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Antagonism of fluorescent *Pseudomonas* BRL-1 against *Curvularia lunata*

S. Sen, K.S. Tahsin and K. Acharya
Molecular and Applied Mycology and Plant Pathology Laboratory,
Department of Botany, University of Calcutta.

Abstract

A potato rhizospheric isolate, fluorescent *Pseudomonas* BRL-1, showed *in vitro* antagonistic activity against *Curvularia lunata*. Microscopic studies of the mycelium after antagonism illustrated hyphal shriveling, swelling, vaculation, short branching and granulation of cytoplasm resulting in lysis of hyphae of *C. lunata*. Association of antagonistic property of the isolate has been found to be coupled with chitinolytic and proteolytic activity as well as by the production of indole acetic acid and siderophore.

Keywords: *fluorescent Pseudomonas BRL-1, Curvularia lunata*

Introduction

Curvularia lunata is an important seed and soil-borne plant pathogen distributed throughout the world. The pathogen mediate leaf spot of *Citrus* sp., *Cymbopogon citrates*, *Musa paradisica*, *Sorghum vulgare* (Chaudhury, 1936; Agarwal and Beliram, 1960; Subramonium, 1953) fruit spot of *Mangifera indica*, *Psidium guajava* (Srivastava *et al.*, 1964; Tilak and Rao, 1968), Karnels spot of *Oryza sativa* (Padmanavan, 1949), cotyledon spot of soybean (Muchovej *et al.*, 1988) blight of Zoysiagrass (Roberts and Tredway, 2008). The species of *Curvularia* along with *Fusarium* pathogen causes viability loss up to 100% in sorghum (Christopher and Clint, 2003). Infection by *C. lunata* in human beings results in allergic fungal sinusis, broncho pulmonary fungal diseases and fatal cerebral phaeohyphomycosis (Rohwedder, *et al.*, 1979; Berry *et al.*, 1981; Carter and Boudreaux, 2004).

Florescent pseudomonads have drawn attention worldwide due to the ability of production of secondary metabolites viz. siderophore (Neilands, 1981), antibiotics (O'Sullivan and O'Gara, 1992; Keel *et al.*, 1992) volatile compound, HCN (Wei *et al.*, 1992) enzymes, phytohormones (O'Sullivan and O'Gara, 1991; Keel *et al.*, 1992) and rhamnolipids (Stanghellini and Miller, 1996). These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneous induction of growth of crop plants. Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, because chemical control is expensive and hazardous to ecosystem. Taking it as an objective, an attempt has been made to isolate a potential antagonistic organism having lethal effect on this pathogenic fungi *C. lunata*.

Materials and Methods

Organisms

The fungal pathogen was obtained from Molecular and Applied Mycology and Plant Pathology Laboratory.

The antagonist was isolated from the rhizosphere of potato and its biocontrol competence was proved against several phytopahogens including *Fusarium oxysporum* (Sen *et al.*, 2005) *Sclerotium rolfsii* (Sen *et al.*, 2006a), *Aspergillus niger* (Sen *et al.*, 2006b), *Mucor hiemalis* (Sen *et al.*, 2006c), *Erwinia carotovora* (Sen *et al.*, 2006d) *Alternaria alternata, Myrithecium rodidum, Colletrotrichum capsisi* (Sen *et al.*, 2006e) etc. The antagonist was subcultured and maintained on TSA medium for subsequent use.

Interaction of fluorescent Pseudomonas BRL-1 against C. lunata in dual solid and liquid culture

During *in vitro* antagonistic study, dual culture technique was performed on peptone glucose agar (PGA) solid (Skidmore and Dikinson. 1976) and liquid (Basha and Ulaganathan, 2002) media. In the former experiment bacterial isolate was streaked on PGA plate in a circular / O shaped and semicircular / U shaped pattern. In the later case 50 ml of PG broth was inoculated with 1ml the bacterial suspension (10^7 cfu ml^{-1}). Then spore suspension of *C. lunata* was subsequently point inoculated at the center of O or U shaped region on the PGA plate and in dual liquid culture technique PG broth was reinoculated with mycelial mat (5mm diameter) of 3 days old culture of *C. lunata*. Sole inoculation with pathogen in both solid and liquid media was served as control. Plates were incubated for 5 days at 30°C and inhibition of colony growth was measured and compared with the control, where as broths were kept at 30°C for 72 hours in a rotary shaker and the mycelial dry weight were estimated. The liquid culture was filtered through pre weighted Watman No. 1 filter paper and was dried for 24 h at 70°C and compared with the control set. Microscopic studies were also performed to detect physical and / or morphological changes of mycelia.

