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Growth Promoting Rhizospheric and Endophytic Bacteria from *Curcuma longa* L. as Biocontrol Agents against Rhizome Rot and Leaf Blight Diseases

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Plant growth promoting rhizobacteria and endophytic bacteria were isolated from different varieties of turmeric (*Curcuma longa* L.) from South India. Totally 50 strains representing, 30 PGPR and 20 endophytic bacteria were identified based on biochemical assays and 16S rDNA sequence analysis. The isolates were screened for antagonistic activity against *Pythium aphanidermatum* (Edson) Fitzp., and *Rhizoctonia solani* Kuhn., causing rhizome rot and leaf blight diseases in turmeric, by dual culture and liquid culture assays. Results revealed that only five isolates of PGPR and four endophytic bacteria showed more than 70% suppression of test pathogens in both assays. The SEM studies of interaction zone showed significant ultrastructural changes of the hyphae like shriveling, breakage and desication of the pathogens by PGPR *B. cereus* (RBacDOB-S24) and endophyte *P. aeruginosa* (BacDOB-E19). Selected isolates showed multiple Plant growth promoting traits. The rhizome bacterization followed by soil application of *B. cereus* (RBacDOB-S24) showed lowest Percent Disease Incidence (PDI) of rhizome rot and leaf blight, 16.4% and 15.5% respectively. Similarly, *P. aeruginosa* (BacDOB-E19) recorded PDI of rhizome rot (17.5%) and leaf blight (17.7%). The treatment of these promising isolates exhibited significant increase in plant height and fresh rhizome yield/plant in comparison with untreated control under greenhouse condition. Thereby, these isolates can be exploited as a potential biocontrol agent for suppressing rhizome rot and leaf blight diseases in turmeric.

Keywords **:** antagonism, biocontrol, growth promotion, *P. aphanidermatum*, *R. solani*

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Turmeric (*Curcuma longa* L., Family-*Zingeberaceae*) is a rhizomatous perennial herb cultivated in Indian sub-continent and middle East countries. Dried rhizomes are used as condiment, dye, drug and for cosmetics. India is the leading producer of turmeric in the world and contributes about 75-80% of the world production followed by China, Myanmar, Nigeria and Bangladesh. The main turmeric producing states in India are Andhra Pradesh, Tamil Nadu, Orissa, West Bengal, Maharashtra, Karnataka and Kerala (Thiripurasundari and Selvarani, 2014). The fungus *P. aphanidermatum* and *R. solani* cause rhizome rot and leaf blight diseases in turmeric plants and reduce commercial value (Park, 1934; Roy, 1992). It has been noted that chemical fungicides like Ridomil, Metalaxyl, Carbendazim (0.1%) and Mancozeb (0.25%) were commonly used to manage rhizome rot and leaf blight diseases (Muthukumar et al., 2011; Rathaiah, 1982). Use of chemical fungicides is of public concern as it causes various human health problems and also pathogens build resisitance against fungicides. The need for adopting environment friendly disease control measures such as biological control strategies are emphasized presently (Hallmann et al., 2009). An alternative to chemical fungicides for the management of plant diseases is the use of soil borne, non-pathogenic rhizospheric or endophytic bacteria.

Plant growth promoting rhizobacteria (PGPR) may induce plant growth promotion by direct or indirect modes of action (Kloeppe et al., 1999). Common PGPR include the strains of *Bacillus*, *Rhizobium*, *Acinetobacter*, *Alcaligenes*, *Azotobacter*, *Arthrobacter*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Burkholderia* (Kloepper et al., 1989). Earlier reports emphasizes the biocontrol potential of PGPRs in agriculture along with growth promotion (Siddiqui, 2005). The PGPR *Bacillus subtilis* and *Burkholderia cepacia* significantly decreased ginger rhizome rot incidence along with increase in yield was reported (Shanmugam et al., 2013).

Endophytes are ubiquitous and have been found in all species of plants. In general, Endophytes could produce different plant hormones to enhance the growth of the host plants (Waqas et al., 2012). Bacterial endophytes colonize the internal tissues of the plant showing no negative effect on their host (Schulz et al., 2006). In comparison to PGPR, endophytes showed better adaptations against biotic and abiotic stresses, that leads to enhanced plant growth (Pillay and Nowak, 1997). Many endophytes constitute the common rhizospheric bacteria (*Burkholderia*, *Pseudomonas* and *Bacillus*) that produce various secondary metabolites, volatile compounds and antibiotics to counter the deleterious effect of pathogens through mechanisms similar to that of PGPR (Lodewyckx et al., 2002).

Endophytic bacteria are promising biocontrol agents as they occupy internal living tissues of plants and due to close proximity to plant pathogens. Endophytic bacteria were used as BCA's against plant pathogenic fungi such as *R. solani*, *Pythium* sp., *Alternaria alternata*, *Fusarium* sp., *Botrytis cinerea*, *Verticillium dahlia*, *Penicillium digitatum*, *Sclerotinkia sclerotiorum*, *B. fabae*, *Colletotrichum gloeosporioides* (Cao et al., 2005). Some endophytic bacteria colonize an ecological niche which makes them suitable as biocontrol agents (Berg et al., 2005).

Direct mode of action of PGPR includes fixation of atmospheric nitrogen, solubalization of minerals, production of phytohormones and enzymes in plants (Bashan and de-Bhashan, 2005) whereas indirect mode includes production of siderophores (Kuffner et al., 2008), production of antibiotics, lytic enzymes such as β-(1,3) glucanase and chitinase, antifungal metabolities that cause lysis of fungal cell wall, competition and inhibition of phytopathogens along with induction of systemic resistance (Ahmad et al., 2008; Compant et al., 2005). Similarly, endophytes reduces the bacterial, fungal, and viral diseases (Berg and Hallmann, 2006; Sturz et al., 2000) by producing siderophores (Lodewyckx et al., 2002) and lytic enzymes (Chernin and Chet, 2002). The endophyes also enhance the plant growth by production of Indole acetic acid (Rana et al., 2011) and Phosphate solubilization activity (Verma et al., 2001; Wakelin et al., 2004).

Several reports are available on the isolation of PGPR and endophytes, and their effects on growth and yield of crops. The biocontrol agents like *T. viride*, *P. chlororaphis* and *B. subtilis* were used for suppression of rhizome rot of turmeric (Kavitha et al., 2012; Ramarethinam and Rajagopal, 1999). Nevertheless, very little information is available on the effect of native multi trait PGPR and endophyes on growth promotion and biocontrol of rhizome and leaf blight diseases of turmeric. This study was taken up to profile the rhizobacteria and endophytic bacteria associated with turmeric and to evaluate their antagonistic activities, biocontrol potentialand plant growth promotion both *in vitro* and *in vivo* conditions against *P. aphanidermatum* and *R. solani* pathogens which cause rhizome rot and leaf blight diseases of turmeric respectively.

Materials and Methods

Sampling. Soil samples were collected from four different states of South India viz., Karnataka, Kerala, Tamilnadu and Andhra Pradesh. Samples were collected from top five cm of soil around healthy turmeric plants adhering to the roots. Collected soil samples (30 nos) were sealed in sterile polythene bags and transferred to ice box for transport. For endophyte isolation, healthy turmeric rhizomes (20 nos) were also collected from the above said regions in polythene bags, labeled and stored in refrigerator at 4ºC in laboratory and processed within 48 h of collection.

Isolation of bacteria from rhizosphere. Ten gram of soil sample was placed in 95 ml sterile water (10^{-1}) and shaken for 10 min. One (1.0) ml of this suspension was transferred into a 9 ml blank (10^{-2}) and serially diluted up to 10^{-10}. About 0.1 ml of each dilution from 10^{-8} to 10^{-10} series was added on Nutrient Agar (NA) medium and incubated at 37ºC for 2-3 days. Morphologically distinct bacterial colonies were isolated and subcultured on NA medium, strains were temporarily cryopreserved at –20 °C in 40% glycerol for further studies.

Isolation of endophytic bacteria from rhizomes. The collected rhizomes were thoroughly washed in running tap water to remove soil particles adhered followed by dipping in phosphate buffer (per L:6.33 g of NaH_2PO_4; 16.5 g of $Na_2HPO_4 \cdot 7H_2O$; 200 ml Tween 40). Distilled water was used to remove foam of Tween 40. Rhizomes were further sterilized by sequential immersion in 70% ethanol for 2 min and in 3.5% sodium hypochloride for 3 min and then rinsed several times in sterile distilled water to remove surface sterilization agents. One gram of rhizome was ground in a sterile mortar and pestle with phosphate buffered saline (PBS) and the solution was made up to 10 ml. Serial dilutions from 10^{-1} to 10^{-4} were prepared and 0.1 ml of aliquots were spread onto NA medium amended with nystatin (50 mg ml^{-1}; Sigma Aldrich, Bengaluru, India) in triplicates under laminar air flow to avoid external contamination and the plates were incubated for 7 days at 37°C. To verify the efficacy of surface sterilization of the rhizomes, 100 µl of the last rinse was added on NA medium and incubated. Morphologically distinct bacterial colonies were selected and pure cultures were preserved in 40% (v/v) glycerol solution at –20°C.

Pathogens. Virulent isolates of *P. aphanidermatum* (Accession No. KT315583) and *R. solani* (Accession No. KT366922) isolated from naturally infected turmeric rhizomes and leaves were obtained from the culture repository of the host Institute Department of Studies in Biotechnology, University of Mysore, Mysuru, India. Actively growing hyphae were successively transferred to the new PDA medium and the cultures were maintained on slants and stored at 4°C.

Characterization of rhizospheric and endophytic bacterial strains. The colony morphology, size, shape, colour and growth pattern of all the bacterial isolates was noted. Biochemical tests viz., Methyl red test, Voges-Proskauer test, Citrate test, Presence of oxidase and catalase, succinic acid, starch hydrolysis, ammonia production, casein hydrolysis were conducted to characterize the isolated bacterial strains (Cappuccino and Sherman, 1992). The Gram's reaction was performed as per standard procedures (Holt et al., 1994). The motility of the bacteria was checked using hanging drop method and for the KOH solubility test, a loop full of bacterial strain was mixed with 3% KOH solution on a clean glass slide for 1 min and observed for formation of a thread like mass. The isolates were grouped based on the results of phenotypic and biochemical characteristics.

For molecular characterization, DNA extraction was done using HipurA Bacterial DNA Purifiation kit of Himedia. DNA was quantified by NanoDrop spectrophotometer (2000C, Thermo Scientific, Tokyo, Japan) and the quality was checked based on absorbance ratio 260/280. The integrity of the DNA was assayed by gel electrophoresis using 0.7% agarose gel. The DNA was amplified using universal primer pair of 16S rDNA, Forward 16S rDNA F 5'-CCAGACTCCTACGGGAGGCAGC-3' and reverse 5'-GCTGACGAGAGCCATGCAGCACC-3' (Sigma Aldrich, Bengaluru, India). The PCR reaction was performed in 50 µl final reaction volume containing 5 µl of 10X PCR buffer, 8 µl of 25 mM $MgCl_2$, 2.5 µl of 1.25 mM dNTP, 0.2 µl of each primer (20 µM), 100 ng of DNA and 0.2 µl Taq DNA polymerase (5 U μl^{-1}) (Sigma-Aldrich, Bengaluru, India) in a thermal cycler (Bio-Rad, CA, USA) programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, primer annealing at 56°C for 45 s, extension at 72°C for 2 min. At the end of the amplification reaction, a final extension step was achieved at 72°C for 10 min. Ten microliters of the PCR products from each PCR reaction were electrophoresed on 1% agarose gel containing 5 mg ml^{-1} of ethidium bromide in a 1XTBE (PH 8.4) along with 100 bp molecular ladder (Sigma-Aldrich, Bengaluru, India) to estimate the size of the PCR products. The electrophoresis was carried out using 100 Volts. The gel was visualized and photographed using Gel Documentation system (Gel Doc 2000, Bio-Rad, CA, USA).

