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Isolation and Identification of Endophytic Bacterial Strains from Sugarcane Stalks and Their *In Vitro* Antagonism against the Red Rot Pathogen

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Forty nine isolates of endophytic bacteria and three isolates of rhizobacteria were isolated from different sugarcane varieties, clones of *Saccharum spontaneum* and *Erianthus* sp. All the bacterial isolates were evaluated for antagonism against the red rot pathogen of sugarcane *Colletotrichum falcatum*. The results showed that seven isolates were effective in inhibiting fungal mycelial growth under *in vitro* conditions. A major proportion of endophytic and rhizosphere isolates belonged to gram-positive rods. Biochemical characterization of the seven efficient antagonistic isolates revealed that three isolates viz., 687-2b1, 71-1-1a and 46-1a2 belonged to *Pseudomonas aeruginosa*, three isolates viz. SS1, SS2 and SS3 belonged to *Pseudomonas fluorescens* and one isolate viz. 312-2b, was *Pseudomonas putida*.

KEY WORDS : Sugarcane, endophytic bacteria, biocontrol, *Colletotrichum falcatum*

During the 1970's specific rhizobacteria applied to seeds were reported to colonize roots and promote plant growth (Kloepper and Schroth, 1978) and these were later termed as plant growth promoting rhizobacteria (PGPR). Most of the reported PGPR strains are from *Pseudomonas* and *Bacillus* species. Fluorescent pseudomonads are known to be antagonistic to different fungal pathogens in various crops. Certain PGPR strains enter into the plant system, colonize root cortex region and stalks and function as endophytes. In general, endophytic bacteria originate from the epiphytic bacterial communities of the rhizosphere and phylloplane as well as from endophyte infested seeds or planting materials. These organisms gain entry into plants through natural openings and wounds and also actively penetrate plant tissues using hydrolytic enzymes such as cellulase and pectinase. Historically, endophytic bacteria from within the plant have been thought to be weakly virulent plant pathogens but have recently been discovered to have several beneficial effects on host plants such as plant growth promotion and increased resistance against plant pathogens and parasites (Hallmann *et al.*, 1997).

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Prevalence of endophytic PGPR strains in sugarcane has been recently established and their antagonistic activity against red rot pathogen was demonstrated. An endophytic strain EP1 induced systemic resistance in sugarcane against the red rot pathogen *Colletotrichum falcatum* (Viswanathan and Samiyappan, 1999a; 2002a). Except for these studies bacterial communities inside the sugarcane stalk tissues for the management of red rot disease have been largely unexplored. Hence studies were carried out on isolation of endophytic strains from sugarcane stalk tissues, testing antagonistic activity of these endophytes against *C. falcatum* and characterization of the efficient strains.

MATERIALS AND METHODS

Isolation of endophytes

For the isolation of endophytes, sugarcane varieties, *Saccharum spontaneum* and *Erianthus* sp. clones were randomly selected from the varietal collections. Healthy sugarcane stalks of 8-10 months age were divided into three portions namely lower, middle and upper portions designated as 1, 2 and 3 and the rind was stripped off using a knife. Tissue cylinders were taken from the various portions of the stalk using a sterile cork borer and placed in different conical flasks containing sterile distilled water. The cylinders were then washed with 0.1% mercuric chloride for 2 minutes and several times

with sterile distilled water. The tissues were finally placed in 100ml conical flasks containing 25ml sterile distilled water and the flasks were shaken in a rotary shaker at 150 rpm for 1 h. One ml of suspension from each conical flask was poured into sterile Petri plates using a micropipette. Melted and cooled Kings' B medium was poured onto these plates, shaken well and allowed to solidify. Three replicates for each sample were maintained. The plates were incubated at 28 ± 2°C for 24-48 h. The organisms grown on KB medium were subcultured onto slants and maintained as pure cultures. Sugarcane rhizosphere strains were also isolated following the procedure reported earlier from soil samples collected from Coimbatore (Viswanathan and Samiyappan, 2002a).

Dual culture to assess *in vitro* antagonism

Fungal discs of *C. falcatum* (Cf671 pathotype isolated from the variety CoC 671) from red rot stock culture collections of Plant Pathology laboratory of the Institute were plated on potato dextrose agar medium at the center of the plates. The fungus was allowed to grow for two days. Bacterial isolates were streaked on either sides of the fungus 2 cm away from the growing mycelium. The plates were incubated for a period of eight days. Measurement of zone of inhibition and fungal growth was taken on 5th and 7th day. Dual culture was also repeated on oatmeal agar with selected efficient strains.

Characterization of efficient strains

Fifty one bacterial cultures were classified as gram positive or gram negative based on Gram staining. The antagonistic strains were identified to the species level based on the biochemical tests viz. gelatin liquefaction, Levan formation, growth at 4°C, 41°C, 45°C and growth in pH 5.7. The efficient strains were categorized as sugarcane endophytes (SCEP) after characterization.