Mode of action of fluorescent Pseudomonas BRL-1

To investigate the inhibitory effect of fluorescent *Pseudomonas* BRL-1 a range of experiments were performed. For the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971). Spores of fungal pathogens were point inoculated on the centre of the petriplate containing 20 ml of PGA. A loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture was strike on petriplate having 20 ml of PGA then inverted over the plates inoculated with the fungal pathogen. Two plates were sealed together (mouth to mouth) with parafilm, control plates consist of fungal pathogens inverted over uninoculated PGA plate. The plates were incubated at 30°C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the pathogen was measured and compared with the control.

Production of hydrogen cyanide was tested qualitatively according the method of Wei et al., (1991). The antagonist was inoculated in TSA medium supplemented with amino acid glycin (4.4 g l^{-1} of medium). 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 the petridish. The petridishes 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.

To test the chitinolytic property of the isolate it was inoculated on LB medium (Gunasekaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicates chitinase activity of the strain (Basha and Ulagnathan, 2002).

IAA production was quantified spectrophotomertically, growing the screened fluorescent *Pseudomonas* in 10 ml of minimal salt media supplemented with 100 mg ml^{-1} of tryptophan, and incubated at 30°C under shaking for 48 h. Broth culture was centrifuged at 7500 rpm for 10 min. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (Gaur et al., 2004).

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect the siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24 h old fluorescent fluorescent *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72 h. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Chemical assay to detect the nature of siderophore

Tetrazolium test were performed to detect the hydroxamate nature. Triphenyltrtrazolium chloride (about 1 mg) was dissolved in a drop of culture supernatant of fluorescent *Pseudomonas* BRL-1, on a tile and a drop of 2 *N* of NaOH added. Immediate development of deep red colour was taken as a positive reaction (Snow, 1954).

Arnow's test was performed to detect catecholate siderophore. This nature of siderophore on reaction, in succession with nitrous acid, molybdate and alkali, yield a pink chromogen that absorbs maximally at 515 nm (Arnow, 1937).

Shenker's spectophotometric test for the detection of carboxylate siderophore. The copper complex formed was observed for absorption maxima between 190-280 nm. There is no specific wavelength at which the copper complex is absorbed. The entire wavelength 190-280 nm was scanned to observe the peak of absorption of siderophore (Shenker, 1992).

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

Results and Discussion

During dual culture, considerable growth inhibition of *C. lunata* by fluorescent *Pseudomonas* BRL-1 was observed both in solid and liquid culture. In solid culture, mycelial growth was restricted near bacterial streak and continued away from it, as evident from figure 1A-1C. Increase in incubation period was proportionate to growth inhibition of *C. lunata* upto 5 days (Fig. 1F). Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching and finally resulting into lysis (Fig. 1E). Co-culture of *C. lunata* and fluorescent *Pseudomonas* BRL-1 in liquid media resulted in 69 percent reduction in the mycelial dry weight when compared

Table1. Different secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Antibiotic Substances	-
HCN production	-
Volatile substances production	-
Chitinase activity	+
Protease activity	++
IAA production	+++

