Plant growth promoting potential of the fungus *Discosia* sp. FIHB 571 from tea rhizosphere tested on chickpea, maize and pea

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Abstract The ITS region sequence of a phosphate-solubilizing fungus isolated from the rhizosphere of tea growing in Kangra valley of Himachal Pradesh showed 96% identity with *Discosia* sp. strain HKUCC 6626 ITS 1, 5.8S rRNA gene and ITS 2 complete sequence, and 28S rRNA gene partial sequence. The fungus exhibited the multiple plant growth promoting attributes of solubilization of inorganic phosphate substrates, production of phytase and siderophores, and biosynthesis of indole acetic acid (IAA)-like auxins. The fungal inoculum significantly increased the root length, shoot length and dry matter in the test plants of maize, pea and chickpea over the uninoculated control under the controlled environment. The plant growth promoting attributes have not been previously studied for the fungus. The fungal strain with its multiple plant growth promoting activities appears attractive towards the development of microbial inoculants.

Keywords *Discosia* sp. · ITS region · Plant growth promotion · Tea

Introduction

Improving tea productivity is imperative to meet the increasing consumers' demand with limited scope for increase in area under cultivation. Low availability of phosphorus is often a limiting factor in the crop productivity [1]. Large quantities of fertilizers are applied annually to augment the availability of phosphorus in the soil. The potential negative effects of chemical fertilizers on the environment have led to the research for their supplementation with microbial inoculants which benefit plant growth by improving the nutrient status of soil, secretion of plant growth promoting hormones, and suppression of soil-borne pathogens [2, 3]. The microorganisms with multiple plant growth promoting activities could be highly effective as the microbial inoculants in agriculture. Earlier studies on the selection of microbial inoculants with multiple plant growth promoting attributes have mainly been limited to rhizobacteria. However, the acidic soil conditions required for tea cultivation are more conducive to the growth of fungi than bacteria [4]. The fungi are also reported to perform better than bacteria as phosphate-solubilizers in acidic soil conditions [5]. Thus the selection of native fungal inoculants with multifarious plant growth promoting activities appears promising for increasing tea productivity. The present paper reports for the first time the plant growth promoting potential of *Discosia* sp. recorded from tea rhizosphere. The fungi belonging to the genus *Discosia* Libert (coelomycetes, anamorphic fungi) are saprobes or parasites of vascular plants reported from many localities distributed throughout the world [6].

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Materials and methods

Isolation and characterization

The fungus was isolated from the soil adhering to the feeder roots of tea (*Camellia sinensis*), growing at the Tea Experimental Farms Banuri, Palampur at an altitude of 1303 m MSL in Himachal Pradesh (India). 100 µl of each serial soil dilutions up to 10^{-4} was spread plated on the modified Pikovskaya (PVK) agar containing 0.5% tricalcium phosphate (TCP) as the source of insoluble phosphate [7]. The morphologically distinct fungal colonies producing prominent zones of TCP solubilization were raised into pure cultures on potato dextrose slants and identified on cultural and microscopic features [8, 9]. One of the phosphate-solubilizing fungi identified as *Discosia* sp. was selected for further studies being the first record from tea rhizosphere and with no previous reports on the plant growth promoting attributes.

The fungal DNA was extracted from the mycelium ground under liquid nitrogen from the 5 days old cultures using Qiagen Plant DNeasy Kit. The amplification of ITS 1, 5.8 ribosomal RNA gene and ITS 2 was achieved using the primers ITS 1: 5' TCC GTA GGT GAA CCT GCGG and ITS 4: GCT GCG TTC ATC GAT GC as described earlier [10]. The sequence of the PCR product was determined by employing the ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kit. The sequence was analyzed using the gapped BLASTn search algorithm and aligned to the nearest neighbours [11]. The phylogenetic tree was constructed using the TREECON software package version 1.3b (Copyright Yves Van de Peer, University of Antwerp, 1994) after aligning the sequences with ClustalW [12] and generating evolutionary distance matrix, inferred by the neighbor-joining method using Kimura's two-parameter model [13].

