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RESEARCH PAPERS

Biopesticide activity of sugarcane associated rhizobacteria: *Ochrobactrum intermedium* strain NH-5 and *Stenotrophomonas maltophilia* strain NH-300 against red rot under field conditions

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Summary. *Colletotrichum falcatum* is the major fungal pathogen causing sugarcane red rot. Four antagonistic bacterial strains exhibiting biocontrol activity against this pathogen in greenhouse conditions were characterized for production of different antifungal metabolites and biocontrol determinants to elucidate the mechanism of action involved in their antagonistic activity. The strains were also evaluated under field conditions to assess their biocontrol potential. All the strains produced hydrogen cyanide (HCN), and volatile and diffusible antibiotics. In addition, the *Ochrobactrum intermedium* strain NH-5 produced siderophores and the broad spectrum antibiotic 2, 4-diacetylphloroglucinol (2,4-DAPG); *Pseudomonas* sp. NH-203 produced siderophores, and *Pseudomonas* sp. NH-276 produced protease. Two strains, *Ochrobactrum intermedium* NH-5 and *Stenotrophomonas maltophilia* NH-300, exhibited good biocontrol activity, suppressing red rot by 44–52% on two sugarcane varieties, SPF-234 and Co-1148, in field experiments. The strains gave consistent results in three consecutive years and showed potential to be used as biopesticides.

Key words: 2, 4-DAPG, siderophores, biocontrol, *Colletotrichum falcatum*, rhizosphere.

Introduction

Sugarcane (*Saccharum officinarum* L.) is a major world industrial crop, which is used in manufacturing of important chemicals and industrial products, including alcohol, sugar, paper and paper board. Many other plants, including potato (*Solanum tuberosum*), tomato (*Lycopersicom esculantum*), maize (*Zea mays*), onion (*Allium cepa*), sunflower (*Helianthus annuus*) and bean (*Phaseolus vulgaris)* can be intercropped with sugarcane in Asia, Brazil and Africa (Sharma *et al*., 1997). Diseases are one of the major constraints responsible for low sugarcane yields. Red rot (caused by *Colletotrichum falcatum* Went) is the most serious disease and causes severe losses in sucrose yield of sugarcane world-wide. Epidemics of red rot caused 29% loss in cane weight and 31% loss in sugar recovery (Alvi *et al*., 2008). Management practices for red rot include cultural practices, crop rotation, fungicide applications, and the use of resistant varieties. At present, there is no single method providing suitable control of red rot under field conditions.

The use of fungicides in plant protection has posed serious consequences in recent years. Their excessive use has resulted in several problems, including persistence of chemical residues in food products and polluted environment. In addition, incorporation of their by-products into soil has reduced soil quality and productivity (Minh *et al*., 2004). Hence, there is a pressing need to utilize fungicide-free disease management strategies, either based on biological agents

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or alternate means to control soil-borne pathogens. With increasing emphasis on organic farming, efforts have been made to screen and identify beneficial microbes. Plant growth promoting rhizobacteria (PGPRs) are being explored extensively for improving plant nutrient acquisition and disease resistance. They are gaining importance due to their increased use as biofertilizers, bioprotectants and soil reclamation agents (Vessey, 2003; Tank, 2009). PGPRs are diverse bacteria belonging to the genera *Bacillus, Pseudomonas, Azotobacter, Azospirilum, Serratia, Ochrobactrum* and *Stenotrophomonas*. They protect plants by producing certain secondary metabolites. Most of these include antibiotic compounds such as 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolnitrin (PRN), surfactin (SRF), iturin (ITR) and phenazine (PHZ) compounds (Haas and Defago, 2005; Kim *et al*., 2010). The metabolite 2, 4-DAPG is a phenolic polyketide with broad spectrum antifungal, antiviral, and nematicidal activity (Bangera and Thomashow, 1999). This compound is mostly produced by the plant–associated pseudomonads and has potential for utilization in agriculture due to its role *in situ* against soil-borne pathogens (Mavrodi *et al*., 2007). In addition to pseudomonads, production of 2, 4-DAPG by *Lysobacter gummosus* has also been reported (Brucker *et al*., 2008). Here, we report the production of this broad spectrum compound by *Ochrobactrum intermedium*.

