Publications

Lists of Publications

1. K. Acharya, M. Rai, N. P. Rai and **S. Sen,** Three new species of *Russula*: Addition to the macrofungi of Sikkim Himalaya. ***Indian Forester,*** 130: 953 – 955, 2004.
2. R. Acharya, M. Mukhia, **S. Sen,** and K. Acharya, Nitric oxide: A common antipathogenic factor of plants. ***Indian Journal of Experimental Biology,*** 43: 100 –103, 2005.
3. **S. Sen,** A. Saha and K. Acharya, *In vitro* inhibition of *Fusarium oxysporum* by florescent *Pseudomonas* BRL-1. ***Journal of Botanical Society of Bengal,*** 59: 31-36, 2005.
4. **S. Sen,** R. Acharya and K. Acharya, *In vitro* antagonistic effect of Fluorescent *Pseudomonas* BRL-1 against *Aspergillus niger*. ***Journal of Mycopathological Research,*** 44: 97-100. 2006.
5. **S. Sen,** A. Saha and K. Acharya, Management of an epidemic disease of *Cymbidium* rot by fluorescent *Pseudomonas*. ***Journal of Mycopathological Research,*** 44: pp. 160, 2006 (Abstract).
6. **S. Sen,** R. Pal, A. Saha and K. Acharya, Optimization of different physiological factors on growth for the development of a cost effective media for large scale production of an antagonistic PGPR fluorescent Pseudomonas BRL-1. ***Journal of Mycopathological Research,*** 44: pp. 184, 2006 (Abstract).
7. **S. Sen,** M. Bhattacharyya, S. Tahsin Khandakar, S. Giri and K. Acharya, Evaluation of biocontrol potentiality and plant growth promoting capability of a fluorescent Pseudomonas BRL-1 against several phytopathogen. ***Journal of Mycopathological Research,*** 44: p. 184-185, 2006 (Abstract).
8. C. K. Maiti, **S. Sen,** A. Karan, S. Ghosh and K. Acharya, Isolation, characterization and screening of fluorescent Pseudomonads population of medicinal plant's rhizosphere against alternaria alternata. ***Journal of Mycopathological Research,*** 44: pp. 185, 2006 (Abstract).
9. **S. Sen,** T. Bose, K. Basu, R. Acharya, N. Samajpati and K. Acharya, *In vitro* antagonistic effect of Fluorescent *Pseudomonas* BRL-1 against *Sclerotium rolfsii*. ***Indian Phytopathology,*** 59: 227-230, 2006.
10. **S. Sen,** A. Saha and K. Acharya, *In vitro* inhibition of *Mucor hiemalis* f. *hiemalis* by florescent *Pseudomonas* BRL-1, ***Ecology and Environment, (In press).***
11. **S. Sen,** R. Acharya, A. Saha, and K. Acharya, A new report of *Cymbidium* pseudobulb rot orchestrated by *Erwinia carotovora, Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis*, ***Plant Disease (In press).***

12. **S. Sen,** S. Ghosh, R. Acharya, A. Saha and K. Acharya, Isolation, characterization and screening of fluorescent pseudomonads against the pathogens of *Cymbidium* pseudobulbs rot, ***Ecology and Environment.*** **(Communicated).**

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(III)

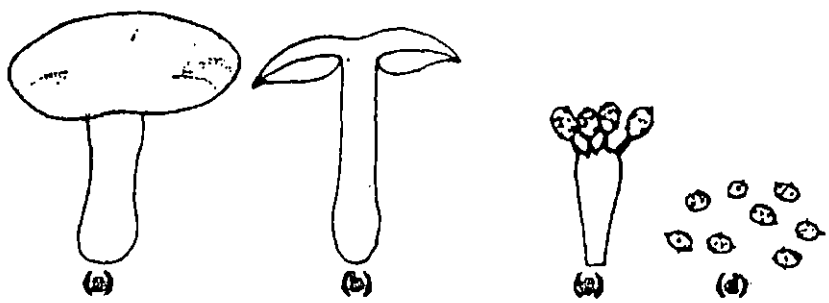
THREE NEW SPECIES OF *RUSSULA* : ADDITION TO THE MACROFUNGI OF SIKKIM HIMALAYA

The Sikkim Himalayas are spread over an area of 7,096 km^2. The general physiography of the region is hilly and the sloping terrain is the major feature over which most habitations, agricultural activity and forest cover may be found. The vertical range is from 1,000 m amsl (foothills), through 4,000 m amsl (timber line) upto 8,500 m amsl (the Kanchanjunga peak) (Rai and Sharma, 1994). The Himalayan ecosystem is known for its richness in biodiversity (Chakraborty, 1997). After Berkeley (1956) very few work have been undertaken regarding the biodiversity of macrofungi of this area (Sarbhoy *et al.*, 1975; Bilgrami *et al.*, 1979, 1991). Three new species of *Russula* have been reported for the first time from this area.

The study materials were collected during field trips in the year 2000 and 2002. The morphological and ecological features were noted and colour photographs were taken. The chemical colour reaction were noted on the fresh collection. After the specimens were brought to the laboratory, their microscopic properties were determined by using an Olympus research microscope. All the microscopic structure were drawn with the help of camera lucida. Then, they are identified according to Ramsbottom (1965), Bessey (1978) and Singer (1986). The voucher specimen has been deposited in Mycological Herbarium of Darjeeling Government College.

Russula ochroleuca Pers. ex. Fr. : Pileus 10 cm, yellow but variously tinged pale lemon yellow colour with orange or brownish at center, convex-umbilicate then flat, slightly depressed, margin thick, curved, sometimes lobed (Fig. 1). Pileus context thick, soft, odour indistinct. Lamellae upto 2 cm broad, thin, adnate,

Fig. 1



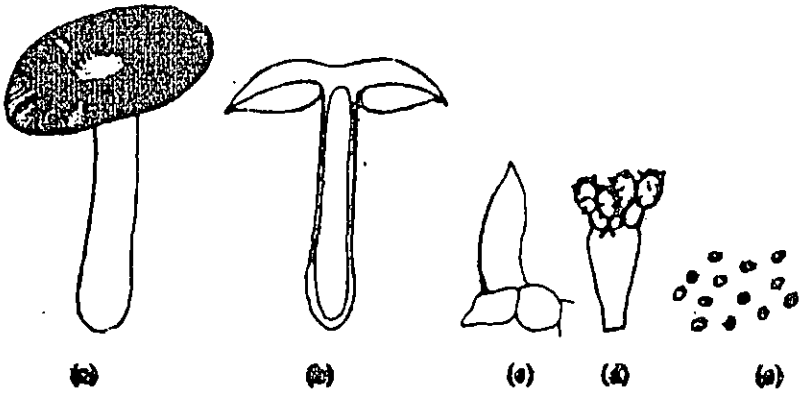
Russula ochroleuca
(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Basidia, (d) Basidiospores.

lamellae of only one length. Stipe 3-7x1.5-2.5 cm, long, cylindrical, central, fistular, white, surface smooth, moist. Annular veil and volva absent. Spore print white. Spore white, ovoid, apiculate 7-9x6.3-9 µ, amyloid. Basidia 20-30x6-8 µ, clavate, thin walled bearing 4 sterigmata along with four basidioles. Subhymenium distinct, psedoparenchymatous, nearly fifty per cent cells are sphaerocysts. Hymenophoral trama regular. Cystidia absent. Pileus cuticle and context made up of septate, branched, interwoven hyphae, clamp connection absent. Collected from the humus forest floor of Darjeeling. The voucher specimen no. DGC/MP/MF-085.