The amplified products were sequenced at Chromous Biotech. Pvt. Ltd, Bengaluru, India. The sequences obtained were blasted using the nucleotide BLAST search at the database of National Center for Biotechnology Information (NCBI, website: http://www.ncbi.nlm.nih.gov). The analysed sequences were submitted to Genbank (NCBI) and accession numbers obtained. Highly homologous sequences were aligned using Clustal-W algorithm (Thompson et al., 1994) and neighbor joining trees were generated by Molecular Evolutionary Genetics Analysis (MEGA) version 6.06 software with 1000 bootstrap replications (Tamura et al., 2011).

In vitro* screening of rhizospheric and endophytic bacterial isolates for antagonism against *P. aphanidermatum* and *R. solani

***Antifungal activity in Dual culture method*:** All rhizospheric and endophytic bacterial isolates were screened for their antagonism in dual culture assays. The pathogen was inoculated in the middle of the petriplate containing PDA medium and bacteria were streaked 3 cm away on either sides of the pathogen and incubated at 28°C for 3 days. The petriplate inoculated with pathogen alone in the absence of antagonist served as control and the experiment was done in triplicates. The radial growth of fungal mycelium on each plate was measured and the percent inhibition of growth over control (absence of antagonists) was determined using the formula:

$$\text{Inhibition of mycella growth (\%)} = \frac{X - Y}{X} \times 100$$

Where, X = mycelia growth of pathogen in absence of antagonists, Y = mycelia growth of pathogen in presence of antagonists

In the vicinity of bacterial colonies the morphology of hyphae of pathogens *P. aphanidermatum* and *R. solani* from PDA plates were observed under scanning electron microscope (SEM). The hyphal samples of pathogens were excised and fixed in 2.5% glutaraldehyde at 4°C for 2 h followed by washing in phosphate buffered saline (PBS) for 4 times, later dehydrated in a graded ethanol series (70%, 80%, 90%, and 100%) ten min each and air dried. It was then coated with gold in a POLARON, AU/PD sputter and scanned in SEM, S-3400N model (Hitachi, Tokyo, Japan) at 5.00 kV and the abnormalities in the fungal hyphae were recorded (Minaxi and Saxena, 2010).

***Antifungal activity in liquid culture*:** Dual liquid culture method was used to test the antifungal activity in potato dextrose broth (PDB). Bacterial isolates that showed more than 70% antagonism in dual culture plates were selected (Table 1). 100ml of PDB was sterilized in 250 ml conical flask and inoculated with 5 × 5 mm disc of pathogenic fungal mycelia and1ml of bacterial culture (OD 0.25 at 590 nm). It was incubated at 28 ± 2°C for five days at 100 rpm. Dry weight of the fungal culture grown with bacterial strains and control (without bacterial strains) were recorded and differences were calculated according to Broekaert et al. (1990) and percent inhibition was calculated.

Plant growth promoting (PGP) traits of rhizospheric and endophytic bacterial isolates. In *in vitro* study for antagonism only five rhizospheric and four endophytic bacteria showed more than 70% inhibition against pathogens (Table 1, 2). These isolates were tested for their plant growth promoting traits. For Indole acetic acid (IAA) production test, each isolate was inoculated to the sterile 15 ml Nutrient broth (NB) amended with L-tryptophan in test tubes and incubated at 28°C for 72 h in the dark (Gordon and Weber, 1951). Subsequently, 2 ml of this broth was centrifuged at 12,000 g for 10 min, followed by addition of 4 ml of Salkawaski reagent (Loper and Schroth, 1986) to the 1 ml of supernatant. The tubes were incubated at 37°C in the dark for 1 h. Development of a pink/red color in the medium indicated IAA production by the organisms.

Production of Hydrogen Cyanide (HCN) was determined in Nutrient agar (NA) supplemented with 4.4 g/l^{-1} of glycine (Lorck, 1948). The slant cultures were streaked on agar and Whatman No.1 filter paper strips dipped in 0.5% picric acid in 2% sodium carbonate solution were inserted from the top of each test tube, sealed with parafilm and incubated at 30°C for 4 days. A change of colour to brown or reddish-brown was recorded as positive (+) reaction.

Siderophore production of rhizospheric and endophytic bacteria was determined as described by Schwyn and Neilands (1987) using Chrome Azurol S (CAS) agar medium. The bacteria were spot inoculated and incubated at 30°C for 3-5 days. Development of yellow–orange halos around the colonies on CAS agar was considered as a positive result.

Phosphate solubalization ability of the strains was detected by spotting them on the Pikovskaya medium containing tricalcium phosphate and incubated at 28 ± 2°C for 2-3 days. Development of clear halo zone around the strains indicated positive result for phosphate solubalization (Pikovskaya, 1948).

Production of hydrolytic enzymes. Production of cell wall degrading enzymes such as protease and cellulase is a common mechanism used by bacteria to inhibit the growth of pathogenic microorganisms. For determining protease production one loop full of bacterial strains was streaked on skimmed milk agar plate (skimmed milk-100 g, peptone-5 g, agar-15 g and distilled water 1000 ml). After 48 h of incubation at 28°C, the development of clear zone around the streak was considered as a positive result. To determine celulolytic activity, carboxymethyl cellulose (CMC) was used in basal medium ($NaNO_3$- 1 g, KCl- 1 g, K_2HPO_4- 1 g, $MgSO_4$- 0.5 g, yeast extract- 0.5 g, agar-15 g, distilled water 1000 ml). The bacteria was streaked on the medium and incubated at 28°C for 3 days. The plates were flooded with 0.01% congo red solution for 15 min and destained using 1% NaCl solution for 5 min. A clear zone indicated the degradation of CMC and the bacteria was positive for cellulase production (Cappuccino and Sherman, 1992).

Evaluation of rhizospheric and endophytic bacteria for growth promotion and disease suppression in green house. our promising bacterial isolates two each from rhizopheric PGPR isolates viz., *P. putida* RBacDOB-S21, *B. cereus* RBacDOB-S24 and endophytic bacteria *P. aeruginosa* BacDOB-E19, *Enterobacter* sp. BacDOB-E21were selected for green house studies based on *in vitro* antagonism studies and PGP traits. Two sets of experiments were performed to analyze the efficacy of the bacterial isolates in controlling the rhizome rot and leaf blight diseases of turmeric under green house condition by using turmeric cultivar 'Erode local' (susceptible). Four replications were maintained for each treatment and each replication consisted of 5 earthen pots (20 cm diameter) in a completely randomized design (CRD) in a green house. The experiment was repeated twice. The talc-based formulation of the rhizospheric and endophytic bacterial isolates was

Table 1. Molecular identification of rhizospheric bacteria isolated from turmeric using 16S rDNA sequences and their antagonistic effect on the pathogenic fungus *Pythium aphanidermatum* and *Rhizoctonia solani*

Sl No	Geographical location (GPS)	Variety of turmeric	Rhizospheric bacterial isolate (PGPR)	Closest related species	% Identity	Accession No.	% Growth inhibition of *P. aphanidermatum*	% Growth Inhibition of *R. solani*
1	Chamaraja nagar, Karnataka 12.0526° N, 77.2865° E	BSR 2	RBacDOB-S1	*Pseudomonas plecoglossicida*	98	KY818291	65.0 ± 0.79^{h}	63.6 ± 0.77^{i}
2	Kollegal, Karnataka 12.1537° N, 77.1111° E	BSR 2	RBacDOB-S4	*Pseudomonas plecoglossicida*	97	KY818292	74.6 ± 0.57^{b}	72.2 ± 0.65^{c}
3	Dharwad, Karnataka 15.4589° N, 75.0078° E	Local	RbacDOB-S6	*Stenotrophomonas* sp.	99	KY883574	71.8 ± 0.37^{d}	65.2 ± 0.77^{h}
4	Dharwad, Karnataka 15.4589° N, 75.0078° E	Local	RBacDOB-S9	*Stenotrophomonas maltophilia*	97	KY883576	67.6 ± 0.33^{f}	67.9 ± 0.57^{f}
5	Dakshina Kannada, Karnataka 12.8438° N, 75.2479° E	Local	RbacDOB-S10	*Pseudomonas monteilii*	99	KY883577	67.3 ± 0.5^{f}	67.5 ± 0.33^{f}
6	Dakshina Kannada, Karnataka 12.8438° N, 75.2479° E	Local	RbacDOB-S11	*Pseudomonas aeruginosa*	97	KY883578	58.9 ± 0.79^{l}	64.2 ± 0.75^{h}
7	Erode, Tamilnadu 11.3410° N, 77.7172° E	Erode Local	RBacDOB-S14	*Pseudomonas aeruginosa*	99	KY883580	56.2 ± 0.56^{n}	53.6 ± 0.33^{o}
8	Chamarajanagar, Karnataka 12.0526° N, 77.2865° E	BSR 1	RbacDOB-S16	*Stenotrophomonas maltophilia*	98	KY883582	73.7 ± 0.73^{c}	71.4 ± 0.37^{d}
9	Chamarajanagar, Karnataka 12.0526° N, 77.2865° E	BSR 1	RBacDOB-18	*Brevibacillus agri*	98	KY883583	57.0 ± 0.32^{m}	59.0 ± 0.54^{l}
10	Hassan, Karnataka 13.0068° N, 76.0996° E	Local	RbacDOB-S20	*Pseudomonas hibiscicola*	97	KY883584	73.2 ± 0.57^{c}	57.6 ± 0.73^{m}
11	Bavanisagar, Tamilnadu 11.4792° N, 77.1341° E	BSR 2	RbacDOB-S21	*Pseudomonas putida*	98	KY883585	79.8 ± 0.97^{a}	74.6 ± 0.59^{b}
12	Dharwad, Karnataka 15.4589° N, 75.0078° E	Local	RbacDOB-S23	*Pseudomonas aeruginosa*	98	KY883586	59.3 ± 0.57^{l}	65.0 ± 0.67^{h}
13	Gobi, Tamilnadu 11.4504° N, 77.4300° E	BSR 2	RbacDOB-S24	*Bacillus cereus*	99	KY883587	79.8 ± 0.37^{a}	76.6 ± 0.87^{a}
14	Coimbatore, Tamilnadu 11.0168° N, 76.9558° E	BSR 2	RBacDOB-S26	*Bacillus thuringiensis*	99	KY883598	66.0 ± 0.33^{g}	66.2 ± 0.33^{g}
15	Coimbatore, Tamilnadu 11.0168° N, 76.9558° E	BSR 2	RbacDOB-S29	*Pseudomonas alcaliphila*	98	KY883588	65.0 ± 0.67^{h}	69.5 ± 0.57^{e}
16	Dharwad, Karnataka 15.4589° N, 75.0078° E	Local	RbacDOB-S30	*Pseudomonas putida*	97	KY883589	60.2 ± 0.77^{k}	60.0 ± 0.81^{k}
17	Kollegal, Karnataka 12.1537° N, 77.1111° E	Salem local	RbacDOB-S35	*Bacillus megaterium*	99	KY883590	57.4 ± 0.61^{m}	59.0 ± 0.57^{l}
18	Calicut, Kerala 11.2588° N, 75.7804° E	Alleppey Finger	RbacDOB-S36	*Stenotrophomonas maltophilia*	97	KY883591	61.1 ± 0.57^{j}	61.4 ± 0.44^{j}
19	Calicut, Kerala 11.2588° N, 75.7804° E	Alleppey Finger	RbacDOB-S40	*Stenotrophomonas maltophilia*	97	KY883592	58.8 ± 0.77^{l}	61.0 ± 0.57^{j}
20	Salem, Tamilnadu 11.6643° N, 78.1460° E	Salem local	RbacDOB-S41	*Pseudomonas plecoglossicida*	98	KY883593	61.8 ± 0.59^{j}	64.2 ± 0.33^{h}
21	Mysore, Karnataka 12.2958° N, 76.6394° E	Local	RbacDOB-S51	*Pseudomonas stutzeri*	98	KY883594	63.4 ± 0.75^{i}	60.0 ± 0.57^{k}
22	H.D.kote, Karnataka 12.0879° N, 76.331° E	Local	RbacDOB-S52	*Ochrobactrum* sp.	98	KY883595	56.0 ± 0.67^{n}	58.2 ± 0.37^{l}
23	Guntur, Andrapradesh 16.3067° N, 80.4365° E	Duggirala	RbacDOB-S53	*Pseudomonas aeruginosa*	97	KY883596	66.7 ± 0.66^{g}	68.9 ± 0.97^{e}
24	Hassan, Karnataka 13.0068° N, 76.0996° E	Local	RbacDOB-S56	*Bacillus cereus*	98	KY883597	56.0 ± 0.57^{n}	57.6 ± 0.91^{m}
25	H.D.Kote, Karnataka 12.0879° N, 76.331° E	Local	RBacDOB-S57	*Exiquebacterium aurantiacum*	99	KY924598	50.1 ± 0.77^{q}	53.0 ± 0.87^{o}

