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Biological Control of Fruit Rot and Die Back Disease of *Capsicum annuum* with *Pseudomonas aeruginosa* WS-1

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ABSTRACT

Colletotrichum capsici is the causal agent of fruit rot and die back disease, the most important diseases of chilli worldwide. A rhizospheric isolate *Pseudomonas aeruginosa* WS-1 successfully showed both *in vitro* (10^7 cells/ml) and *in vivo* (3×10^8 cfu/ml) antagonistic activity against the pathogen *C. capsici*. On dual plate culture, the antagonist effectively suppressed the growth of the fungal pathogen. Light microscopy of mycelium from the interacting region showed hyphal shriveling, hyphal deformities, tip swelling, short branching and granulation of cytoplasm resulting in lysis of hyphae. The antifungal activity of this isolate is correlated with the production of siderophores and HCN. The strain also produces mycolytic enzyme-like proteases and chitinases, which are well known to lyse the cell walls of the fungal pathogen. The efficacy of *P. aeruginosa* WS-1 application under greenhouse conditions was comparable to that of fungicide (carbendazim) to manage the disease. This result suggests that *P. aeruginosa* WS-1 has the potential to be used as a biopesticide for effective management of fruit rot and die back disease of chilli in India.

Keywords: antagonist, chilli, *Colletotrichum capsici*
Abbreviations: CAS, Chrome Azurol S; **CFU**, colony forming unit; **HCN**, hydrocyanic acid; **PDA**, potato dextrose agar; **PGA**, peptone glucose agar; **TSA**, tryptic soy agar

INTRODUCTION

Chilli is considered to be one of the most important crops in the tropics. The area cultivated with chilli worldwide is about 17,64,284 ha for producing fresh chilli, and around 18,37,419 ha for producing dried chilli; a total area of 36,01,703 ha with a total production of 2,99,08,102 t (FAO 2007). The most important producers and exporters of chilli include China, India, Mexico, Morocco, Pakistan, Thailand and Turkey. Chilli has many culinary advantages. It comprises numerous chemicals including steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fiber and mineral elements (Bosland and Votava 2003). Many chilli constituents are important for their nutritional value, flavor, aroma, texture and colour. Chillies are low in sodium and cholesterol-free, rich in vitamins A and C, and are a good source of potassium, folic acid and vitamin E. Fresh green chilli peppers contain more vitamin C than citrus fruits and fresh red chilli has more vitamin A than carrots (Osuna-García *et al*. 1998; Marin *et al*. 2004). Two chemical groups produced by chilli are capsaicinoids and carotenoids. The capsaicinoids are alkaloids that make hot chilli pungent. A large number of carotenoids provide high nutritional value and the colour to chilli (Britton and Hornero-Méndez 1997; Hornero-Méndez *et al*. 2002; Pérez-Gálvez *et al*. 2003). Diseases caused by fungi, bacteria and viruses are the major constraints to chilli production. Anthracnose disease caused by *Colletotrichum* species, bacterial wilt caused by *Pseudomonas solanacearum*, and mosaic disease caused by *Chilli veinal mottle virus* (CVMV) or *Cucumber mosaic virus* (CMV) are the most serious destructive diseases of chilli (Isaac 1992).

The chilli (*Capsicum annuum*) in India suffers from two serious fungal diseases – ripe fruit rot and die back, both caused by same fungus, *Colletotrichum capsici* (Syd.) Butler and Bisby (Sing 2000). The diseases caused by *Colletotrichum* species are some of the most economically important diseases reducing marketability yield from 10-80% of the crop production in some developing countries (Poonpolgul and Kumphai 2007). The fungus survives in the field in plant debris and secondary spread takes place by windborne conidia. Seeds from badly diseased fruits also carry the primary inocula (Sing 2000). The disease protection measures of plants are still restricted to the application of various chemical fungicides which result in fungicide tolerance if a single compound is relied upon too heavily. Management of *C. capsici* in chilli is limited by efficient seed transmission and lack of effectiveness of many earlier chemicals (Maneb, Ziram, Perenox, Blitox-50 etc.) (Sing 2000; Smith 2000). It can be controlled by a combination of cultural practices and fungicide application such as quarantine, removal of infected plant debris, sowing disease-free seeds, sanitation procedure, seed treatment and spraying of chemical compounds during the crop cycle.