Stenotrophomonas and *Ochrobactrum* strains have been isolated from various sources, mainly plant rhizospheres, clinical material and aquatic habitats (Dunne *et al*., 1997; Ryan *et al*., 2009; Imran *et al*., 2010; Hassan *et al*., 2010a). Our previous work demonstrated that certain sugarcane associated rhizobacteria inhibited the growth of the pathogen *Colletotrichum falcatum* on PDA plates *in vitro* and reduced red rot infection *in vivo* (Hassan *et al*., 2010a; 2011). These antagonistic bacteria include Gram positive and Gram negative isolates of *Pseudomonas*, *Bacillus* and *Ochrobactrum*. In the present study, we selected four strains, including *O. intermedium* strain NH-5, *Pseudomonas* sp. strains NH-203 and NH-276 and *S. maltophilia* strain NH-300, capable of suppressing the red rot pathogen *in vitro* and *in vivo*, to investigate the different metabolites produced by these antagonistic strains, and evaluate their suppressive effects on red rot of sugarcane under field conditions.

Materials and methods

PGPR strains and fungal pathogen isolate

The PGPR strains *O. intermedium* NH-5, *S. maltophilia* NH-300, two *Pseudomonas* sp. strains NH-203, NH-276, and the virulent fungus *C. falcatum* previously isolated from diseased stalks and rhizospheres of sugarcane (Hassan *et al*., 2010a) were obtained from the Pakistan Collection of Microbial Cells and Cultures (PCMC), Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan. A well-characterized biocontrol agent *P. fluorescens* CHA0 (Voisard *et al*., 1989) was used as positive control in the study.

Activity of cell free supernatants of bioantagonists

Activity of cell free supernatants of bioantagonists was determined using the methods of Naureen *et al*., (2009). One hundred μL volumes of freshly grown bacteria were inoculated into 500 mL capacity flasks containing 100 mL of LB broth (Bertani, 1951). The flasks were incubated for 72 h at 28±2°C with shaking of 190 rpm. Cells were separated by centrifuging in 50 mL falcon tubes at 13,000 rpm for 9 min. The supernatant was passed through a syringe filter (0.22 µm) and added, at variable concentrations of 5–25% v/v, to PDA (Oxoid) in Petri plates. A 5 mm mycelial disk of freshly grown *C. falcatum* was placed at the center of each Petri plate containing PDA amended with supernatant. LB broth was added instead of cultural filtrate in the negative controls. All the plates were placed in an incubator at 28±2°C for 5–6 d and diameter of the fungus colony in each plate was measured and compared with controls. The proportional inhibition was calculated using the following formula; % Inhibition = [1 – (Fungal growth/control growth)] × 100 (Kazempour, 2004). The experiment was conducted in three replicates and repeated three times.

Production of HCN, protease and siderophores

HCN production was determined by plate assay, as reported by Miller and Higgins (1970) and Hassan *et al*. (2011), with some modifications. Bacterial cells were inoculated onto King's medium B (KMB) agar plates containing 4.4 g L^{-1} glycine. A piece of Whatman filter paper was soaked in a solution consisting of 1%

sodium carbonate and 0.5% picric acid and placed in the upper lids of inoculated plates which were then incubated for 72 h at 28 ± 2°C. HCN production was detected by observing the change in colour of these filter papers from yellow to brown, and by scoring the colour change using a 0–2 scale (0, none; 1, little; 2, strong) as described by Ahmadzadeh *et al*., (2006).

Protease production by bacterial strains was tested as described by Denizci *et al*., (2004), (Hassan *et al*., 2011). The bacteria were inoculated onto skim milk agar (SMA) plates which were incubated at 28 ± 2°C for 48–72 h. The protease producing strains made halo zones around the bacterial colonies. Production of protease was semi quantified by measuring the diameter of solublization zones (Ahmadzadeh *et al*., 2006).

Ability of the PGPR strains to produce siderophores was evaluated by inoculating them into Petri dishes containing Chrome azurol S (CAS) medium as described by Schwyn and Neilands (1987). One drop of the bacterial culture grown in LB broth for 24 h at 28 ± 2°C was inoculated into each plate, which were then incubated for 8 d. Bacteria that were capable to produce siderophores grew and formed a purple halos in the dark bluish medium. The siderophore production was semi quantified by measuring the diameter of purple zones.

