SHORT COMMUNICATION

BIOLOGICAL CONTROL OF PATHOGENS CAUSING THE *CYMBIDIUM* PSEUDOBULB ROT COMPLEX USING FLUORESCENT *PSEUDOMONAS* STRAIN BRL-1

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SUMMARY

Cymbidium, a commonly grown orchid of the Eastern Himalayas of India, suffers from huge crop losses due to an epidemic of pseudobulb rot since 1995. The disease is caused by the synergistic activity of three pathogens, *Fusarium oxysporum*, *Mucor hiemalis* f. sp. *hiemalis* and *Pectobacterium carotovorum* subsp. *carotovorum*. A rhizosphere bacterium, identified as a fluorescent *Pseudomonas*, named BRL-1, showed both *in vitro* and *in vivo* antagonistic activity against these pathogens. Our results show that antimicrobial activity of the isolate might be linked with the production of siderophores, proteases, chitinases and indole acetic acid (IAA). A talc-based formulation of the antagonist did not only protect *Cymbidium* plants from the disease but improved also its growth.

Key words: Biocontrol, antagonism, *Fusarium oxysporum*, *Mucor hiemalis* f. sp. *hiemalis*, *Pectobacterium carotovorum subsp. carotovorum.*

Cymbidium is widely grown throughout the cooler parts of the world, and is one of the most popular orchids cultivated commercially in India, in Darjeeling and its adjoining area. Since 1995, most of the orchid growers in this region suffer from huge losses due to an epidemic called pseudobulb rot that develops during monsoon months. More than 15 species and about 25 hybrid varieties of *Cymbidium* are commercially grown in this region, all of which are susceptible to pseudobulb rot. The disease is epidemic during the wet season and its spread is favoured by wind and rain. Some 69% of the nurseries inspected in the affected regions were contaminated at a rate of 60-100%. Three pathogens were consistently isolated from the diseased samples: namely *Pectobacterium carotovorum* subsp. *carotovorum, Fusarium oxysporum* and *Mucor hiemalis* f. sp. *hiemalis*, in the early, middle and later phases of disease progression, respectively. The apparent synergistic activity of the three pathogens seems to be the cause of the uncontrolled epidemics (Sen *et al.*, 2006).

Several chemical control measures have been implemented by orchid growers, so far with no significant achievements. Rhizosphere bacteria like the fluorescent pseudomonads *Bacillus subtilis* and *Streptomyces* sp. are alleged plant disease suppressors (Ryder *et al.,* 1994). In our previous work we have screened 54 fluorescent pseudomonads against the agents of *Cymbidium* rot using an *in vitro* system. Six of them (BRL-1, Dj-5, Sg-5, Sg-1, BB-9 and Sili-15) showed positive antagonistic activity (Sen *et al.,* 2007). Now attempts have been made to identify the strain with the highest antagonistic potential both *in vitro* and *in vivo.*

The causal agents of *Cymbidium* rot (Sen *et al.,* 2006) and the six pre-screened antagonists (Sen *et al.,* 2007) were obtained from our laboratory culture stock. The comparative *in vitro* antagonistic effects of the six fluorescent pseudomonads were tested against fungal pathogens on peptone glucose agar (PGA) following the method of Skidmore and Dickinson (1970). This was done by streaking a 24-h-old culture of the test strain in a circle 'O'- and semicircle 'U'-shaped pattern, whereas the fungal pathogens were point inoculated at the centre of the plate. Plates without antagonists served as control. The plates were incubated at 30°C for 5 days and growth inhibition measured. Fungal mycelium growing towards the zone of interaction was observed under a light microscope and abnormalities in the hyphae were recorded. To test the inhibitory effect of antagonists against *P. carotovorum* subsp. *carotovorum*, it was streaked straight onto nutrient agar plate and the test strain streaked perpendicular to the bacterium in such a way that they did not touch each other. Inoculated plates were again incubated at 30°C for 48 h (Dubey and Maheshwari, 2002). For the strain which showed effective antagonistic activity in the comparative *in vitro* antagonism test, further experiments were designed to determine its mode of action towards the causal agents of *Cymbidium* rot *in vivo.*

To test the chitinolytic property of a potential antagonistic fluorescent *Pseudomonas* species, it was inoculat-

