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Research Article

Evaluation of epiphytic microflora as antagonists of red rot pathogen, *Colletotrichum falcatum* in sugarcane under subtropical conditions

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ABSTRACT: Epiphytic microflora isolated from different plant parts of healthy sugarcane plants at different growth stages were assessed for their ability to control fungal red rot pathogen of sugarcane both *in vitro* and *in vivo*. Microbial population was higher in the month of August with highest on cane stalk followed by buds and dry leaves. A total of 112 bacterial isolates representing 16 morphotypes, 63 fungal and 35 actinomycete isolates representing 7 morphotypes each and 21 mould isolates of 3 morphotypes were purified from the collection of isolates obtained from different plant parts at different periods of plant growth stage and environment condition. These 231 microbial isolates were screened *in vitro* for their antifungal activity against *Colletotrichum falcatum* using dual culture technique. None of the mould isolates could inhibit *C. falcatum* growth, while 12 bacterial isolates, 4 fungal isolates and 5 actinomycete isolates were found to be antagonistic to *C. falcatum*. Biochemical and 16SrRNA partial sequence characterization of 12 bacterial isolates led to identification of *Pseudomonas*, *Serratia, Bacillus*, *Paenibacillus*, *Gluconacetobacter*, *Serratia marscens* (DQ144501) and *Paenibacillus macerans* (DQ144502). Based on colony morphology, spore arrangement and structure characteristics the fungal and actinomycete isolates were identified to of *Aspergillus*, *Trichoderma* and *Penicillium* and of *Streptomyces* and *Saccharopolyspora* sp., respectively. The strongest inhibition of *C. falcatum* was obtained with actinomycete isolates and *Serratia marscens* both under *in vitro* and *in vivo* conditions, that can serve to be part of integrated disease management (IDM) of red rot in sugarcane in subtropics.

KEY WORDS: Epiphytes, Biocontrol, *Colletotrichum falcatum, Serratia marscens*, *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *Streptomyces* sp., S*accharopolyspora* sp., sugarcane

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INTRODUCTION

Sugarcane (*Saccharum* spp.), is cultivated in an area of around 4.0 million ha with average productivity of 60 t ha^{-1} and provides major economic security to the farmer. Red-rot caused by the fungus *Colletotrichum falcatum* is the prime disease of sugarcane in tropical belts and is the major production constraint for sugarcane production in the country (Alexander and Viswanathan, 1996). The changing races of fungus makes the commercial sugarcane varieties susceptible to red rot. In India, due to red rot outbreaks, many high sugar commercial varieties of sugarcane (Co312, Co997, Co1148, CoJ64, CoC671 etc.) have been eliminated. The sugar industry in India suffers losses of more than US$ 500 million every year due to red rot disease (Padmanaban *et al*., 1996; Viswanathan *et al.,* 1997). Disease is mainly sett-borne with secondary infections through leaf lamina and buds. Moist hot air therapy (MHAT), dilute acid treatments have been tried, but with little success as the presence of tough external rind makes the cane unamenable for chemicals to enter and control the pathogen.

Resistance to red-rot is monitored by inoculating *C. falcatum* spores in the growing canes at 6-month-old stage by plug-method (Singh and Singh, 1989) and spread of disease is monitored after 6 weeks for assigning disease related character. The susceptible varieties are not recommended for cultivation, however, there are certain susceptible varieties like CoJ 64 and CoS 767 which are cultivated by farmers due to their high sugar (18% brix) and early maturing (10 month) characteristics. Such varieties have been showing some kind of unexplained field resistance to secondary stage infection of *C. falcatum*. Since, secondary stage infections are mainly through leaf-lamina, buds and root-primordia on the nodal region, we at Indian Institute of Sugarcane Research, Lucknow started working on a hypothesis that probably epiphytic micro-

flora of such varieties may have some role in checking secondary infection of *C. falcatum*. Since sugarcane plant houses a plethora of micro-organisms in its rhizosphere and as endophyte and epiphytes, the present work aims to (i) isolate the predominant naturally occurring epiphytic microbial isolates that can check the proliferation of *C. falcatum*, (ii) identify and utilize them for managing the red rot disease.