Production of diffusible and volatile antibiotics

Antibiotic production was examined as described by Montealegre *et al*. (2003). To detect production of volatile antibiotics, bacteria were grown on LB agar and *C. falcatum* was grown on PDA (oxoid) in separate Petri plates. Plates containing the fungus or test bacteria were then placed face to face, avoiding physical contact between bacteria and fungus, and then sealing the pairs of plates with Parafilm. For diffusible antibiotics, bacteria were grown on PDA plates covered with cellophane membranes for 72 h. The membranes were then removed and fungus was inoculated on the plates. Proportional fungal inhibition was calculated as described above.

The experiment was conducted in three replicates and repeated three times.

HPLC analysis of antibiotics

The broad spectrum antibiotics 2, 4 diacetylphloroglucinol (2, 4-DAPG), pyoluteorin and pyrrolnitrin were extracted and analyzed by high performance liquid chromatography assay (HPLC) as described by Bonsall *et al.* (1997). The bacteria were grown in 50 mL capacity flasks each containing 10 mL LB broth, separately for extraction of each antibiotic. 2,4-DAPG was extracted by acidifying the bacterial broth cells with 10% trifluoroacetic acid (TFA) to pH 2.0 followed by vigorous shaking for 3 h and separating the cells by centrifugation at 10,000 rpm at 4°C. The cell-free acidified supernatant was extracted with two fold volumes of ethyl acetate.

Pyrrolnitrin and pyoluteorin were also extracted in the same way, except that the cells were separated before acidification with 1M HCL. The crude extracts were dried, dissolved in methanol and analyzed on an HPLC Varian (Prostar; 1100 Series), consisting of pressure module 6000 psi, UV detector and a C18 reverse-phase column.

The solvent conditions, flow rate and monitoring peak maxima included 10% acetonitrile -0.1% trifluoroacetic acid, 1.0 mL min^{-1} and 270 nm for 2,4-DAPG; 45% H_2O, 30% acetonitrile and 25%-methanol, 1 mL min^{-1} and 225 nm for pyrrolnitrin; and acetonitrile-methanol-water (7:5:8), 1.5 mL min^{-1}, and 310 nm for pyoluteorin. Linear gradient was used for detection of 2, 4-DAPG while isocratic gradient was used for detection of pyrrolnitrin and pyoluteorin. Purified antibiotics extracted from the reference strain *P. fluorescens* CHA0 were used as standards. Synthetic 2, 4- DAPG (Toronto Research Chemicals) and pyrrolnitrin (Sigma) were also used as standards.

Detection of *Phl* D gene involved in 2, 4-DAPG synthesis

The *PhlD* gene conserved in all 2, 4-DAPG producing bacteria was targeted for amplification with the two set of primers *Phl2a* 5′–GAG GAC GTC GAA GAC CAC CA–3′, *Phl2b* 5′–ACC GCA GCA TCG TGT ATG AG–3′ and BPF2, 5′–ACC CAC CGC AGC ATC GTT TAT GAG C –3′, BPR4, CCG CCG GTA TGG AAG ATG AAA AAG TC from the genomic DNA of *O. intermedium* NH-5 and the reference strain *P. fluorescens* CHA0. A 25 µL reaction mixture consisting of 1 × Taq DNA polymerase buffer (Ferments), 8–10 ng of the DNA, 15–25 ng of each primer, 1.5 mM $MgCl_2$, 200 µM of each, dATP, dCTP, dGTP, and dTTP (Fermentas), and 1.0 U of Taq polymerase (Fermentas) was amplified in a thermocycler (Eppendorf) with

the following conditions; Initial denaturation at 95°C for 5 min followed by 30 cycles [94°C for 1 min, 60 or 67°C (*Phl2a*, *2b* or BPF2, BPR4) for 1 min, 72°C for 1 min] and final extension 72°C for 5 min. The PCR product was analyzed on a 1.2% agarose gel, visualized with a UV transilluminator and compared with 1kb ladder (Fermentas).

Field evaluations

Location, varieties and experimental designs

The antagonistic strains were evaluated in the field at the Shakarganj Sugar Research Institute (SSRI), Jhang (Pakistan) for three consecutive years. Two sugarcane varieties SPF-234 and Co-1148, both susceptible to sugarcane red rot, were used. Field experiments were designed on the basis of different fungal inoculation methods, using "stalk inoculation" or "soil inoculation". In "stalk inoculation" experiments, *C. falcatum* was inoculated into above ground stalks of sugarcane plants to separate the pathogen from bacterial strains and test the ability of the strains to suppress disease through the mechanism of induced systemic resistance. In "soil inoculation" experiments, the fungus was inoculated into the soil.