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ed on LB medium supplemented with 0.5% colloidal chitin. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicated chitinase activity of the strain (Basha and Ulagnathan, 2002). Production of hydrolytic enzymes was qualitatively assayed in minimal medium containing gelatine, starch, pectin and carboxymethyl cellulose (CMC). Plates were incubated for 48 h at 30°C and formation of a clear zone around bacterial colonies was read as positive (Gaur *et al.*, 2004). Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect siderophore production. CAS agar (blue agar) was inoculated at the centre of the plate with a 24 h old culture of the fluorescent *Pseudomonas* and kept for incubation at 30°C for 72 h. The change of the blue colour of the medium to orange or the presence of a yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore. Production of hydrogen cyanide was tested qualitatively according the method of Wei *et al.* (1991). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of a Petri dish. The Petri dishes were then sealed with parafilm and incubated at 30°C for 4 days. A change of colour of the filter paper strip from yellow to light brown, to brown or reddish brown was recorded as weak, moderate or strong cyanogenic potential, respectively. Indole acetic acid (IAA) production was quantified spectrophotometrically, growing the strain in 10 ml of minimal salt media supplemented with 100 µg/ml of tryptophan, and incubated at 30°C under shaking for 48 h. Broth culture was centrifuged at 7,500 rpm for 10 min. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent were added and incubated at 30°C for 25 min. Absorption was read at 530 nm and IAA levels were quantified, using a standard curve (Gaur *et al.*, 2004).

A talc-based formulation of the antagonist was prepared using a method developed by Vidyasekaran and Muthamilan (1995) modified. Ten gram carboxy methyl cellulose (CMC) per kg of sterile talc was used as adhesive. The bacterial suspension ($8x10^9$ cfu/ml^{-1}) was mixed with sterile talc (400 ml/kg^{-1}) and air dried (approximately to 35% w/w, moisture content). The formulation was stored at 4°C for up to 180 days. The antagonist in the talc-based formulation was monitored *in vitro* with respect to its shelf-life and viability under this storage condition. Survival of the bacterial population in the formulation was assayed at 30 days intervals using King's medium B in a dilution plate assay according to Vidyasekaran and Muthamilan (1995).

In vivo evaluation of talc-based formulation of a potential antagonist was performed in clay pots. Healthy, 2-month-old *Cymbidium* plantlets from a nursery were washed thoroughly with tap water and sown in pots filled with 3 kg non-sterile soil (3 part loam and 1 part mature cow manure). Inocula of fungal and bacterial pathogens were prepared in potato dextrose broth and nutrient broth, respectively and incubated in a rotary shaker at 150 rpm at 25±2°C. After 48 h incubation the bacterial culture was centrifuged (7,000 rpm for 15 min at 4°C). Pellets were washed with, and resuspended in sterile distilled water to a concentration of 10^7 cfu/ml^{-1} (haemocytometer cell count). For fungal inoculum, after 96 h incubation at 30°C, cultures were filtered through cheesecloth to remove the mycelial mass. Microconidial (*F. oxysporum*) and zoospore (*M. hiemalis* f. sp. *hiemalis*) densities in the filtrate were determined using a haemocytometer and adjusted to 10^6 conidia or spores per ml^{-1}. Soils was infested with the inocula before plantlets were transplanted in the pots and incubated for 2 days at 25±2°C. Two sets of treatments were de-

Table 1. Comparative analysis of *in vitro* inhibitory effect of six screened fluorescent *Pseudomonas* strains cultured as semicircular ('U'- shaped) and circular ('O'- shaped) streak, around *Mucor hiemalis f. sp. hiemalis*.

