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Research Article

Plant growth-promoting and antagonistic endophytic bacteria from the medicinal plant *Tinospora cordifolia* stem

Pratibha Vyas* and Ramanpreet Kaur

Microbiology Domain, School of Bioengineering and Biosciences, Lovely Professional University, Jala ndhar-Delhi GT Road, Phagwara-144411, Punjab, India

ABSTRACT

Endophytic bacteria residing within the plant tissues are poorly investigated microorganisms. A total of 35 endophytic bacteria isolated from *Tinospora cordifolia* stem tissue samples collected from 3 different districts of Punjab, India were screened for phosphate solubilization, IAA-like auxin production, ACC-deaminase activity, and production of siderophores, hydrogen cyanide and ammonia. Out of these bacteria, 11 showing multiple plant growth-promoting activities were screened for anatagonism against phytopathogens *Fusarium moniliforme*, *Alternaria alternata* and *Curvularia lunata.* One isolate TCA2 showed broad-spectrum activity with 38% growth inhibition in *Alternaria alternata*, 35% inhibition in *Fusarium moniliforme* and 25% inhibition in *Curvularia lunata.* On the basis of 16S rDNA gene sequencing, the isolate TCA2 has been identified as *Pseudomonas* sp. The maize plants inoculated with the bacterial isolate TCA2 showed 33.1%, 31.6%, and 38.4% inc reased root length, shoot length and dry weight, respectively, over the respective inoculated controls in pots under natural conditions. In the present studies, *Pseudomonas* sp. TCA2 with multiple plant growth-promoting activities has been selected as a sui table candidate for the development of microbial inoculants.

Keywords: Antagonism; Endophytes; plant growth promotion; *Tinospora cordifolia*

INTRODUCTION

Tinospora, a herbaceous, glabrous and deciduous plant with the common name 'Giloy' is a multipurpos e medicinal plant (Mittal *et al.,* 2013). It is easily growing plant throughout the India and described as "Amrita" in classical literature of ayurvedic system of medicine. The whole plant is medically important. It is considered as bitter tonic to cure the skin infections, irritability, diabetes, diarrhoea and dysenteric diseases. It can be used as anticancer, antiviral, antiallergic and anti - inflammatory agent. The microorganisms living inside the plant tissues without causing apparent harm termed as 'Endophytes' are potential source of various novel compounds enhancing the plant growth and eliminating plant pathogens, which can be utilized for sustainable agriculture. Endophytic microorganisms are not only the promising source of growth metabolites but also enable the plant to resist stress like conditions.

One of the major challenges for agriculture in the twenty-first century is the sustainable crop production. Extensive use of pesticides and chemical fertilizers has led to the buildup of pesticide resistance among pathogens and health risks to mankind (Aktar et al., 2009; Krauss et al., 2010). Control of plant diseases has remained a major challenge for improving crop productivity. Therefore, it is important to find alternative control approaches for crop diseases in response to continued use of pesticides. Use of antagonistic bacteria for biological control of phytopathogens appears to be an eco-friendly alternative to the use of chemical fungicides and pesticides (Fischer et al., 2010). Microorganisms produce several metabolites including antibiotics, siderophores, hydrogen cyanide and enzymes that reducing the growth and/or activity of plant pathogens. Effective selection of bacteria with multiple plant growth-promoting and broad-spectrum antagonistic activity will be beneficial in developing effective bio control inoculants.

Endophytic microorganisms have the potential to produce plant growth-promoting metabolites including phytohormones, enzymes like ACC-deaminase, organic acids aiding in phosphate solubilization, siderophores, celluloses and chitinases (Mittal et al., 2013). The application of endophytic microorganisms with multiple plant growth-promoting activities and biocontrol mechanisms could be beneficial in reducing the use of chemical fertilizers and pesticides for sustainable agriculture in the fragile ecosystems.

Plants which possess ethanobotanical history offer enormous opportunities for the recovery of novel en-

* Corresponding Author
Email: vyasp2000@yahoo.co.in
Conta ct: +91-8699007301
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dophytic microorganisms. There is complex relationship between endophytes and host plant. No reports are available on the endophytic bacteria isolated from *Tinospora cordifolia* antagonistic against plant pathogens and plant growth promotion. Therefore, present study was aimed at isolating endophytic bacteria from *Tinospora cordifolia* with antagonistic and multiple plant growth-promoting activities against plant fungal pathogens. This is the first report on endophytic bacteria associated with *Tinospora cordifolia* with antagonistic and plant growth-promoting ability.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Endophytic bacteria were isolated from sterilized stems of *Tinospora cordifolia* growing at three different districts of Jalandhar, Punjab in India. Stems were collected from healthy plants, cut into small segments of 4 cm approximately, washed with distilled water. The stem sections were surface sterilized by dipping for 30 s in ethanol (75%) followed by 0.2% $HgCl_2$ for 8 min and washed with sterilized distilled water five (Tan et al., 2015). The segments were cut into thin slices (1–2 mm) and incubated on agar-solidified trypticase soy agar in the dark at 28°C for 4–10 days. The bacterial isolates growing in the immediate vicinity of plant tissue were purified and preserved under 30% glycerol for further studies.