Biological control is an important method in the management of plant pathogens. Advantages include: reduction of dependence of high-risk chemicals for disease control or other ecological and economical benefits (Bale *et al.* 2008). The diversity of naturally occurring microorganisms of the rhizosphere and phyllosphere and their potential for biological control of plant pathogens have been examined extensively (Jayraj *et al*. 2007). Pseudomonads are considered to be an important rhizosphere organism, wherein considerable research is underway globally to exploit its potential. Fluorescent pseudomonads help in the maintenance of soil health, protect crops from pathogens and are metabolically and functionally more diverse than *Bacillus subtilis*, *B. lichiniformis*, *Trichoderma harzianum*, *T. virens*, etc. (Choudhury *et al*. 2009). A number of fluorescent pseudomonads have been reported to have *in vitro* and *in vivo* biocontrol potential against a wide range of phytopathogens (Hodges *et al.* 1994; Fanny and Pfender 1997; Gupta *et al.* 2002; Kishore *et al.* 2005; Mansoor *et al.* 2007).

In this study, attempts have been made to evaluate the *in*

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Fig. 1 Diseased leaf and fruits of chilli.

vitro and *in vivo* antagonistic activity of a bacterium, *Pseudomonas aeruginosa* WS-1 against this lethal phytopathogenic fungi *C. capsici*.

MATERIALS AND METHODS

Materials

All the culture media, carboxy methyl cellulose, pectin, starch, gelatin were purchased from HiMedia, Mumbai, India. Colloidal chitin was obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemical used were of analytical grade.

Organisms

The pathogenic organism was isolated from the diseased leaves and fruits of *C. annuum* (**Fig. 1**) as a pure culture on potato dextrose agar (PDA) medium. Diseased leaf tissues were cut into 3-mm pieces using a sterile scalpel. These were surface sterilized with 0.1% mercuric chloride solution for 30 sec, washed repeatedly in sterile distilled water and then aseptically transferred onto PDA plates. The plates were incubated at 28°C for 5-7 days. Aseptically bits of mycelia were taken from the margin of colonies on PDA medium. These were subcultured on fresh PDA medium. The purity of the culture was checked microscopically comparing the mycelia and conidial characters of the organism with those observed during a histopathological study. The culture was maintained in the same medium and stored at 4°C for further study. *P. aeruginosa* WS-1 was isolated from the rhizosphere of healthy *Withenia somnifera* from the medicinal plant garden of Narendrapur Ramakrishna Mission Ashrama, West Bengal, India by a serial dilution method. It was identified as per Bergey's manual and further confirmed by Microbial Type Culture Collection (MTCC), Chandigarh, India (Maity *et al.* 2007). The antagonist was subcultured and maintained on tryptic soy agar (TSA) medium for subsequent use. All cultures were maintained in the laboratory of Molecular and Applied Mycology and Plant Pathology, Department of Botany, University of Calcutta, India.

Dual culture bioassay

The antagonist *P. aeruginosa* WS-1 from a 24-h old culture (10^7 cells/ml) was streaked on peptone (1%), glucose (2%) and agar (2%) (PGA) plate in a circular (O) and semicircular (U) pattern. Then mycelial disc (5 mm diameter) of 3-day old *C. capsici* culture was subsequently inoculated at the center of the O- or U-shaped region on the PGA plate (Skidmore and Dickinson 1976). Inoculation only with the pathogen served as the control. Plates were incubated in triplicate at 30°C for 5 days and the diameter of colony growth was measured at 24-h intervals. Phase contrast microscopic (Zeiss AX10) studies were also performed to detect physical and/or morphological changes of mycelia.

In vitro antifungal bioassay

1. Chitinase bioassay

P. aeruginosa WS-1 was inoculated on LB plates supplemented with 0.5% colloidal chitin. Plates were incubated at 30°C for three days. Enzymatic activity was identified by the development of clear halo zones around the bacterial spots (Basha and Ulagnathan 2002).

2. Detection of hydrolytic enzymes

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxymethyl cellulose (CMC) for protease, amylase, pectinase and cellulase. Plates were incubated for 48 h at 30°C and the formation of a clear zone around bacterial colonies was interpreted as being positive (Gaur *et al.* 2004).