***Russula sororia* (Fr.) Romell.** : Pileus 5.4 cm, dark brown or cigar brown at center, yellowish brown at edge, sometimes with olive markings, subglobose to convex, depressed (Fig. 2). Pileus context thin, soft, fruity smell. Lamellae 2.7 cm long, finely adnexed, lamellae of one length. Stipe fistular, central, 5x1.5 cm, cylindrical, white, surface smooth. Annular veil and volva absent. Spore print creamy white. Spores 6.5-8.5x5.7 µ, amyloid (positive test with Melger regent), pale cream-colored, ovoid with conical obtuse or truncated warts. Basidia with four sterigmata with four basidioles. Subhymenium distinct with fifty percent of cells spherocysts. Hymenophoral trama is intermixed with cystidia. Collected from the forest floor of broadleaf trees in Lebong area, Darjeeling in mid-June. The voucher specimen no. is DGC/MP/MF-098.

***Russula xerampelina* Fr.**: Pileus 10 cm, purple to crimson pale lilac red with central blackish often brownish wine colored, convex then flat, slightly viscous when damp but shiny in nature, velvety, margin thin (Fig. 3). Pileus context thin soft; lamellae upto 2 cm broad, thin, adnate, lamellae of one length only. Stipe cylindrical, central, smooth, fistular 7x2.2 cm whitish tinged with glowing red-pink. Annular veil and volva absent. Spore print white, spore ovoid with obtuse on conical warts, 8-9x5-8 µ, amyloid. Basidia thin walled with four sterigmata along four

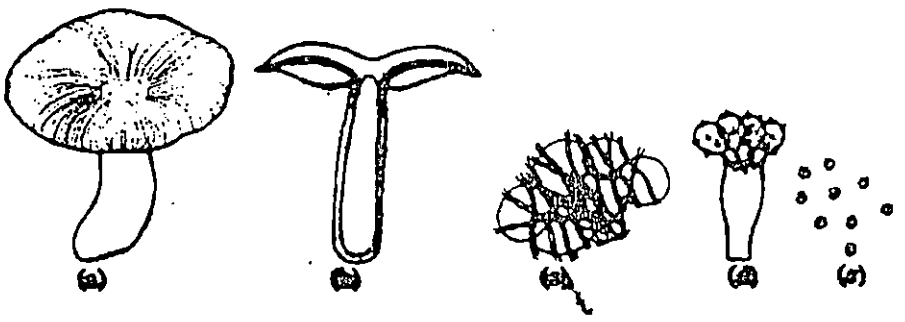
Fig. 2



Russula sororia

(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Cystidia, (d) Basidia, (e) Basidiospores

Fig. 3



Russula xerampelina
(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Trammel cells, (d) Basidia, (e) Basidiospores.

basidioles. Subhymenium distinct with nearly fifty per cent of sphaerocysts cell. Hymenophoral trama regular, cystidia absent, grayish green reaction with ferrous sulphate. Pileus cuticale and context made up of thin walled cells. Spore amyloid (positive test with Melger regent). Collected from the Lebong forest area on June. The voucher specimens no. DGC/ MP/MF - 080.

References

Berkeley, M.J. (1956). Decades of Fungi, Decas 1-62, Nos. 1-620. *Hooker's London, J. Bot.* 3-8: 1844-1856.

Bessey, E.A. (1978). *Morphology and Taxonomy of Fungi*. Vikas Publising House Pvt. Ltd., New Delhi.

Bilgrami, K.S., S. Jamaluddin and M.A. Rizwi (1979). *Fungi of India (Part I) :- List and References*. Today and Tomorrow's Printers and Publishers, New Delhi.

Bilgrami, K.S., S. Jamaluddin and M.A. Rizwi (1991). *Fungi of India*. Today and Tomorrow's Printers and Publishers, New Delhi.

Chakraborty, T. (1997). *Himalayan Paryavaran*, 5: 67-69.

Sarbhoy, A.K., Girdharilal and J.L. Varshney (1975). *Fungi of India*. Navajug Traders, New Delhi.

Singer, R. (1986). *The Agaricales in Modern Taxonomy*. Bishen Singh Mahendra Pal Singh, Dehra Dun. VIII, 981 pp.

Ramsbottom, J. (1965). *A Handbook of the Larger British Fungi*. Alden & Mowbray Ltd. Great Britain.

Rai, L.K. and E. Sharma (1994). *Medicinal Plants of the Sikkim Himalaya : Status, Uses and Potential*. Bishen Singh Mahendra Pal Singh, Dehra Dun. pp. 15-17.

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Nitric oxide: A common antipathogenic factor of plants

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In the present study, nitric oxide synthase/nitric oxide (NOS/NO) status was tested in the host plants infected with fungi, bacteria and virus. In each case cytosolic nitric oxide synthase (Cyt-NOS) of diseased plants was inhibited and inhibition was competitive in nature in respect to l-arginine, the substrate for the enzymic activity. Elevation of host nitric oxide (NO) level before infection using nitric oxide (NO) donor protected disease initiation significantly. The nature of enzyme kinetics and the manner of disease protection by nitric oxide donor (NO-donor) was similar in all the three cases of infection. It was concluded that nitric oxide was a common antipathogenic factor of plants.

Keywords: *Nitric oxide/nitric oxide synthase, Plant defense*

Disease resistance in plants is usually associated with different kinds of passive and active responses. Current concept of active defense mechanism includes programmed cell death (PCD)[1], hypersensitive reaction (HR)[2,3] production of different diffusible signaling molecules like salicylic acid (SA), jasmonic acid (JA), ethylene etc[4] and ultimately the establishment of systemic acquired resistance (SAR)[3,5]. Recently a new concept ie, the concept of nitric oxide (NO) has been introduced in this field[6,7]. It is a potent signaling molecule[8]. NO is well studied in animal system and was first identified as endothelial derived relaxing factor (EDRF)[9]. It is a versatile and powerful effector of animal redox regulated signaling system and immune responses[10] and it also functions as a second messenger of insulin[11]. Nitric oxide synthase (NOS) is the key enzyme for the production of NO from L-arginine in a system[10,11]. In contrast to extensive studies throughout the last two decades or more on role of NO in the mammalian immunity, recently its involvement in the plant defense system has been addressed[6,7]. Some basic analogies at the molecular level regarding the signal transduction of NO in animals to that of plants focus interest to study the involvement of this molecule in plant defense mechanism[12]. It has been reported that NO is produced by plants[6,7]. It mediates leaf expansion[13], root growth[14], phytoalexin production[15] and defense responses against pathogens[12]. In our earlier papers, we have reported the status of NOS in different healthy and diseased plants[16], impact of disease initiation on host NOS[17] and involvement of NOS in host pathogen interaction[18]. In this report we have demonstrated NO as universal effector molecule of plant defense responses. By inhibiting the host NOS activity and thereby restraining NO production, a pathogen makes its way more effective to establish a disease. Furthermore, this finding has been supported by opposite experiments where elevated level of host NO provide significant disease protection after challenging with efficient pathogen (s).

Chemicals – N^G methyl-l-arginine acetate ester (NAME) was purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Collection of healthy and diseased plants – Three different host plants of *Brassica campestris* L. var. sarson Prain, *Citrus aurantifolia* Swingle and *Ammomum subulatum* Roxburg were collected from field in their healthy as well as pathologically disordered condition caused by *Alternaria brassicae* (Bark.) Sacc., *Xanthomonas citri* Hasse. and chirke (mosaic streak) virus, a fungal, bacterial and viral pathogen, respectively.

Assay of NOS activity of intact tissue and homogenates – The reaction mixture containing intact leaf tissue (100 mg) or tissue homogenate (200 – 300 μg of protein) or cytosolic fraction (150 – 200 μg of protein) or soluble particulate fraction (250 – 400 μg of protein) of both healthy and diseased sample was incubated with 10 μM of l-arginine, 64 mM of haemoglobin, in a total volume of 2.5 ml Tris – HCl buffer (pH 7.4) for different periods at 25° ± 1°C. At different time periods, portions of reaction mixture was centrifuged at 10,000 × g for 5 min and NO content of the supernatant was determined and compared with an appropriate control set.