Plant growth promoting activities

The bacterial isolate was initially screened for phosphate solubilization on modified Pikovskaya agar (Gupta et al., 1994). Tricalcium phosphate solubilization was estimated using vanado molybdate method after growing bacterium for 5 days at 28°C in National Botanical Research Institute's phosphate (NBRIP) broth as described earlier (Nautiyal, 1999). The method of Loper and Schroth (1986) was used to estimate IAA-like auxins production in nutrient broth wirh 0.1% tryptophan. Screening for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was carried out by growing the bacteria on DF salts minimal medium containing ACC as nitrogen source (Jacobson et al., 1994). Production of siderophores was detected on chrome azurol sulfonate (CAS) agar plates as described (Schwyn and Neilands 1987). Hydrogen cyanide production was determined at 28°C after 48 h incubation on glycine supplemented nutrient agar plates (Bakker and Schippers 1987). Production of ammonia was detected following Cappuccino and Sherman (1992).

Screening for antagonism on solid medium by dual plate assay

The bacterial isolates were evaluated for antagonism against fungal pathogens on yeast extract medium by dual culture plate assay. The pathogenic isolates of *Fusarium moniliforme* strain 1100 (MTCC 156), *Curvularia lunata* strain 716 (MTCC 283), *Alternaria alternata* strain 6663 (MTCC 1362), procured from Microbial Type Culture Collection (MTCC), Chandigarh, India were grown for 5 days on potato dextrose agar at 28 °C. The bacterial isolates were grown on nutrient agar for 48 h at 28°C. The bacterial isolates were streaked in a file near the periphery in separate yeast malt extract agar plates. The agar discs nearly 6 mm diameter with full mycelium growth for each pathogen were seeded perpendicular to the bacterial streak on the opposite side near the plate periphery. The Petri plates without bacterial inoculation were uesed as control. The plates in triplicates were incubated for 7 days at 28 °C. The following formula was used to calculate the reduction in fungal growth:

$$Reduction\ in\ mycelium\ growth(\%) = \frac{(Control - Treatment)}{Control} \times 100$$

Treatment = mycelial growth of fungus in plates with bacterium

Control = mycelial growth of fungus in plates without bacterium

Identification of bacterial isolate TCA2 by 16S rRNA gene sequencing

The bacterial isolate was identified based on 16S rRNA gene sequencing. DNA isolation was was done using the Qiagen DNeasy Plant Mini Kit. The method used for gene amplification, thermocycling conditions, cloning, and analysis of the sequence has been described earlier (Gulati et al., 2008). The sequences were aligned with ClustalW and MEGA software package version 7 with Kimura's two-parameter model was used to calculate the evolutionary distance of TCA2 and its related taxa.

Plant growth promotion experiments

The bacterial isolate TCA2 was evaluated for plant growth promotion as described earlier (Gulati et al., 2009, Vyas et al., 2010). The seeds of maize (*Zea mays*) were sterilized for 3 min with 20% sodium hypochlorite, washed thrice with steriled distilled water, dipped in 48 h old bacterial culture for 30 min and sown in 15-cm diameter plastic pots filled with unsterilized garden soil with two seeds per pot. For control, seeds dipped in sterilized nutrient broth only were sown in pots. The experiment had two treatments with 4 replicates each. After placing the pots in a randomized block design under natural conditions for 30 days, the data were collected on root length, shoot length, and total dry weight. To determine total dry weight, the plants were dried in an oven for at 70°C for 2 days till constant weight.