Phosphate solubilization

Estimation of inorganic phosphate solubilization was done in NBRIP broth containing 0.5% TCP, aluminium phosphate (AP), iron phosphate (FP), Mussoorie rock phosphate (MRP), North Carolina rock phosphate (NCRP) or Udaipur rock phosphate (URP) as previously described [10]. The spectrophotometric vanado-molybdate method was used to determine total phosphorus in the rock phosphates [14].

Phytase production

Production of phytases by the fungus was determined on phytate screening medium after 5 days incubation at 28°C [15].

Production of IAA-like auxins

The fungus was grown in Czapek-Dox liquid medium with 0.1% DL-tryptophan in a Innova Model 4230 refrigerated incubator shaker (New Brunswick Scientific, Edison, NJ) for 5 days at 180 rpm. Estimation of IAA-like auxins in the culture filtrate was first done using colorimetric technique with Salkowski reagent [16]. Quantification of indole derivatives in ethyl acetate extract of the culture filtrate was done on Merck Hitachi La Chrom 7000 HPLC system (Merck, Darmstadt, Germany), equipped with a PDA detector and Lichrosphere RP-18 column (4.6×250 mm, 5 µm; Merck, Darmstadt, Germany) as described earlier [17]. A mobile phase of 60% solvent A (0.5% acetic acid) and 40 % solvent B (100% acetonitrile) was employed at a flow rate of 1 ml/min in the present studies. Eluates were detected using an isocratic system and identified by retention time and co-chromatography by spiking the sample with authentic indole compounds. The indole derivatives were quantified by reference to the peak areas obtained for authentic standards for tryptophan (try), indole acetic acid (IAA), indole pyruvic acid (IPA), indole-acetaldehyde (IAAld), indole acetamide (IAM), and indole acetonitrile (IAN). The samples were analyzed in triplicate.

Siderophore production

The universal chemical assay was used for siderophore detection in CAS agar plates [18]. Quantitative estimation was done in the culture filtrate of 5 days old fungus grown at 28°C in iron-free Czapek-Dox liquid medium by CAS-shuttle assay [19].

Plant test

The influence of the fungus on plant height and biomass was studied in three test plants – *Zea mays* var. Girija, *Pisum sativum* var. Palam Priya, and *Cicer arietinum* var. HPG 17. Ten milliliter of the fungal suspension (10^5 cfu ml⁻¹) was applied around the surface sterilized pre-germinated seeds in 150 mm diameter plastic pots filled with sterilized vermiculite. The pots were placed in a complete randomized design with six replications in the Environment Control Room under a mixed incandescent and fluorescent illumination of 550 µM photon/m²/s with a 16 h photoperiod at $25 \pm 2^\circ\text{C}$, and 50–60% RH. The pots were maintained at 60% water holding capacity by watering daily with sterile distilled water and weekly with 10 times diluted Hoagland's solution [20]. Data were recorded on root length, shoot length and total dry weight after 30 days of inoculum application. The plants were oven-dried at 70°C for 3 days to determine the total dry weight.

Experimental design and data analysis

The data were checked for normality and subjected to analysis of variance (ANOVA) using the STATISTICA data analysis software system version 7 (StatSoft® Inc., Tulsa, USA 2004). The mean of the treatments was compared by CD value at $P = 0.01$.

Results and discussion

Isolation and characterization

Thirteen phosphate-solubilizing fungi were identified as *Aspergillus niger*, *A. versicolor*, *Discosia* sp., *Eupenicillium parvum*, *Gliocladium roseum*, *G. virens*, *G. viride*, *Penicillium auranteogrisium*, *P. decumbens*, *P. lanosum*, *P. purpurogenum*, *P. griseofulvum*, and *Trichoderma pseudokoningii* on the basis of cultural and microscopic features from tea rhizosphere. *Discosia* sp. not previously reported from tea rhizosphere formed appressed, greenish-black colonies with regular striations from periphery to center and blackish-brown on reverse side; mycelium immersed, branched, septate, pale brown; conidia straight or slightly curved, dorsiventral, 3–4 euseptate, smooth, tapered to a truncate base, apex obtuse, hyaline to pale brown, showing resemblance to the known descriptions for the genus *Discosia*. A single band of ~600 bp was obtained on amplification of the ITS region of the fungal mycelium. The sequence of 496 bp ITS region of the fungus was deposited in the NCBI GenBank (Accession No. DQ 536523). The fungus showed highest similarity at 96% to *Discosia* sp. strain HKUCC

6626 from the unidentified dead leaf from Hong Kong (Fig. 1) [21].