Strains		Colony diameter (in cm) of *M. hiemalis f. sp. hiemalis* after incubation period of				
		0h	24h	48h	72h	96h
Control		0 ± 0.00	1.97 ± 0.16	3.65 ± 0.4	5.77 ± 0.53	7.62 ± 0.51
BRL-1	U	0 ± 0.00	0.66 ± 0.12	0.93 ± 0.09	1.67 ± 0.08	2.05 ± 0.08
	O	0 ± 0.00	0.4 ± 0.09	0.76 ± 0.21	0.92 ± 0.2	1.06 ± 0.16
Dj-5	U	0 ± 0.00	0.91 ± 0.09	1.81 ± 0.31	2.22 ± 0.48	2.83 ± 0.38
	O	0 ± 0.00	0.5 ± 0.04	1 ± 0.1	1.25 ± 0.05	1.55 ± 0.05
BB9	U	0 ± 0.00	1.3 ± 0.14	2.62 ± 0.41	3.82 ± 0.23	4.2 ± 0.3
	O	0 ± 0.00	0.92 ± 0.14	1.57 ± 0.41	2.02 ± 0.23	2.15 ± 0.3
Sg-5	U	0 ± 0.00	1.47 ± 0.04	2.4 ± 0.12	3.85 ± 0.04	4.4 ± 0.07
	O	0 ± 0.00	1 ± 0.04	1.5 ± 0.12	1.92 ± 0.04	2.5 ± 0.07
Sg-1	U	0 ± 0.00	1.1 ± 0.27	2.03 ± 0.2	3.83 ± 0.22	4.4 ± 0.34
	O	0 ± 0.00	1.02 ± 0.27	1.65 ± 0.2	1.95 ± 0.22	2.37 ± 0.34
Sili-15	U	0 ± 0.00	1.37 ± 0.11	2.12 ± 0.16	3.57 ± 0.22	4.62 ± 0.14
	O	0 ± 0.00	0.75 ± 0.11	1.77 ± 0.16	2.67 ± 0.22	2.87 ± 0.14

vised: (i) soil was infested with all three pathogens and *Cymbidium* bulbs and roots were uniformly coated with the talc-based formulation of the antagonist; (ii) soil was infested with all three pathogens before transplanting *Cymbidium* plantlets. Each treatment consisted of 30 plants (3 plants per pot and 10 replicates). One set of 30 plants was not inoculated and served as controls. Disease intensity was assayed periodically (15 days interval up to 60 days) as the percentage of pseudobulb and roots infected by the pathogens. Four randomly selected bulbs per treatment were uprooted and intensity of disease recorded.

In an *in vitro* assay six pre-screened antagonists were evaluated against all the pathogens by dual plate culture technique. In dual culture, growth inhibitions of fungal pathogens (*F. oxysporum* Schlecht and *M. hiemalis* f. sp. *hiemalis*) were variably inhibited by all the six strains, as shown by a clear inhibition of colony growth (Table 1 and 2). In all cases, the mycelia in the interaction zone stopped growing and gradually lost vigour. The bacterial pathogen (*P. carotovorum subsp. carotovorum*) was also inhibited by the antagonists (Fig. 1). The fluorescent *Pseudomonas* strain BRL-1 appeared to be the strongest antagonist. Effects of this bacterium were hyphal shrivelling, swelling, fragmentation, short branching, coagulation of cytoplasm and, ultimately, lysis (Fig. 2b and 2d).

The production of different secondary metabolites by BRL-1 in an *in vitro* system (siderophores, hydrolytic enzymes protease and chitinase and IAA) is presented in Table 3. Trivedi *et al.* (2008) reported that siderophores and chitinase are responsible for growth inhibition of plant pathogens, while Sharaf and Farrag (2004) reported that IAA reduces spore germination, mycelial dry weight and protein content of pathogenic fungi, thus preventing significantly any chance for disease induction by soil pathogens. Our study complements their findings by demonstrating considerable growth inhibition and morphological abnormalities of the hyphae by strain BRL-1.

Plants of treatment (i) where the bulbs were treated with the talc-based formulation of the antagonist and potted into pathogen-infested soil showed only 5-6% disease (Fig. 3). It was observed that the plants of this set were not only significantly protected from the disease but showed also healthy and vigorous growth. Plants from treatment (ii) where bulbs were potted into pathogen infested soil only, showed gradual increase in disease intensity, which reached more than 73% at 60 days after planting (Fig. 3). Plants of this set showed