Statistical analyses

For conducting the experiments, randomized block design was used. The data was analyzed by analysis of variance (ANOVA) using the XLSTAT 2016. Unless stated otherwise, all experiments were repeated twice and values are means of three replicates. Treatment means

Table 1: Plant growth promoting attributes of endophytic bacteria isolated from Tinospora cordifolia stem

Isolate	Plant growth-promoting attributes					
	P-solubilization (µg/ml)	IAA-like auxin (µg/ml)	HCN production	Ammonia production	ACC-deaminase activity	Siderophore production (% units)
TCA2	383.7±4.41	24.8±1.48	+++	++	+	53.8±2.74
TCA3	225.7±5.21	17.3±0.88	-	+	++	10.7±0.88
TCA5	227.7±4.05	5.8±0.93	+	++	+	12.7±1.20
TCA9	235.3±4.97	14.3±1.45	+	+	-	21.5±1.44
TCA18	249.7±5.36	25.3±1.76	-	-	+	13.3±1.45
TCA21	129.0±3.21	6.8±0.72	-	+	+	6.5±0.76
TCA22	324.3±2.60	15.8±0.92	++	-	+	18.3±1.20
TCA25	127.7±3.28	3.8±0.44	-	+	+	20.3±1.85
TCA29	252.7±3.84	12.7±0.88	-	-	++	44.7±1.85
TCA30	83.3±2.96	9.0±1.63	-	+	-	18.7±1.76
TCA34	111.0±3.21	33.5±2.04	-	-	+	12.3±0.67

Values represent the mean of three replicates ± standard error. +++ very strong activity, ++ moderate activity, + weak activity

Table 2: Effect of Pseudomonas sp. TCA2 on growth promotion of maize in soil after 45 days of inoculation in pots under natural conditions

Treatment	Growth Parameter (cm)		
	Root Length	Shoot Length	Dry weight
Control	21.18	35.62	0.276
Pseudomonas sp. TCA2	28.19* (33.1)	46.87* (31.6)	0.382*(38.4)
Fisher's LSD at 5%	1.8	1.65	0.06

Values are the Mean of eight replicates. *Significantly different from control at P < 0.05. Values in parentheses are the % increase over control.

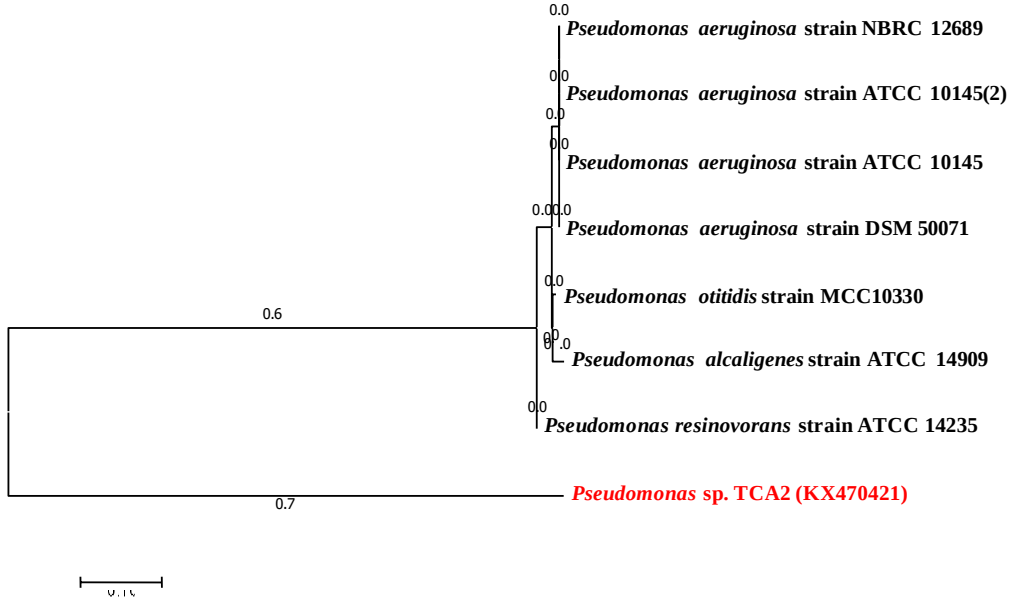

Figure 1: Phylogenetic tree constructed on the basis of 16S rRNA gene sequences showing relationship among *Pseudomonas* sp. TCA2 and nearest neighbours drawn using the neighbour-joining method

were compared at p values of 0.5 by critical difference (CD).