MATERIALS AND METHODS

Epiphytic micro-flora of sugarcane

Sugarcane variety CoJ 64 growing at farm of Indian Institute of Sugarcane Research, Lucknow (located at 2605'N, 8006'E, 111m above sea level) was selected for sampling different plant parts (Sehgal *et al.,* 1990). Sugarcane leaf is attached to stalk through leaf sheath with a transverse mark at the joint of leaf lamina and leaf sheath. The first leaf in which this transverse mark becomes visible is called LTM (Lateral Transverse Mark) leaf and is considered as an indicator leaf for physiological/biochemical studies. Other leaves on sugarcane stalk are numbered relative to LTM leaf. The planting of the crop was done in February 2005 and the plant samples were drawn at 3 stages of crop growth representing different seasonal temperatures of 40–45°C (May; 3 month crop), 30–35°C (August; 6 month crop) and 4–7°C (December 15, 10 month crop). Ten intact plants were uprooted and brought to laboratory. After giving a mild washing with sterilized water for removing dust, different leaves (–2, LTM, +2, +4, last green leaf and dried leaf), buds, stem and root pieces were cut using a sterilized knife or a pair of scissors and were carefully transferred to aseptic sealable plastic bags. The experiment was started by composting the samples and three replicates were taken with 5 leaves of each kind, 20 buds and 5 canes for each replicate.

Processing of leaf samples

Five leaf pieces measuring 3 cm^2 were cut from each leaf randomly covering up to the tip and in total 25 such pieces from 5 leaves were transferred to a sterilized 500 ml Erlenmeyer flask containing 100 ml of saline–PO_4 buffer. Cane pieces measuring 5 cm were cut with sterilized sharp knife and the ends were sealed with paraffin film. Five such cane pieces were transferred to 50 ml of saline–PO_4 buffer. Similarly, 20 buds and five grams of roots were transferred to 50 ml and 100 ml of saline-PO_4 buffer, respectively. All the flasks were shaken for 1hr at 100 rpm using an incubator shaker for preparing suspension of epiphytic micro-flora.

Isolation of epiphytic micro-flora associated with cane parts

Serial dilutions of suspension containing epiphytic micro-flora of leaf, bud, stem and roots were plated on Nutrient Agar (NA), Trypton Yeast-Extract Mannitol (TYM), King's B (KB), Tryptic Soy Agar (TSA) growth media for bacterial enumeration, Czapkdox Agar (CA) and Potato Dextrose Agar (PDA) for fungal and Actinomycete Agar for actinomycete counts. Three dilutions for each sample in duplicate were plated and the plates were incubated at 30°C for 3–7 days. Observations were recorded for total microbial counts and the fraction of most predominant ones. Colonies of bacteria, fungi and actinomycete appearing different on the basis of color, size and colony margin pattern were purified by streaking on the respective media and maintained on same culture plates as well as at –80°C with 40% glycerol. For *in vitro* assays the suspensions of different microbial isolates containing 10^7–10^8 cells 10 (L^{-1}) were prepared by suspending isolates in saline–PO_4 buffer.

Culturing of *Colletotrichum falcatum*

A virulent isolate of *Colletotrichum falcatum* (Cf 09), from IISR, Lucknow was used for this studies. The culture was grown on oat meal agar media plates, containing oatmeal 30 g and agar-agar 16g L^{-1}, at 28°C for 15 days for complete sporulation. Spore suspension was prepared by adding 5 ml of saline–PO_4 buffer to the Petri plate, shaking and collecting it in a sterilized vial. The spore counts in the suspension were done by haemocytometer and by dilution plating on oat-meal agar. Depending upon counts, the spore suspension was diluted to maintain approximately 10^7 spores per 10^{-1} and of suspension.

In vitro screening of microbial isolates for antifungal activity

Dual inoculation method was followed for initial screening. The common medium on which both *C. falcatum* and different microbial bioagents could grow efficiently was found to be TYM and King's B agar. Therefore, these two growth media have been used for all *in vitro* assays. Fungal spores absorbed on disc of sterilized Whatman filter paper (No. 10) was kept in the center of the medium plate and after incubating for 24hr at 30°C, the bacterial suspension discs were placed on either side of the fungal disc. The plates were incubated at 30°C for 7–10 days and the inhibition of growing fungal culture was taken as positive antifungal activity of different bacterial, fungal and actinomycete isolates.

