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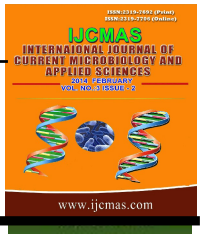

Original Research Article

Isolation, Biochemical and PGP characterization of endophytic *Pseudomonas aeruginosa* isolated from chilli red fruit antagonistic against chilli anthracnose disease

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A B S T R A C T

Nineteen endophytic bacteria strains were isolated from red fruit of chilli and screened for their antagonistic potential against *Colletotrichum gloeosporioides*, cause anthracnose in chilli. Ten strains i.e., CEFR-1, CEFR-3, CEFR-4, CEFR-5, CEFR-6, CEFR-7, CEFR-9, CEFR10, CEFR-11, and CEFR-12 were identified as fungal antagonistic strains against *Colletotrichum* and most promising bio control isolates. All 10 isolates were characterized for the production of Siderophore, fluorescence, Indole acetic acid (IAA), Hydrocyanic acid (HCN), Ammonia and solubilisation of phosphorus. All 10 isolates were tested for extracellular enzymes (hydrolytic enzymes)-amylase, cellulase, protease, and Catalase. This study evaluates the selection of suitable endophytic bacterial isolates for their potential plant growth promoting and bio control traits of anthracnose of chilli.

Keywords

Endophytic bacteria; Antagonistic potential; Anthracnose; *Colletotrichum sp;*

Introduction

Beneficial plant-microbe interactions that promote plant health and development have been the subject of considerable study. Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday, 1989; Schulz & Boyle, 2006). Nearly 3,00,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel *et al.*, 2004). Bacterial endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg *et al.*, 2006). Indeed, numerous studies reported the capacity if endophytes to control plant pathogens insects and nematodes (Sturz & Matheson, 1996; Duijff *et al.*, 1997; Krishnamurthy & Gnanamanickam, 1997, Azevedo *et al.*, 2000, Hallmann *et al.*, 1997, 1998). Endophytes also accelerate seedlings emergence, promote plant establishment under adverse conditions (Chanway 1997, Bent & Chanway 1998). Chilli (*Capsicum annuum* L) is one of the major spice crops grown in India. There are various diseases of chilli caused by

Bacteria, Virus, and Fungi. Anthracnose caused by *Colletotrichum capsici* and *C.gloesporioides* are the most devastating fungal pathogens in several chilli growing areas of the country (Po-Po-Than *et al.* 2008). Although there are several reports on the biological control of the diseases caused by *Colletotrichum* sp. in chillies (Hegde & Kulkarni 2001) efficient fungal antagonistic endophytic microorganisms strains have not been identified for the sustainable management of Anthracnose. This communication aims at isolation of natural microflora from chilli red fruit, screening them for the antagonistic effects against *C.gloesporioides*. Biological control is ecologically safe and it will not pollute the environment. The tendency of endophytic microorganisms showing fungal antagonism helps to develop bio fungicide as a substitute for chemical fungicide (Opoku *et al.*, Mann *et al.*, 2008). However there is no research yet reported of controlling Chilli anthracnose with endophytic bacteria. This communication aims to evaluate endophytic bacteria isolate from red fruit of chilli for biological control of anthracnose in chilli.

Materials and Methods

Isolation of endophytic bacterial strains

Endophytic bacteria isolated from *C. frutescence* variety US 341 commonly grown disease resistant varieties in Guntur district. Healthy chilli plant at fruit ripening stage was collected from Amaravathi village in Guntur district in zip lock covers under aseptic condition. Ripened fruits, green fruits, stem and leaves were separated thoroughly washed with tap water to remove adhering dust, fertilizers, soil and debris then washed with sterile distilled water. Ripened fruits were disinfected with H_2O_2 for 2min, rinsed with 70% ethanol for 5min followed by 3% sodium hypochlorite rinse for ten times (5min each rinse) in sterile saline water. To confirm the surface disinfection procedure successful and to verify that no biological contamination on the surface, sterility checks were carried out as per the procedure of Yingwu Shi *et al*(2009). After the confirmation of surface disinfestations without biological contamination, sample of plant material was aseptically weighed one gram and then macerate. Macerated tissue was placed in a tube containing 9ml sterile 1% NaCl solution, and serially diluted upto 10^{-10}. 0.1 ml of each dilution was spread with a sterile glass rod over surface of nutrient agar (NA) supplemented with 2.0 g/L sucrose. Petri plates were incubated at 28^0C for 7 days. Colony counts recorded by standard methods and isolated each colony on agar slant separately.