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Determination of NO – Nitric oxide was determined according to Jia *et al.*[19] using scanning Beckman spectrophotometer (Model DU6). Typically, NO content was determined by adding 64 m*M* of oxyhemoglobin to the isolated supernatant and the formation of NO was quantified by determining the conversion of oxyhemoglobin to methemoglobin[19].

Preparation of tissue homogenate – Leaf tissue (100 mg) of diseased and healthy plants was taken separately in a morter with 1 ml of chilled 50 m*M* ofTris-HCl buffer (*p*H 7.4) and homogenized by pestle. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°C and then the supernatant was further centrifuged at 20,000 × *g* for 30 min at 4°C for the separation of cytosolic and particulate fractions. Both the cytosolic and particulate fractions were collected separately and stored at 0°C. Before further use, particulate fraction was resuspended in equal volume of the same buffer. All the experiments were done at 4°C. Protein was estimated using Folin-ciocalteu reagent, according to the method of Lowry *et al*[20].

Determination of enzyme kinetics – K_m and V_{max} values of the cytosolic NOS were determined by Lineweaver-Burk Plot.

Statistical analyses – Results presented as mean ± SD (Standard deviation) of at least six individual experiments. Data were analyzed by Student's *t* test and values of $P< 0.01$ were considered significant.

In each cases of pathological conditions irrespective of fungi, bacteria and virus attack, the host NOS activity of the intact tissue declined, as determined by NO production from 0.594 ± 0.027 to 0.333 ± 0.012; 0.462 ± 0.024 to 0.379 ± 0.035 and 0.810 ± 0.051 to 0.525 ± 0.033 nmole/100 g of tissue/hr, respectively when compared with healthy subjects. To determine whether the broken cell preparation could possess NOS activity, tissue homogenate and broken cells was prepared from diseased plants as described earlier and observed enzyme inhibition as found in the intact or whole tissue of disease plants. Addition of 10 μM of NAME to the reaction mixture in all cases completely inhibited NO production. Furthermore, both the particulate and soluble cytosolic fractions of healthy tissue homogenate prepared by ultracentrifugation contained detectable amount of NOS activity. But in case of diseased host only the cytosolic NOS activity was inhibited. The basal NOS activity of the cytosolic fractions of healthy plants were 1.107 ± 0.063; 1.95 ± 0.02; 2.08 ± 0.033 nmole NO produced/mg protein/hr (n=3), respectively, decreased to 0.888 ± 0.022; 1.70 ± 0.04 and 1.69 ± 0.049 nmole NO produced/mg protein/hr, respectively in case of diseased plants. In contrast, NOS activity of the particulate fraction of the host tissue was 0.490 ± 0.016; 0.908 ± 0.029 and 0.751 ± 0.087 nmole NO produced/mg protein/hr respectively at their healthy condition was likely to be changed to 0.475 ± 0.027; 0.900 ± 0.046 and 0.760 ± 0.021 nmole NO produced/mg protein/hr, respectively at their diseased condition.

Lineweaver-Bark plot of the cytosolic NOS activity of the diseased and healthy plants demonstrated that inhibition of NOS activity during diseased phase was related to the increase of K_m of l-arginine from 0.480, 0.344, 0.442 μM (n=6) to 1.0, 0.485, 0.757 μM, respectively when compared to diseased phase with simultaneous decrease of V_{max} from 1.05, 1.98 and 2.17 nmole NO produced/mg protein/hr to 0.869, 1.84 and 1.96 nmole NO produced/mg protein/hr (Table 1). Addition of 10 μM N^G nitro-l-arginine-methyl ester

Table 1 — Effect of diseased condition of the plants on NOS activity of cytosolic fraction. Cytosolic fraction of healthy and diseased plant tissue homogenate (n = 6) was incubated with different concentration of l-arginine for 1 hr. The NOS activity of the cytosolic fractions were analyzed by Lineweaver-Burk plot as described in materials and methods

Host name	Cytosolic fraction of	K_m (μM)	V_{max} (nmole NO produced/mg protein/hr)
Brassica campestris	A	0.480 ± 0.037	1.05 ± 0.05
	B	1.00 ± 0.06*	0.869 ± 0.056*
Citrus aurantifolia	A	0.344 ± 0.042	1.98 ± 0.017
	B	0.485 ± 0.036*	1.84 ± 0.016*
Ammomum subulatum	A	0.442 ± 0.047	2.17 ± 0.039
	B	0.656 ± 0.023*	2.04 ± 0.018*

A – Healthy plant; and B – Diseased plant.
*Significant at $P<0.01$

(NAME) to reaction mixture containing various concentrations of l-arginine (added for the demonstration of K_m and V_{max}) completely inhibited NO production. Durner *et al*[12] have reported that NO induces PR-1 gene expression through salicylic acid. Our study complements their findings by demonstrating the present work that pathogen might somehow be blocking the NOS activity mediating a competitive inhibition and thus, inhibiting the defense gene expression to make the plant more vulnerable for susceptibility. Interestingly, the NOS activity was found to be present in both particulate and cytosolic fraction, but results suggested that only cytosolic NOS participates in this mechanism. Further work on pathogen blocking the NOS activity is going on.

Since NOS activity was inhibited in a similar manner in each cases of infection, we tested the possibility of elevated level of NO to protect plants from disease initiation. Sodium nitroprusside (SNP) is well known for its NO generating character. Other workers[2] have also demonstrated the production of NO (~2 μM) from SNP (0.5 mM). To determine whether SNP could be substantiated for NO production of plants against infection, we experimented the effect of SNP on the progression of pathogenic activity. Administration of SNP (0.1 mg/ml) by foliar spray before 24 hr of infection protected fungal diseases up to 72% and bacterial diseases up to 65% as resulted from cell count of micro-HR detected by Evans Blue staining[21]. Since SNP did not generate NO spontaneously in aqueous medium[22], its effect was quite less. However, there are many other NO donors available which are known to generate NO spontaneously in aqueous medium[21] and it is possible that some of these NO donors may be useful for protection of the disease. Mechanism of this NO protection might be related to self amplifying character of NO as described by Klessig[4]. Therefore, NO donor can possibly be used as an antipathogenic agent and feasibility of using NO donors as a routine adjunct in the crop field can be considered. Thus, a new concept of internal scenario of diseased plants has been exposed in this paper denoting: (i) insufficient production of NO through suppression of cytosolic NOS activity: (ii) exhibition of a competitive inhibition of a cytosolic NOS; and (iii) partial protection from disease by elevating host NO level using NO donor which furnish strength to conclude that NO may play a direct protective role as a general antipathogenic molecule to resist disease occurrence in plants. Further work is in progress to evaluate the potentiality of other NO donors on disease protection in the field.

References

1 Greenberg J T, Guo A, Klessig D F & Ausubel F M, Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense function, *Cell*, 77 (1994) 551.

2 Delledonne M, Xia Y, Dixon R A & Lamb C, Nitric oxide functions as a signal in plant defense resistance, *Nature*, 394 (1998) 585.

3 Hausladen A & Stamler J S, Nitric oxide in plant immunity, *Proc Natl Acad Sci USA*, 95 (1998) 10345.

4 Klessig D F, Durner J, Noad R, Navarre D A, Wendehenne D, Kumar D, Zhou J M, Shah J, Zhang S, Kachroo P, Trifa Y, Pontier D, Lam E & Silva H, Nitric oxide and salicylic acid in plant defense, *Proc Natl Acad Sci USA*, 97 (2000) 8849.

5 Shirasu K, Dixon R A & Lamb C, Signal transduction in plant immunity, *Curr Opinion Immunol*, 8 (1996) 3.

6 Ninnemann H & Maier J, Indications for the occurrence of nitric oxide synthase in fungi and plants and the involvement in photoconidiation of *Neurospora crassa*, *Phytochem Phytobiol*, 64 (1996) 393.

7 Wildt J, Kley D, Rockel A. Rockel P & Segschneider H, Emission of NO from higher plant species, *J Geophys Res*, 107 (1997) 5919.