The extent of antifungal activity of bioagents was estimated by plate and broth culture inhibition assays. For plate inhibition assay 0.1 ml of *C. falcatum* spore suspension was plated on King's B agar plate and after it dried, the microbial disc was placed in the center. Fungal inhibition zone around the bacterial disc was measured after incubating at 30°C for 7 days. For broth culture inhibition assay 20ml of King's B broth was inoculated with different bioagents. After incubation of 10 days at 30°C the cultures were centrifuged at 6000-g for 10 min and passed through a 0.2m filter. The culture filtrate was inoculated by 0.1ml *C. falcatum* spore suspension and the flasks were incubated for 10 days at 30°C. The developed fungal mycelium of *C. falcatum* was separated by filtering it through pre-weighed Whatmann (no. 1) filter paper and thus the weight of the developed mycelia was recorded. All the assays were performed three times in duplicate. Direct microscopic observation of the plates or temporary slides of the *C. falcatum* mycelia was performed using Leica phase contrast microscope.

Enzyme assays in culture filtrate

Culture supernatant was assayed for carboxymethyl cellulase (CMCase), chitinase, and protease activities. For CMCase activity, 50mM 2-(Nmorpholino) ethanesulfonic acid (MES) buffer, pH 5.0 was mixed with 0.2% CMC and incubated at 37°C. Reducing sugars liberated due to CMCase activities were determined by the method of Nelson (1944) with a glucose standard. One unit of CMCase activity was defined as the amount of enzyme that released 1mM glucose reducing equivalent min^{-1} ml^{-1} culture. Chitinase activity was determined by incubation of culture supernatant with 50mM citrate buffer, pH 5.0, and 0.2% chitin at 37°C. Liberated N-acetylglucosamine equivalents were determined by the method of Reissing *et al.* (1955). One unit of chitinase activity was the amount of enzyme that released 1mM N-acetylglucosamine equivalent min^{-1} ml^{-1} culture. Protease activity was determined by incubating culture filtrate in 100mM potassium phosphate buffer, pH 7.0, plus 0.4% azocoll at 37°C (Chavira *et al.,* 1984). One unit of protease activity was the amount of enzyme that increased absorbance at 520nm 1 unit hr^{-1} ml^{-1} culture filtrate. Lower limits of detection were 2.3U for CMCase and 0.3U for chitinase activity, and 0.001U for protease activity.

Characterization of potent biocontrol agents and developing antibiotic resistant variant of bio-agents

Potential bacterial biocontrol agents were characterized using morpho-physical and biochemical techniques as per Bergey's Manual of Determinative Bacteriology (1984). Further, by using MicroSeqTm500-16SrDNA bacterial sequencing kit, 16SrDNA amplification and sequencing was done using Eppendorf thermal cycler and capillary based ABI Prism sequencer and its data software, respectively. Upon blasting the DNA sequences in existing database and based on the sequence matching, the bacterial cultures were identified. Colony, mycelial, sporangium and biochemical characteristics were used for fungal (Pitt and Cocking, 1986) and actinomycete identification (Bergey's Manual of Determinative Bacteriology, 1984). Selection from naturally occurring variants of a culture was used to develop the antibiotic resistant marker for the survival studies. It was achieved by plating bacterial isolates on King's B agar containing kanamycin (100g ml^{-1}) as most of the isolates were sensitive to 50g ml^{-1} concentration. For fungal and actinomycete, sodium azide (200g ml^{-1}) was used as selection pressure in their respective growth media. All the antibiotic resistant marked bioagents were tested again for their antifungal activity under *in vitro* condition.

***In vivo* testing of biocontrol agents for suppressing red rot disease**

Crop culture and treatments

Two set of experiments were performed for studying the effect of bio-agents on red-rot disease development under pot culture. Soil for filling earthen pots was collected from 0 to 15 cm layer of five different fields at IISR farm. Soil of the IISR farm belong to fine loamy, non-calcareous, mixed hypothermic udic ustochrepts. Before filling the pots, considering the standard recommendation of fertilizer application for sugarcane (per hectare N:P:K:150:60:60) 0.15 g nitrogen kg^{-1} soil in the form of urea, 0.3 g phosphorous kg^{-1} soil through single super phosphate and 1.6 g potassium kg^{-1} soil through murate of potash was mixed in the soil. Each pot was filled with 8.5 kg of soil and approximately 60% available soil moisture was maintained through out the growth cycle by watering pots as and when required. Pots were placed completely randomized in a net-house under natural light from February to September,. The mean ambient temperature during this period was 24.5°C with a mean minimum of 15.9°C and mean maximum of 33.1°C. The mean period of bright sunshine was 7.5 h day^{-1} with solar radiations of approximately 19.5 MJ m^{-2} day^{-1}.