3. Production of volatile substance (HCN) and its effect on growth of C. capsici

Production of a volatile compound, hydrogen cyanide (HCN), was tested qualitatively according the method of Wei *et al.* (1991). *P. aeruginosa* WS-1 was inoculated in TSA medium supplemented with amino acid glycine (4.4 g/l of medium). A strip of sterilized filter paper saturated with a solution containing 0.5% picric acid (yellow) and 2% sodium carbonate was placed in the upper lid of the 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, brown or reddish brown was recorded as weak, moderate or strong cyanogenic potential, respectively. The inhibitory effect of the volatile compound (HCN) on the growth of *C. capsici* was determined by the 'inverted plate technique' of Dennis and Webster (1971). Strain WS-1 was grown in Petri dishes containing TSA for 12 h. Agar discs (5 mm diameter) cut from a 4-days-old culture of *C. capsici* was placed at the center of another Petri dish containing PDA. Then both plates inoculated with fungal pathogen and bacterial antagonist were placed in an inverted position and sealed together (mouth to mouth) with parafilm. Control plates containing *C. capsici* were inverted over uninoculated TSA plates. The plates were incubated at 30°C for 5 days and the radial growth of the test pathogen was recorded.

4. Siderophore production

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 24-h-old culture of WS-1 and kept for incubation at 30°C for 72 h. The change of blue colour of the medium to orange or the presence of a yellow to light orange halo surrounding the bacterial colony indicated the production of siderophores.

P. aeruginosa WS-1 treatment and challenge inoculation in greenhouse

Chilli seedlings (30-days old) were planted in earthen pots (15 cm diameter) filled with sterilized potting soil (10 kg) at two seedlings per pot. 30 days after planting, 25 ml of *P. aeruginosa* WS-1 suspension (3×10^8 cfu/ml) per pot was poured. 1 day after the treatment plants were inoculated by spraying the spore suspension of *C. capsici* at a concentration of 1×10^6 conidia/ml. A second set of plants without prior treatment with antagonist were inoculated with pathogen at the same cfu. In another set pathogen-inoculated plants treated with a foliar spray of 0.1% carbendazim served as the fungicide control. Plants neither treated with biocontrol nor with pathogen served as controls. All plants were maintained in a greenhouse at 28 ± 2°C having 85% RH. All treatments were replicated three times. Quantification of disease severity was determined on a 0-5 scale (0: healthy leaves; 1: spot appears on the upper surface; 2: spot touches both sides; 3: spot coalesces > 1 cm; 4: infected leaves distorted and defoliated; 5: plant dies).

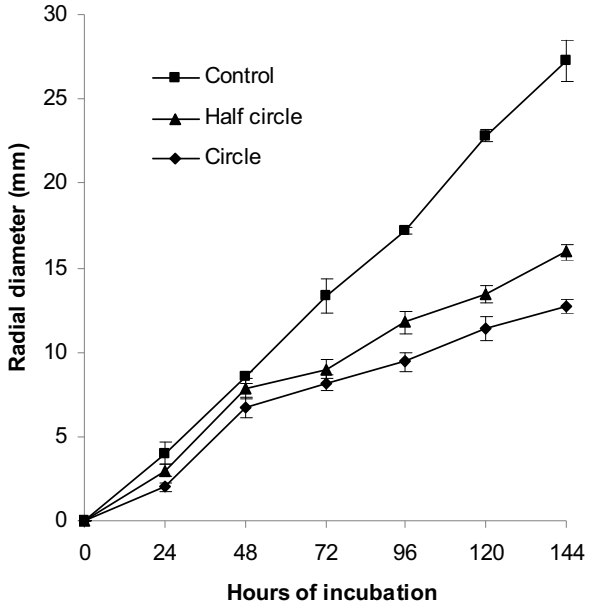

Fig. 2 Inhibition of *C. capsici* by *P. aeruginosa* WS-1 under dual plate culture using circular (O) and semicircular (U) method. Data represents the mean of triplicate sets of experiments. The vertical bars indicate the standard deviation (SD).

Statistical analysis

Each experiment was repeated three times. Data was combined and subjected to ANOVA (SPSS 11.5 for Windows) and compared using Tukey's multiple range test (P =0.05).

RESULTS

In vitro interaction of *P. aeruginosa* WS-1 against *C. capsici* in dual solid culture

In dual culture, significant growth inhibition of *C. capsici* by *P. aeruginosa* WS-1 was observed in solid culture medium. Growth of mycelium was restricted near bacterial streaks and continued away from it. An increase in incubation period was proportionate to growth inhibition of *C. capsici* up to 6 days. Quantitatively, *P. aeruginosa* WS-1 demonstrated 53.21 and 41.57% inhibition, respectively for

Table 1 Secondary metabolites production and enzymatic activity of *P. aeruginosa* WS-1.