The experiments were laid out in randomized complete block designs with the individual plot size of 10 m^2 (2m × 5m), and were established each year, by sowing new sugarcane plants in a different area of the same field, applying the same treatments each year.

Sowing of plants, cultural practices and treatments

The plants were established into a clay to clay loam soil of pH = 7.9, electrical conductivity = 1.2 dS m^{-1}, total soluble salts = 744.3 mg L^{-1}, organic matter = 0.39%, nitrogen = 0.013 ppm, phosphorus = 7.4 ppm and potassium = 144.7 ppm.

The plants were established by putting sugarcane setts in wide furrows with row-to-row distance of 0.75 m. The N: P: K fertilizer was applied at the equivalent of 150:100:100 kg ha^{-1} (Maqsood *et al*., 2000; Hassan et al. 2011). Plants were irrigated fortnightly during February-April, and irrigation interval was variable in May-August depending on the rainfall/climatic conditions and moisture content of the soil. The treatments in each trial included; (i) *O. intermedium* strain NH-5, (ii) *Pseudomonas* sp. NH-203, (iii) *Pseudomonas* sp NH-276, (iv) *S. maltophilia* NH-300 (v) 0.85% saline (negative control) (vi) *P. fluorescens* CHA0; a well characterized biocontrol agent (Voisard *et al*., 1989; Viswanathan and Samiyappan, 2008, Hassan et al. 2011).

Pathogen inoculation

In "soil inoculation" experiments, *C. falcatum* was inoculated by mixing infected stalks with soil before sowing the crop as described by Viswanathan and Samiyappan (2008) and Hassan *et al.* (2012). In "stalk inoculation" experiments, *C. falcatum* was inoculated to plants 6 months after planting and 1 month after the last bacterial application, by making a hole at the 3rd above-ground node of each cane, placing a 1 mL suspension of the fungal conidia (10^6 conidia mL^{-1}) using a syringe, followed by sealing the inoculation hole with cellophane membrane and cotton (Srinivasan and Bhat, 1961; Hassan *et al*., 2010a).

Bacterial inoculation

Bacteria were inoculated into LB broth and incubated in a shaking incubator for 24–48 h. The cells were separated by centrifuging at 12,000 rpm for 3 min, then suspended in in 0.87% saline to give a cell density of 10^9 CFU mL^{-1} (Hassan *et al*., 2011). Five mL of this saline suspension was drenched twice (4 and 5 months after planting) in the rhizosphere of each sugarcane plant. There were 90–92 plants in each experiment and a total volume of 450 mL of the bacterial inoculumn was used for each plot.

Disease assessment

In "stalk inoculation" experiments, disease was assessed twice i.e. at 30 and 60 d after pathogen inoculation. The plant stems were split longitudinally and the disease severity was scored on 0–9 scale based on the different parameters as described in Table 1 (Srinivasan and Bhat 1961; Hussnain *et al*., 2007). In "soil inoculation" experiments, disease was assessed by sorting the diseased plants and counting the number of infected tillers in each treatment. A total of 50–60 plants containing 1–2 tillers per plant in each replicate were randomly selected and assessed for the development of red rot. Disease symptoms were observed twice at 8 and 10 months (harvesting time) of the (crop harvest stage). The disease incidence and proportion of disease suppression were calculated using the following formulas.

$$\text{Disease incidence} = \frac{\text{Infected tillers}}{\text{Total tillers}} \times 100$$

Table 1. Parameters used for determining red rot severity scores on sugarcane, caused by *Colletotrichum falcatum*.

Parameter	Observation	Score
Condition of the shoot	Green	0
	Yellow/ dry	1
Lesion extent	Very rare lesion	1
	Lesion spreading, but not covering the entire cane area	2
	Lesion covering entire inner cane area	3
White spots	Restricted	1
	Progressive	2
Nodal transgression	If 1 node crossed by fungal pathogen	1
	If 2 nodes crossed by fungal pathogen	2
	If 3 nodes crossed by fungal pathogen	3

$$\% \text{ Disease suppression} = 1 - \frac{\text{Disease in treatment}}{\text{Disease in control}} \times 100$$

Statistical analyses

All the data were subjected to analysis of variance analysis (ANOVA) using standard procedures for randomized complete block designs and MSTAT-C computer software. The data for varieties and time periods was combined over each year. Mean values were separated and compared using LSD tests.