Experiment I

Healthy canes were given moist hot air treatment (MHAT) at 54°C for 2 hrs. As a common practice for

disease induction, single bud sets were treated with *C. falcatum* spore suspension (10^8 spores ml^{-1}) by mixing the setts with fungal inoculum in sterilized poly-bags. After 48 hr of incubation at 30°C, the *C. falcatum* inoculated setts were treated with bioagents by overnight dipping the setts in culture suspension (10^8 cells ml^{-1}) of different bio-agents. At planting, 5 ml of culture suspension was also added in the adjacent soil of the setts. Autoclaved fungal suspension and subsequently water was used for control treatment.

Experiment II

Red rot infected canes of sugarcane variety CoJ 64 having fungal infection in its buds were taken from the IISR field trial. Infected single bud setts were treated with bioagents by overnight dipping the setts in culture suspension (10^8 cells ml^{-1}) of different bio-agents. At planting, 5 ml of culture suspension was also added in the adjacent soil of the setts. Water was used for control treatment.

Eight single bud cane setts were planted horizontally in each pot. In all, there were 39 pots with 13 treatments (12 bioagents + 1 control) in 3 replications. Different treatments were- T1: *Serratia marcens*; T2: *Pseudomonas fluorescens*; T3: *Paenibacillus macerans*; T4: *Bacillus subtilis*; T5: *Lactobacillus* spp.; T6: *Gluconacetobacter diazotrophicus*; T7: *Aspergillus terreus*; T8: *Trichoderma reesi*; T9: *Trichoderma harzianum;* T10: *Penicillium* spp. T11: *Streptomyces* spp; T12: *Saccharopolyspora* spp. and T13: Control. The growing plants in pots were sprayed with the same bioagent suspension after 3 months of planting using mist sprayer and 25 ml culture broth mixed with 50 ml of sterilized water was used for spraying each pot.

Red rot disease development and its severity index

Canes growing in pots were harvested by cutting from the base and were split open to note the disease development. The severity of the disease was ranked using severity index that depends on parameters such as condition of green top, transgression of disease through internodes, development of white patches and nodal necrosis. It is ranked on a 0-9 scale where 0-2 is for resistant phenotype (R), 2-4 for moderate resistant (MR) 4-6 for moderate susceptible (MS), 6-8 for susceptible (S) and >8 is highly sensitive (HS) phenotypes.

Population of biocontrol agents in soil, on setts and plant surface

Before planting two setts from each bioagent treatment were shaken with 50 ml of saline-PO^4 buffer and similarly at harvest different plant parts (leaf, stalk and bud) and soil attached to roots (5g each) were also suspended in 50 ml saline PO^4 buffer. All the suspensions were diluted and plated on respective culture growth medium with kanamycin/sodium azide. Microbial counts were made after incubating the plates at 30°C for 5-7 days. Re-isolated biocontrol agents were tested for their *in vitro* antifungal property and other biochemical/ genetic characteristics.

Statistical analyses

Data are expressed on an oven-dry soil or plant weight basis. One way analysis of variance with Duncan Multiple Range Test (DMRT) as post hoc analysis was used to compare the means (Snedecar & Cochran, 1967). Microbial count data were log transformed before analysis of variance.

RESULTS AND DISCUSSION

Selection of biocontrol agents suppressing *Colletotrichum falcatum*

Variable number and type of microbial population was observed at different plant parts and growth stages (Table 1). Invariably, the microbial population was more in the month of August with highest on cane stalk followed by buds and dry leaves. A total of 112 bacterial isolates representing 16 morphotypes, 63 fungal and 35 actinomycete isolates representing 7 morphotypes each and 21 mould isolates of 3 morphotypes were purified from the collection of isolates obtained from different plant parts at different periods of plant growth stage and environment condition (Table 1). These 231 microbial isolates were screened in vitro for their antifungal activity against *Colletotrichum falcatum* using dual culture technique. None of the mould isolates could inhibit *C. falcatum* growth, while 12 bacterial isolates, 4 fungal isolates and 5 actinomycete isolates were found to be antagonistic to *C. falcatum*. Upon biochemical and 16SrRNA partial sequence characterization 12 bacterial isolates were identified to 6 genera namely *Pseudomonas, Serratia, Bacillus, Paenibacillus* and *Gluconacetobacter* and the partial sequence of 2 genera *Serratia marscens* (DQ144501) and *Paenibacillus macerans* (DQ144502) have been submitted to NCBI Genbank (Suman *et al.,* 2005a and b). Based on colony morphology, spore arrangement and structure characteristics the fungal and actinomycete isolates were identified to 3 (*Aspergillus, Trichoderma* and *Penicillium*) and 2 (*Streptomyces* and *Saccharopolyspora*) genera, respectively. The strongest inhibition of *C. falcatum* was observed by actinomycete isolates and *Serratia marscens*.