Metabolites	Rate of production
Siderophore production	+++
Volatile substances (HCN) production	+++
Protease activity	++
Chitinase activity	+
Amylase activity	-
Pectinase activity	-
Cellulase activity	-

'+++' Stronger production, '++' Moderate production, '+' Low production, '-'No production.

circular and semi-circular streaks after 144 h of incubation (**Fig. 2**). A phase contrast microscopic study of the mycelia from the interacting zone showed hyphal shriveling and deformities, tip swelling, short branching and finally lysis (**Fig. 3**).

In vitro antifungal bioassay

Morphological abnormalities of hyphae in dual culture forced us to detect the production of different secondary metabolites by *P. aeruginosa* WS-1. The antagonist produced mainly chitinase, protease, hydrogen cyanide, volatile compounds and siderophores (**Table 1**). The strain formed clear zones around spots when they were grown in chitin and gelatin media which confirms chitinase and protease activity, respectively. The volatile compound produced by *P. aeruginosa* WS-1 inhibited the growth of *C. capsici* by up to 57%. Furthermore, after 24 h of incubation the colour of the picric acid-saturated filter paper strips became light brown and with increasing incubation time the colour of the filter strips changed to reddish-brown which indicates strong cyanogenic activity of the strain. The blue colour of the CAS medium was due to the Fe-dye complex and when siderophores produced by WS-1 the iron released from the Fe-dye complex resulted in a change of colour to orange, which indicates the hydroxamate nature of siderophores.

P. aeruginosa WS-1 treatment and challenge inoculation in a greenhouse

The data of disease incidence on chilli in 2009 are summarized in **Table 2**. Disease incidence (DI) was signifi-

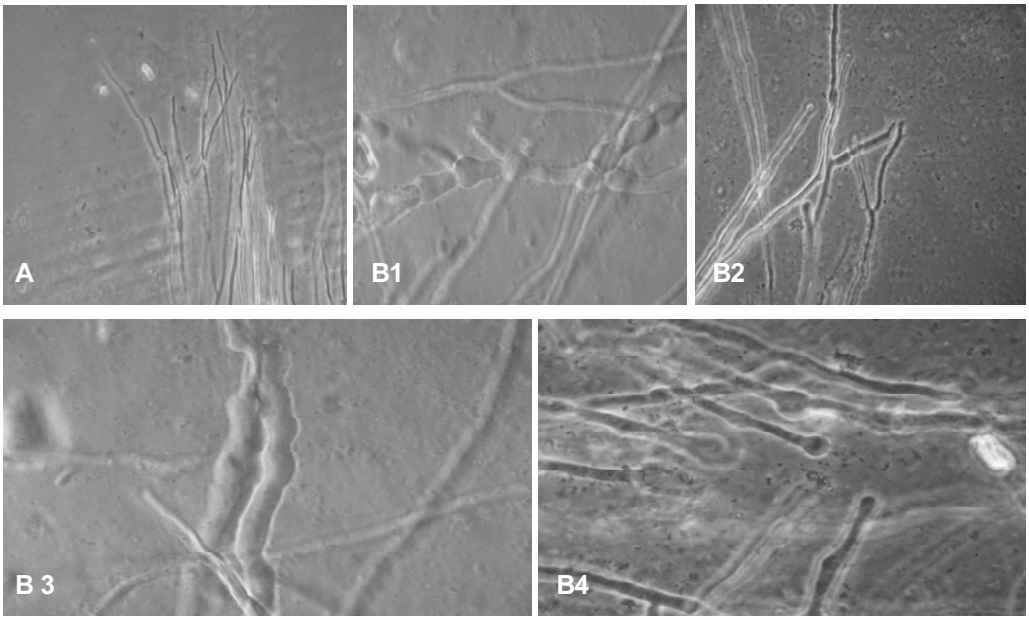

Fig. 3 Phase contrast microscopic observation of mycelium inhibited by *P. aeruginosa* WS-1. A: Hyphae of control plate showing normal hyphal structure. B (1-4): Hyphae of treated plate showing: 1. Short branching, 2. Tip swelling, 3. Hyphal shriveling, 4. Hyphal deformities.

Table 2 Green house evaluation of *P. aeruginosa* WS-1 on fruit rot and die back disease index (DI) in chilli.

