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In vitro antagonistic effect of Fluorescent *Pseudomonas* BRL-1 against *Aspergillus niger*

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Fluorescent *Pseudomonas* BRL-1, a rhizosphere isolate, showed *in vitro* antagonistic activity against *Aspergillus niger*. Microscopic examination after antagonism showed hyphal shriveling, swelling, vaculation, shortbranching and granulation of cytoplasm resulting in lysis of hyphae of *Aspergillus niger*. Correlation of antifungal activity of this isolate has been found to be linked with the production of siderophore, proteases and chitinases.

Key Words : Fluorescent *Pseudomonas*, *Aspergillus niger*

INTRODUCTION

Fluorescent pseudomonads have been recognized as an excellent bio-control agent against several soil borne pathogens by producing secondary metabolites siderophores, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Gupta *et al.*, 2001). These ultimately reduce the harmful rhizopathogen as well as enhance the plant growth. Biological control of plant diseases with bacterial antagonist is a potential alternative to chemical control because chemical control is expensive and drastically harmful to ruin the basic ecological principles of the soil. The application of rhizospheric bacteria as bio-control agents for crop protection is an effective alternative to the use of chemical fungicides.

Aspergillus niger, popularly known as the black mold is considered a 'weed of laboratory' as it often contaminates the bacteriological and mycological cultures. *A. niger* causes calyx end rot of dates, branches mold of grapes, pomegranate rot, black mold of peach, crown rot of peanut and storage rot of onion, apple, potato etc. Considering the above fact attempts have been made to isolate a soil bacterium showing antagonistic activity against *A. niger* to be used as a potential biocontrol agent.

MATERIALS AND METHODS

Isolation of fluorescent pseudomonads

A strain of fluorescent *Pseudomonas* BRL-1 was isolated from the rhizosphere of potato and was characterized according to Bergy's Manual of Determinative Bacteriology (Holt *et al.*, 1994). It was maintained on Tryptic Soy Agar (TSA) medium.

Interaction of fluorescent Pseudomonas BRL-1 against A. niger in dual plate culture

The antagonistic effect of fluorescent *Pseudomonas* BRL-1 were tested against *A. niger* supplied from the laboratory of Molecular and Applied Mycology and Plant Pathology, University of Calcutta on Peptone Glucose Agar (PGA) plates using dual culture technique (Randhawa *et al.*, 2002). This was done by streaking the fluorescent *Pseudomonas* BRL-1 in a circle and semicircle/U shaped pattern, where as spores of *A. niger* was point inoculated in

the centre on the plates. The fungal pathogen inoculated centrally on PGA plate, but uninoculated by fluorescent *Pseudomonas* BRL-1 served as control. The inoculated plates were incubated at 30°C for 6 days and inhibition of colony growth was measured.

Interaction of fluorescent Pseudomonas BRL-1 against A. niger in dual liquid culture

To test the antifungal activity of the fluorescent *Pseudomonas* BRL-1, dual liquid culture method was employed. 1 ml of freshly grown fluorescent *Pseudomonas* BRL-1 culture (containing 10^7 cfu/ml) and an agar block (5 mm diameter) of 5 days old culture of *A.niger* was inoculated onto 50 ml of Peptone Glucose medium in 250 ml of conical flask. The culture was incubated on a rotary shaker at 30°C for 72 h. For the control experiments the fungal pathogen were grown alone. Experiments were performed thrice taking triplicate for every set (Basha and Ulaganathan, 2002). A drop of fungal culture form each flask was taken out and placed on a clean glass slide and stained with lactophenol and cotton blue and observed under Leica DMLS Research Microscope.

Differences in dry weights between the fungal cultures grown with fluorescent *Pseudomonas* BRL-1 strain or the control culture grown without any bacterium were recorded (Basha and Ulaganathan, 2002). For this 72 h. old dual cultures were filtered through the pre weighted Whatman No. 1 filter paper. It was dried for 24 h at 70°C and weights were measured.

Inhibitory effect of fluorescent Pseudomonas BRL-1

To investigate the inhibitory effect on fluorescent *Pseudomonas* BRL-1 for the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971). Spores of *A.niger* was point inoculated on the centre of the Petriplate containing 20ml 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 plate consist of *A.niger* 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 test pathogen was measured and compared with the control.