Table 1: Sugarcane associated microbial population as influenced by plant parts and seasonal temperature

Plant parts	Bacteria			Fungi			Actinomycete			Mould		
	3MAP (43°C)	6MAP (30°C)	10MAP (5°C)	3MAP (43°C)	6MAP (30°C)	10MAP (5°C)	3MAP (43°C)	6MAP (30°C)	10MAP (5°C)	3MAP (43°C)	6MAP (30°C)	10MAP (5°C)
Leaf [1]												
–2	28	32	4	2	2	5	3	2	2	3	4	ND
LTM	30	43	6	3	3	6	3	5	2	37	39	2
+2	97	134	28	5	8	6	6	5	5	51	58	5
+4	98	129	32	3	6	6	8	10	7	59	57	10
Last green	66	93	12	4	7	7	14	12	8	42	37	17
Dry-trash	740	$1.02x10^3$	725	40	52	44	32	38	22	72	58	51
Stalk[2]	$1.12x10^3$	$1.43x10^3$	$8.46x10^3$	$1.58x10^3$	$5.41x10^3$	$3.98x10^3$	12	14	10	40	37	35
Bud[3]	$1.17x10^3$	$1.23x10^3$	$7.50x10^3$	645	$1.04x10^3$	906	10	12	7	ND	ND	ND
Root[4]	$3.67x10^6$	$6.89x10^7$	$1.92x10^7$	$3.62x10^3$	$4.65x10^3$	$4.25x10^3$	635	689	609	$1.9x10^3$	$6.4x10^3$	$3.6x10^3$

* Mean of 3 experiments; MAP: months after planting of cane setts; 1 and 2: counts cm^{-2}; 3: counts bud^{-1}; 4: counts g^{-1} root

Antifungal potential of biocontrol agents

C. falcatum inhibition zone formed by different microbial isolates ranged from 10 to 32.7 mm (Table 2). The maximum zone of inhibition was shown by actinomycete isolates and *Serratia marscens* (30-32.7mm) and minimum by *Gluconacetobacter diazotrophicus* (10mm). *C. falcatum* inhibition in broth culture was assessed by measuring its mycelia weight in the culture filtrates of biocontrol agents (Table 2). Mycelia weight was lowest with actinomycete isolates (1.2–1.3 mg) followed by *Serratia marscens* and *Pseudomonas fluorescens* isolate (2.7–3.13 mg) compared to 35.6mg in the control where no biocontrol agent was cultured. The mycelia weight varied from 8.4–10.5 mg in the treatments where fungal bioagents were used.

A time course investigation of the interaction between biocontrol agents and *C. falcatum* by light microscopy of the culture plates showed the formation of the abnormal forms of mycelia such as swelling, curling, multiple branching, degraded protoplasm and cell wall. These ultimately led to the hyphal death as observed by the clear zone formed between biocontrol agent and the fungus.

Biocontrol agents mediated suppression of sugarcane red rot

There was only 12% germination in *C. falcatum* inoculated setts (Cf–Control) compared to 100% in healthy cane sett treatment (blank) (Table 3). Treatment with biocontrol agents improved the germination of infected setts by 67 to 96%. The germination vigor, which takes care of germination% and plant height, was high and varied from 1704 to 3648 units in biocontrol agents' treatments compared to 216 in Cf-Control and 3500 in blank treatment. All the germinated setts of Cf-Control treatment developed red rot disease and the disease severity index was ranked as highly sensitive (Table 3), whereas disease induction was 14 to 65% in different biocontrol agents treatment. The disease was also less severe as disease severity index varied from sensitive in *Lactobacillus* spp. treatment to resistant in the case of *S. marscens, P. fluorescens, G. diazotrophicus* and *T. harzianum* treatments.