Antagonism of endophytic bacteria against *Colletotrichum* spp.

Lyophilized fungal cultures were purchased from MTCC Chandigarh. Cultures were grown in laboratory as per the instructions and specific media given by MTCC Chandigarh. Chilli red fruit endophytic bacterial isolates were assayed for antifungal activities against *C.gloesporioides* (MTCC3439), by dual culture method using Potato Dextrose Agar (PDA) medium. Endophytic isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the centre of the medium. The barrier between endophytic isolate and fungi indicated the antagonist interaction between them. Antagonist activity was investigated for 4 to 7 days after incubation at room temperature. The value of inhibition was measured using the

formula 1-(a/b) x 100% (a: distance between fungi in the centre of Petri dish to endophytic isolate, b: distance between fungi in the center of Petri dish to blank are without endophytic isolate) described by Kumar *et al.* (2002).

Production of Secondary metabolites by endophytes

Indole-3-acetic acid

IAA was determined by the method of Patten and Glick (2002). The endophytic bacteria were grown in Luria broth supplemented with L-tryptophan (1µg ml^{-1}) for 72 h. At the end of the incubation, cultures were centrifuged at 10,000g for 10 min and the supernatant was collected. One ml of this culture filtrate was allowed to react with 2ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) at 28± 2^0C for 30 min. At the end of the incubation, pink color development indicates the presence of IAA.

Phosphate solubilisation

Solubilisation of tri-calcium phosphate was detected in Pikovskaya's agar (Wasyu di *et al.,* 2011). Each endophytic isolate was streaked on the surface of Pikovskaya agar medium and phosphate solubilising activity was estimated after 1 to 5 days of incubation at room temperature. Phosphate solubilisation activity was determined by the development of the clear zone around bacterial colony.

Hydrocyanic acid (HCN) production

Hydrogen cyanide (HCN) production from glycine production from glycine was tested following the procedure of Donate-Correa *et al*(2005). Tryptic Soya Agar (TSA) supplemented with glycine (4.4 g L^{-1}) was prepared and autoclaved for 15 min at 121^0C and poured on petri dishes. The medium was maintained in a refrigerator overnight and allowed to be at room temperature for about 30 min before inoculation. A loop full of 24 h old broth culture of the isolates was inoculated to the plates. Sterile filter paper soaked with 0.5% (w/v) picric acid was fixed to the underside of the Petridis lids. The plates were then sealed with parafilm and incubated for 5-7 days at 30^0C. A change in the color of the filter strip from yellow to brown or reddish-brown was regarded as indication of cyanogenic potential.

Siderophore production

Siderophore production was tested qualitatively using Chrome Azurol S medium (CAS-medium) as described by Husen (2003). Each endophytic isolate was streaked on the surface of CAS medium and incubated at room temperature for 1 to 3 days. Siderophore production was indicated by orange halos around the colonies after the incubation Test was done in two replicates.

Fluorescence production

The protocol of king *et al.* (1954) was used for fluorescence production. Endophytic bacteria were streaked on King's B agar and incubated at 28±2^0C for 48h. At the end of the incubation the plates were observed under UV light for production of fluorescence.

Ammonia production

Endophytic isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water separately and incubated for 48–72 h at 36 ± 2°C.

Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Analysis of hydrolytic enzyme activity

Antagonistic endophytic bacterial isolates were analysed for production of four enzymes i.e. protease, amylase, cellulase and lipase by plate method as described by Kasana *et al*(2008).

Proteolytic activity of the cultures was studied in a medium containing skimmed milk. Zone of precipitation of paracasein around the colonies in the next 48h were taken as evidence of Proteolytic activity.

The presence of amylolytic activity was determined using starch agar medium. After inoculation of endophytic bacteria and incubation at 28^0C for 5 days, the plates were flooded with 0.3% I_2 and 0.6% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$ with 0.5% carboxymethylcellulose and 2% agar were surface inoculated. Iodine solution was used to detect cellulase activity. The clear zone formation around the growing colony was considered as positive.