Results

The four native bacterial strains exhibiting *in vitro* and *in vivo* biocontrol activity against *C. falcatum* causing sugarcane red rot (Hassan *et al*., 2010a) produced various types of secondary metabolites, and suppressed red rot on both sugarcane varieties SPF-234 and Co-1148 under field conditions.

Inhibition of fungus by cell-free supernatants, volatile and diffusible antibiotics

Mycelial growth of *C. falcatum* was inhibited by 14–52% by the cell free supernatants and inhibitory factors of the antagonistic bacteria *O. intermedium* strain NH-5, and by 22–46% by metabolites of *S. maltophilia* strain NH-300. Cell-free supernatants of *O. intermedium* strain NH-5 caused 52% inhibition of the fungus and volatile antibiotics gave 33% inhibition. The *S. maltophilia* strain NH-300 inhibited *C. falcatum* by up to 46% by producing diffusible antibiotics. The effects of metabolites of all the strains on inhibition of *C. falcatum* were statistically significant (Table 2).

Production of HCN, protease and siderophores

All the tested bacterial strains and the reference strain *P. fluorescens* CHA0 produced HCN. The hydrolytic enzyme protease was only produced by the strain NH-276 while two strains, *O. intermedium* NH-5 and *Pseudomonas* sp. NH-203, produced siderophores (Table 3).

Biochemical identification of 2, 4-DAPG

The native strain *O. intermedium* NH-5 and reference strain *P. fluorescens* CHA0 produced variable quantities of DAPG 0.4 and 0.1 μg mL^{-1} of 10^8 CFU respectively as (Table 3). None of the native strains produced pyoluteorin and pyrrolnitrin.

Absence of the *PhlD* gene in *Ochrobactrum intermedium*

No band corresponding to the *PhlD* gene was observed in the test strain (Figure 1). This indicates the presence of a different biosynthetic pathway for 2, 4-DAPG in *O. intermedium*.

Field performance of the antagonistic strains

Year 1

In the "soil inoculation", the antagonistic bacteria *S. maltophilia* strain NH-300 suppressed red rot by 35%, and *O. intermedium* strain NH-5 gave 32% suppression of the disease. The reference strain *P. fluorescens* CHA0 suppressed disease by 40%.

In the "stalk inoculation" experiment, disease suppression was greatest from *O. intermedium* strain NH-5, with 53% disease suppression of red rot, followed by the reference strain *P. fluorescens* strain CHA0 which gave 50% disease suppression. The *S. maltophilia* strain NH-300 caused 43% disease sup-

Table 2. *In vitro* inhibition of *Colletotrichum falcatum* by the extracellular metabolites and antibiotics produced by antagonistic bacteria.

Strain	% Inhibition of *C. falcatum*				
	Extracellular metabolites			Antibiotics	
	5%	15%	25%	Diffusible	Volatile
Ochrobactrum intermedium NH-5	14.7 b[a]	35 b	52 b	45 a	32.5 a
Pseudomonas sp. NH-203	12.3 b	26.7 c	36 d	6.7 b	10.8 c
Pseudomonas sp. NH-276	6 c	38 a	55 a	5.2 b	11.7 bc
Stenotrophomonas maltophilia NH-300	22 a	37 ab	46 c	46 a	16.7 b
Pseudomonas fluorescens CHA0	ND	ND	ND	ND	ND
Control (d.H_20/ LB)	0 d	0 d	0 e	0 b	0 d

[a] The mean values of three replications bearing different letters in the same column are significantly different from each other at $P<0.05$.

Table 3. Production of different antifungal metabolites by antagonistic bacteria.