Table 1. Continued

Sl No	Geographical location (GPS)	Variety of turmeric	Rhizospheric bacterial isolate (PGPR)	Closest related species	% Identity	Accession No.	% Growth inhibition of *P. aphanidermatum*	% Growth Inhibition of *R. solani*
26	Wayanad, Kerala 11.6854° N, 76.1320° E	Alleppey Finger	RBacDOB-S62	*Acinetobacter* sp.	97	KY971450	50.1±0.57^{q}	52.6±0.81^{o}
27	Kadapa, Andhra Pradesh 14.4674° N, 78.8241° E	Tekurpeta	RBac-DOBS70	*Enterobacter* sp.	99	KY971459	70.1±0.73^{e}	69.0±0.57^{e}
28	Kadapa, Andhra Pradesh 14.4674° N, 78.8241° E	Sugandham	RbacDOB-S72	*Rhizobium pusense*	98	KY883605	63.1±0.57^{i}	61.8±0.53^{j}
29	Calicut, Kerala 11.2588° N, 75.7804° E	Alleppey Finger	RBacDOB-S74	*Brevibacillus brostelensis*	99	KY982872	54.6±0.63^{o}	54.6±0.97^{n}
30	Salem, Tamilnadu 11.6643° N, 78.1460° E	Salem local	RBacDOB-S78	*Alcaligenes faecalis*	99	KY982875	53.0±0.97^{p}	57.0±0.85^{m}

Values are the mean of three independent replicates (n = 3). ± indicate standard errors. Mean followed by the same letter (s) within the same column are not significantly ($P \leq 0.05$) different according to Tukey's HSD.

prepared containing population densities of 3×10^8 bacteria/g talc powder (Shanmugam et al., 2011). The talc based formulations 20 g/L of each rhizospheric bacteria *P. putida* RBacDOB-S21, *B. cereus* RBacDOB-S24 and endophytic bacterial isolates *P. aeruginosa* BacDOB-E19, *Enterobacteria* sp. BacDOB-E21 were applied as rhizome treatment. The rhizomes were surface sterilized with 2% sodium hypochlorite for 1 min and soaked in sterile distilled water containing 20 g/l formulation. The suspension was drained off after 12 h and the rhizomes were air dried overnight under a sterile air stream. The rhizomes with three nodes were planted in earthen pots containing sterilized soil of 5 kg.

For first set of experiment, the pathogen *P. aphanidermatum* was multiplied on sand-corn meal medium and the rhizomes were infected after 30 days of planting at a ratio of 1:19 (sand-maize inoculum: soil), i.e., 300 g having 16×10^4 cfu g^{-1} of medium per pot (Shanmugam et al., 2013). For second set, the 30 day old BCA treated turmeric plants were challenge inoculated with *R. solani* by inserting young immature sclerotia, 2 sclerotia per sheath (Sriraj et al., 2014).

Soil applications (8 g) of biocontrol formulation containing 3×10^8 bacteria/g talc powder was applied three times upto 90 days at intervals of 15 days for first set of plants (*P. aphanidermatum* inoculated). For the second set of plants (*R. solani* inoculated) soil application of bacteria was followed by foliar spray of rhizospheric and endophytic bacteria at 10^8 spores/ml suspended in water.

Carbendazim (0.1%) + Mancozeb (0.25%) combination was applied for rhizome treatment and soil drenching (20 ml) and for *R. solani* inoculated plants the rhizome and soil treatment was followed by spray of Carbendazim (0.1%) + Mancozeb (0.25%) served as fungicide control. The rhizomes without treatment and pathogens treated alone served as controls. The control plants showed systemic infection in 4-6 weeks of inoculation at temperature of 20-30°C. The disease severity on rhizome was assessed and the PDI was calculated as described below

$$PDI = \frac{\text{Number of infected plants}}{\text{Total number of inoculated plants}} \times 100$$

The intensity of leaf blight disease was recorded after seven days of inoculation, with 0-9 scale of the Standard Evaluation System of rice, IRRI (2002) and expressed as percent disease index (Sriraj et al., 2014).

$$PDI = \frac{\text{Sum of individual ratings}}{\text{Total number of plants observed}} \times \frac{100}{\text{Maximum grade}}$$

A separate set with four treatments along with untreated control and pathogenic control was maintained for rhizome colonization assay and growth promotion studies. The plant length and fresh rhizome yield of the plants were recorded at the time of harvest.

Rhizome colonization assay by Confocal Microscopy. BCA (PGPR *B. cereus* and *P. aeruginosa*) treated turmeric rhizomes (as explained earlier) of 60 days old, were removed intact from the soil. The rhizomes were thoroughly washed in running tap water followed by distilled water. The rhizomes were surface sterilized with 2% (w/v) sodium hypochoride solution for 30 s. Experiments were performed twice, and rhizomes from three plants were

Table 2. Molecular identification of endophytic bacteria isolated from turmeric rhizome using 16S rDNA region and their antagonistic effect on the pathogenic fungus *P. aphanidermatum* and *R. solani*

Sl No.	Geographical location (GPS)	Variety of turmeric	Endophytic bacterial isolate	Closest related sps.	% Identity	Accession No.	% Growth inhibition of *P. aphanider matum*	% Growth inhibition of *R. solani*
1	Chamaraja nagar, Karnataka 12.0526° N, 77.2865° E	BSR 2	BacDOB-E2	*Alcaligenes faecalis*	97	KY883599	64.0±0.57[g]	62.6±0.67[i]
2	Hassan, Karnataka 13.0068° N, 76.0996° E	Local	BacDOB-E3	*Pseudomonas* sp.	99	KY883600	61.6±0.44[h]	65.2±0.33[h]
3	Kollegal, Karnataka 12.1537° N, 77.1111° E	BSR 2	BacDOB-E4	*Pseudomonas aeruginosa*	99	KY883601	69.0±0.77[e]	67.0±0.57[g]
4	Dharwad, Karnataka 15.4589° N, 75.0078° E	Local	BacDOB-E5	*Citrobacter* sp.	99	KY883602	70.8±0.57[d]	68.2±0.43[f]
5	H.D Kote Karnataka 12.0879° N, 76.331° E	Local	BacDOB-E7	*Terribacillus saccharophilus*	98	KY883603	57.0±0.63[k]	59.4±0.57[l]
6	Mysore, Karnataka 12.2958° N, 76.6394° E	Local	BacDOB-E8	*Pseudomonas aeruginosa*	99	KY883604	69.6±0.49[e]	68.9±0.33[f]
7	Dandeli, Karnataka 15.2497° N, 74.6174° E	Local	BacDOB-E9	*Pseudomonas plecoglossicida*	97	KY883606	66.3±0.57[f]	69.5±0.37[e]
8	Madikeri, Karnataka 12.4244° N, 75.7382° E	Local	BacDOB-E11	*Pseudomonas aeruginosa*	99	KY883607	58.9±0.63[i]	60.2±0.57[k]
9	Kollegal, Karnataka 12.1537° N, 77.1111° E	BSR 1	BacDOB-E12	*Acinetobacter* sp.	98	KY924605	73.2±0.73[c]	71.6±0.33[d]
10	Coimbatore, Tamilnadu 11.0168° N, 76.9558° E	BSR 2	BacDOB-E14	*Alcaligenes* sp.	99	KY776473	66.8±0.51[e]	65.8±0.57[h]
11	Salem, Tamilnadu 11.6643° N, 78.1460° E	Salem local	BacDOB-E15	*Pseudomonas aeruginosa*	99	KY883608	73.8±0.61[c]	72.6±0.77[c]
12	Gobi, Tamilnadu 11.4504° N, 77.4300° E	Erode local	BacDOB-E17	*Pseudomonas aeruginosa*	99	KY924595	58.0±0.33[j]	60.0±0.79[k]
13	Bavanisagar, Tamilnadu 11.4792° N, 77.1341° E	BSR 2	BacDOB-E18	*Arthrobacter* sp.	97	KY924596	73.2±0.43[c]	66.6±0.74[g]
14	Erode, Tamilnadu 11.3410° N, 77.7172° E	BSR 1	BacDOB-E19	*Pseudomonas aeruginosa*	99	KY924597	76.9±0.57[a]	74.6±0.53[a]
15	Sathyamangalam, Tamilnadu 11.5048° N, 77.2384° E	BSR 2	BacDOB-E20	*Bacillus cereus*	99	KY924599	59.3±0.63[i]	63.0±0.57[i]
16	Guntur, Andrapradesh 16.3067° N, 80.4365° E	Duggirala	BacDOB-E21	*Enterobacter* sp.	98	KY924600	75.7 ±0.57[b]	73.4±0.87[b]
17	Kadapa, Andrapradesh 14.4674° N, 78.8241° E	Sugandham	BacDOB-E22	*Bacillus cereus*	98	KY924601	66.0±0.83[e]	68.2±0.47[f]
18	Wayanad, Kerala 11.6854° N, 76.1320° E	Alleppey Finger	BacDOB-E34	*Acinetobacter* sp.	97	KY924606	57.0±0.47[k]	54.5±0.53[n]
19	Wayanad, Kerala 11.6854° N, 76.1320° E	Alleppey Finger	BacDOB-E47	*Enterobacter* sp.	98	KY924602	61.2±0.77[h]	62.0±0.59[j]
20	Calicut, Kerala 11.2588° N, 75.7804° E	Alleppey Finger	BacDOB-E52	*Klebsiella* sp.	99	KY986971	57.2±0.33[k]	58.6±0.77[m]

Values are the mean of three independent replicates (n = 3). ± indicate standard errors. Mean followed by the same letter (s) within the same column are not significantly ($P \leq 0.05$) different according to Tukey's HSD.

analyzed for each data. The rhizome material (1 cm) was transferred to trichloroacetic acid fixation solution (0.15% (wt/vol) trichloroacetic acid in 4:1 (vol/vol) ethanol/chloroform). Sections from rhizome were hand cut about 1 cm from the surface and approximately 50 µm thick segments were mounted on a microscope slide. Bacteria in rhizome segments were stained by 5 µl of Ethidium bromide (EtBr 1.25 mg ml^{-1}) (Someya, 1995). Subsequently, segments were incubated at room temperature for 10 min. After incubation the segments were mounted on clean glass slides and examined immediately. Confocal fluorescence images were recorded on Advanced Spectral Confocal Microscope System-LSM 710 (Carl Zeiss, Jena, Germany). It was excited with a 514-nm laser line and detected at 552-693 nm, Channels EtBr and T-PMT were used (Hansen et al., 1997).