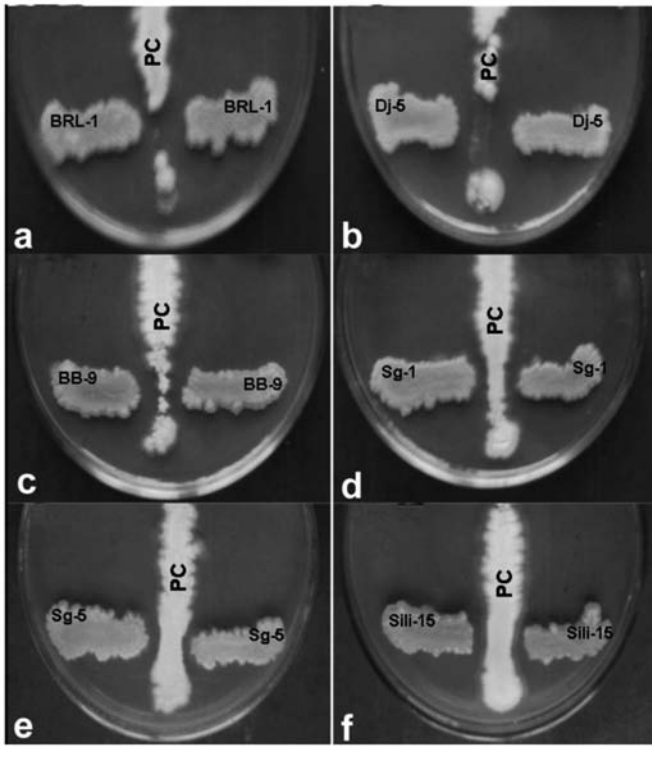

Fig. 1. Growth inhibition of *Pectobacterium carotovorum subsp. carotovorum* (PC) by the six screened fluorescent *Pseudomonas* (see also text).

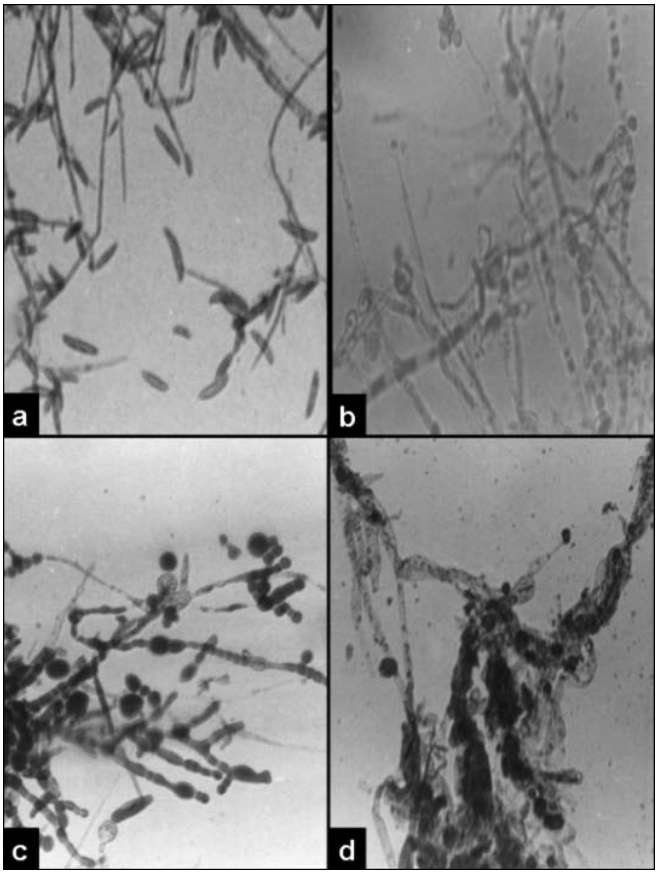

Fig. 2. Effects of strain BRL-1 on mycelia of tested fungi. a. *F. oxysporum* mycelium grown on PGA (control); b. distorted mycelium of *F. oxysporum* present in the interacting zone when grown with the antagonist; c. mycelium of *Mucor hiemalis f. sp. hiemalis* grown on PGA (control); d. heavily damaged mycelium of *Mucor hiemalis f. hiemalis* found at the zone of interaction.

Table 2. Comparative analysis of *in vitro* inhibitory effect of six screened fluorescent *Pseudomonas* strains cultured as semicircular ('U'- shaped) and circular ('O'- shaped) streak, around *Fusarium oxysporum*.