Strain	Production of inhibitory factors					
	HCN[a]	Protease zone diameter (mm)	Siderophores zone diameter (mm)	PLT[b]	PRN[b]	2, 4-DAPG[b] (µg mL^{-1})
Ochrobactrum intermedium NH-5	2	0	8.2	--	--	0.4
Pseudomonas sp. NH-203	1	0	3.6	--	--	--
Pseudomonas sp. NH-276	2	7.2	0	--	--	--
Stenotrophomonas maltophilia NH-300	2	--	0	--	--	--
Pseudomonas fluorescens CHA0	2	10.6	9.7	+	+	0.1
Control (d.H_20/ LB)	0	0	0	--	--	--

[a] HCN production by plate assay: 1, brown pigmentation on half portion of filter paper; 2, complete brown pigmentation on filter paper indicates HCN production.
[b] Pyoluteorin (PLT), Pyrrolnitrin (PRN) and 2, 4- DAPG production by HPLC: +, positive production; --, no production.

pression in this experiment. The effect of the other bacterial strains on red rot suppression was statistically significant (Tables 5 and 7).

Year 2

In the second year of field experiments, plants treated with strain *O. intermedium* NH-5 had the least red rot with a disease severity score of 3.7 and disease suppression 56% in the "stalk inoculation" which was statistically equivalent to disease reduction of the positive control strain CHAO. In the "soil inoculation" experiment, activity of *S. maltophilia* strain NH-300 was the greatest, and equivalent to the positive control with 60% disease suppression. The *O. intermedium* strain NH-5 suppressed the disease by 43%. Ability of all the strains to suppress red rot was significantly different (Tables 5 and 7).

Year 3

During this year, the antagonistic bacterium *O. intermedium* strain NH-5 caused 47% red rot suppression in the "stalk inoculation" experiment. This

efficacy was second to that of positive control strain CHA0, which suppressed the disease by 60%. The strain *S. maltophilia* NH-300 suppressed the disease by up to 42%. A similar pattern of performance was observed in the "soil inoculation" experiment, but there was a high level of disease suppression (Table 7). The effects of all strains on red rot was were statistically significant (Tables 5 and 7).

The biocontrol efficacy of the bacterial strains was similar on both sugarcane varieties, but differed with respect to the time period throughout the three years of field experiments (Tables 4 and 6).

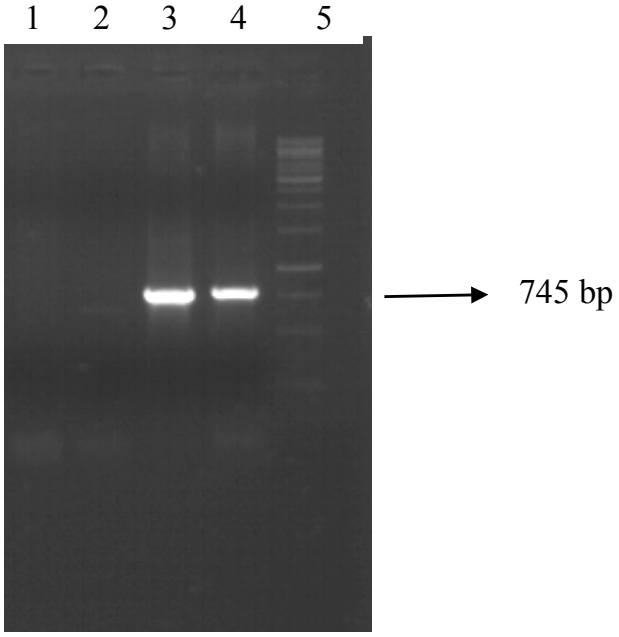

Figure 1. Amplification of *PhlD* gene involved in synthesis of 2,4-diacetylphloroglucinol (DAPG). Lane 1, 2, *Ochrobactrum intermedium* strain NH-5; lane 3, 4, *Pseudomonas fluorescence* CHA0; lane 5, 1kb Ladder (Fermentas).

Discussion

Biological control of red rot of sugarcane using plant growth promoting rhizobacteria (PGPR) is an appealing alternative management strategy to other control measures. The biocontrol agents belonging to *Bacillus* and *Pseudomonas* are well characterized and documented (Ongena and Jacques, 2008; Ahmadzadeh and Tehrani, 2009). However, use of antagonistic strains belonging to *Ochrobactrum* and *Stenotrophomonas* has been rarely reported.

This paper is first to report on the potential of *O. intermedium* and *S. maltophilia* strains for control the red rot of sugarcane under field conditions. However, the occurrence of these two antagonistic bacteria has been shown to be rare (0.3% of the antagonistic bacteria) in the rhizosphere of sugarcane (Hassan *et al*., 2010a).