Statistical analysis. Statistical analyses were performed using Ans. SPSS, Version 17 (Chicago, IL, USA) and MS-Exel version 2007 (Microsoft, Washington, DC, USA). A completely randomized design was used for all the experiments, with 3 replications for each treatment. Differences between experimental outcomes were analysed using Tukey's HSD test and $P \leq 0.05$ was considered not significantly different.

Results

Morphological and Biochemical traits of rhizospheric and endophytic bacteria isolated from turmeric. A total of 30 PGPR isolates from Rhizosphere and 20 endophytic bacteria from different geographic regions of South India viz., Karnataka, Kerala, Tamilnadu and Andhra Pradesh states were obtained. The isolates belonged to *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Alcaligenes*, *Acinetobacter*, *Ochrobactrum*, *Exiquebacterium*, Rhizobium, *Klebsiella*, *Citrobacter* and *Terribacillus* species. All the isolates were motile rods. Out of 50 isolates, 43 were Gram-negative while 7 were Gram-positive. Besides, 12 for methyl red test, 42 for citrate utilization, 33 for oxidase, 4 for VP, 22 for succinic acid production, 1 for starch hydrolysis, 31

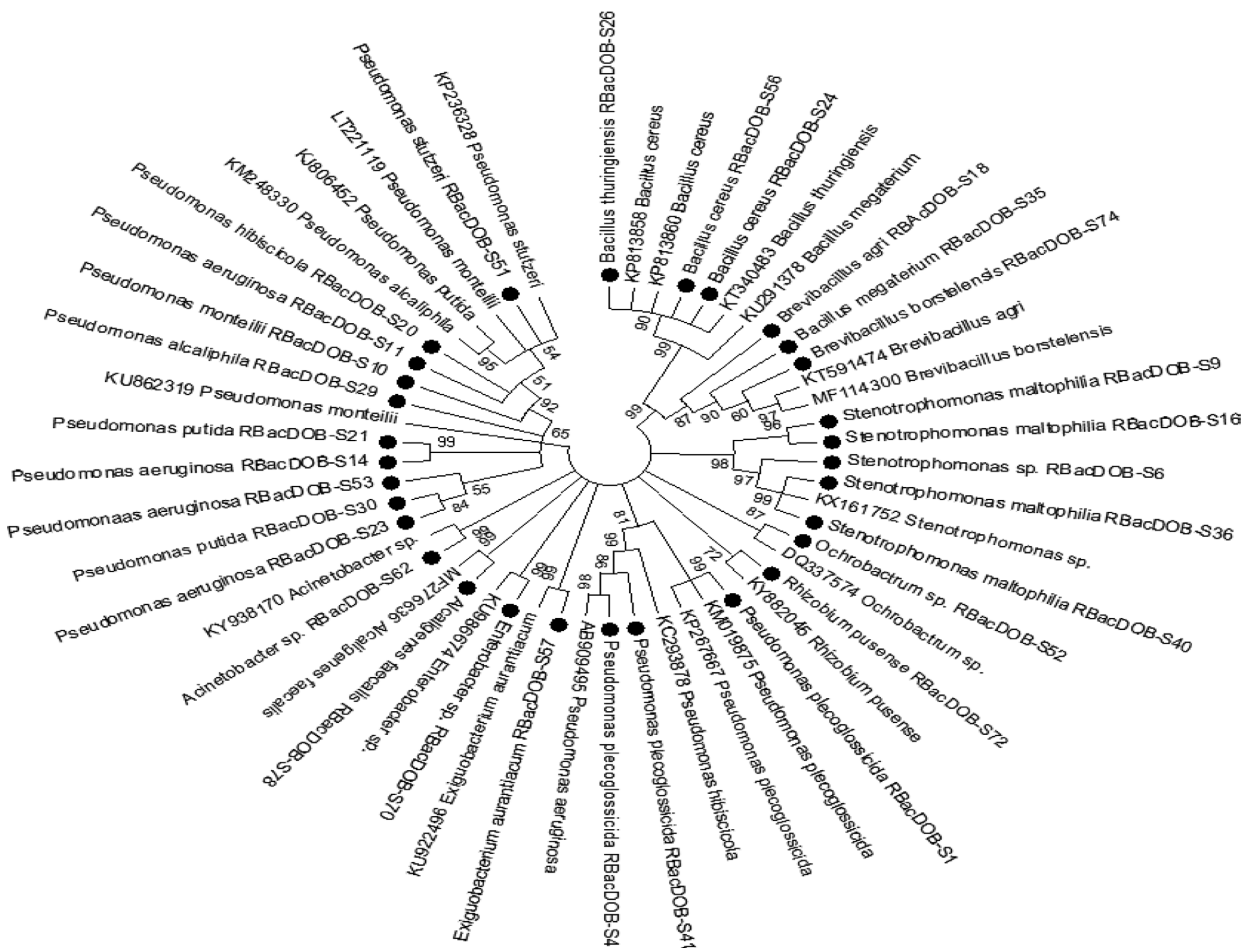

Fig. 1. Neighbor-joining tree based on analysis of partial 16 s rDNA nucleotide sequences of rhizospheric bacterial isolates of turmeric (●-symbol represents bacteria isolated in present study).The data of type strains of related species were from GenBank database. Numbers above and below the nodes indicate bootstrap values generated after 1000 replications.

isolates for NH3 production, 41 isolates for casein hydrolysis were found positive, while all the isolates were positive for catalase production (Supplementary Table 1).

Molecular characterization of rhizospheric and endophytic bacteria. The quality of genomic DNA of rhizospheric and endophytic bacteria was good as evident from the ratio of 260/280, which was 1.72. All the bacterial isolates the DNA was subjected to PCR amplification with specific primer for the 16s rDNA region which generated bands ranging from 630-700 bp. The sequences of 30 rhizospheric and 20 endophytic bacterial isolates showed 97-99% similarity with the species in Genebank during Blast analysis. The Blast search confirmed the presence of *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Alcaligenes*, *Acinetobacter*, *Ochrobactrum*, *Exiquebacterium*, *Rhizobium*, *Klebsiella*, *Citrobacter* and *Terribacillus* species. All the 50 bacterial sequences were submitted to Genebank (NCBI) and their accession numbers were obtained (Table 1, 2).

The Phylogenetic trees of rhizospheric and endophytic bacterial isolates constructed from 16s rDNA sequences along with the related reference species retrieved from Genbank of NCBI confirmed these isolates belong to *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Alcaligenes*, *Acinetobacter*, *Ochrobactrum*, *Exiquebacterium*, *Rhizobium*, *Klebsiella*, *Citrobacter* and *Terribacillus* species by clustering of each of the isolate to its corresponding group (Fig. 1, 2).

***In vitro* antagonism.** All the isolates were screened against *P. aphanidermatum* and *R. solani* by dual culture method (Fig. 1, 2). Five PGPR isolates viz., RBacDOB-S4, RBacDOB-S16, RBacDOB-S21, RBacDOB-S24, RBacDOB-S70 out of 30 rhizosoheric bacterial isolates and four endophytic bacteria BacDOB-E12, BacDOB-E15, BacDOB-E19, BacDOB-E21 out of 20 exhibited > 70% growth inhibition against both the pathogens (Table 1). SEM studies showed that the endophytes cause deformities in the mycelia of both *P. aphanidermatum* and *R. solani* pathogens. The deformities included hyphal fragmentation, perforation, desiccation of hyphae and mycelia degenera-

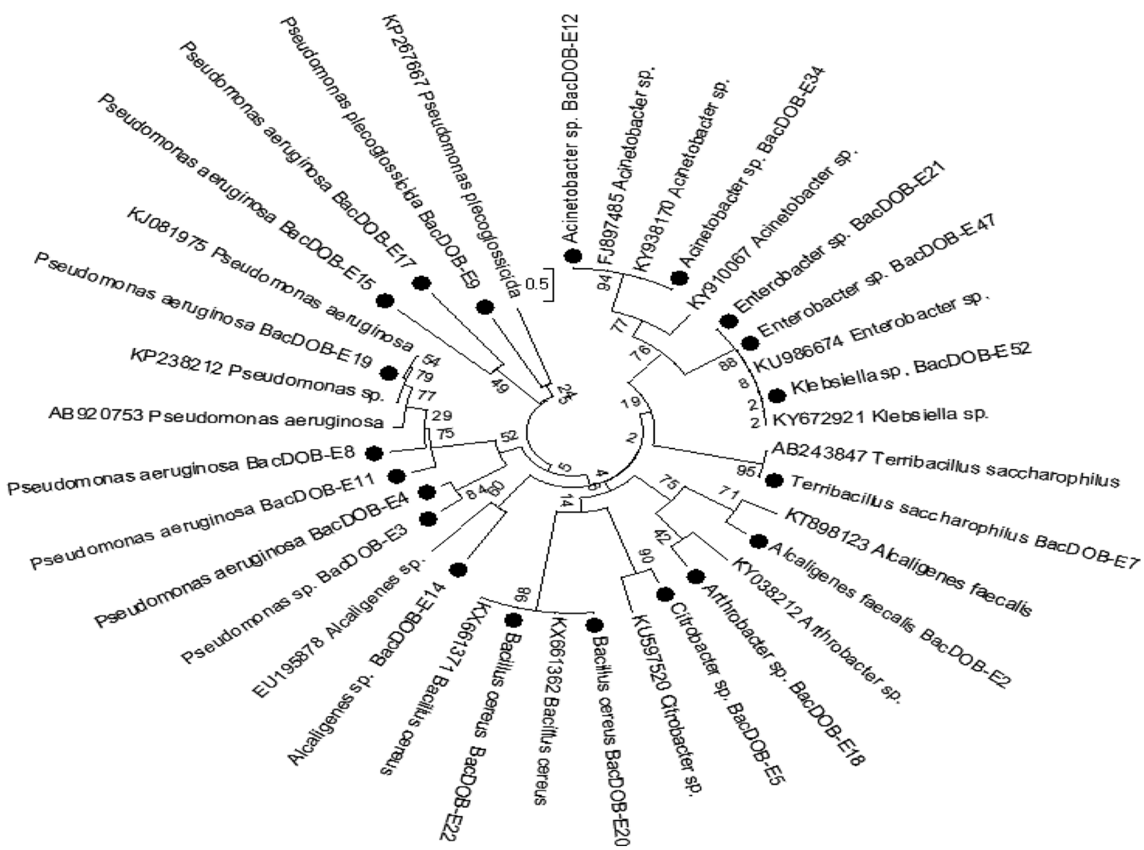

Fig. 2. Neighbor-joining tree based on analysis of partial 16s rDNA nucleotide sequences of endophytic isolates of turmeric (●- symbol represents bacteria isolated in present study). The data of type strains of related species were from GenBank database. Numbers above and below the nodes indicate bootstrap values generated after 1000 replications.