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

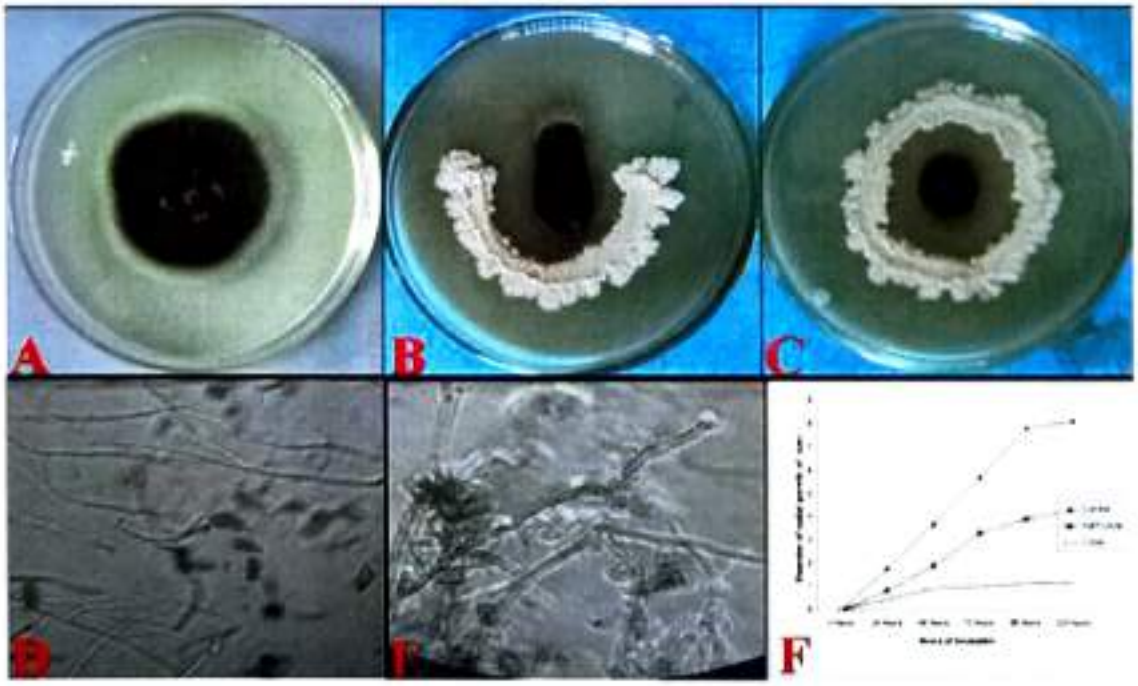

Fig 1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *Curvularia lunata*. (A) Control; *C. lunata* colony in pure culture showing more rapid growth at 30°C; (B) Colony of *C. lunata* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist and (C) Colony *C. lunata* showing restricted growth due to the bacterium inoculated as a circular streak. (D) Microscopic observations of mycelium from set 'A' (control) (E) Microscopic observations of mycelium from set 'C' (treated) (F) Incubation period verses radial growth of the fungi in control and treated sets. Data represents the mean of triplicate sets of experiments.

to the control. Among different biochemical tests performed for the detection of inhibitory effect of fluorescent *Pseudomonas* BRL-1, the isolate showed significant protease, chitinase, IAA and siderophore producing activity (Table 1). The antagonist has been found to produce chitinase and protease, when it grown in chitin and gelatin media respectively, as a sole carbon source. Moreover, Lorito *et al.*, (1994) and Dunne *et al.*, (1997) reported earlier that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease could result in the degradation of the structural matrix of the fungal cell wall. So the reduction of mycelial dry weight might be coincided by the chitinase and protease activity. In general Indole acetic acid is universally accepted as a plant growth promoter. The level of IAA was quantified spectrophotomatrically. It was found that the isolate produce 24 $\mu g\ ml^{-1}$ IAA at 48 h of incubation, increase in incubation period was proportionate to gradual decline in the production level of IAA upto 6 days. Infact, Chirst and Mosinger (1989) reported that this phytohormone could also induce resistance through PR protein production; Sharaf and Farrag (2004) reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1. The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Alstrom and Burns, 1989) CAS agar (blue agar) plate assay indicated the hydroxamate nature of siderophore production by fluorescent *Pseudomonas* BRL-1 as indicated by orange halo around the colony. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the siderophore production. The isolate produced hydroxamate siderophore as evidenced by positive tetrazolium test and negative in case of Arnow and Shenker's tests. Siderophore have been shown to play a role in increased growth response of certain plants to treat the planting material with fluorescent pseudomonads (Ahl *et al.*, 1986; Kloepper and Schroth, 1981; Scher and Bakker, 1982). The response is thought to involve suppression of deleterious rhizospheric microorganism (Ahl *et al.*, 1986; Kloepper and Schroth, 1981; Schippers *et al.*, 1987).

Thus the present study concluded that the fluorescent *Pseudomonas* BRL-1 showed considerable antagonistic property through combined and / or individual effect of siderophore, proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen. These observations and further study might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential biocontrol agent against *C. lunata*.

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