8 Ignaroo L J, Nitric oxide: A novel signal transduction mechanism for transcelluler communication, *Hypertension*, 16 (1990) 477.

9 Ignaroo L J, Buga M, Wood K S, Byrns R E & Chaudhuri G, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, *Proc Natl Acad Sci USA*, 84 (1987) 9265.

10 Klessig D F & Durner J, Nitric oxide as a signal in plants, *Curr Opinion Plant Biol*, 2 (1999) 369.

11 Kahn N N, Acharya K, Bhattacharya S, Acharya R, Mazumder S, Bawmann A W & Sinha A K, Nitric oxide: The "Second messenger" of insulin. *IUBMB Life*, 49 (2000) 441.

12 Durner J, Wendehenne D & Klessig D F, Defense gene induction in tobacco by nitric oxide, cGMP and cADP ribose, *Proc Natl Acad Sci USA*, 95 (1998) 10328.

13 Klepper L, NOx evolution by soybean leave treated with salicylic acid and selected derivatives, *Pest Biochem Physiol*, 39 (1991) 43.

14 Cueto M, Hernandez-Perera O, Martin R, Benture M L, Rodrigo J, Lamas S & Galvano M P, Presence of nitric oxide synthase in roots and nodules of *Lupinus albus*, *FEBS Lett*, 398 (1996) 159.

15 Noritake T, Kawakita K & Doke N, Nitric oxide induces phytoalexin accumulation in potato tuber tissue, *Plant Cell Physiol*, 37 (1996) 113.

16 Acharya K, Pokhrel B, Sherpa A R, Katel O N & Acharya R, Inhibition of nitric oxide synthesis an important step in plant pathogenesis, *Bull Pure Appl Sci*, 20 (2001) 7.

17 Acharya K & Acharya R, Nitric oxide: The signal molecule of plant defense blocked during pathogenesis, *Indian J Pure Appl Biol*, 17 (2002) 128.

18 Acharya K & Acharya R, Involvement of nitric oxide synthase in *Solanum tuberosum – Phytopthora infestans* interaction, *J Mycopathol Res*, 40 (2002) 29.

19 Jia L, Bonaventua C, Bonaventura J & Stamler S J, S-nitrosohemoglobin: A dynamic activity of blood involved in vascular control. *Nature*, 380 (1996) 221.

20 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with folin-phenol reagent, *J Biol Chem*, 193 (1951) 265.
21 Dietrich R A, Delaney T P, Uknes S J, Ward E R, Ryals J A & Dangl J, *Arabidopsis* mutants stimulating disease resistance response, *Cell*, 77 (1994) 565.
22 Hogg N, Struck A, Goss S P A, Santanam N, Joseph J, Parthasarathy S & Kalyanaraman B, Inhibition macrophase dependent low density lipoprotein oxidation by nitric oxide donors, *J Lipid Res*, 36 (1995) 1756.

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In vitro inhibition of *Fusarium oxysporum* by fluorescent *Pseudomonas* BRL-1

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

Key words: Fluorescent *Pseudomonas*, *Fusarium oxysporum*.

Introduction

Fusarium oxysporum Schlecht. is an economically important soilborn plant pathogen which mediate many diseases like pod rot, wilt, root rot of *Archis hypogaea* (Mustafee, 1971), fruit rot of *Citrullus vulgaris* (Mathur and Mathur, 1958), wilt of *Dolichos biflorus* (Chakravarti and Kumar, 1972), wilt of *Helianthus annuus* (Ghodajkal *et al.*, 1976), wilt of *Musa paradisica* (Malik and Mehta, 1963), wilt of *Piper bitle* (Dastur, 1935), dry rot of rhizome of *Zingiber officinali* (Gupta and Sohi, 1967) etc. causes persistent and significant losses in crop yields. In recent years, fluorescent Pseudomonads have drawn attention world wide because of production of secondary metabolites such as siderophore (Neilands, *et al.*, 1991), antibiotics (Keel *et al.*, 1992; O'Sullivan 1981) volatile compound, HCN (Wei et al., 1991) enzyme and phytohormones (Keel *et al.*, 1992; O'Sullivan *et al.*, 1991). These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneously induced plant growth. Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, because chemical control is expensive and hazardous to soil ecosystem.

Here attempt has been made to evaluate *in vitro* antagonistic activity of an isolate of florescent *Pseudomonas* BRL-1 against this lethal phytopathogenic fungi *Fusarium oxysporum*.

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 F. oxysporum in dual plate culture

The antagonistic effect of fluorescent *Pseudomonas* BRL-1 were tested against *F. oxysporum* supplied

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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 / O shaped and semicircle / U shaped pattern, where as spore suspension of *F. oxysporum* 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 F. oxysporum in dual liquid culture

To test the antifungal activity of the fluorescent *Pseudomonas* BRL-1, dual liquid culture method was employed (Basha and Ulagnathan, 2002). One ml of 24 hours grown fluorescent *Pseudomonas* BRL-1 culture (containing 10^7 cfu / ml) and an agar block (5 mm diameter) of 5 days old culture of *F. oxysporum* was inoculated onto 50 ml of Peptone Glucose Broth medium in 250 ml of conical flask. The culture was incubated on a rotary shaker at 30°C for 72 hours. For the control experiments the fungal pathogen was grown alone. Experiments were performed thrice taking triplicate for every set. A drop of fungal culture from 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.

The differences in dry weights (72 hours old culture was dried for 24 hours at 70°C) between the fungal cultures grown with fluorescent *Pseudomonas* BRL-1 strain or the control culture grown without any bacterium were recorded (Basha and Ulagnathan, 2002).

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). An agar block (5 mm diameter) of 4 days old culture of *F. oxysporum* placed on the centre of the Petri plate containing 20 ml of PGA. A loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture was strike on Petri plate having 20 ml of PGA then inverted over the plates inoculated with the fungal pathogen *F. oxysporum*. Two plates were sealed together (mouth to mouth) with parafilm, control plate consist of *F. oxysporum* 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 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.

To test the production of IAA, active culture of fluorescent *Pseudomonas* BRL-1 was inoculated in 10 ml of minimal salt media supplemented with 100 µg / ml of tryptophan, and incubated at 30°C under shaking for 48 hours. Culture broth was centrifuged at 7500 rpm for 10 minuets. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and contents incubated at 30°C for 25 minutes. Absorption was read at 530 nm and levels quantified from standard curve of IAA.

To test the chitololytic property of fluorescent *Pseudomonas* BRL-1 it was inoculated on LB medium (Gunasekaaran, 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 (Frandberg and Schnurer, 1994).

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (Schwyn and Neilands, 1987) to detect the siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24 hours old fluorescent fluorescent *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72 hours. 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.

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 hours 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 *F. oxysporum* occurred due to antagonistic effect of fluorescent *Pseudomonas* BRL-1, which was evident with clear inhibition of colony diameter. The results are presented in Table1 and depicted in figure 1. At 48 hours of incubation, *F. oxysporum* was strongly inhibited by fluorescent *Pseudomonas* BRL-1 and simultaneous increase in incubation time corresponded escalation in percent growth inhibition. 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 (figure 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 68% reduction in dry weight of the culture grown with fluorescent *Pseudomonas* BRL-1 strain when compared to the control.

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, IAA investigated for their effect on *F. oxysporum in vitro* (Table 2).

The results (Table 2) showed fluorescent *Pseudomonas* BRL-1 produced mainly siderophore, protease,

Table 1. *In vitro* antagonistic activity of fluorescent *Pseudomonas* BRL-1, cultured as semi-circular and circular streaks, on *Fusarium oxysporum* point-inoculated in the center PGA plates incubated at 30°C.