In the other experiment where infected cane setts were used, the germination was only 10% in Cf-Control treatment, whereas the treatment of infected cane setts by different biocontrol agents improved the germination up to 32% (Table 4). The *Lactobacillus* and actinomycete isolates treatments could not improve the germination. The germination vigor varied from 170 to 864 units in different bioagent treatments compared to Cf-Control treatment, where the germinated plants developed red rot disease and succumbed immediately. Induction of red rot disease varied from 12 to 100% in different biocontrol agents' treatments. Disease severity index was highly sensitive (HS) in *Lactobacillus* and sensitive (S) to moderated resistant (MR) in different bioagent treatments (Table 3). Overall among the different biocontrol agents *S. marscens, P. fluorescens, G. diazotrophicus, T. reesi* and *Penicillium* treatments were found to be the best

Table 2: Antifungal activity of different biocontrol agents against *Colletotrichum falcatum*

Sl. No.	Biocontrol agents	*Colletotrichum falcatum*	
		Inhibition zone (mm)	Mycelial weight (mg)
1	*Serratia marscens*	30.0^{ab} ± 0.58	2.70 ± 0.10 (92%)
2	*Pseudomonas fluorescens*	27.7^{b} ± 0.33	3.13 ± 0.15 (91%)
3	*Paenibacillus macerans*	20.7^{c} ± 0.33	5.90 ± 0.26 (83%)
4	*Bacillus* spp.	19.0^{c} ± 0.58	6.67 ± 0.35 (81%)
5	*Lactobacillus* spp.	13.7^{d} ± 0.88	7.80 ± 0.30 (78%)
6	*Gluconacetobacter diazotrophicus*	10.0^{e} ± 0.58	10.8 ± 0.26 (70%)
7	*Aspergillus terreus*	20.3^{c} ± 0.88	9.63 ± 0.40 (73%)
8	*Trichoderma reesi*	19.0^{c} ± 1.15	10.4 ± 0.40 (72%)
9	*Trichoderma harzianum*	20.0^{c} ± 1.15	10.5 ± 0.56 (70%)
10	*Penicillium* spp.	20.3^{c} ± 1.33	8.40 ± 0.40 (76%)
11	*Streptomyces* spp.	30.3^{ab} ± 1.33	1.20 ± 0.20 (97%)
12	*Saccharopolyspora* spp.	32.7^{a} ± 1.43	1.30 ± 0.20 (96%)
13	Control	0.00	35.6 ± 0.72

Table 3: Effect of biocontrol agents on *C. falcatum* infected healthy sugarcane setts

Sl. No.	Biocontrol agents	Cf-infected healthy setts		Red Rot Disease	
		Germination (%)	Germination vigor	Induction (%)	Reaction
1	*Serratia marscens*	92^{a}	3588^{a}	23^{ab}	R
2	*Pseudomonas fluorescens*	96^{a}	3648^{a}	17^{a}	R
3	*Paenibacillus macerans*	75^{c}	2400^{c}	39^{c}	MS
4	*Bacillus* spp.	79^{bc}	2765^{b}	47^{cd}	MS
5	*Lactobacillus* spp.	71^{c}	1704^{e}	65^{d}	S
6	*Gluconacetobacter diazotrophicus*	92^{a}	3588^{a}	14^{a}	R
7	*Aspergillus terreus*	71^{c}	1917^{d}	29b	MR
8	*Trichoderma reesi*	79^{bc}	2765^{b}	26^{b}	MR
9	*Trichoderma harzianum*	67^{c}	2490^{c}	20^{a}	MR
10	*Penicillium* spp.	83^{b}	2490^{c}	20^{a}	MR
11	*Streptomyces* spp.	87^{b}	2088^{d}	48^{cd}	MS
12	*Saccharopolyspora* spp.	83^{b}	2241^{cd}	45^{cd}	MS
13	Control–*Cf*	12^{d}	216^{f}	100^{e}	HS
14	Control-blank	100^{a}	3500^{a}	–	–

S: Sensitive; MS: Moderate Sensitive; HS: Highly Sensitive; R: Resistant; MR: Moderate Resistant
Control-*Cf*: Healthy sugarcane setts infected by *Cf* but not treated by any biocontrol agent
Control-blank: Healthy setts not treated by Cf and/or any biocontrol agent
Values in each column with same letter do not differ significantly at $p \leq 0.05$ by Duncan's Multiple Range Test.