Strains		Colony diameter (in cm) of *F. oxysporum* after incubation period of					
		0h	24h	48h	72h	96h	120h
Control		0 ± 0.00	0.9 ± 0.00	2.3 ± 0.11	3.8 ± 0.17	4.9 ± 0.15	6.2 ± 0.22
BRL-1	U	0 ± 0.00	0.16 ± 0.08	0.42 ± 0.11	0.9 ± 0.2	1.2 ± 0.18	1.26 ± 0.19
	O	0 ± 0.00	0 ± 0.00	0.49 ± 0.03	0.75 ± 0.12	0.88 ± 0.01	0.96 ± 0.02
Dj-5	U	0 ± 0.00	0.4 ± 0.03	0.7 ± 0.12	1.2 ± 0.00	1.75 ± 0.05	1.99 ± 0.02
	O	0 ± 0.00	0.25 ± 0.05	0.65 ± 0.00	0.98 ± 0.00	1.36 ± 0.05	1.5 ± 0.00
BB9	U	0 ± 0.00	0.37 ± 0.08	0.75 ± 0.23	1.7 ± 0.38	2.05 ± 0.47	2.19 ± 0.58
	O	0 ± 0.00	0.36 ± 0.09	0.82 ± 0.16	1.2 ± 0.16	1.5 ± 0.2	1.72 ± 0.16
Sg-5	U	0 ± 0.00	0.7 ± 0.08	1.6 ± 0.14	1.76 ± 0.16	1.96 ± 0.12	2.2 ± 0.08
	O	0 ± 0.00	0.8 ± 0.04	1.23 ± 0.16	1.6 ± 0.14	1.8 ± 0.16	1.9 ± 0.12
Sg-1	U	0 ± 0.00	0.67 ± 0.14	1.47 ± 0.14	1.67 ± 0.19	2.02 ± 0.33	2.47 ± 0.48
	O	0 ± 0.00	0.8 ± 0.11	1.45 ± 0.16	1.77 ± 0.19	1.96 ± 0.27	2.3 ± 0.25
Sili-15	U	0 ± 0.00	0.55 ± 0.11	1.5 ± 0.15	2.05 ± 0.16	2.35 ± 0.3	2.67 ± 0.4
	O	0 ± 0.00	0.88 ± 0.24	1.63 ± 0.12	1.92 ± 0.12	2.23 ± 0.12	2.56 ± 0.09

Table 3. Secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1.

Siderophore production	+++
IAA production	+++
Protease activity	++
Chitinase activity	+
Amylase activity	-
Pectinase activity	-
Cellulase activity	-
HCN production	-

'+++' Strong production; '++' Moderate production; '+' Low production; '-' No production.

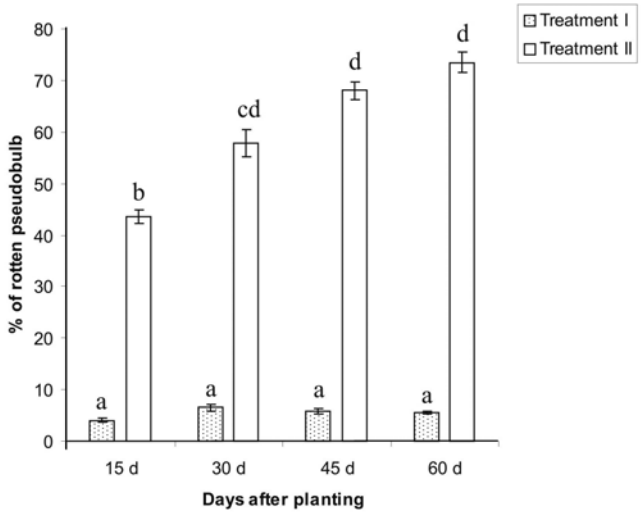

Fig. 3. *In vivo* effect of a talc-based formulation of strain BRL-1 for controlling *Cymbidium* rot. Values are the mean of four plants, randomly sampled. Columns denoted by a different letter differ significantly at $p \leq 0.05$. Analysis by one-way ANOVA. Bars represent standard error (SE).

hollow and fibrous condition of both pseudobulb and root system.

On the basis of these studies it can be concluded that the fluorescent *Pseudomonas* BRL-1 isolate has antagonistic properties, possibly effected by siderophores, proteolytic enzymes, IAA and chitinolytic activity. It was also evident that application of the talc-based formulation of the antagonist not only protected plants from the disease but also induced more vigorous growth, thus proving useful for the control of *Cymbidium* pseudobulb rot in practice.

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