Cell-free supernatants, volatile and diffusible antibiotics of the antagonistic strains inhibited the *C.*

Table 4. Analysis of variance table for the bacterial strain biocontrol activity against *Colletotrichum falcatum* on plants of two sugarcane varieties in "stalk inoculation" experiments.

Source	Year 1-3 (combined)	Year-1	Year-2	Year-3
Year × Strain	**	---	---	---
Varieties (A)	†NS	†NS	†NS	†NS
Strain (B)	**	**	**	**
Time period (C)	**	†NS	**	**
Variety × Strain (AB)	†NS	†NS	†NS	†NS
Variety × Time period (AC)	†NS	†NS	†NS	*
Strain × Time period (BC)	†NS	†NS	†NS	†NS
Variety × Strain × Time period (ABC)	†NS	†NS	†NS	†NS

* Significant at P=0.05.
* Significant P=0.01.
† NS= Non significant.

Table 5. Effects of the antagonistic bacteria on the incidence of red rot (caused by *Colletotrichum falcatum*) on sugarcane in "stalk inoculation" experiments.

Strain	Year-1		Year-2		Year-3	
	Disease score	% Disease suppression	Disease score	% Disease suppression	Disease score	% Disease suppression
Ochrobactrum intermedium NH-5	4 e	53	3.7 d	56	4.4 c	47
Pseudomonas sp NH-203	7.8 b	8	7.5 b	19	7.8 b	6
Pseudomonas sp NH-276	7 c	17	7.6 b	10	8.1 ab	3
Stenotrophomonas maltophilia NH-300	4.8 d	43	4.3 c	49	4.8 c	42
Pseudomonas fluorescens CHA0	4.2 e	50	3.3 d	61	3.3 d	60
Control	8.5 a	0	8.4 a	0	8.3 a	0

The mean values of three replications bearing different letters in the same column are significantly different from each other.

Table 6. Analysis of variance table for biocontrol activity of bacterial the strains against *Colletotrichum falcatum* on two varieties of sugarcane plants in "soil inoculation" experiments.

Source	Year 1-3 (combined)	Year-1	Year-2	Year-3
Year × Strain	**	---	---	----
Varieties (A)	†NS	†NS	†NS	†NS
Strain (B)	**	**	**	**
Time period (C)	**	**	**	**
Variety × Strain (AB)	†NS	**	**	†NS
Variety × Time period (AC)	†NS	†NS	†NS	†NS
Strain × Time period (BC)	†NS	*	†NS	†NS
Variety × Strain × Time period (ABC)	†NS	†NS	†NS	†NS

* Significant at P=0.05.
** Significant at P=0.01.
† NS= Non significant.

falcatum on test plates. The efficacy of such secondary metabolites has been reported previously (Naureen *et al*., 2009, Hassan *et al*., 2010b). Metabolites of the antagonistic bacteria *O. intermedium* strain NH-5 and *S. maltophilia* strain NH-300 caused the maximum inhibition of the *C. falcatum in vitro*, and these strains also showed good potential to suppress the disease *in vivo*. Hence, an association was found between the inhibition of pathogen *in vitro* and disease control under field conditions. The strain *O. intermedium* NH-5 also produced HCN, siderophores and 2, 4-diacetylphloroglucinol, which are compounds produced by a number of potent biocontrol agents (Bloemberg and Lugtenberg, 2001). All the other strains produced one or more of these metabolites, but not 2, 4-DAPG. Moreover, these strains were unable to significantly reduce red rot under field conditions. These findings support previous reports that 2, 4-DAPG producing strains are capable of protecting the plants from a broad range of pathogens, including fungi, viruses and nematodes (De Souza *et al*., 2003).

Table 7. Effect of the antagonistic bacteria on the incidence of red rot disease caused by *Colletotrichum falcatum* on sugarcane in "soil inoculation" experiments.

Strain	Year-1		Year-2		Year-3	
	Disease incidence (%)	Disease suppression (%)	Disease Incidence (%)	Disease suppression (%)	Disease incidence (%)	Disease suppression (%)
Ochrobactrum intermedium NH-5	23.3 d	32	25.3 d	43	19.2 d	58
Pseudomonas sp. NH-203	28.9 c	13	31.1 c	29	34.4 b	24
Pseudomonas sp. NH-276	32.1 b	4	33 b	24	32.2 c	29
Stenotrophomonas maltophilia NH-300	21.7 e	35	17.2 e	60	20.5 d	55
Pseudomonas fluorescens CHA0	20.1 f	40	17.6 e	60	17.1 e	62
Control	34.5 a	0	43.8 a	0	45.2 a	0

The mean values of three replications bearing different letters in the same column are significantly different from each other.