RESULTS AND DISCUSSION

Isolation and screening for plant growth promoting attributes

A total of 35 bacteria were isolated from 6 stem samples of *Tinospora cordifolia* collected from three different districts of Punjab in India: 12 isolates (TCA1 - TCA12) from Banga, 17 isolates (TCA13-TCA29) from Nawanshahar, and six isolates (TCA30-TCA35) from Jalandhar, Punjab, India. Out of these, 11 bacterial isolates showed plant growth-promoting activities of

phosphate solubilization, auxin production, siderophore production (Table 2). ACC-deaminase enzyme activity was shown by nine isolates on DF salts minimal medium, ammonia production by seven isolates and HCN production by four isolates (Table 1). Endophytic *Pseudomonas* sp. from rhizome of *Zingiber officinale* showed IAA production, ACC-deaminase and siderophore production (Jasim et al., 2014). However, no reports are available on plant growth-promoting and antagonistic activities against fungal phytopathogens by *Pseudomonas* from *Tinospora cordifolia.* The bacterial isolates also showed strong HCN production as indicated by orange-brown colour of filter paper discs placed in the lids of the plates with sodium carbonate in picric acid.

Screening for antagonistic activity

Eleven endophytic bacterial isolates showing several plant growth-promoting activities were screened for antagonistic activity against fungal phyopathogen by dual plate assay. Out of eleven bacterial isolates, five isolates showed anatagonistic activity ranging from 11 to 38 % against *Alternaria alternate*, two isolates ranging from 10 to 40% against *Fusarium moniliforme* and three isolates ranging from 7 to 25% against *Curvularia lunata*. The antagonistic activity was not observed for six isolates against *Alternaria alternate*, nine isolates against *Fusarium moniliforme* and eight isolates against *Curvularia lunata*. Only one isolate TCA2 showed broad-spectrum activity with 38% growth inhibition in *Alternaria alternata*, 35% inhibition in *Fusarium moniliforme* and 25% inhibition in *Curvularia lunata*. Microscopic examination showed that all three fungi growing close to bacterial culture TCA2 had broken hyphae and ruptured spores, whereas control fungi showed normal growth of mycelium as well as spores. Considerable decrease in the mycelial growth and morphological alterations of fungal hyphae and spores might be connected to production of siderophores, HCN or enzymes. The studies carried out by light microscopy are in agreement with earlier studies in which endophytic *Bacillus subtilis* caused the morphological alterations in the fungal hyphae (Nongkhlaw et al., 2016). The phytopathogenic fungi cause considerable economic losses in agriculture crops. Antagonistic activity of *Pseudomonas* spp. inhibiting *Alternaria alternaata, Fusarium solani*, *Rhizoctonia solani*, *Sclerotinia minor* and *S. sclerotiorum* through the production of siderophore and HCN has been reported (Bakker and Schi ppers 1987, Pandey et al., 2006, Fischer et al., 2010, Abdallah et al., 2016) as also reported in the present studies. In addition to indirectly affecting plant growth through the inhibition of phytopathogens, the strain TCA2 can also enhance plant growth directly by phosphate solubilisation, auxin production in soil.

Identification of the select endophytic bacterial isolate

The bacterial isolate TCA2 showing multiple plant growth promoting attributes and broad-spectrum antagonistic activity was identified on the basis of morphological features, biochemical features and 16S rRNA gene sequencing. The bacterial isolate was Gram negative, motile, rod shaped, positive for citrate utilization, catalase and oxidase whereas negative for indole, methyl red and Voges Proskauer, and urease.

The results of a BLAST search of 1516-bp partial 16S rRNA gene sequence of TCA2 showed 99% homology with *Pseudomonas* sp. DSM 5001. The phylogenetic tree constructed on the basis of 16S rRNA gene sequence of TCA2 and its nearest neighbours formed four groups with TCA2 forming an outgroup (Fig. 1). The 16S rRNA gene sequence of the isolate was deposited in NCBI GenBank with the accession number KX470421. Species of *Pseudomonas* are the common inhabitant of rhizosphere and plant tissues but no reports are available on the isolation of *Pseudomonas* spp. from the medicinal plants *Tinospora.*

Effect on plant growth

A significant increase in maize growth was observed in the plants inoculated with *Pseudomonas* sp. TCA2 in comparison to the uninoculated plants after 45 days of inoculation under natural conditions (Table 2). The increase in root length was 33.1%, shoot length was 31.6%, and dry matter was 38.4% in inoculated plants over uninoculated control. *Pseudomonas* spp. strains with different plant growth-promoting activities have been reported to enhance growth in many plants (Mehnaz and Lazarovits 2006, Oteino et al., 2015, Vyas and Gulati 2009).

CONCLUSION

The present study has selected an endophytic bacterium *Pseudomonas* sp. TCA2 with broad-spectrum antagonistic activity against fungal pathogens and multiple plant growth-promoting activity. The bacterial isolate also enhanced the growth of the test plant, showing it as a potential candidate to be used as a plant growth promoting microbial inoculants after testing in fields.

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