The lipase activity of bacterial isolates was determined by following diffusion agar methods, i.e. nutrient agar medium was supplemented with 0.01% of $CaCl_2.H_2O$. Tween 80 sterilised for 20 min at 120^oC was added to the molten agar medium at 45^0C to give a final concentration of 1%. The medium was shaken until the Tween 80 had dissolved completely and then was poured onto Petri plates. Presence of opaque halo zone around the colonies was considered as positive.

Identification of endophytic bacterial isolates

Biochemical characterization

10 strains were characterized according to Bergey's manual of determinative bacteriology (Holt *et al.*, 1994).

Molecular identification of the isolates

Pure cultures of potential fungal antagonistic CEFR were grown until log phase and genomic DNA were isolated essentially according to Bazzicalupo and Fani (1995). The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) and 27F (5´ AGAGTTTGATCMTGGCTC AG-3´) as per the conditions described by Pandey *et al.* (2005). The PCR product was sequenced at Indian Institute of Horticulture Research, Hasserghat, Bangalore. The sequences obtained were compared with those from the GenBank using the BLAST program (Alschul *et al.* 1990) and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree.(Astchul SF *et al.* 2010 and Tamura K *et al.* 2007).

Effect of *endophytic bacterial strain* on plant growth promotion (*in vitro*)

Seed treatment and nursery experiments

Seeds of chilli were treated with the 48-h-old culture (approximately 10^8 CFU/ml) of the selected isolate of CEFR-3 for 30 min and were shade-dried at 28 + 28C for 1 h.

The treated seeds (100) were sown in pots containing coco peat in a greenhouse. Observations were recorded on germination percentage in the beginning, root length, shoot length and wet weight of the seedlings after 1 month of sowing by removing 10 seedlings from each replication.

Results and Discussion

The analysis is done which is focused on

Isolation of endophytic bacterial strains

A total of 19 endophytic bacteria were isolated from red fruit of chilli before harvest and named as CEFR-1 to CEFR-19.

Antagonism of endophytic bacteria of chilli red fruit (CEFR) against *Colletotrichum* sp.

The results of the dual culture technique indicated that out of 19 endophytic bacteria isolates 10 endophytes inhibited growth *C.gloesporioides* significantly (Table-1). Maximum inhibition 65.44% was recorded by CEFR-3. (Fig 1).

Plant growth promoting properties

The isolates that show antagonism against *C. gloeosporioides* was further tested for their PGP activities such as IAA, Siderophore production, Phosphate solubilisation and Ammonia production (Table-2) The results revealed that all 10 isolates were positive to IAA, Siderophore production, Phosphate solubilisation and Ammonia production. Except CEFR 6, none of the isolates was found negative for HCN production. All strains identified as potential IAA producers (Table 2) however CEFR-3 showed higher production of IAA (17µg/ml). In our study all antagonistic endophytes produce IAA, Siderophore, ammonia, phosphate solubilisation.

Hydrolytic enzyme activity

The isolates that show antagonism against *C. gloeosporioides* is tested for hydrolytic enzymes production (Table-2). The results revealed that most of the isolates were positive for catalase, protease and Lipase. Negative for cellulose and amylase.

Identification of endophytic bacterial isolates

By biochemical characterization all strains identified as *Pseudomonas* genera. CEFR-3 showed potential antagonism against Colletotrichum and with promising plant growth promotion activities (Table 3). The strain was characterized by 16s r-DNA and identified as *Pseudomonas aeruginosa.*The strain was submitted to GEN BANK NCBI (ID No. 1605949). CEFR-3 showed 100% similarity with *Pseudomonas aeruginosa* PAO1 strain and 99% similarity with *Pseudomonas aeruginosa* (fig-2).

Effect of endophytic bacterial strain on plant growth promotion (*in vitro*)

Invitro growth parameters root length, shoot leaf area determined for 100 seedlings at an interval of 4 weeks after sowing. Inoculation of pot trays containing chilli seeds with *Pseudomonas aeruginosa* (CEFR-3) lead to a significant increase in growth parameters of chilli seedlings (table 4) *Pseudomonas aeruginosa* (CEFR-3) increased root length, shoot length, fresh weight and seedling vigor.(fig 3).

Last couple of years it was reported that *C. gloeosporioides* showed wide

Table.1 Antagonistic effect of chilli red fruit endophytes (CEFR) against *Colletotrichum sp* on PDA media by dual culture method.