RESULTS

A total of 51 isolates were isolated from sugarcane stalk tissues and rhizosphere soil samples. Among the 12 hybrid sugarcane varieties, Co 419 and CoC 92061 yielded higher number of endophytic isolates of six and eight, respectively (Table 1). In other varieties the number of isolates varied from one to four. The clone IND 85-576 (*S. spontaneum*) yielded seven isolates. The isolates from sugarcane varieties/clones were designated with number and/or name of the variety/ clone, and position of sampling of the stalk. The hybrid clones Co 6907, Co 8371, Co 86032, Co 86035, Co 93009, CoPant 84211, CoS 109 and Q28, the *S. spontaneum* clones SES 15116 and SES 98B and the *Erianthus* sp clones IJ 76-366, IJ 76-390, IND 90-826 and IND 99-994 did not yield any endophytic bacteria. From rhizosphere soil samples three bacterial isolates were obtained.

All the 51 isolates were tested for their antagonism against the sugarcane red rot pathogen on potato dextrose agar. All the isolates inhibited the pathogenic growth in the solid medium. However only seven isolates, viz. 687-2b1, 46-1a2, 312-2b, 71-11a, SS1, SS2 and SS3 showed strong antagonistic activity against the pathogen with clear inhibition zones (Table 2). The standard biocontrol strains Pf1 and FP7 showed inhibition zones of 10 and 11 mm, respectively. Among the effective isolates, the rhizosphere strains exhibited larger inhibition zones (12-13 mm) than standard strains. In other strains the inhibition zones ranged from 7mm to 8mm. Subsequently, antagonism of the seven effective isolates was repeated on oatmeal agar. In this test also all the isolates except 46-1a2 showed antagonism in the same manner as the standard strains (Table 3). All the isolates exhibited almost similar inhibition zones with the isolate 687-2b1 recording slightly larger inhibition zone of 12mm.

Gram staining was performed for all the isolates to group them based on gram stain results. Among the 51 isolates, 39 were gram-positive rods, seven were gram-negative rods, one gram positive coccus, and one gram positive coccobacillus. All the three isolates from rhizosphere were gram negative rods. In general, the proportion of gram positive bacterial endophytes was higher than that of gram negative bacteria (Table 4).

Table - 1 : Isolates of endophytes isolated from different sugarcane varieties/clones

Variety/genotype	Isolates
BO 91	91-1b, 91-4b
Co 1148	1148-1b,1148-2a,1148-2b,1148-3a
Co 312	312-2b
Co 419	419-1b, 419-2a, 419-2b, 419-3a, 419-3b, 419-4a
Co 6304	6304-3a
CoA 71-7	71-7-1a
CoC 671	671-1a1, 671-1a2, 671-1a3
CoC 92061	92061-1a, 92061-1b, 92061-2a, 920613a, 92061-3b, 92061-3c, 92061-4a, 92061-5b
CoJ 46	46-1a2
CoJ 64	64-2a, 64-3a
CoS 687	687-1a, 687-2b$_1$, 687-2b2, 687-3b
NCo 310	310-1a2
IND84-406	406-1a, 406-2b
IND85-576	576-1a, 576-1b, 576-2a, 576-2b, 576-3a, 576-3b, 576-4b
IJ76-66	66-1b
IK76-66	76-66-1a
Rhizosphere soil	SS1, SS2, SS3

Table - 2 : Antagonism of sugarcane endophytes to *Colletotrichum falcatum*

Isolates	*C. falcatum* mycelial growth (mm)		Zone of inhibition (mm)
	5 th day	7 th day	
91-1b	28.0	40.0	-
91-4b	30.5	39.5	-
1148-1b	27.5	38.5	-
1148-2a	28.0	40.0	-
1148-2b	30.0	40.0	-
1148-3a	29.0	40.0	-
312-2b	23.0	30.5	7
419-1b	29.5	39.5	-
419-2a	28.0	39.0	-
419-2b	28.5	39.5	-
419-3a	28.5	39.5	-
419-3b	30.0	40.0	-
419-4a	28.0	40.0	-
6304-3a	28.0	39.5	-
71-1-1a	23.0	29.0	8
671-1a1	29.5	38.5	-
671-1a2	30.0	40.0	-
671-1a3	28.0	38.5	-
92061-1a	29.0	40.0	-
92061-1b	30.0	38.5	-
92061-2a	27.5	37.5	-
92061-3a	29.0	45.0	-
92061-3b	28.5	40.5	-
92061-3c	30.0	39.5	-
92061-4a	27.5	40.0	-
92061-5b	30.0	40.5	-
46-1a2	23.0	30.5	7
64-2a	29.0	39.0	-
64-3a	30.0	41.0	-
687-2b1	21.0	30.5	8
687-2b2	30.0	39.0	-
687-3b	30.0	41.0	-
84-406-1a	29.5	39.0	-
84-406-2b	31.0	39.0	-
85-576-1a	29.5	38.5	-
85-576-1b	28.5	38.5	-
85-576-2a	30.0	40.0	-
85-876-2b	30.0	40.0	-
85-876-3a	29.0	38.0	-
85-876-3b	30.5	38.5	-
85-876-4b	29.0	38.0	-
IJ76-66-1a	28.5	38.5	-
IK76-66-1b	30.0	40.0	-
310-1a2	30.0	40.0	-
SS1	22.0	8.0	13
SS2	22.5	28.5	12
SS3	22.5	28.5	13
Pf1	22.5	29.5	10
FP7	20.5	28.5	11
Control	30.0	45.0	-