Treatments	DI (2009)
Control (Inoculum only)	4.225 ± 0.25 a
Fungicide control	0.990 ± 0.17 b
P. aeruginosa WS-1 treated	1.363 ± 0.14 b

Different letters indicate significant differences according to Tukey's test at $P < 0.0001$

cantly reduced with the bacterial and fungicide treatment compared to the control ($P < 0.0001$, **Table 2**). The highest DI (= 4.225) was observed in control and the lowest DI (= 0.990) was observed in the fungicide-treated set. The efficacy of *P. aeruginosa* WS-1 application (DI = 1.363) was equivalent to standard fungicide application in controlling the disease severity in the greenhouse study.

DISCUSSION

Colletotrichum disease is one of the major economic constrains to chilli production worldwide, especially in tropical and subtropical regions (Than *et al.* 2008). In the absence of resistance cultivars, chemical control offers the only viable solution for disease management. However, fungicide tolerance often arises quickly if a single compound is relied upon too heavily (Stub 1991; Smith 2000). Under field conditions only organo-sulphur and copper oxychloride compounds are extensively used. Practical limitations of these fungicides are: (i) they are preventive and not curative, and (ii) require several applications up to the fruit ripening stage (Thind and Jhooty 1987). The strobilurin fungicides azoxystrobin, trifloxystrobin and pyraclostrobin have recently been labeled for anthracnose of chilli, but only preliminary reports are available on the efficacy of these fungicides against the severe form of the disease (Alexander and Waldenmaier 2002; Lewis an Miller 2003). Furthermore, there are numerous reports on negative affects of using chemicals on farmers' income and health, and toxic contamination to the environment (Voorrips *et al.* 2004). Besides that, some pesticides can be hardly cleaned from nature and have the potential to have adverse effects or destroy useful microorganisms which have positive effects in fertility of soil and growth of plants. To lower or avoid side effects, biological control is an alternative and proper choice in pest management. Control of plant pathogenic fungi by antagonistic bacteria and fungi have been widely studied in the past two decades. Most of these studies dealt with antagonists controlling soil-borne pathogens and to a lesser extent, foliar pathogens. The potential for biological control of *Colletotrichum* species had been suggested as early as 1976 by Lenne and Parbery (1976). Antagonistic bacterial strains (DGg13 and BB133) were found to effectively control *C. capsici* in Thailand (Intanoo and Chamswarng 2007). So far, biological controls for *Colletotrichum* diseases of chilli have not received much attention. In *in vitro P. aeruginosa* WS-1 reduced the growth rate of *C. capsici.* Microscopic observation of mycelium from the interacting zone showed abnormalities which might be correlated with the production of secondary metabolites like siderophores, HCN and lytic enzymes like protease and chitinase. Production of various antifungal metabolites by fluorescent pseudomonads and their destructive effect on plant pathogenic fungi are well known (Upadhyay and Jayaswal 1992; Dowling and O'Gara 1994; Bano and Musarrat 2002). Lorito *et al.* (1994) and Dunne *et al.* (1997) reported that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase or protease could result in the degradation of a structural matrix of fungal cell walls. Cessation of growth was also observed in *C. capsici* in the presence of volatiles. Direct retardation of fungal growth by HCN is thought to be one of the major causes of inhibition due to the blocking of terminal cytochrome-c oxidase in the respiratory chain and binding to metalo enzymes (Blumer and Hass 2000). Siderophore production has been reported to protect tomato plants from *Pythium* by *P. aeruginosa* 7NSK2 (Buysens *et al.* 1996). According to several studies, HCN, siderophore, chitinase and protease produced by fluorescent pseudomonads are known to inhibit the growth of some fungal pathogens (Seong *et al.* 1992; Lim and Kim 1995; Bhatia *et al.* 2003). However, a successful biocontrol agent should have a broad range of target pathogens and could be used against several diseases both in protected and in field crops (Jensen *et al.* 2007). *P. aeruginosa* WS-1 has also been reported earlier by our group as a strong antagonist of *Alternaria alternata*, *A. longipes* and *A. steviae* under *in vitro* conditions (Chatterjee *et al.* 2007; Maiti *et al.* 2007, 2008). Treatment with *P. aeruginosa* WS-1 as part of an *in planta* experiment showed a significant protection from the disease at the greenhouse level.

In conclusion, the evaluation of *P. aeruginosa* WS-1 on biocontrol activity in the fruit rot and die back disease-chilli pathosystem showed promising results, with up to 68% reduction in disease severity. Thus *P. aeruginosa* WS-1 can be a potential tool in an integrated control system against chilli *Colletotrichum* disease in India. Moreover, further analysis focusing on the specific plant microbe interactions using molecular tools and fluorescent microscopy is necessary for a better understanding of the nature of this biocontrol system.

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