Isolate Name	CEFR-1	CEFR-3	CEFR-4	CEFR-5	CEFR-6	CEFR-7	CEFR-9	CEFR-10	CEFR-11	CEFR-12
C.gloeosporioides	54.54 %	**65.44 %**	59.09 %	58.18 %	60.00 %	60.90 %	57.27 %	48.18 %	53.63 %	60.90 %

Table.2 Plant growth promoting and Hydrolytic enzyme properties of CEFR - 3 isolates

Isolate Name	IAA µg/ml	Ammonia	Siderophore	HCN	Phosphate Solubilisation	Fluorescence	Protease	Catalase	Cellulose	Lipase	Amylase
CEFR-1	11	+	+	-	+	+	+	+	-	+	-
CEFR-3	17	+	+	-	+	+	+	+	-	+	-
CEFR-4	13	+	+	-	+	+	+	+	-	+	-
CEFR-5	13	+	+	-	+	+	+	+	-	+	-
CEFR-6	15	+	+	+	+	+	+	+	-	+	-
CEFR-7	11	+	+	-	+	+	+	+	-	+	-
CEFR-9	15	+	+	-	+	+	+	+	-	+	-
CEFR-10	10	+	+	-	+	+	+	+	-	+	-
CEFR-11	12	+	+	-	+	+	+	+	-	+	-
CEFR-12	15	+	+	-	+	+	+	+	-	+	-

pathogenicity on various hosts. Occurrence of anthracnose by *C. gloeosporioides* on different sp of plant such as Indian fig cactus (Kim *et al.* 2000); *Olea europaea* (Sergeeva *et al.* 2008); *Blepharocalyx salicifolius* (Larran *et al.* 2011); *Allium cepa* (Sikirou *et al.* 2011) and *Jatropha curcas* (Kwon *et al.* 2012); *Aloe vera* (Avasthi *et al.* 2011); Bell pepper (Gupta *et al.* 2009); *Jasminum grandiflorum* (Sharma *et al.* 2012); *Boehravia diffusa* L (Ajay Kumar Gautam *et al.* 2012); *Edilanthus tithymaloides* (Gautam *et al.* 2012); Noni (Hubballi *et al.* 2012); *Garcinia indicia* (Jadhav *et al*, 2009) and *Syzygium aromaticum* (Jadhav *et al*, 2008). The production of one or more secondary metabolites might have an impact on antagonism towards *C. gloeosporioides*. The proposed mechanisms to provide a protective effect on the root and suppression of pathogens are plant hormones like IAA (Loper and Schroth 1986) and other plant growth promoting substance like Siderophore (Leong 1986).

Table.3 Biochemical characterizations of different isolates of Bacterial endophytes

Biochemical test	**CEFR-1**	**CEFR-3**	**CEFR-4**	**CEFR-5**	**CEFR-6**	**CEFR-7**	**CEFR-9**	**CEFR-10**	**CEFR-11**	**CEFR-12**
Gram stain	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Florescent Pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment	Produce pigment
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Oxidase test	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Catalase test	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
H_2Sproduction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Urea test	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve
Nitrate reduced	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Vogas-proskauer	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate utilization	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Growth temp.	20-40	20-40	20-40	20-40	20-40	20-40	20-40	20-40	20-40	20-40
Starch hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Gelatin hydrolysis	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	-ve	+ ve
Tyrosine hydrolysis	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Tween 80 hydrolysis	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Casein hydrolysis	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Sugar Fermentation										
L-arabinose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Cellobiose	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
Ethanol	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Glycerol	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Fructose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Inulin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
D-mannitol	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
L-rhamnose	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
Maltose	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
Lactose	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
Raffinose	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
D-Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
D-sorbitol	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sucrose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Salicin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Trehalose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Xylose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

Table.4 Effect of *Pseudomonas aeruginosa* (CEFR-3) cell suspension of growth of chilli seedlings.

Time period	Name	Root length	Shoot length	No. Leaves	Fresh weight	Seedlings vigor index#
After one month	Control	6	6.2	4	0.11	1037
	Pseudomonas aeruginosa (CEFR-3)	8.7	7	6	0.27	1475.80

*Values are mean of three replicates. ± SE

#Seedlings viour index= seedling length (cm) × germination percentage.

Fig.1 Phylogenetic Tree constructed using Neighbor Joining Method of CEFR-3.