Colony diameter (in cm) of F. oxysporum after incubation period of						
	0 h	24 h	48 h	72 h	96 h	120 h
Control	0.00	0.80 ± 0.10	2.28 ± 0.02	3.75 ± 0.07	5.00 ± 0.21	7.47 ± 0.53
Semi-circular	0.00	0.27 ± 0.10	0.74 ± 0.15	1.09 ± 0.21	1.41 ± 0.12	1.51 ± 0.02
Circular	0.00	0.00	0.52 ± 0.03	0.78 ± 0.12	1.01 ± 0.01	1.02 ± 0.02

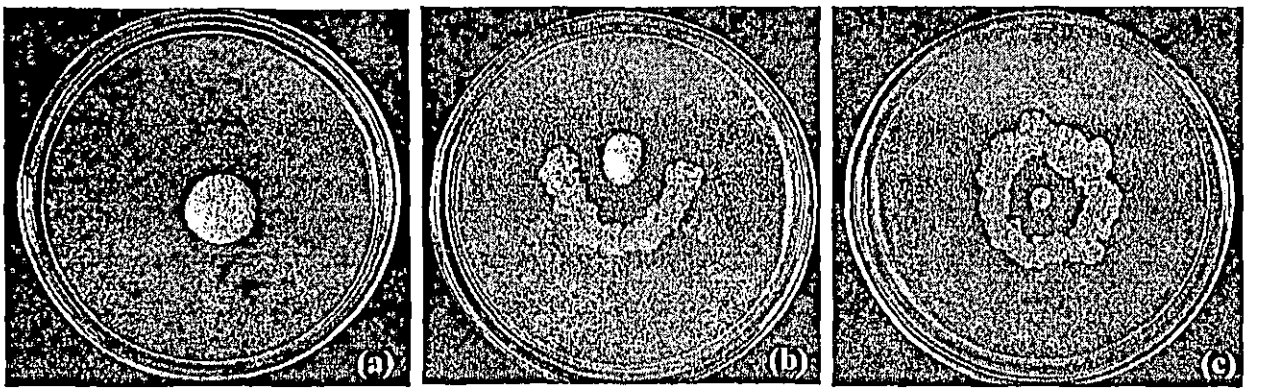


Fig.1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *F. oxysporum.* (a) Control; *F. oxysporum* colony in pure culture showing more rapid growth at 30°C; (b) Colony of *F. oxysporum* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist fluorescent *Pseudomonas* BRL-1 and (c) Colony of *F. oxysporum* showing restricted growth due to fluorescent *Pseudomonas* BRL-1 inoculated as a circular streak .

Table 2. Showing 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

chitinase and IAA.

The isolate was further studied for role of chitinase and protease activity, characterization of siderophore for their nature and binding properties and IAA production.

Mycolytic enzymes consist of chitinase, protease etc. based formulations have been used to control fungal plant pathogens (Despande, 1999). Fluorescent

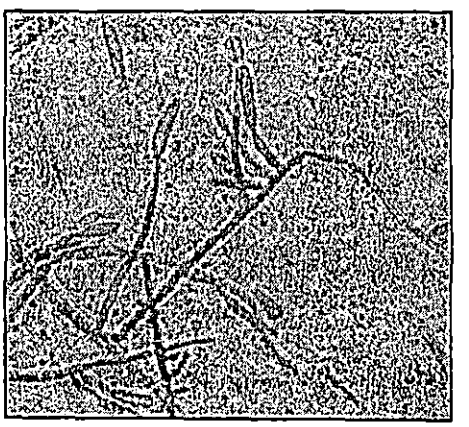
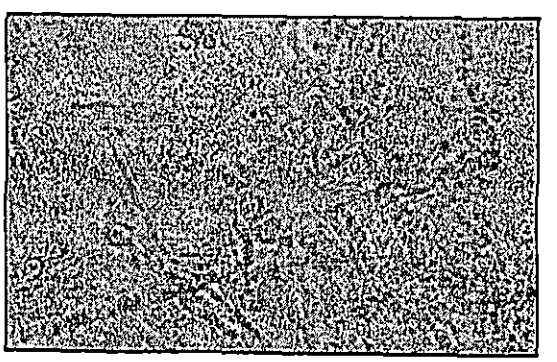


Fig. 2. Microscopic observations of mycelium inhibited by fluorescent *Pseudomonas* BRL-1 strain. Mycelium of *F. oxysporum* (a) grown on Peptone Glucose Agar (PGA) media (Control),



(b) present in the inhibition zone, when grown with fluorescent *Pseudomonas* BRL-1 on PGA.

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 siderophore production.

The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Despande , 1999).

In general Indole acetic acid is universally accepted as a plant growth promoter. Other than that, it has been reported by Chirst and Mosinger (1989) that this phytohormone could also induce resistance through PR protein production. Again in the year 2004, Sharaf and Farrag 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 soil pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1.

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, 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 (Rameshkumar *et al.*, 2002). These observations and further studies might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against *F. oxysporum.*

References

Arora, N.K., Kang, S.C. and Maheshwari, D.K. 2001. Isolation of siderophore producing strains of *Rhizobium meliloti* 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.

Chakravarti, B. P. and Anil Kumar T. B. 1972. Wilt of *Dolichos biforus* caused by *Fusarium oxysporum. Ind. Phytopathol.* 25: 583.

Christ, U. and Mosinger, E. 1989. Pathogenesis related protein of tomato: Induction by *Phytophthora infestance* and other biotic and abiotic inducers and correlations with resistance. *Phyisolo.Mol. Plant Pathol* 35:53-65.

Dastur J F, Diseases of Pan (*Piper bitle*) in the central provinces 1935. *Proc. Ind. Acac. Sci.* 1: 778-815.

Dennis, C. and Webster, J. 1971. Antagonistic properties of species group of *Trichoderma* II. Production of volatile antibiotics. *Trans. Bri. Mycolo. Soc.* 82: 41-48.

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

Frandberg, E. and Schnurer, J. 1994. Chitinolytic properties of Bacillus Pabuli K1[J] *.J Appl Bacteriol.*76: 361-367.

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.

Ghodajkal, B. N., Ganacharya, N. M., Bindu, K. J. and Jahav, V. T. 1976. Sunflower wilt, anew disease from India. *Curr. Sci.* 45: 844.

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

Gupta, G. K. and Sohi, H. S. 1967. Fungal foes that menace ginger crop. *Ind. Fmg.* 16: 15.

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

Keel C. U., Schnider, M., Maurhofer, C., Voisard, J., Laville. U., Burger, P., Wirthner, D., Haas and Défago,

G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol, *Mol Plant-Microbe Interact.* 5: 4-13.

Malik, P. C. and Mehta, P. P. 1963. Banana wilt in Bihar. *Sci and Cult,* 29: 245.

Mathur, R. L. and Mathur, B. L. 1958. Fruit rot of *Citrullus vulgaris* var. *fistulosus* L.,*Ind Phytopathol,* 11: 66-69.

Mustafee, T. P. 1971. Fiber deteriorating microflora of Ramie, *Sci and Cul.* 37: 386-387.

Neilands J B. 1981. Microbial iron compounds. *Annl. Rev. Biochem.* 50: 715-731.

O'Sullivan, M., Stephens, P. M. and O'Gara, F. 1991. Extracellular protease production by fluorescent *Pseudomonas* spp. and the colonization of sugarbeetroots and soil, *Soil Biol Biochem.* 23: 623-627.

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. Curr. 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.

Sharaf, E.F. and Farrag, A.A. 2004. Induced resistance in tomato plants by IAA against Fusarium oxysporum lycopersici *Pol J Microbiol.* 53: 111-116.

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.

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/l 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.