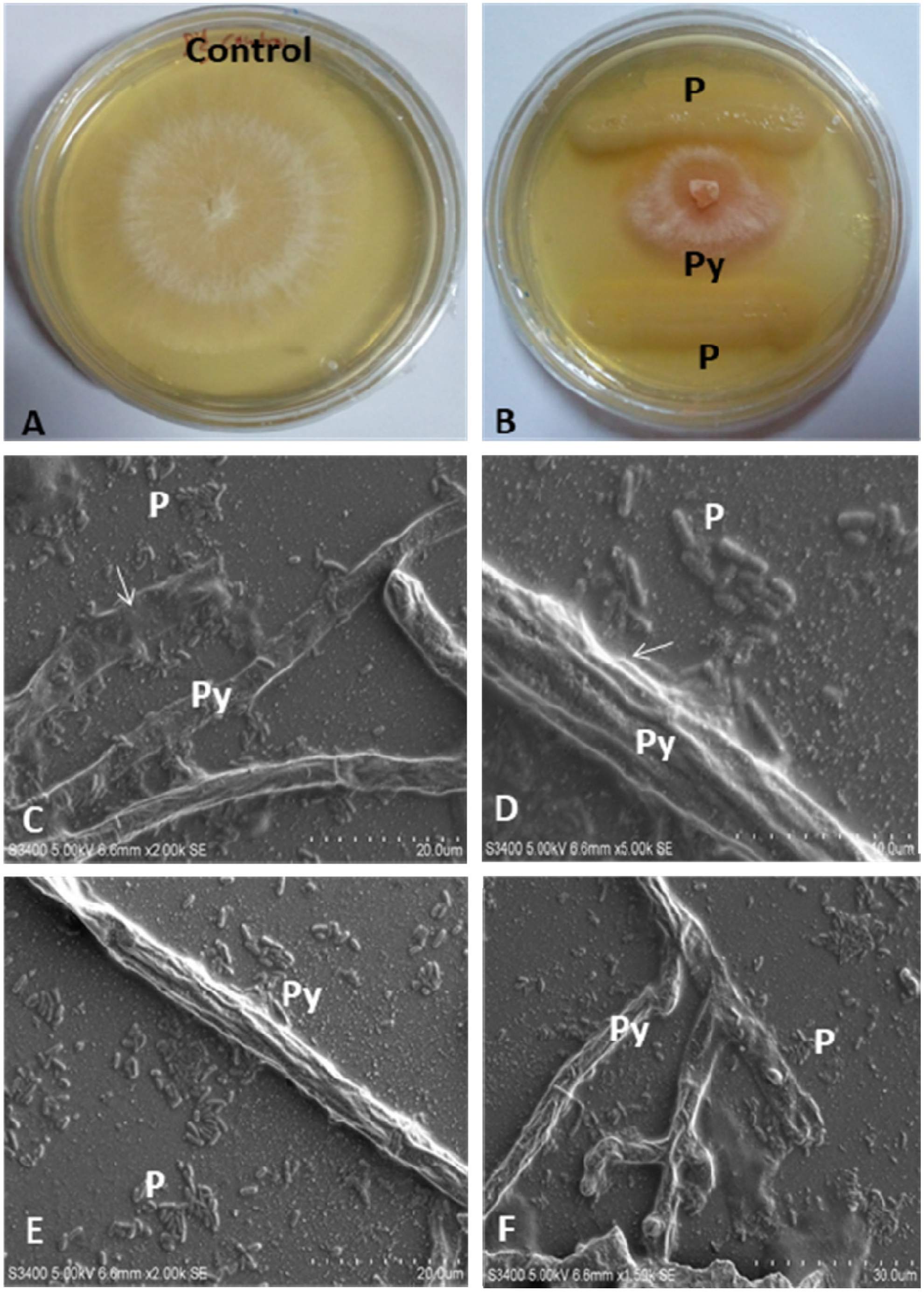

Fig. 3. Photographs of dual culture tests, A- *P. aphanidermatum* control, B-Dual culture of pathogen and endophytic bacterial isolate; Scanning electron micrograph showing morphological changes in *P. aphanidermatum* mycelia inhibited by endophytic bacterial isolate *P. aeruginosa* BacDOB-E19; C- Breakage of hyphae, D and E- Lysis of hyphae, F-shows shrivelling and desication of the mycelium of *P. aphanidermatum*. Py = *P. aphanidermatum*, P = *P. aeruginosa.*

tion (Fig. 3, 4) which finally resulted in fungal death.

In liquid dual culture assays *B. cereus* RBacDOB-S24 showed 86% and 84% growth inhibition of *P. aphanidermatum* and *R. solani*, followed by endophytic *P. aeruginosa* BacDOB- E19 that showed 85% and 82% growth inhibition respectively (Fig. 5, 6).

***In vitro* evaluation of plant growth promoting potentials.** All the five rhizospheric PGPR isolates *viz*., RBacDOB-S4, RBacDOB-S16, RBacDOB-S21, RBacDOB-S24, RBacDOB-S70 and four endophytic bacterial isolates BacDOB-E12, BacDOB-E15, BacDOB-E19, BacDOB-E21 were able to produce IAA with l-tryptophan as a precursor. Except BacDOB-E12, the other isolates exhibited positive results for HCN production, while three isolates solubilized inorganic phosphate $Ca_3(PO_4)$ and four isolates were positive to cellulase activity. PGPR isolates RBacDOB-S21, RBacDOB-S24 and endophytic bacterial

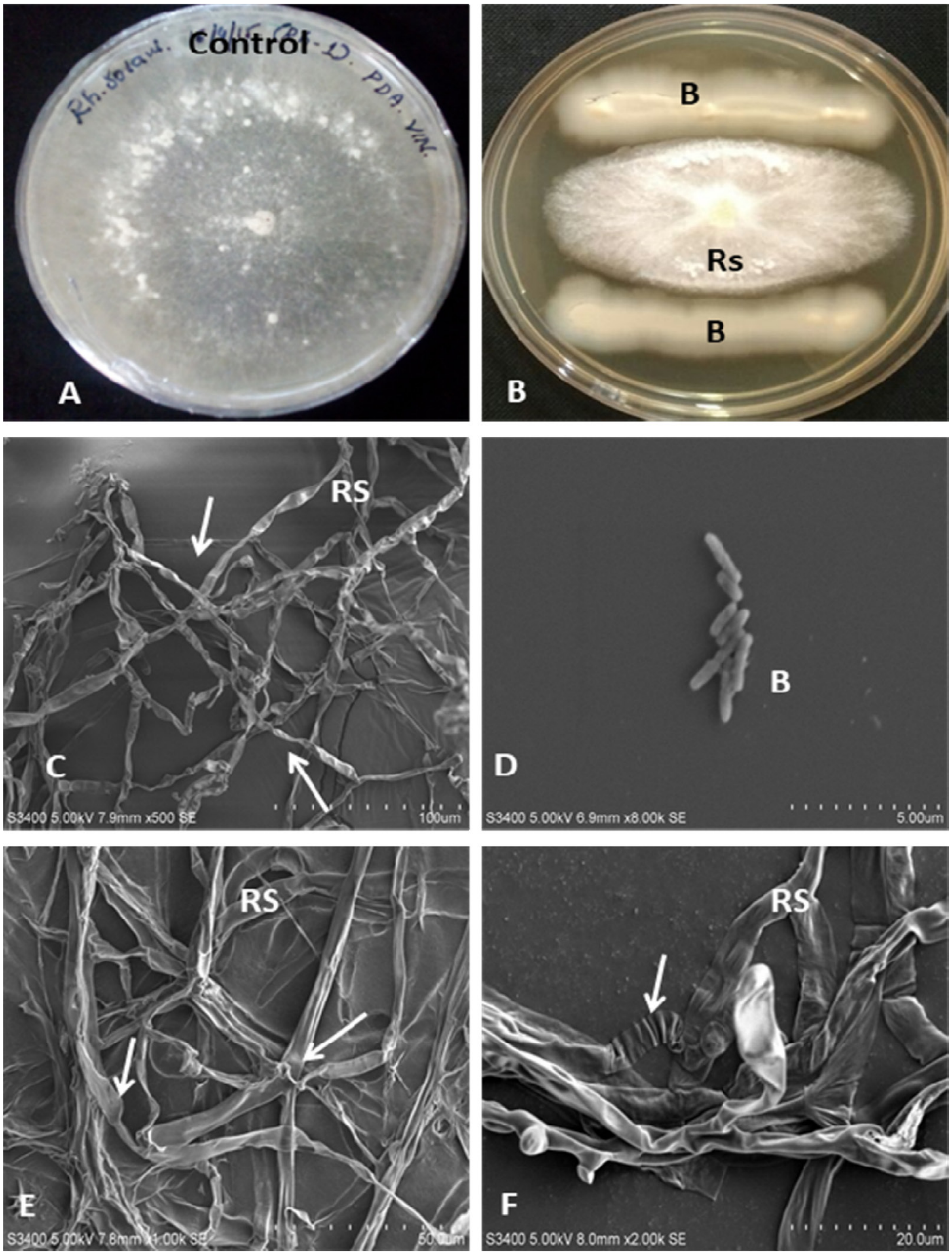

Fig. 4. Photographs of dual culture tests, A- *R. solani* control, B-Dual culture of pathogen and rhizospheric bacterial isolate RBacDOB-S24 *B. cereus*; Scanning electron micrograph showing morphological changes in *R. solani* mycelia inhibited by rhizospheric bacterial isolate *B. cereus* RBacDOB-S24; C- Breakage of hyphae, E and F- Arrow shows shrivelling and desication of the mycelium of *R. solani*. RS = *R. solani*, B = *B. Cereus.*

isolates BacDOB-E19, BacDOB-E21 were positive for multiple PGP traits viz., IAA, HCN, Siderophore production, inorganic phosphate solubalization, production of cellulase and protease (Table 3, Supplementary Fig. 1).

Greenhouse experiments. The severity of leaf blight and rhizome rot disease was markedly reduced in the four individual treatments of rhizospheric PGPR isolates RBacDOB-S21, RBacDOB-S24; endophytic bacterial isolates BacDOB-E19 and BacDOB-E21. Isolate RBacDOB-S24 significantly reduced the disease incidence of rhizome rot and leaf blight (by 16.4% and 15.5% respectively), followed by the endophytic bacterial isolate BacDOB-E19 reduced the disease incidence of rhizome rot and leaf blight (by 17.5% to 17.7%) respectively. The rhizospheric PGPR isolate RBacDOB-S24 enhanced the plant height to 89.09 and 86.71 cm; fresh rhizome weight to 392 and 339 g against *P. aphanidermatum* and *R. solani* pathogens

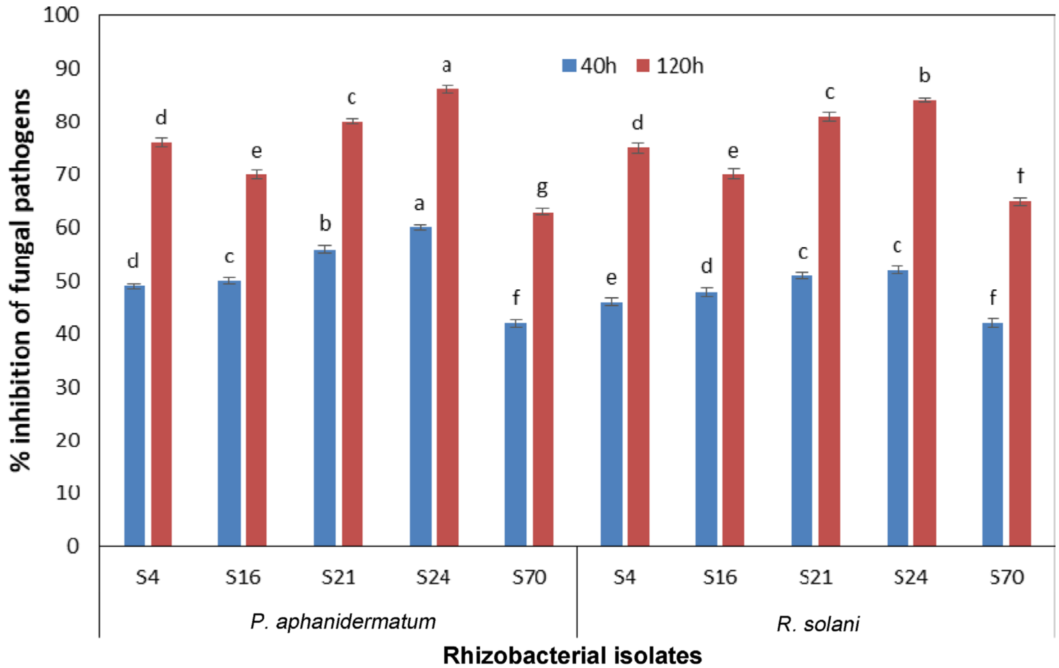

Fig. 5. Antagonistic activity of rhizospheric bacterial isolates against *P. aphanidermatum* and *R. solani* at two different time intervals in dual liquid culture assay.Py1 and Rh1 = % inhibition of fungi after 48 h; Py2 and Rh2 = % inhibition of fungi after 120 h. Each value is the mean for four replicates (n = 4) and bars sharing the same letters are not significantly different ($P \leq 0.05$) according to Turkey's HSD. The vertical bars indicates the standard error.