Production of hydrogen cyanide was tested qualitatively according the method of Wei *et al.*, (1991). The fluorescent *Pseudomonas* BRL-1 was inoculated in TSA medium supplemented with amino acid glycin (4.4 g/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 fluorescent *Pseudomonas* BRL-1 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 indicated chitinase activity of the strain (Basha and Ulagnathan, 2002).

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 *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72h. 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. Siderophore excreted into the culture medium was determined by spectophotometry. Concentration was calculated using absorption maximum and the molar absorption coefficient (λ_{max} = 400 nm and ε = 20,000 M^{-1} cm^{-1}) according to the method of Meyer and Abdallah (1978).

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

In dual culture, significant growth inhibition of *A.niger* occurred due to antagonistic effect of fluorescent *Pseudomonas* BRL-1, which was evident with clear inhibition of colony diameter. At 48h of incubation, *A.niger* was strongly inhibited by fluorescent *Pseudomonas* BRL-1 and simultaneous increase in incubation time corresponded escalation in percent growth inhibition (Fig. 1). After 96h of incubation the mycelia growing toward the interaction zone, which was prominently evident in semicircular/U-shaped streak stopped, and the mycelia gradually lost vigor. Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching and finally resulting into lysis (Fig. 2). The difference in dry weights between the fungal culture grown with and without fluorescent *Pseudomonas* BRL-1 strain was recorded. There was more than 50% reduction in dry weight of the culture grown with fluorescent *Pseudomonas* BRL-1 strain when compared to the control.

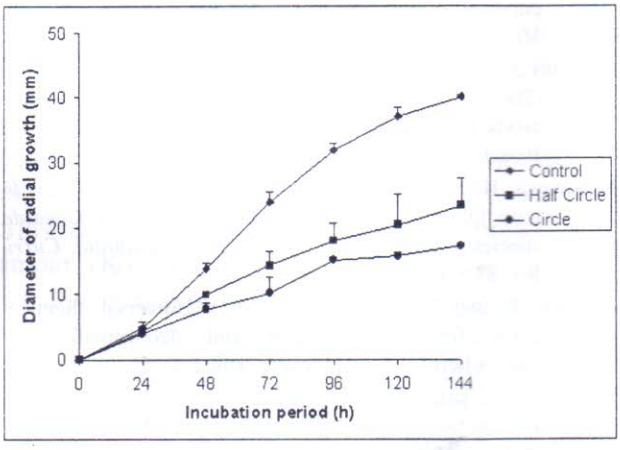

Fig. 1 : *In vitro* antagonistic activity of Fluorescent *Pseudomonas* BRL-1, cultured as semi-circular and circular streaks, on *Aspergillus niger* point-inoculated in the centre of PGA plates incubated at 30°C.

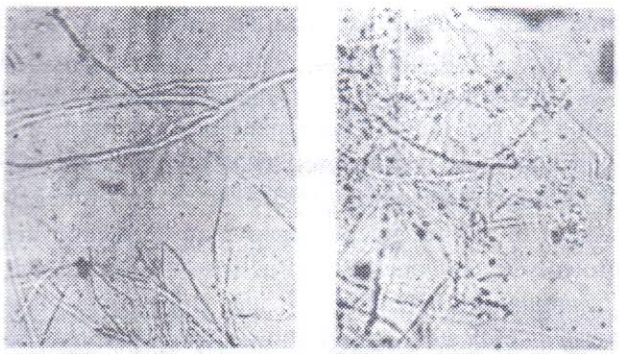

Fig. 2 : Microscopic observation sof mycelium inhibited by Fluorescent *Pseudomonas* BRL-1 strain. Mycelium of *A. niger* (a) grown on Peptone Glucose Agar (PGA) media (Control), (b) present in the inhibition zone, when grown with Fluorescent *Pseudomonas* BRL-1 on PGA.

Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the bacterium (Arora *et al.*, 2001). Further study was made to screened for the production of different secondary metabolites including siderophore, hydrogencyanide, antimicrobial compound, volatiles, hydrolytic enzymes investigated for their effect on *A.niger in vitro* (Table 1). The results (Table 1) showed fluorescent *Pseudomonas* BRL-1 produced mainly siderophore, protease and chitinase.