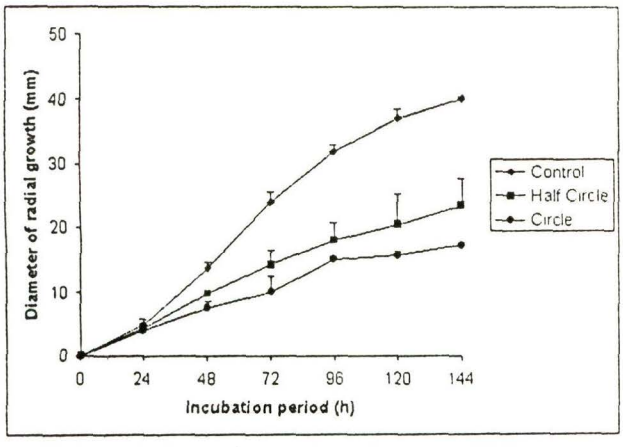


Fig. 1 : *In vitro* antagonistic activity of Fluorescent *Pseudomonas* BRL-1, cultured as semi-circular and circular streaks, on *Aspergillus niger* point-inoculated in the centre of PGA plates incubated at 30°C.

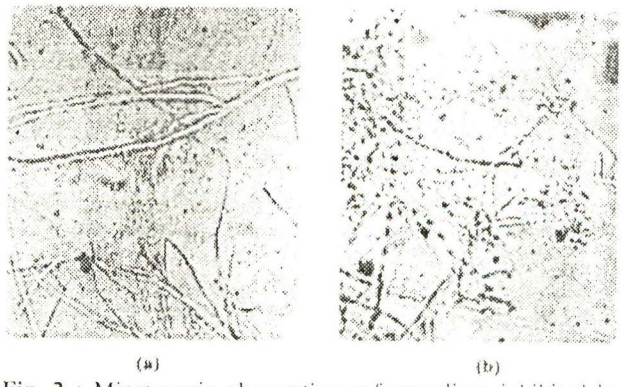


Fig. 2 : Microscopic observation sof mycelium inhibited by Fluorescent *Pseudomonas* BRL-1 strain. Mycelium of *A. niger* (a) grown on Peptone Glucose Agar (PGA) media (Control), (b) present in the inhibition zone, when grown with Fluorescent *Pseudomonas* BRL-1 on PGA.

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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IV. 2 : Special dispersion statistics and sequential sampling plan for frog eye leaf spot disease caused by *Cercospora capsici* in chilli

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Iwao's patchiness regression technique was used to study the spatial distribution of *Cercospora capsici* infecting chilli leaves in its five growth stages *viz.*, nursery, vegetative, flowering, podding and fruit ripening stages. The dispersion statistics indicated that the basic components of the disease lesion population aggregate and they were contagiously distributed in chilli at all growth stages. Botanical extracts spray did not alter the basic distribution pattern of disease. The dispersion statistics obtained from pooled data enabled to arrive at sequential sampling plan that requires a maximum of 58 leaves sample for assessing the threshold level of diseases in order to initiate control measures. A preliminary economic threshold of one lesion per leaf was used to prepare the sequential sampling plan for timing the initiation for using of plant extract to control the disease. It is a cost effective ecofriendly quantitative method in plant disease management.

IV. 3 : Integrated management approach against sigatoka leaf spot of Banana

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Leaf spot or sigatoka disease of banana causes considerable yield loss in every year and it may goes up to 50% or more in severe cases. The disease has a great importance in West Bengal as the commercial cultivation of the crop increases rapidly during recent years. Different management approaches have been taken in this investigation including chemical fungicides, botanical oils and a bacterium inoculants (*Pseudomonas fluorescens*). Among the 10 fungicides assessed, propiconazole showed best performance against the disease followed by chlorathalonil and tridemorph. Five botanical oils tested, of which neem oil was found to be the best to reduce the disease severity. Performance of the oil was as good as some chemical fungicide (eg. propineb) when applied @ 0.1% level. The bacterium *Pseudomonas fluorescens* when applied @ 5 g formulation (10^8 cfu/g) per litre, per cent disease index (PDI) was estimated as 17.83 as compared to 27.47 in the control sets. Yield of the crop was recorded during harvest where propiconazole gave the best result (30.82 t/ha). Productivity per ha of the neem treated plot and *P. florescens* plot were 27.12 t and 25.80 t respectively, whereas yield of the control plot was 21.66 t/ha. Yield increases over control were calculated 42.88%, 25.20% and 19.11% from propiconazole, neem oil and *Pseudomonas fluorescens* treated plot respectively.

IV. 4 : Management of an epidemic disease of *Cymbidium* rot by fluorescent *Pseudomonas*

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Orchid is an important crop for earning foreign exchange as it is one of the most beautiful ornamental plant, and also play a significant role in agricultural economy. In Darjeeling and its adjoining area it has been found that the commercially important part i.e., pseudobulb and roots of *Cymbidium* are attacked by a combination of two fungal pathogens namely *Fusarium oxysporum* Schlecht. and *Mucor hiemalis* Wehmer.f. *hiemalis*, and one pathogenic bacteria *Erwinia*. These three pathogens might play a synergistic effect to increase the severity of this disease. It was estimated by the Himalayan Orchid Society that the loss is more than million rupees during this last five years. Furthermore we have isolated an antagonist PGPR fluorescent *Pseudomonas* BRL-1 which showed significant inhibition over the growth of pathogens *in vitro* by the production of siderophore, chitinase, protease and IAA. An application of this antagonist in the nursery level protected plants upto 80%. Routine application of this biocontrol agent in the field might help the horticulturist to protect this commercially important plant and might boost up the economy of this area.

IV. 5 : Diseases of Oak Tasar host plants (*Quercus* spp.) in Manipur

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Quercus serrata and *Quercus acutissima* are the two common oak plants for rearing Oak Tasar silkworm (*Antheraea proylei*) in Manipur. These plants are occasionally attacked by fungal diseases. In the present investigation three fungal diseases namely Sooty mold (*Chaetophoma quircifolia*), Powdery mildew (*Phyllactenia corylea*) and Rust disease (*Cronartium quercum*) were found to attack mostly from March to November in a year. Survey data from five plantation sites of Oak tasar plantation farm revealed that disease incidence and disease severity increase from May to July and gradually decrease in the winter season. These diseases are mostly air borne as screening of seeds didn't reveal the presence of any of these pathogens on Oak tasar seeds. Effect of epidemiological factors on the development of these diseases on Oak Tasar host plants is discussed in the full paper.

IV. 6 : Effect of pre and post emergence herbicides on fibre yield and soil microbial population under a jute crop in an alluvial soil

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A field experiment was conducted in an alluvial soil (*Typic Ustocrept*) to study the effect of two pre (Trifluralin and Fluchloralin) and one post emergence (Quizalofop ethyl) herbicides on fibre yield and soil microbial population in jute crop. The fibre yield of jute increased significantly with herbicides application over control. The maximum fibre yield (37.9 q/ha) was recorded with pre emergence herbicide "Trifluralin" @ 0.75 kg a.i./ha which was significantly superior over other treatments. The total population of bacteria, fungi and actinomycetes decreased drastically after 7 days of application of these herbicides compared to initial status as well as control. The microbial population increased gradually after 15 days onwards and reached to the initial status at harvest i.e. after 120 days.

IV. 7 : Leaf rot of Betelvine : host range of, medium for isolation and technique for bioassay of fungicides against the pathogen

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Leaf rot of Betelvine caused by *Phytophthora nicotianae* is prevalent in all betelvine growing areas of West Bengal. In laboratory condition the pathogen infected brinjal, tomato, sweet pepper, red pepper, cucumber and guava fruits and leaves of black pepper. On artificial inoculation it infected red pepper (chilli) causing foot rot symptom in field condition. *Phytophthora nicotianae* isolated from fruit rot of brinjal, guava, leaf rot of black pepper, foot rot of *Hibiscus subdariffa*, foot rot and leaf blight of sesamum and *Phytophthora capsici* from foot rot of chilli infected betelvine leaves in laboratory and in field conditions. *Phytophthora* spp. from all the hosts were successfully isolated in Oat meal agar medium amended with vancomycin (200 ppm), pimaricin (10 ppm) and carbendazim (25 ppm). An easy technique for bioassay of fungicides against *Phytophthora nicotianae* has been standardized. When agar disc with mycelial growth of *Phytophthora nicotianae* was put in water in half-submerged codition, the fungus produced good mycelial growth with abundant sporangia and the sporangia thus formed germinated in water. Sensitivity of this fungus towards fungicides was successfully tested by putting agar disc mycelial growth in different concentration of a particular fungicide and subsequently recording the extent of mycelial growth, sporangia formation and sporangial germination. Copper oxychloride, copper hydroxide, mancozeb, thiram, combination product of mancozeb + metalaxyl, copper oxychloride + metalaxyl, cymoxanil + mancozeb showed good inhibition against all *Phytophthora* species.