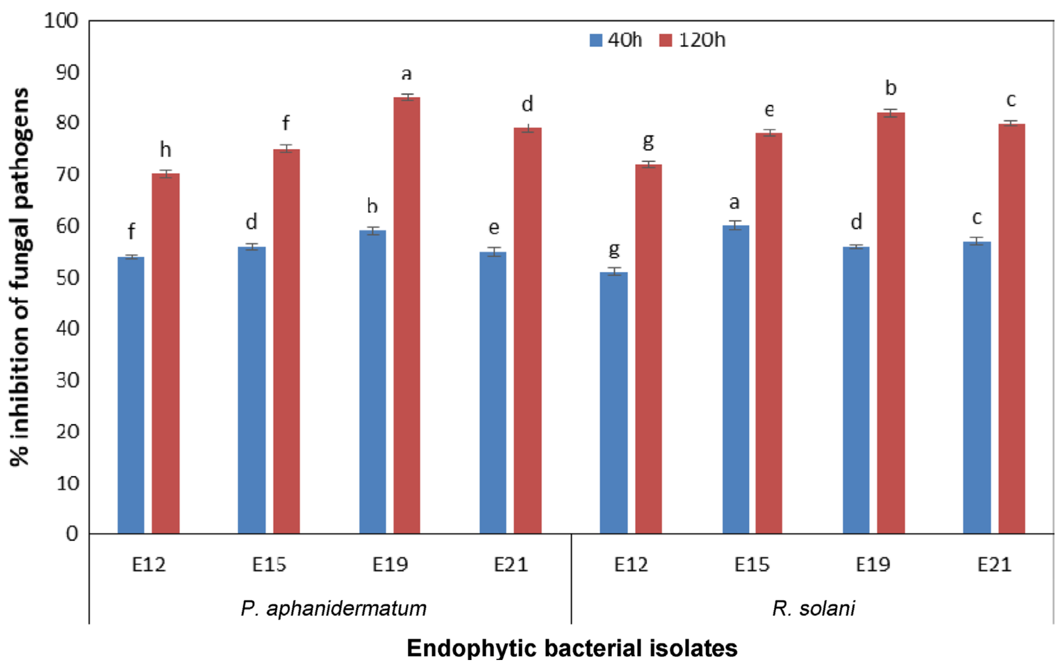

Fig. 6. Antagonistic activity of endophytic bacterial isolates against *P. aphanidermatum* and *R. solani* at two different time intervals in dual liquid culture assay. Py1 and Rh1 = % inhibition of fungi after 48 h; Py2 and Rh2 = % inhibition of fungi after120 h. Each value is the mean for four replicates (n = 4) and bars sharing the same letters are not significantly different ($P \leq 0.05$) according to Turkey's HSD. The vertical bars indicates the standard error.

respectively, followed by the endophytic isolate BacDOB-E19 enhanced the plant height to 82.75 and 80.37 cm; fresh rhizome weight 375 and 305 g respectively when compared to untreated control (Supplementary Table 2, Table 4, Fig. 7).

Rhizome colonization assay by Confocal Microscopy. The colonization in turmeric rhizomes was analysed by confocal microscopy. The results revealed that the PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa*BacDOB-E19 treated rhizomes showed colonization between the cells (Fig. 8).

Discussion

In this study, 30 PGPR were isolated from turmeric rhizosphere and 20 endophytes from healthy rhizome. The identity of the isolates was confirmed by morphological,

Table 3. Characterization of selected rhizospheric and endophytic bacterial isolates for plant growth promoting potentials

Sl. No.	Isolate No.	Species Identified	IAA production	HCN production	Phosphate solubilization	Cellulase activity	Protease activity	Siderophore production
		Rhizospheric bacterial isolates						
1	RBacDOB-S4	*Pseudomonas plecoglossicida*	+	+	+	+	+	+
2	RBacDOB-S16	*Stenotrophomonas maltophilia*	+	+	+	+	+	+
3	RBacDOB-S21	*Pseudomonas putida*	+	+	+	+	+	+
4	RBacDOB-S24	*Bacillus cereus*	+	+	+	+	+	−
5	RBacDOB-S70	*Enterobacter* sp.	+	+	+	−	+	+
		Endophytic bacterial isolates						
1	BacDOB-E12	*Acinetobacter* sp.	+	+	+	−	+	+
2	BacDOB-E15	*Pseudomonas aeruginosa*	+	+	+	+	+	+
3	BacDOB-E19	*Pseudomonas aeruginosa*	+	+	+	+	+	+
4	BacDOB-E21	*Enterobacter* sp.	+	+	+	−	+	+

+: represents positive, −: represents negative.

Table 4. Management of rhizome rot and leaf blight diseases of turmeric caused by *P. aphanidermatum* and *R. solani* by rhizospheric and endophytic bacterial isolates in green house

Treatment	Rhizome rot			Leaf blight		
	Fresh rhizome weight (g)	Plant length (Cms)	PDI	Fresh rhizome weight (g)	Plant length (Cms)	PDI
Rhizospheric bacterial isolates						
B. cereus RBacDOB-S24	392±2.73^{a}	89.09±1.73^{a}	16.4±0.79^{f}	339±1.47^{a}	86.71±0.37^{a}	15.5±0.57^{f}
P. putida RBacDOB-S21	381±2.51^{b}	83.17±1.37^{b}	18.2±0.47^{d}	327±1.74^{b}	82.57±0.75^{b}	16.6±0.73^{e}
Endophytic bacterial isolates						
P. aeruginosa BacDOB-E19	375±1.79^{c}	82.75±1.27^{c}	17.5±0.73^{e}	305±1.72^{c}	80.37±0.77^{c}	17.7±0.32^{d}
Enterobacteria BacDOB-E21	362±2.23^{d}	81.65±1.57^{d}	20.7±0.43^{b}	290±1.23^{d}	78.60±1.31^{d}	18.8±1.07^{c}
Carbendazim (0.1%) + Mancozeb (0.25%)	279±1.97^{e}	65.47±0.97^{e}	19.4±0.71^{c}	257±1.27^{e}	63.75±0.75^{e}	21.1±0.33^{b}
Uninoculated control	257±1.79^{f}	52.75±0.73^{f}	0.0	247±1.73^{f}	50.57±0.39^{f}	0.0
Pathogenic control	207±1.73^{g}	41.59±0.77^{g}	79.0±0.54^{a}	207±1.43^{g}	41.75±0.62^{g}	77.7±0.75^{a}

The values are mean of three replications ± SE. Mean followed by the same letter (s) within the same column are not significantly ($P \leq 0.05$) different according to Tukey's HSD.

biochemical and 16S rDNA sequences. The 16S rDNA sequences were submitted to Genbank (NCBI) (Table 1). A majority of the isolates belonged to *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Alcaligenes*, *Acinetobacter*, *Ochrobactrum*, *Exiquebacterium*, Rhizobium, *Klebsiella*, *Citrobacter* and *Terribacillus* species. All the isolates (30 PGPR and 20 endophytes) were screened for nutrient solubilization, biochemical traits and antagonism in order to select the isolates that showed the most promising results with regard to growth promotion and biocontrol of rhizome rot and leaf blight diseases in turmeric plants.

Many of the soil-borne fungal diseases have been successfully controlled by the use of antagonists (Weller, 1988). The *in vitro* screening of rhizosphere bacterial isolates and endophytic bacteria for antagonism against *P. aphanidermatum* an *R. solani* indicated that nine isolates exhibited > 70% inhibition (Table 1, 2) of both the pathogens in dual culture and liquid culture assays. Endophytic bacteria, used as whole cells (Rajendran and Samiyappan, 2008) and cell-free culture filtrates (Li et al., 2012) suppressed some plant pathogenic fungi due to antimicrobial compounds that cause alteration in structural architect and lysis of mycelia (Yuan et al., 2012). Our SEM results also revealed the morphological deformities of mycelia of both the pathogens. Similar observations have been reported in *Pythium myriotylum* due to the effect of extracellular metabolites by *Bacillus* sp. (Jimtha et al., 2016).

The rhizospheric and endophytic isolates have also exhibited significant plant growth promoting traits. Bacteria producing IAA promotes plant growth directly by increas-

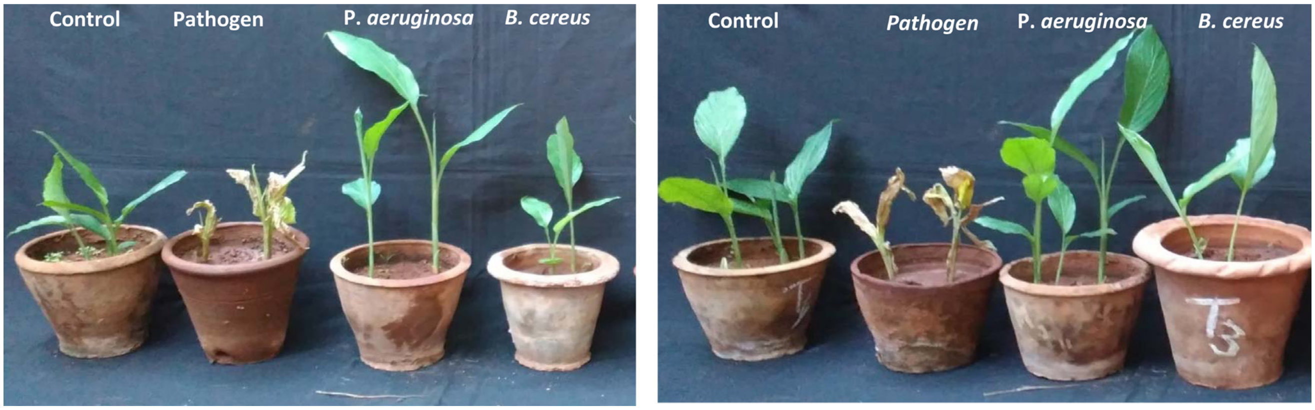

Fig. 7. Performance of antagonistic microbes against rhizome rot (*P. aphanidermatum*) and leaf blight (*R. solani*) diseases in green house; A- Growth promotion of selected PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 antagonist, along with *P. aphanidermatum* pathogenic control and Untreated control. B- Growth promotion of selected PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 antagonist, along with *R. solani* pathogenic control and Untreated control.