Table - 3 : Antagonism of selected isolates of endophytes to *Colletotrichum falcatum* on oatmeal agar

Isolates	Mycelial growth		Zone of inhibition (mm)
	5th day (mm)	7th day (mm)	
Control	30.0	44.0	
312-2b	20.5	32.5	11
71-1-1a	22.0	30.0	8
46-1a2	28.5	40.5	-
687-2b1	23.0	32.0	12
SS1	21.0	30.0	10
SS2	22.5	29.0	9
SS3	22.0	31.5	10
Pf1	21.5	31.5	10
FP7	21.5	30.0	11

Table - 4 : Categorization of sugarcane endophytes by Gram staining

Gram positive	91-1b, 91-4b, 1148-1b, 1148-2a, 1148-2b, 1148-3a, 419-1b, 419-2a, 419-3a, 419-3b, 419-4a, 63043a, 671-1a$_1$, 671-3a, 92061-1a, 92061-1b, 92061-2a, 92061-3a, 92061-3b, 92061-3c, 92061-4a, 92061-5b, 64-2a, 64-3a, 84-406-1a, 84-406-2b, 85-576-1a, 85-5761b, 85-576-2a, 85-576-2b, 85-576-3a, 85-576-3b, 85-576-4b, IJ76-66-1b, IK76-66-1a, 310-1a$_2$.
Gram negative	312-2b, 419-2b, 71-1-1a, 671-1a$_2$, 46-1a$_2$, 687-1a,687-2b$_1$, 687-2b$_2$,687-3b, SS1, SS2, SS3

Seven antagonistic isolates were characterized based on different biochemical assays. The strain 312-2b (SCEP2) which showed growth at 4°C and pH5.7 and negative reaction for gelatin liquefaction was grouped as *Pseudomonas putida*. The strain viz., 71-1-1a (SCEP3), 46-1a2 (SCEP4) and 687-2b1 (SCEP5) showing positive to gelatin liquefaction and growth at 45°C were grouped under *Pseudomonas aeruginosa* .All the three rhizosphere strains (SS1 - SS3) which were positive to gelatin liquefaction and negative to growth at 45° C were grouped as *Pseudomonas fluorescens*. Isolate SS3 also showed positive to levan formation (Table 5)

DISCUSSION

Endophytic bacteria colonize an ecological niche similar to that of plant pathogens, especially vascular wilt pathogens. Once established within the plant tissue, endophytic bacteria can be transmitted vegetatively, as reported for *Acetobacter diazotrophicus* for two successive cuttings of sugarcane (Dong *et al.*, 1994). Kloepper *et al.* (1992) found that five of six rhizobacteria, which induced systemic resistance in cucumber, exhibited both external and internal colonization. Exploiting an additional microbial habitat

Table - 5 : Biochemical characterization of sugarcane endophytes antagonistic to *C. falcatum*

Efficient isolates	Gelatin liquefaction	Levan formation	Growth at 4° C	Growth at 45° C	Growth at pH5.7	Species identified
312-2b	-	-	+	-	+	*Pseudomonas putida*
71-1-1a	+	-	-	+	-	*P. aeruginosa*
46-1a2	+	-	-	+	-	*P. aeruginosa*
687-2b1	+	-	-	+	-	*P. aeruginosa*
SS1	+	-	+	-	-	*P. fluorescens*
SS2	+	-	+	-	-	*P. fluorescens*
SS3	+	+	+	-	-	*P. fluorescens*
Pf1	+	-	+	-	-	*P. fluorescens*
FP7	+	-	+	-	-	*P. fluorescens*