Screening for plant growth promoting attributes

The fungus showed the multiple activities for plant growth promotion. Appearance of a clear halo around the fungal colony on modified PVK agar indicated phosphate-solubilization by the fungus [7]. A significant difference was recorded in the solubilization of various substrates with a high solubilization of TCP and NCRP (Table 1). The solubilization was significantly higher for TCP, NCRP, FP, URP and AP, while it was nonsignificant for MRP over the control. The majority of phosphate-solubilizing microorganisms are able to solubilize Ca-P complexes but only a few species of *Aspergillus* and *Penicillium* are reported to solubilize Fe-P and Al-P complexes [22, 23]. However, *Discosia* sp. solubilized these complexes and could thus also prove effective in alfisols in which phosphates are mainly complexed to Al and Fe ions. The solubilization of TCP, NCRP, URP and FP by the fungus was comparatively much higher in comparison to 214, 110, 6, and 8 $\mu\text{g P}_2\text{O}_5/\text{ml}$ solubilized from these substrates, respectively, by *Eupenicillium parvum* recently reported from the tea soils [10]. The results on the solubilization of inorganic phosphates by *Discosia* sp. corroborated the earlier reports that rock, iron and aluminium phosphates are less amenable to microbial solubilization than TCP [3, 24]. A significant decline in the pH of medium was recorded during solubilization of different phosphate substrates, which suggested secretion of organic acids by the fungus [24, 25]. However, no relationship could be ascertained between the quantity of phosphate solubilization

Fig. 1 Phylogenetic tree showing relationship among *Discosia* sp. FIHB 571 and representatives of some related taxa, based on analysis of ITS region. The numbers on the nodes indicate how often (no. of times, %) the species to the right are grouped together in 100 bootstrap samples. *Aspergillus flavipes* was used as an outgroup. Bar, 0.1 substitution per site.

Table 1 Phosphate-solubilization by *Discosia* sp. FIHB 571 in NBRIP broth after 5 days incubation at 28°C with initial pH 6.8

Phosphate source	Phosphate solubilization over control ($\mu\text{g P}_2\text{O}_5/\text{ml}$)	Final pH of medium
Tricalcium phosphate	628.3 (62.8)	3.5
Aluminium phosphate	13.4 (1.2)	3.0
Iron phosphate	24.6 (1.3)	3.0
Mussoorie rock phosphate	3.4 (0.7)	4.3
Udaipur rock phosphate	15.1 (2.2)	3.6
North Carolina rock phosphate	292.1 (40.9)	3.4
S.E.M. $\pm$	1.0 (0.1)	0.1
CD ($P = 0.01$)	4.5 (0.6)	0.2

Total phosphorus content in Udaipur rock phosphate, Mussoorie rock phosphate, and North Carolina rock phosphate is 14.0, 9.1, and 14.3 %, respectively. Values in parentheses indicate per cent phosphate solubilization over control. Each value represents the mean of three replicates.

Table 2 Plant growth promoting activities of *Discosia* sp. FIHB 571 after 5 days incubation at 28°C

Attributes		Activity
Phytate solubilization zone (mm)		21 $\pm$ 1.2
Siderophore zone (mm)		14 $\pm$ 1.1
Siderophore units (%)		68 $\pm$ 1.7
Indole derivatives:		
Colorimetric (Salkowski reagent) ($\mu\text{g/ml}$)		8.5 $\pm$ 1.20
IAAld		2.1 $\pm$ 0.30
IAA		0.3 $\pm$ 0.01
IAM		0.7 $\pm$ 0.05
IPA		7.0 $\pm$ 0.60

Each value represents mean $\pm$ S.D. of three replicates

and the acidity of medium as decline in pH was at par in the solubilization of TCP and NCRP, though solubilization was significantly higher for TCP than for NCRP. It has been reported earlier that release of soluble phosphate is not necessarily correlated with acidity [26].