Table 4: Effect of biocontrol agents on *Colletotrichum falcatum* diseased sugarcane setts

Sl. No.	Biocontrol agents	Cf-infected healthy setts		Red Rot Disease	
		Germination (%)	Germination vigor	Induction (%)	Reaction
1	*Serratia marscens*	32[b]	864[b]	12[a]	MR
2	*Pseudomonas fluorescens*	30[b]	840[b]	14[a]	MR
3	*Paenibacillus macerans*	20[c]	380[e]	60[c]	S
4	*Bacillus* spp.	24[c]	504[d]	50[c]	S
5	*Lactobacillus* spp.	10[d]	170[f]	100[d]	HS
6	*Gluconacetobacter diazotrophicus*	22[c]	660[c]	20[a]	MR
7	*Aspergillus terreus*	18[cd]	378[e]	25[b]	MR
8	*Trichoderma reesi*	20[c]	500[d]	20[a]	MR
9	*Trichoderma harzianum*	24[c]	576[d]	17[a]	MR
10	*Penicillium* spp.	25[c]	700[c]	14[a]	MR
11	*Streptomyces* spp.	10[d]	140[f]	50[c]	MS
12	*Saccharopolyspora* spp.	10[d]	170[f]	33[b]	MS
13	Control - *Cf*	0	–	–	HS
14	Control - blank	97[a]	3492[a]	–	–

Cf: Colletotrichum falcatum; S: Sensitive; MS: Moderate Sensitive; HS: Highly Sensitive; R: Resistant; MR: Moderate Resistant
Control-*Cf*: Healthy sugarcane setts infected by *Cf* but not treated by any biocontrol agent
Control-blank: Healthy setts not treated by Cf and/or any biocontrol agent
Values in each column with same letter do not differ significantly at $p \leq 0.05$ by Duncan's Multiple Range Test.

in checking red rot disease and improving germination vigor and plant growth.

Survival of biocontrol agents

Survival of potent 5 microbial biocontrol agents (*S. marscens, P. fluorescens, G. diazotrophicus, T. reesi* and *Penicillium*) on different plant parts and soil was studied at the harvest stage in December. Sufficient number of microbial population was present on all the plant surfaces and even in the adjoining rhizospheric soil around the setts (Table 4). The re-isolated biocontrol agents showed positive *in vitro* antifungal property and were similar to the original isolates based on biochemical/ genetic characteristics.

In the recent years many reports concerning epiphytes with an adverse effect on plant pathogens have been published (Campant *et al.,* 2005; Janisiewicz *et al.,* 1992; Sholberg *et al.,* 1995; Sinigaglia *et al.,* 1998). The antagonistic effect of the microflora is explained not only by their production of antibiotic substances/ inhibitory allelochemicals, but also by competition for ecological niche, nutrients and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens and/or abiotic stresses (Chalutz *et al.,* 1988; Droby *et al.,* 1989; Janisiewicz 1987; Chand-Goyal and Spotts 1996, 1997). Interactions among epiphytes can play an important role in a plant's defense responses. The nature of the interactions depends, among other things on the qualitative-quantitative composition of epiphytic communities. In an attempt to identify potential biocontrol agent for controlling red rot disease in sugarcane our results demonstrate that a variety of bacterial, fungal and actinomycete isolates colonize different parts of the sugarcane plant. A total of 231 different microbial isolates representing 33 morphotypes were purified from different plant parts of sugarcane. A notable feature of epiphytic microbial population is their variation in size or type even on different leaves of same age and having identical appearance of the same plant (Kinkel, 1997). Availability of nutrients, sugars and selected metabolites on the plant parts govern the variable distribution of the epiphytic microbial population (Mercier and Lindow, 2000). Therefore, the microbial population of the plants could be modified by changing the nutrient status of the plant surface and this has implication for the biological control of plant

pathogens. In this study, approximately 64% of the isolates purified as epiphytic microflora strongly inhibited *C. falcatum* under *in vitro* condition. Six identified bacterial isolates belonged to *Pseudomonas, Serratia, Bacillus, Paenibacillus* and *Gluconacetobacter* sp. some of which have been used as biocontrol agents in other crops also. Four fungal agents were identified to *Aspergillus, Trichoderma* and *Penicillium* group and two actinomycete isolates were placed in *Streptomyces* and *Saccharopolyspora* group. These microbial isolates are known to produce a variety of bioactive compounds which makes a firm basis for their antagonistic principle against pathogens. Co-culturing of *C. falcatum* and biocontrol agents was avoided as in case of fungal and actinomycete biocontrol agents it is difficult to segregate the contribution of each in the total mycelial weight observed. Rather culturing of *C. falcatum* in the culture filtrate of different biocontrol agents observed a reduction of 70 to 97% in the mycelial growth, indicating that production of bioactive compounds was mainly responsible for inhibiting *C. falcatum* growth and not the direct contact as evidenced by the effect of culture filtrates on *C. falcatum* growth. Nautiyal *et al.* (2006) have shown that co-culturing of *Bacillus* individually and a microbial consortium of 3 isolates with *Fusarium moniliformis* and *C. falcatum* inhibited fungal growth ranging from 47 to 70%.