for biocontrol purposes might enhance overall biocontrol efficacy and increase consistency in performance, since the endophytic agent could avoid unfavourable conditions prevailing in the soil environment by entering and localizing in the intercellular spaces of the epidermal cells of root tissues. Endophytic bacteria have shown significant control of diseases such as *Fusarium vasinfectum* in cotton (Chen *et al.*, 1995; Van Buren *et al.*, 1993), *Verticillium albo-atrum, Rhizoctonia solani* and *Clavibacter michiganesis* subsp. *sepedonicum* in potato (Nowak *et al.*, 1995), *Sclerotium rolfsii* in bean (Pleban *et al.*, 1995), *Rhizoctonia solani, Pythium myriotylum, Gauemannomyces graminis,* and *Helerobasidium annosum* in rice (Mukhopadhyay *et al.*, 1996), and *Fusarium moniliformae* in maize (Hinton and Bacon, 1995). In addition, *Pseudomonas fluorescens* 89B-27 and *Serratia marcescens* 90-166 induced resistance in cucumber to *Pseudomonas syringae* pv. *lachrymans* as well as to the fungal pathogens *F. oxysporum* f. sp. *cucumerinum* and *Colletotrichum orbiculare* (Liu *et al.*, 1995).

The present studies revealed survival of useful bacterial antagonists in sugarcane stalk tissues. The efficient strains were as effective as standard biocontrol strains Pf1 and FP7 in inhibiting fungal growth *in vitro*. Recently attempts were made to isolate rhizosphere/ endophytic strains of fluorescent pseudomonads and to identify antagonistic strains against the red rot pathogen. Efficient antagonistic strains isolated from sugarcane rhizosphere, viz. KKM1, VPT4 and VPT10, and endophytic strain EP1 were found to be inducing systemic resistance against *C. falcatum* in disease susceptible varieties. Application of fluorescent pseudomonads to rhizosphere region had induced several defence related enzymes such as chitinase, ß-1,3- glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase in sugarcane stalks which arrested the pathogen colonization and spread in the stalk (Viswanathan and Samiyappan, 1999a, b; 2001; 2002a,b). Gillis *et al.* (1989) found *Acetobacter diazotrophicus,* a nitrogen fixing bacterium in large numbers in roots and stems of sugarcane plants from many areas in Brazil. This bacterium has been found in many sugarcane varieties in several regions of Brazil as well as from Mexico, Cuba and Australia with numbers ranging from 10^3 to10^7 per g in roots, basal and apical stems, leaves and in sugarcane trash. Another endophyte *Herbaspirillum* species has been found to occur in parts of many graminaceous crops including the roots and aerial tissues of rice and *Pennisetum purpureum* and in the roots of various forage grasses, sugarcane, maize and in many weeds in sugarcane and maize fields (Boddey and Dobereiner, 1995). This information on association of endophytic nitrogen fixing diazotrophs in sugarcane suggests that the bacteria reside inside the stalks and the plant is benefited by way of biological nitrogen fixation. If the introduced antagonistic bacteria infect and colonize niches of the red rot pathogen in sugarcane stalks, pathogen colonization and build up would be reduced significantly. The efficient endophytic strains were identified as *Pseudomonas aeruginosa* and *Bacillus cereus* and rhizosphere strains as *Pseudomonas fluorescens*. Previous studies of Viswanathan and Samiyappan (1999a) revealed association of *Pseudomonas fluorescens* strain in sugarcane stalk.

In addition to biological control, endophytic bacteria improved plant growth in different crops like potato (Sturz, 1995) and rice (Hurek *et al.*, 1994). Hallmann *et al.* (1997) speculated that the observed plant growth promotion in different crops might have been caused by enhanced plant mineral uptake and improved plant water relationships associated with the colonization of endophytic strains. Some strains of *Pseudomonas, Enterobacter, Staphylococcus, Azotobacter* and *Azospirillum* produce plant growth regulators such as ethylene, auxins or cytokinins and have, therefore, been considered as causal agents for altering plant growth and development. In addition to a direct mechanism for growth promotion, plant growth promotion is also thought to be due to the suppression of deleterious

microflora by the introduced endophyte (Kloepper *et al.*, 1991; Leifert *et al.*, 1994). The beneficial effects of bacterial endophytes, however, vary and appear to operate through similar mechanisms as described for plant growth promoting rhizobacteria (Kloepper *et al.*, 1991; Hoflich *et al.*, 1994). However, because of the different habitats colonized, endophytes offer another tool for developing biological control strategies. By integrating the use of bacterial endophytes with rhizosphere antagonists, a holistic biological control system could be developed that works against the pathogen like red rot infecting the stalk and surviving in the soil. Talc-based formulations of *Pseudomonas* strains antagonistic to sugarcane red rot were prepared and application through sett treatment and soil application was standardized in different field trials (Viswanathan and Samiyappan, 1999a). Since application methods have been standardized for the biocontrol bacteria in sugarcane, the identified endophytic strains could be mass multiplied following the standardized procedure for commercial exploitation.

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