ing root surface area and length by stimulating plant cell elongation or by affecting the cell division thereby providing greater access to soil nutrients by plants (Glick, 1995). In plant growth promotion analysis endophytic strains *P. aeruginosa* BacDOB-E19 and PGPR strains *B. cereus* RbacDOB-S24 produced significant amount of IAA, earlier reported in *B. cereus* (Rana et al., 2011) and *P. putida* (Jasim et al., 2014). Production of siderophores, indirectly influence the plant growth by binding to the available form of iron in the rhizosphere making it unavailable to the phytopathogens and protecting the plant health. Siderophore production by *Bacillus* sp. and *Pseudomonas* sp. in this study evidenced for one of the biocontrol mechanism similar to previous reports (Jasim et al., 2014; Kumar et al., 2016). Siderophore produced by *Pseudomonas* sp. has been reported to be an important mechanism of biological control of *Pythium* diseases (Matthijs et al., 2007). The results supported that endophytic and PGPR bacterial strains viz., *B. cereus*, *P. aeruginosa*, *P. putida* solubilize phosphate as reported previously for *Bacillus* sp. and *P. putida* (Forchetti et al., 2007; Pandey et al., 2006). In the present study, *B. cereus* and *P. aeruginosa* strains showed production of HCN similar to the previous results reported for *Pseudomonas* strains that controlled the plant root pathogens including *F. oxysporum* and *R. solani* by production of siderophores, HCN and lytic enzymes (Nagrajkumar et al., 2004). The selected strains *B. cereus* and *P. aeruginosa* produced cell wall degrading enzymes such as cellulases and proteases, which reported earlier as important in breakdown of cell walls of oomycete pathogens such as *Phytopthora* (Valois et al., 1996) and *Pythium* spp. (El-Tarabily et al., 2009). Similarly, the lytic enzyme production by rhizospheric *P. fluorescence* are known to be involved in the control of pathogens like *F. oxysporum* and *R. solani* (Nagrajkumar et al., 2004).

The four promising biocontrol agents (BCA's), two from rhizosphere and two from endophytes were then tested in the green house for their disease suppression and plant growth promotion abilities compared to untreated and pathogenic controls. Green house results suggested that the PGPR PGPR *B. cereus* and endophyte *P. aeruginosa* showed significant disease reduction also enhanced the yield of turmeric when compared to untreated control. Similar to our reports on turmeric, there are several studies on growth promotion by PGPR in other crops like maize (Egamberdiyeva, 2007), tomato (Almaghrabi et al., 2013), common bean (Martins et al., 2013) and ginger (Dinesh et al., 2015) have been reported. Endophyte *B. cereus* and *P. fluorescens* possesses biocontrol potential in crops like cotton and chilli, against root rot and damping off caused by *R. solani* and *P. aphanidermatum* respectively (Muthukumar et al., 2011; Pleban et al., 1997). Bacterial endophytes viz., *B. cereus*, *B. thuringiensis*, *B. pumilis*, *P. putida* and *Clavibacter michiganensis*, isolated from turmeric rhizomes exhibited PGP traits and antifungal activity against *F. solani*, *A. pullulans*, *Alternaria alternata* and *B. fulva* pathogens (Kumar et al., 2016). The culture supernatant of *B. cereus* QQ308 was active against numerous plant pathogenic fungi and has used in biological control (Chang et al., 2007). The potential of *Bacillus* cereus as a biocontrol agent against *Fusarium solani* causing rhizome rot in turmeric has been reported previously (Chauhan et al., 2016). Control of anthracnose rot caused by *Colletotrichum acutatum* in harvested loquat fruit inducing disease

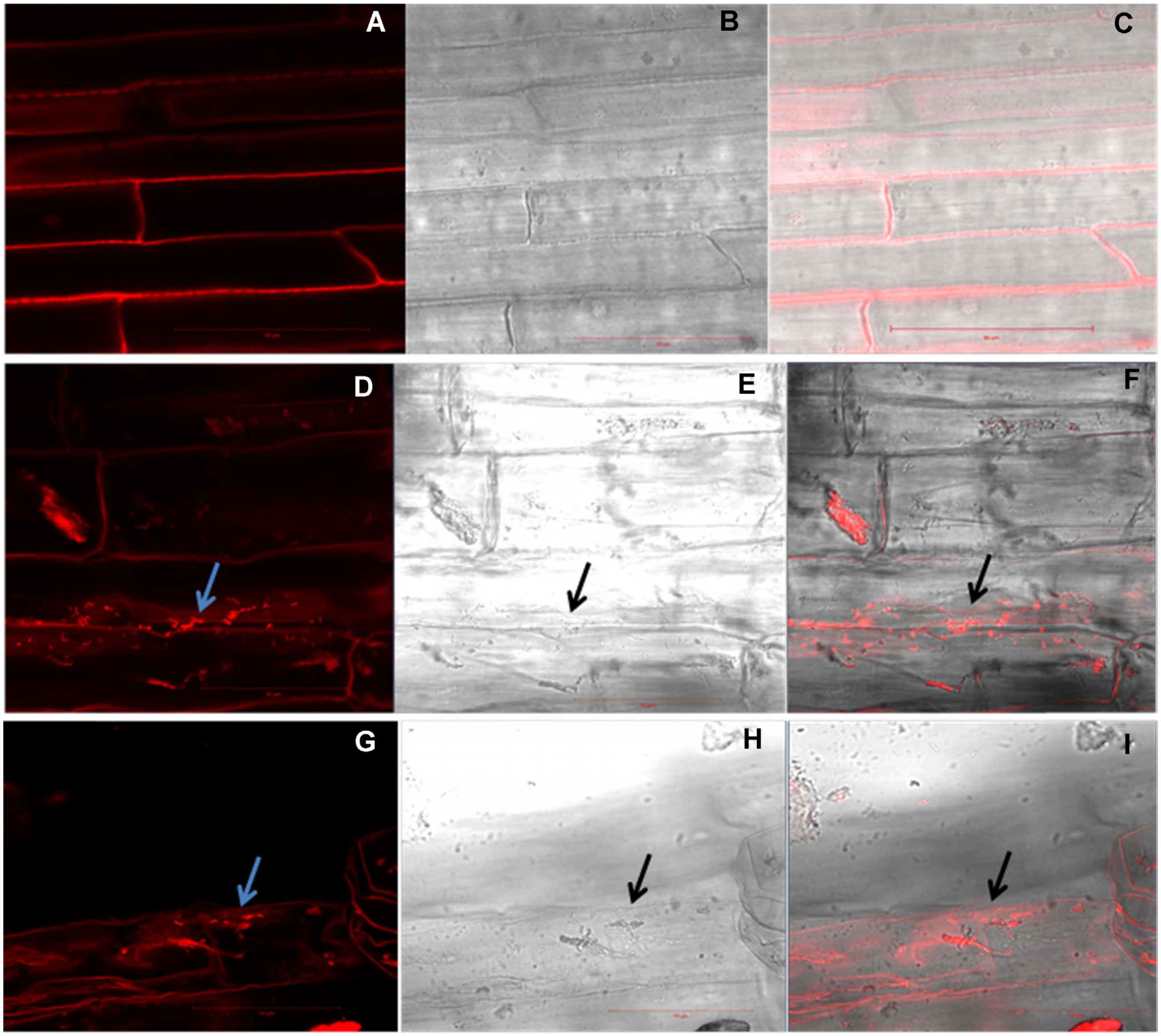

Fig. 8. Confocal microscopy observations of PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 treated 60 day old turmeric rhizome segments for colonization. (A-C) Control (untreated turmeric rhizomes), (D-F) PGPR *B. cereus* RBacDOB-S24 treated rhizomes showing colonization between the cells (arrowheads), [(F) the overlay of fluroscence image (D) and T PMT field image (E)]. (G-I) Endophyte *P. aeruginosa* BacDOB-E19 treated rhizomes showing colonization between the cells (arrowheads) [(I) the overlay of fluroscence image (G) and T PMT field image (H)] (scale bars: 50 µm).

by biocontrol agent *B. cereus* was reported (Wang et al., 2014). The endophytes viz., *P. aeruginosa*, *P. putida* and *B. megaterium* associated with black pepper were reported as effective antagonists for biological control of *Phytophthora* foot rot which recorded over 70% disease suppression in green house trials (Aravind et al., 2009).

The PGPR isolated from rhizosphere of ginger viz., *B. amyloliquefaciens* and *S. marcescens* markedly reduced the soft rot incidence of ginger rhizome caused by *P. myriotylum* and showed marked increase in rhizome yield compared to chemical treatments (Dinesh et al., 2015). The potent PGPR strains should fulfill at least two of the criteria such as colonization, plant growth stimulation and biocontrol (Beneduzi et al., 2012). Nevertheless, in present study PGPR *B. cereus* and endophyte *P. aeruginosa* both possess direct PGP activities like IAA production and phosphate solubilization and indirect PGP activities like antifungal activity, siderophore, HCN production and produce lytic enzymes protease and cellulase. *In vivo* evidence suggest that PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 suppressed the disease incidence of rhizome rot and leaf blight significantly and expressed high yield. Hence, these strains can be explored as potential biocontrol agents in order to control the rhizome rot and leaf blight diseases in turmeric which helps to reform the chemical fungicide based disease management approaches.

The present study revealed the importance of isolating, screening of bacteria for multiple PGP and biocontrol traits through greenhouse experiments in turmeric. In this study, based on *in vitro* experiments, two strains viz., PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 (Out of the 30 PGPR and 20 endophytes from turmeric) exhibited multiple plant growth promoting traits. The results of green house evidenced these strains suppressed the disease incidence of rhizome rot and leaf blight significantly, and markedly enhanced the yield in turmeric compared to untreated control and chemical treatments like Carbendazim- mancozeb. Also, *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 treated rhizomes showed colonization in the cells. The study confirms the potential of PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 as biocontrol agents (BCA's) for sustainable turmeric cultivation. For the best of our knowledge, this is the first report on the strains PGPR *B. cereus* RBacDOB-S24 and endophyte *P. aeruginosa* BacDOB-E19 as biocontrol agents (BCA's) against *P. aphanidermatum* and *R. solani* pathogens of turmeric. Further studies concerning field applications and stable bioformulations are in progress.

Conflicts of Interest

The authors declare that they have no conflict of interests regarding the publication of this paper.

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References

Ahmad, F., Ahmad, I. and Khan, M. S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* 163:173-181.

Almaghrabi, O. A., Massoud, S. I. and Abdelmoneim, T. S. 2013. Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. *Saudi J. Biol. Sci.* 20:57-61.

Aravind, R., Kumar, A., Eapen, S. J. and Ramana, K. V. 2009. Endophytic bacterial flora in root and stem tissues of black pepper (*Piper nigrum* L.) genotype: isolation, identification and evaluation against *Phytophthora capsici*. *Lett. Appl. Microbiol*. 48:58-64.

Bashan, Y. and De-Bashan, L. E. 2005. Plant growth-promoting. In: *Encyclopedia of soils in the environment*. Vol. 1, pp. 103-115.

Beneduzi, A., Ambrosini, A. and Passaglia, L. M. 2012. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and bio control agents. *Genet. Mol. Biol.* 35:1044-1051.

Berg, G. and Hallmann, J. 2006. Control of plant pathogenic fungi with bacterial endophytes. In: *Microbial root endophytes*, pp. 53-69. Springer Berlin Heidelberg.

Berg, G., Krechel, A., Ditz, M., Faupel, A., Sikora, R. A., Ulrich, A. and Hallmann, J. 2005. Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol. Ecol.* 51:215-229.

