20 seedlings were assessed for various plant growth parameters. The fungus was re-isolated from the roots of the remaining five seedlings, which were also examined for endophytic colonization.

At harvest, the seedlings were freed from adhering growth substrate by gentle shaking and rinsing with sterile distilled water. Plant growth parameters (plant height, leaf number, shoot and root biomass and total root length) were measured. Biomass was measured after drying at 40 °C for 48 h. The root/shoot (R/S) ratio was calculated from the respective dry weights. Re-isolation of the fungus from the roots was performed as described above.

2.4. Determination of endophytic colonization

A portion of the fine roots from inoculated and uninoculated plants were examined to evaluate endophytic colonization by the fungus. The roots were cleared in 2.5% (w/v) KOH at 90 °C for 70 min, washed, acidified with 5N HCl and stained with 0.05% (w/v) trypan blue in lactoglycerol. The stained roots were examined under an Olympus BX51 bright field microscope for the presence of fungal structures.

2.5. Qualitative estimation of phosphate solubilization

For qualitative estimation of P solubilization, the fungus was cultured on Pikovskaya's (PVK) medium (Pikovskaya, 1948) containing 2 g l^{-1} of either $Ca_3(PO_4)_2$, $AlPO_4$ or $FePO_4$. For better visualization of the solubilization zone, the pH indicator dye bromocresol green was added to the medium prior to sterilization (Gadagi and Sa, 2002). The cultures (in triplicate) were incubated at 28 °C for 7 d. A clear zone around the fungal colony representing phosphate solubilization was measured (Singh et al., 2011). The solubilizing index was calculated according to Fankem et al. (2006) using the formula:

$$\text{Solubilizing index (SI)} = \frac{\text{Colony diameter} + \text{Clearing zone}}{\text{Colony diameter}}$$