In vivo studies revealed that all the antagonists reduced the root rot of sesame significantly, when applied as soil drenching or in combination of seed soaking and soil drenching. Combination of seed soaking and soil drenching gave better results than the soil drenching alone. Here among the antagonists maximum disease control was observed by the *T. harzianum* which gave up to 66.17% of sesame root rot disease control followed by isolate BS-12 (up to 65.27%) and BS-17 (up to 65.25%).

P-VIII. 4 : EFFECT OF DIFFERENT LEVELS OF IRRIGATION AND SOURCES OF SULPHUR ON NODULE NUMBER, WEIGHT AND YIELD OF SUMMER GROUNDNUT (*ARACHIS HYPOGAEA*)

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Groundnut is one of the leading oilseed crops in the global agricultural scenario. India, though ranks 2nd in groundnut production, but its productivity is far lower than most of the groundnut producing countries like China, America and Argentina. The main reasons advocated for such low productivity are lack of irrigation facilities and inadequate fertilizer application. Among the chemical fertilizers, sulphur is a major one.

Keeping in mind the above view points, the experiment was conducted during pre-kharif season of 2003 and 2004 at Jagulia Instructional Farm, B.C.K.V., Mohanpur, Nadia on sandy loam soil under irrigated upland condition. The experiment was conducted in a split-plot design having 4 levels or irrigation (Rainfed, one irrigation at flower initiation, two irrigations at flower initiation + pegging, and three irrigations at flower initiation + pegging + pod development) in the main plot and 4 sources of S (Elemental S, single super phosphate, ammonium sulphate and gypsum) applied at 40 kg/ha in the subplot. The experiment was replicated thrice. Spacing adopted were 30 cm (row to row) and 10 cm between plants. The seed rate and variety were 60 kg/ha and SB-XI respectively. Crop received 20 kg N, 40 kg P_2O_5 and 20 kg K_2O/ha applied as basal.

Experimental results revealed that number of nodules, dry weight of nodules and yield of groundnut crop were greatly improved by different levels of irrigation. Three irrigations level recorded the maximum nodule number and dry weight with a corresponding highest seed yield of groundnut. Different sources of sulphur influenced the number and dry weight of nodules and the seed yield of groundnut significantly. Gypsum proved to be the best performer in this regard. The interaction between irrigation levels and sources of sulphur was found to be significant on groundnut yield and three irrigation with gypsum was found to be the best.

P-VIII. 5 : INTEGRATED MANAGEMENT OF BLACK-ROT DISEASE OF CAULIFLOWER IN ORISSA

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The efficacy of bacterial antagonists *Pseudomonas fluorescens* and *Azotobacter chroococcum*, chemical like strptocycline and a botanical neem cake was studied for the management of black-rot disease of cauliflower. Field trials were conducted for three consecutive Rabi seasons 2001-02, 2002-03 and 2003-04 in the experimental field of All India Coordinated Vegetable Improvement Project, OUAT, Bhubaneswar with the variety Himlata-2. The trial was laid out in randomized block design with four replications. The row to row spacing of 60 cm and plant to plant spacing of 40 cm was adopted in the plot size of 8.1 sq.m. (3.0 m × 2.7 m) with the fertilizer dose of 150 : 75 : 75 kg N : P_2O_5 : K_2O/ha. Among the different treatments, seed treatment with sterptocycline (100 ppm) for 15 minutes followed by seedling dip (100 ppm) for 15 minutes before planting and three sprays of streptocycline (200 ppm) at 10 days intervals starting from 15 days after planting proved very effective in management of black-rot with mean PDI of 4.55 %, maximum curd yield of 220.23 q/ha and registering 81.84 % disease control and 75.71 % increase in curd yield over control. The same treatment gave maximum cost benefit ratio of 1:10.85 with net return of Rs.27,505.00. Seed treatment with *Azotobacter chroococcum* (1.5 g/kg), soil application of the culture (250 g/50 kg FYM/ha) and slurry culture drenching (after 15 days of planting) was found to be next best in respect of disease control (54.69 %) followed by use of *Pseudomonas fluroescens* as seed treatment (10 g/kg), seedling dip (0.2 % spore suspension for 30 minutes before planting) and foliar spraying (three sprays of 0.2 % spore suspension at 10 days interval starting from 15 days after planting) registered 37.71 % disease control with mean cost benefit ratio of 1:5.68. However, the control plots recorded maximum disease incidence of 25.6 % with lowest curd yield of 125.47 q/ha.

P-VIII. 6 : OPTIMIZATION OF DIFFERENT PHYSIOLOGICAL FACTORS ON GROWTH FOR THE DEVELOPMENT OF A COST EFFECTIVE MEDIA FOR LARGE SCALE PRODUCTION OF AN ANTAGONISTIC PGPR FLUORESCENT *PSEUDOMONAS* BRL-1

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Considerable research efforts are underway globally to exploit the potential of fluorescent Pseudomonads in maintenance of soil health and as crop protectants, since they represent not only a dominant bacterial group of rhizosphre ecosystem but are also metabolically and functionally most versatile. Our isolate, fluorescent *Pseudomonas* BRL-1, showed significant antagonistic activity against several phytopathogens like *Fusarium oxysporum, Sclerotium rolfsii, Myrothecium roridum, Colletotrichum capsici* and *Curvularia lunata* and also having PGPR activity. Correlation of antifungal activity of these isolates have been found to be linked with the production of siderophore, protease, chitinase and IAA. The objective of this study was to standardize a suitable cost effective fermentational technique for the large-scale production and powder formulation of the said strain. Various factors influencing the growth of the strain were worked out by keeping all the factors constant except the one was varied. The factors considered were pH of the medium, time period, temperature, aeration, inoculum volume and selection of suitable carbon and nitrogen sources.

Survival of the fluorescent *Pseudomonas* BRL-1 monitored at room temperature in a talc based formulation showed viable population up to 120 days. The population was drastically reduced after 120 days of storage. The results indicate that this efficient rhizospheric bacteria could be commercially produced in this cost effective media composition and could be maintained in talc based powdered form for at least five months.

P-VIII. 7 : EVALUATION OF BIOCONTROL POTENTIALITY AND PLANT GROWTH PROMOTING CAPABILITY OF A FLUORESCENT *PSEUDOMONAS* BRL-1 AGAINST SEVERAL PHYTOPATHOGEN

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Considerable research efforts are underway globally to exploit the potential of fluorescent Pseudomonads in maintenance of soil health and as crop protectants, since they represent not only a dominant bacterial group in the rhizosphere ecosystem but are also metabolically and functionally most versatile.

In this study we have evaluated the biocontrol potentiality and PGPR activity of an isolate, fluorescent *Pseudomonas* BRL-1. In dual culture, significant growth inhibition of several phytopathogens like *Fusarium oxysporum, Mucor hiemalis, Sclerocium rolfsii, Myrothecium roridum, Colletotrichum capsici, Alternaria alternata* etc. occurred due to the antagonistic effect of fluorescent *Pseudomonas* BRL-1. Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycellial deformities, swelling, fragmentation, short branching and finally resulting into lysis.

Morphological abnormalities of the hyphae occurred due to the production of secondary metabolites mainly siderophore which is of hydroxamate in nature. The strain produce mycolitic enzyme like chitinase and protease, which are well known to lyse the cell wall of fungal pathogen. Furthermore the isolate produces significant amount of IAA which functions as a plant growth promoter as well as prevent the growth of pathogen.