The development of a clear zone around the fungal colony in calcium phytate plates exhibited the additional ability of *Discosia* sp. to release P from phytic acid, which is a predominant form of organic phosphates present in the soil (Table 2) [27, 28]. Thus the fungus could also be useful in releasing phosphorus from the pruning litter used as mulch as a common practice in tea cultivation [29]. The litter decomposing ability of *Discosia* strain is reported to be relatively high among the interior and surface fungal colonizers of beech leaves [30].

Plant growth promotion is also influenced by the production of plant growth promoting hormones by the microorganisms in the rhizosphere [31]. The present studies also showed the secretion of IAA by the fungus in the presence of tryptophan (Table 2). The root exudates of various plants contain rich supplies of tryptophan utilized by the microorganisms for synthesis and release of auxins as secondary

metabolites in the rhizosphere [31, 32]. The presence of IAA, IAAld, IAM, and IPA in the culture filtrates suggested the synthesis of IAA through different biosynthetic pathways by the fungus (Fig. 2) [17].

Siderophore production is another important trait of the microorganisms that influences plant growth through the suppression of fungal pathogens by rendering iron unavailable by binding in the rhizosphere. In the present studies, *Discosia* sp. produced orange halo on CAS agar, indicating the siderophore-mediated iron removal from the ternary complex CAS-Fe (III) HDTMA (Table 2). The supernatant of CAS-shuttle assay also showed a strong positive reaction for siderophore activity in the culture filtrates as reported for *Trichoderma* spp. with a high biocontrol activity [33].

Effect on plant growth

An incremental effect on growth was observed in maize, pea and chickpea on inoculation indicated a broad spectrum plant growth promoting activity by the fungus (Table 3). The fungus significantly increased the root length, shoot

Fig. 2 HPLC chromatograms of authentic indole compounds (A) and the ethyl acetate extract of fungal supernatant of *Discosia* sp. FIHB 571 grown in Czapek-Dox liquid medium with tryptophan (B). The retention time of IAAI, IAM, IAA, IPA, Trp, and IAN as indicated was 1.36, 2.93, 3.23, 4.16, 6.05, and 7.49, respectively. Peaks # 1, 4, 5, and 6 co-eluted with IAAI, IAM, IAA, and IPA, respectively. The peaks were identified by performing co-chromatography by spiking the sample with authentic corresponding compounds. The peaks # 2, 3, 7, and 8 remain to be determined.

Table 3 Effect of *Discosia* sp. FIHB 571 on growth of test plants after 30 days in environment control rooms

Growth parameter	<i>Zea mays</i> var. Girija		<i>Pisum sativum</i> var. Palam Priya		<i>Cicer arietinum</i> var. HPG 17	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
Root length (cm)	25.40	29.6*	24.1	28.1*	20.3	22.1*
Shoot length (cm)	34.00	41.1*	22.1	26.8*	16.6	21.0*
Dry wt. (g/plant)	0.35	0.62*	0.25	0.32*	0.10	0.14*

Each value represents mean of nine replicates;

* significantly different from the control at $P < 0.01$

length, and total dry matter of all the test plants over the uninoculated control. Earlier many fungi including *Aspergillus*, *Penicillium*, *Trichoderma* and *Gliocladium* have been reported to enhance plant growth through phosphate-solubilizing and biocontrol activities [34–37]. With the multiple plant-promoting activities of phosphate-solubilization, production of IAA-like auxins and siderophore, and incremental growth influence in the test plants, *Discosia* sp. appears potentially promising for further testing as a bioinoculant in the tea soil, where conditions are much more complex than those prevailing in vitro. The chances of successful application of the fungus would appear high as it is to be deployed in the same milieu from where it has been isolated.

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