Table 1 : Showing different secondary metabolities production and enzymatic activity of Fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Portease activity	++
Chitinase activity	+
HCN production	—
Volatile substances production	—
Antibiotic substances	—

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

Mycolytic enzymes consisting of chitinase, protease etc. based formulations have been used to control fungal plant pathogens (Deshpande, 1999). Fluorescent *Pseudomonas* BRL-1 has been found to

produce chitinase and protease, when they were grown in chitin and gelatin media respectively, as a sole carbon source. As the fungal cell wall contains chitin as the major component and chitinase are well known to lyse the cell walls of both live and dead fungi (Ueno *et al.,* 1990). The reduction in dry weight of the fungal pathogen may be due to the proteolytic and chitinolytic activity of fluorescent *Pseudomonas* BRL-1. CAS agar (blue agar) plate assay indicate siderophore production by fluorescent *Pseudomonas* BRL-1. The strain forms colonies with an orange halo. 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 hydroxamate nature of siderophore. The isolate did not produce catecholate or carboxylate siderophore, the positive result for pyoveridine nature of the hydroxamate siderophore was also evident by their absorption maxima between 407 and 413 nm.

The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Arora *et al.,* 2001).

On the basis of these studies it can be concluded that the fluorescent *Pseudomonas* BRL-1 isolate is showing significant antagonistic property through combined and/or individual effect of siderophore, production of proteolytic enzyme, 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 (Remeshkumar *et al.,* 2002). These observations and further studies might help in developing this fluorescent *Pseudomonas* BRL-1 as potential rhizospheric biocontrol agent against *A.niger.*

REFERENCES

Arora, N. K., Kang, S. C. and Maheshwari, D. K. (2001). Isolation of siderophore producing strains of *Rhizobium meloloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of ground nut. *Curr. Sci.* **81**:673-677.

Basha, S. and Ulagnathan, K. (2002). Antagonism of *Bacillus* species (strain BC121) toward *Curvularia lunata. Curr. Sci.* **82** : 1457-1463.

Bilgrami, K. S., Jamaluddin, S. and Rizwi, M. A. (1991). In *Fungi of India List and References.* Todays and Tomorrow's Printing Publishers. New Delhi. pp. 454-456.

Dennis, C. and Webster, J. (1971). Antagonistic properties of species group of *Trichoderma* II. Production of volatile antibiotics. Transaction of British Mycological Society. **82** : 41-48.

Despande, M. V. (1999). Mycopesticide production by fermentation : Potential and chanllenges. *Crit. Rev. Microbiol.* **25** : 229.

Gaur, R., Shani, N., Kawaljeet, Johri, B. N., Rossi, P. and Aragno, M. (2004). Diacetylphloroglucinol producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Curr. Sci.* **86** : 453-457.

Gunasekaaran, P. (1995). In *Laboratory Manual In Microbiology.* New Age International (P) Limited, New Delhi. pp. 125.

Gupta, C. P., Dubey, S. C., Kang, S. C. and Maheshwari D. K. (2001). Antibiosis-mediated necrotrophic effect of *Pseudomonas* GRC2 against two fungal plant pathogen. *Curri. Sci.* **81** : 91-94.

Holt, G. J., Krieg, R. N., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994) In *Bergy's manual of Determinative Bacteriology.* Willims & Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp. 151-168.

Meyer, J. M. and Abdallah, M. A. (1978). The florescent pigment of *Pseudomonas fluorescens* Biosynthesis, purification and physical-chemical properties. *J. Gen. Microbiol.* **107** : 319-328.

Rameshkumar, N., Thirumalai Arasu, V. and Gunasekaran, P. (2002). Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria *Pseudomonas fluorescens. Curr. Sci* **82** : 1463-1466.

Randhawa, H. S., Sandhu, R. S. and Kowshik, T. (2002). *In vitro* inhibition of *Aspergillus fumigatus* by *Candida* species, especially *C. albicans* and *C. glabrata. Curri. Sci.* **87** : 860-865.

Schwyn, B. and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Annal. Bichem.* **160** : 47-56.

Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. (1990). Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. *J. Gen. Appl. Microbiol.* **36** : 377.

Wei, G., Kloepper, J. W. and Sadik, T. (1991). Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth promoting rhizobacteria. *Phytopathology.* **81** : 1508-1512.

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