Broekaert, W. F., Terras, F. R., Cammue, B. P. and Vanderleyden, J. 1990. An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* 69:55-59.

Cao, L., Qiu, Z., You, J., Tan, H. and Zhou, S. 2005. Isolation and characterization of endophytic streptomycete antagonists of *Fusarium* wilt pathogen from surface-sterilized banana roots. *FEMS Microbiol. Lett.* 247:147-152.

Cappuccino, J. C. and Sherman, N. 1992. Microbiology: a Laboratory Manual. 3rd ed. pp. 125-179. Benjamin/Cummings Pub. Co., NY, USA.

Chang, W. T., Chen, Y. C. and Jao, C. L. 2007. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Bioresour. Technol.* 98:1224-1230.

Chauhan, A. K., Maheshwari, D. K., Kim, K. and Bajpai, V. K. 2016. Termitarium-inhabiting *Bacillus endophyticus* TSH42 and Bacillus cereus TSH77 colonizing *Curcuma longa* L.: isolation, characterization, and evaluation of their biocontrol and plant-growth-promoting activities. *Can. J. Microbiol.* 62:880-892.

Chernin, L. and Chet, I. 2002. Microbial enzymes in biocontrol of plant pathogens and pests. In: *Enzymes in the environ-*

ment: activity, ecology, and applications, pp. 171-225. Marcel Dekker, NY, USA.

Compant, S., Duffy, B., Nowak, J., Clément, C. and Barka, E. A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71:4951-4959.

Dinesh, R., Anandaraj, M., Kumar, A., Bini, Y. K., Subila, K. P. and Aravind, R. 2015. Isolation, characterization, and evaluation of multi-trait plant growth promoting rhizobacteria for their growth promoting and disease suppressing effects on ginger. *Microbiol. Res*. 173:34-43.

Egamberdiyeva, D. 2007. The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Appl. Soil Ecol.* 36:184-189.

El-Tarabily, K. A., Nassar, A. H., Hardy, G. S. J. and Sivasithamparam, K. 2009. Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *J. Appl. Microbiol*. 106:13-26.

Forchetti, G., Masciarelli, O., Alemano, S., Alvarez, D. and Abdala, G. 2007. Endophytic bacteria in sunflower (*Helianthus annuus* L.): isolation, characterization, and production of jasmonates and abscisic acid in culture medium. *Appl. Microbiol. Biotechnol.* 76:1145-1152.

Glick, B. R. 1995. The enhancement of plant growth by free living bacteria. *Can. J. Microbiol.* 41:109-114.

Gordon, S. A. and Weber, R. P. 1951. Colorimetric estimation of indoleacetic acid. *Plant Physiol.* 26:192-195.

Hallmann, J., Davies, K. G. and Sikora, R. 2009. Biological control using microbial pathogens, endophytes and antagonists. In: *Root-Knot Nematodes*, pp. 380-411. CABI, Wallingford, UK.

Hansen, M., Kragelund, L., Nybroe, O. and Sorensen, J. 1997. Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiol. Ecol*. 23:353-360.

Holt, J. G., Krieg, N. R. and Sneath, P. H. A. 1994. Berger's manual of determinative bacteriology. 9th ed. Williams & Wilkins, Baltimore, MD, USA.

IRRI. 2002. Standard Evaluation system for rice. International Rice Research Institute, Manila, Philippines. p.19.

Jasim, B., Joseph, A. A., John, C. J., Mathew, J. and Radhakrishnan, E. K. 2014. Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zingiber officinale*. *3 Biotech* 4:197-204.

Jimtha, J. C., Jishma, P., Arathy, G. B., Anisha, C. and Radhakrishnan, E. K. 2016. Identification of plant growth promoting Rhizosphere *Bacillus* sp. WG4 antagonistic to *Pythium myriotylum* and its enhanced antifungal effect in association with *Trichoderma*. *J. Soil Sci. Plant Nutr.* 16:578-590.

Kavitha, K., Nakkeeran, S. and Chandrasekar, G. 2012. Rhizobacterial-mediated induction of defense enzymes to enhance the resistance of turmeric (*Curcuma longa* L) to *Pythium aphanidermatum* causing rhizome rot. *Arch. Phytopathology Plant Protect.* 45:199-219.

Kloeppe, J. W., Rodriguez-Kabana, R., Zehnder, A. W., Murphy, J. F., Sikora, E. and Fernandez, C. 1999. Plant root-bacterial interactions in biological control of soil borne diseases and potential extension to systemic and foliar diseases. *Australas. Plant Pathol.* 28:21-26.

Kloepper, J. W., Lifshitz, R. and Zablotowicz, R. M. 1989. Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol*. 7:39-44.

Kuffner, M., Puschenreiter, M., Wieshammer, G., Gorfer, M. and Sessitsch, A. 2008. Rhizosphere bacteria affect growth and metal uptake of heavy metal accumulating willows. *Plant Soil* 304:35-44.

Kumar, A., Singh, R., Yadav, A., Giri, D. D., Singh, P. K. and Pandey, K. D. 2016. Isolation and characterization of bacterial endophytes of *Curcuma longa* L. *3 Biotech* 6:1-8.

Li, H., Wang, X., Han, M., Zhao, Z., Wang, M., Tang, Q., Liu, C., Kemp, B., Gu, Y., Shuang, J. and Xue, Y. 2012. Endophytic *Bacillus subtilis* ZZ120 and its potential application in control of replant diseases. *Afr. J. Biotechnol.* 11:231-242.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R., Taghavi, S., Mezgeay, M. and der Lelie, D. V. 2002. Endophytic bacteria and their potential applications. *CRC Crit. Rev. Plant Sci.* 21:583-606.

Loper, J. E. and Schroth, M. N. 1986. Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. *Phytopathology* 76:386-389.

Lorck, H. 1948. Production of hydrocyanic acid by bacteria. *Physiol. Plant.* 1:142-146.

Martins, S. J., de Medeiros, F. H. V., de Souza, R. M., de Resende, M. L. V. and Ribeiro, P. M. 2013. Biological control of bacterial wilt of common bean by plant growth-promoting rhizobacteria. *Biol. Control* 66:65-71.

Matthijs, S., Tehrani, K. A., Laus, G., Jackson, R. W., Cooper, R. M. and Cornelis, P. 2007. Thioquinolobactin, a *Pseudomonas* siderophore with antifungal and anti-*Pythium* activity. *Environ. Microbiol.* 9:425-434.

Minaxi and Saxena, J. 2010. Characterization of *Pseudomonas aeruginosa* RM-3 as a potential biocontrol agent. *Mycopathologia* 170:181-193.

Muthukumar, A., Eswaran, A. and Sangeetha, G. 2011. Induction of systemic resistance by mixtures of fungal and endophytic bacterial isolates against *Pythium aphanidermatum*. *Acta Physiol. Plant*. 33:1933-1944.

Nagrajkumar, M., Bhaskaran, R. and Velazhahan, R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani,* the rice sheath of blight pathogen. *Microbiol. Res.* 159:73-81.

Pandey, A., Trivedi, P., Kumar, B. and Palni, L. M. S. 2006. Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. *Curr. Microbiol.*

53:102-107.

Park, M. 1934. Report on the work of the mycology division. In: *Administrative report of directorate of agriculture*, pp. 126-133. Ceylon.

Pikovskaya, R. I. 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* 17:362-370.

Pillay, V. K. and Nowak, J. 1997. Inoculum density, temperature, and genotype effects on in vitro growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicon esculentum* L.) seedlings inoculated with a pseudomonad bacterium. *Can. J. Microbiol.* 43:354-361.

Pleban, S., Chernin, L. and Chet, I. 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. *Lett. App. Microbiol.* 25:284-288.

Rajendran, L. and Samiyappan, R. 2008. Endophytic *Bacillus* species confer increased resistance in cotton against damping off disease caused by *Rhizoctonia solani*. *Plant Pathol. J.* 7:1-12.

Ramarethinam, S. and Rajagopal, B. 1999. Efficacy of *Trichoderma* sp. organic amendments and seed dressing fungicides on rhizome rot of turmeric. *Pestology* 23:21-22.

Rana, A., Saharan, B., Joshi, M., Prasanna, R., Kumar, K. and Nain, L. 2011. Identification of multi-trait PGPR isolates and evaluating their potential as inoculants for wheat. *Ann. Microbiol.* 61:893-900.

Rathaiah, Y. 1982. Ridomil for control of rhizome rot of turmeric. *Indian Phytopathol*. 35:297-299.

Roy, A. K. 1992. Severity of *Rhizoctonia solani* on the leaves of rice and turmeric. *Indian Phytopathol*. 45:344-347.

Minaxi and Saxena, J. 2010. Characterization of *Pseudomonas aeruginosa* RM-3 as a potential biocontrol agent. *Mycopathologia* 170:181-193.

Schulz, B. J. E., Boyle, C. J. C. and Sieber, T. N. 2006. Microbial root endophytes, pp. 1-13. Springer-Verlag, Berlin.

Schwyn, B. and Neilands, J. B. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160:47-56.

Shanmugam, V., Gupta, A. K., Kanoujia, S. and Naruka, N. D. S. 2011. Selection and differentiation of *Bacillus* spp. Antagonistic to *Fusarium oxysporum* f.sp. *lycopersici and Alternaria solani* infecting tomato. *Folia Microbiol. (Praha)* 56:170-177.

Shanmugam, V., Gupta, S. and Dohroo, N. P. 2013. Selection of a compatible biocontrol strain mixture based on co-cultivation to control rhizome rot of ginger. *Crop Prot.* 43:119-127.

Siddiqui, Z. A. 2005. PGPR: prospective biocontrol agents of plant pathogens. In *PGPR: biocontrol and biofertilization*, pp. 111-142. Springer, Netherlands.

Someya, T. 1995. Three-dimensional observation of soil bacteria in organic debris with a confocal laser scanning microscope. *Soil Microorganisms* 46:61-69.

Sriraj, P. P., Sundravadana, S. and Alice, D. 2014. Efficacy of fungicides, botanicals and bioagents against *Rhizoctonia solani* inciting leaf blight on turmeric (*Curcuma longa* L.). *Afr. J. Microbiol. Res.* 8:3284-3294.

Sturz, A. V., Christie, B. R. and Nowak, J. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *CRC Crit. Rev. Plant Sci.* 19:1-30.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731-2739.

Thiripurasundari, K. and Selvarani, K. 2014. Production of turmeric in India: an analysis. *Int. J. Bus. Manag*. 2:229.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22:4673-4680.

Valois, D., Fayad, K., Barasubiye, T., Garon, M., Dery, C., Brzezinski, R. and Beaulieu, C. 1996. Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. *Appl. Environ. Microbiol.* 62:1630-1635.

Verma, S. C., Ladha, J. K. and Tripathi, A. K. 2001. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J. Biotechnol.* 91:127-141.

Wakelin, S. A., Warren, R. A., Harvey, P. R. and Ryder, M. H. 2004. Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. *Biol. Fert. Soils* 40:36-43.

Wang, X., Wang, L., Wang, J., Jin, P., Liu, H. and Zheng, Y. 2014. *Bacillus cereus* AR156-induced resistance to *Colletotrichum acutatum* is associated with priming of defense responses in loquat fruit. *PLoS One* 9:e112494.

Waqas, M., Khan, A. L., Kamran, M., Hamayun, M., Kang, S. M., Kim, Y. H. and Lee, I. J. 2012. Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules* 17:10754-10773.

Weller, D. M. 1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26:379-407.

Yuan, J., Raza, W., Shen, Q. and Huang, Q. 2012. Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. *Appl. Environ. Microbiol.* 78:5942-5944.