The 2, 4-DAPG-producing biocontrol agent *O. intermedium* strain NH-5 significantly suppressed disease in the "stalk inoculation" experiments, while the strain *S. maltophilia* NH-300 exhibited best biocontrol activity in the "soil inoculation" experiments. Biological suppression of sugarcane red rot by the *Pseudomonas* spp. has already been reported by Viswanathan and Samiyappan (2008) and Hassan et al. 2011. However, the present study is the first report to demonstrate the potential of strains belonging to the bacterial genera *Ochrobactrum* and *Stenotrophomonas* for suppression of this disease under field conditions.

Variation in the activity of these antagonistic strains may be related to the production of variable secondary metabolites and hence, adoption of variable mechanism to suppress the disease. In the "stalk inoculation" experiments, the pathogen was inoculated in a way that avoided direct contact with the antagonistic bacterial strains. In the "soil inoculation" experiments, both the pathogen and the antagonistic bacteria were inoculated in soil to facilitate direct physical contact. The mechanism of disease suppression in which a biocontrol agent acts without contact with the pathogen is termed induced systemic resistance (ISR). ISR is long-lasting resistance and protects host plants against a broad range of pests (Heil and Bostok, 2002). Low performance of *O. intermedium* NH-5 in the "soil inoculation" experiments compared to the "stalk inoculation" experiments indicates the bacteria which suppress disease by the ISR mechanism are likely to be the most efficacious. Involvement of ISR in management of soil-borne diseases by PGPR in many crops has been well documented (Fallahzadeh *et al*., 2009; Verhagen *et al*., 2010). Performance of the strains for control of red rot was similar for both of the sugarcane varieties used in this study. However, the efficacy of the strains to control disease decreased with increasing time. This could have been due to the rapid progression of the pathogen over time. These findings suggest that multiple applications of antagonists after a suitable time intervals may be necessary. There were no significant 'variety' effects and the disease levels in the second time period of assessment were greater in general than the first. The effects of all strains and their mutual interactions is shown in Tables 4 and 6.

This study reports the production of 2,4 DAPG by the *O. intermedium* strain NH-5 and has demonstrated the potential of *O. intermedium* NH-5 and *S. maltophilia* NH-300 for control of red rot in sugarcane crops.

The role of 2,4 DAPG-producing strains in suppressing plant diseases has been widely reported (Meziane *et al*., 2005). This compound 2, 4 DAPG is mostly produced by the pseudomonads, and the *phlD* gene has been recognized as marker for the 2, 4 DAPG-producing pseudomonads (McSpadden *et al*., 2001). Absence of the *phlD* gene suggests presence of a biosynthetic pathway different from that in pseudomonads. It will be important to explore the genes involved in DAPG production in *O. intermedium*. and to identify antifungal metabolites involved in the antagonistic activity of the *S. maltophilia* NH-300.

The detection of virulence genes, genomic analysis and comparison with the other strains of *Stenotrophomonas* and *Ochrobacterum* spp., which are either human opportunistic or plant associated (Crossman *et al*., 2008; Teyssier *et al*., 2005), should be investigated before registration of these strains as biopesticides.

The strains belonging to genera *Ochrobactrum* and *Stenotrophomonas* are being utilized as PGPRs, but few strains also have bivalent interaction with plant or human hosts. They colonize human tissues and cause diseases in humans. Hence, there is need to carry out risk assessment before registering them as bioinoculants or biopesticides (Berg *et al*., 2005).

In conclusion, the potent antagonistic strains *O. intermedium* NH-5 produced HCN, siderophores and the antibiotic 2, 4-DAPG, while *S. maltophilia* NH-300 produced only HCN. These strains suppressed the red rot of sugarcane under field conditions, irrespective of the fungal inoculation methods used in this study. Moreover, these strains showed consistency in performance in three consecutive years of field evaluations. These results suggest that these strains are good candidates for biopesticide development. However, additional investigations are required, including toxicity assays, risk assessment, as well as development of suitable formulations and application methods compatible with conventional sugarcane crop management.

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