Light microscopy observations of the clearing zone formed between biocontrol agents and *C. falcatum* indicated the development of abnormal forms in the *C. falcatum* mycelium. There were alteration and distortion of the hyphal cell wall resulting in the formation of swelling, curling, branching and ultimately lysed or empty tube like structure. Someya and Kataoka (1999) have shown the development of such abnormal forms in Cyclamen pathogen *Fusarium oxysporum* and *Rhizoctonia solani* by an isolate of *Serratia marscens*. Ultimately loss of cytoplasmic content and cell lysis was responsible for its death. In a prolonged interaction of pathogen and biocontrol agent, the cells of *Serratia marscens* were seen in the empty tube like mycelium of *C. falcatum*. Nautiyal *et al.* (2006) have demonstrated a similar mechanism in the lysis of *Fusarium moniliformis* causing sugarcane wilt by the *Bacillus* isolates from cow milk. The production of extracellular enzymes and bioactive compounds are mainly involved in the lysis of cell wall of phytopathogenic fungi (Glick and Bashan, 1997; Raajmakers *et al.,* 2002). Using light microscopy Huang and Chan (2008) have shown that the chitinase acitivity of *Bacillus elliptica* alone and in synergism with fungicides could inhibit conidial germination of *A. brassicicola, A. longipes* and *C. gloeoporioides*. Viswanathan and Samiyappan (1999 a,b) have also demonstrated the early and increased expression of peroxidase and chitinase enzymes by fluorescent pseudomonas probably mediated induced systemic resistance in sugarcane and resulted in significant disease suppression

Biopriming plants with plant growth promoting microorganisms (PGPM) can also provide systemic resistance against a broad spectrum of plant pathogens. Diseases of fungal, bacterial, and viral origin and in some instances even damage caused by insects and nematodes can be reduced after application of PGPM. Manifestation of ISR is dependent on the combination of host plant and bacterial strain. PGPM-elicited ISR was first observed on carnation (*Dianthus caryophillus*) with reduced susceptibility to wilt caused by *Fusarium* sp. and on cucumber (*Cucumis sativus*) with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare*. Some other similar examples where ISR was triggered by biological agents are: *P. fluorescens* EP1 against red rot caused by *Colletotrichum falcatum* on sugarcane, *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* on grapevine and *Verticllium dahliae* on tomato, *P. denitrificans* 1-15 and *P. putida* 5-48 against *Ceratocystis fagacearum* on oak, *P. fluorescens* 63-28 against *F. oxysporum* f. sp. *radicis-lycopersici* on tomato and *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* on pea roots and *Bacilluspumilus* SE34 against *F. oxysporum* f. sp. *pisi* on pea roots and *F. oxysporum* f. sp. *vasinfectum* on cotton roots. *Trichoderma viride* isolated from cowpea phylloplane hyperasitised the mycelium of *Colletotrichum truncatum*, causal agent of brown blotch disease of cowpea *in vitro*. *T. viride* treatment in the form of a seed dip in a spore suspension and soil drenching with a spore suspension were very effective in reducing infection from brown blotch infected seeds.

Presence of high population of these biocontrol agents as estimated, using intrinsic antibiotic/drug resistant marker, on different plant parts at harvest stage (approximately after 8 months) clearly indicate that these microbial forms have well colonized the sugarcane epiphytic plane and are habituated of the sugarcane ecosystem. Mercier and Lindow (2000) have shown the role and specificity of leaf sugars in colonization of plants by bacterial epiphytes. Plants with variable sugar exudation pattern vary in their epiphytic microbial population carrying capacity as pea and corn which had the lowest amount of leaf surface sugars had the lowest counts of epiphytic microflora compared to beans and tomato, which harbored high amounts of sugars. The proportion of antagonistic microflora in whole epiphytic

communities was higher in the resistant clones and the hybrid than in the susceptible clones, with the microflora having a more restrictive effect on the development of the pathogen.

To sum up, irrespective of the mechanisms underlying interactions among the plant, saprophytic epiphytes, and the pathogen, several epiphytic bacterial and fungal isolates of sugarcane were able to check the red rot pathogen, *C. falcatum*, both under *in vitro* and in vivo condition. Further, detailed study on their inoculation effects in field trials both on disease management and in turn on plant growth and productivity shall make these bioagents be part of integrated disease management (IDM) of red rot in sugarcane, which is the prime concern for sugarcane growing farmers in subtropics.

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