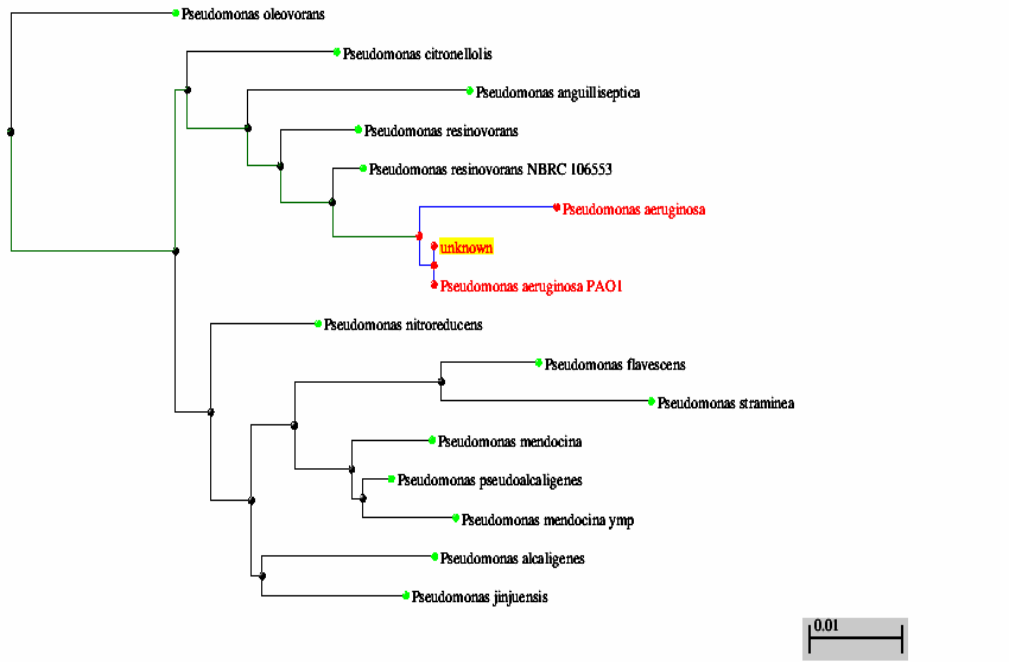

Fig.2 Plant growth promotion of *Capsicum frutescence* var.960 by *Pseudomonas aeruginosa* (CEFR-3)

*Values are mean of three replicates. ± SE

IAA production by some sweet potato endophytes could influence the host growth in low fertile soils (Zareen Khan *et al.* 2009) and stimulates root development, improves mineral and water uptake by the system (Gyaneshwar P *et al.* 2001 :Ladha JK *et al.* 2000: Rosenblueth M *et al.* 2006 ;Stoltzfus JR *et al.* 2006). IAA also triggers level of protection against external adverse conditions by enhancing different cellular defence systems (Bianco *et al.* 2006). Phosphate solubilization by plant-associated bacteria has also been well documented (Son H *et al.* 2005). In present study *Pseudomonas aeruginosa* (CEFR-3) is consider being a potential integrated isolate for controlling phytopathogen and enhances plant growth because *Pseudomonas aeruginosa* (CEFR-3) showed positive response to antagonism as well as plant growth properties. It was considered that microbial metabolites may have an active role in resistance to disease by functioning as signals mediating a crosstalk between the endophyte and its host (Kleopper *et al.* 1980; Cao *et al.* 2005: Graner *et al.* 2003). Buchenaier (1998) also stated that lytic enzymes secreted by bacteria are suspected to play an important role in suppression of pathogens. The majority of antagonistic endophytic bacteria are gram-negative and belong to the group of fluorescent pseudomonads, which are effective Biocontrol agents (Bloemberg and Lugtenberg 2001; Whipps 2001). Antagonistic isolates have been reported to occur in *Pseudomonas chlororaphis, P. fluorescens, P. graminis, P. putida, P. tolaasii* and *P. veronii* (Chen *et al* 1995; Adhikari *et al* 2001; Krechel *et al*). These examples illustrated the high potential of endophytic bacteria in fungal pathogen control. This is the first report of using endophytic bacteria isolated from Andhra Pradesh for minimising growth of the *Colletotrichum* as well as possessing PGP properties. The antagonistic bacteria are now under investigation for the development of suitable field conditions. Formulation and application that meet common farming practice still need to be developed. Promising bio control agents and strategies that enhance overall control efficacy should be explored.

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