The fluorescent *Pseudomonas* BRL-1 showing antagonistic activity through combined and/ or individual effect of siderophore, production of mycolitic enzyme and IAA activity. These observation and further studies might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against different pathogens.

P-VIII. 8 : ISOLATION, CHARACTERIZATION AND SCREENING OF FLUORESCENT PSEUDOMONADS POPULATION OF MEDICINAL PLANT'S RHIZOSPHERE AGAINST *ALTERNARIA ALTERNATA*

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In recent years fluorescent Pseudomonads have drawn attention world wide because of production of secondary metabolites such as siderophores, antibiotics, volatile compound, HCN, enzymes and phytohormons. Here, the phenotypic diversity of 100 fluorescent *Pseudomonas* strain isolated from the rhizosphere of different medicinal plants were compared. Vast ranges of biochemical, morphological, cultural and physiological homology were evaluated. The following tests were performed: proteolysis of gelatin, nitrate reductase, oxidase test, arginine dihydrolase production, lipase production, production of gas from carbohydrates (such as glucose, sucrose, lactose), urease production, levan formation from sucrose, H_2S formation, catalase, methyl red and Voges-Proskawer test. Morphological and cultural characteristics were examined on 4 days old culture. All the isolates were rod shaped, aerobic, gram negative; as regard to their enzymatic activity they were oxidase positive, nitrate positive and arginine dihydrolase positive they were levan positive and showed negative result in Voges-Proskauer test. Among all the Fluorescent Pseudomonad native isolates, 26 different isolates showed antagonistic activity against *Alternaria alternata*, out of which GS9, GS11, CA9, CA17, SR9, SR14, WS10, WS11 and RS11 were found to be most potent antagonist.

P-VIII. 9 : BIOLOGICAL CONTROL OF RHIZOME ROT DISEASE OF TURMERIC

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Turmeric (*Curcuma longa* Linn.) an important rhizomatous spice crop being cultivated in India, since ancient times, is affected by some diseases of which rhizome rot disease caused by *Pythium* sp is most important. The three biocontrol agents viz., *Trichoderma viride*, *Pseudomonas fluorescence* and *Bacillus subtilis* and Farm Yard Manure (FYM) were used in different combinations both as seed treatment and soil application to develop an effective biological control method against rhizome rot disease of turmeric. Combination of seed treatments with both *Trichoderma viride* and *Pseudomonas fluorescence* along with FYM showed highest disease reduction (63.65% less disease) as compared to check than other treatments. The soil application of *Trichoderma* and *Pseudomonas* along with FYM as well as combination of seed treatment and soil application with these two bio-control agents also found to give very good result in terms of per cent reduction in rot over control. Regarding rhizome yield, combined seed and soil treatment with both *Trichoderma* and *Pseudomonas* along with FYM was the best, recording the highest rhizome yield. *Bacillus subtilis* had less pronounced effect on disease incidence and rhizome yield.

P-VIII. 10 : POTENTIAL DETERRENCE OF *TRICHODERMA* SPP. AGAINST FUSARIAL WILT OF TOMATO

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Wilt of tomato (*Lycopersicon esculentum*) is a most dreadful disease caused by *Fusarium oxysporum* leading to an enormous economic loss to the growers as a consequence. An attempt was made to control the disease biologically with the application of antagonists viz. *Trichoderma viride, T. lignorum, T. harzianum, T. hamatum and T. reesei*. The effect of volatile and non-volatile antibiotics of *Trichoderma* origin on growth inhibition of the wilt pathogen was studied. *T. harzianum* showed maximum growth inhibition (91%) of the pathogen through mycoparasitism followed by *T. viride* and the non-volatiles produced by these antagonists exhibited their excellent antagonism to the growth of the pathogen (100%) under *in vitro* condition. *Trichoderma* spp produced siderophore, iron-chelating compounds that contribute much towards enhancement of their competitive behaviour for nutrition with the target pathogenic fungi and as such offer their greater antagonistic potentiality. Overall experimental studies clearly indicate *T. harzianum, T. viride* and *T. hamatum* are capable of retarding the growth of the pathogen while *T. lignorum* and *T. reesei* are comparatively less efficient to this effect.

P-VIII. 11 : EVALUATION OF ANTAGONISTIC POTENTIAL OF SOME WILD AND MUTANT ISOLATES OF *TRICHODERMA HARZIANUM* AGAINST *MACROPHOMINA PHASEOLINA* (TASSI) GOID CAUSING STEM ROT OF JUTE

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Six gamma irradiated mutant isolates of *Trichoderma harzianum* and their two wild biotypes were evaluated for antagonistic potential against *Macrophomina phaseolina* causing stem rot of jute. All the mutant isolates were more effective in inhibiting the mycelial growth of the pathgogen than their corresponding wild biotypes in dual culture and two mutant isolates namely $75^{Th}4IV$ and $125^{Th}41$ were most effective in inhibiting pathogen growth by 78%. The highest production of volatile compound was recorded in case of $150^{Th}31$ which was evidenced by 49% inhibition of mycelial growth of the pathogen over control. The maximum inhibition of mycelial growth of the pathogen through production of nonvolatile antibiotic was recorded in case of Th4, $150^{Th}31$ and $75^{Th}41V$. The mutant isolate $75^{Th}41V$ was also found to produce greater quantity of extracellualr enzymes like β-1,3 glucanase, β-1,4 endoglucanase and chitinase when Czapek Dox broth was partially induced with mycelial powder of *M. phaseolina*. *In vivo* trial was conduced to determine the biocontrol potential against *M. phaseolina* incited stem rot of jute and observations on disease incidence were taken at fortnightly interval up to 8 weeks. At 56 days after sowing all the mutants were superior to their respective wild biotypes in non-sterilized soil, but they were highly variable in their disesase management potential. However, the mutants $75^{Th}41V$ and 125th 41 were found most promisoing in reducing stem rot infestation in both-sterlized and unsterilized soil.

P-VIII. 12 : DIVERSITY OF WILD EDIBLE MUSHROOMS AND THEIR ECONOMIC VALUES IN SHEPOUMARAMTH NAGA AREAS UNDER SENAPATI DISTRICT OF MANIPUR

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Out of the total twenty-four different edible fungi collected and identified in local dialects, the present work focused on nine locally well known mushrooms. Detail information's in regard to habit, habitats,

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In vitro antagonistic effect of fluorescent *Pseudomonas* BRL-1 against *Sclerotium rolfsii*

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Key words: Fluorescent *Pseudomonas, Sclerotium rolfsii*

Sclerotium rolfsii Sacc. is an important soil-borne plant pathogen distributed throughout the world, having broad host range and persists for longer period in soil by resistant resting structures (sclerotia) (3). Use of synthetic fungicides and crop management strategies were unsuccessful in eradicating this pathogen completely, and the former causing environmental hazards and chronic health problem (2).

In recent years, florescent Pseudomonads have drawn attention worldwide owing the ability of production of secondary metabolites such as siderophore (16), antibiotics (12, 17), volatile compound, HCN (24), enzymes and phytohormones (12, 17). 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 phytopathogenic fungi *Sclerotium rolfsii.*

Soil bacterial isolates were made from the rhizosphere soil samples of potato following serial dilution technique in King's B media (13). All the isolates were subcultured and maintained on Tryptic Soy Agar (TSA) medium. Screening for the antagonistic activity was carried out by following the method of Huang and Hoes (1976) (11). An agar block of 5mm diameter was placed at the center of the PGA plate and allowed to grow for 48 hours. A loopful of bacterial culture was streaked at the periphery of the plate and incubated at 30°C for five days. Development of inhibition zone around the bacterial colony was observed. Out of the 96 isolates tested 6 showed inhibition against *S. rolfsii,* of which strain BRL-1 showed the highest level of inhibition and selected for further study. This strain was characterized morphologically and biochemically following Bergey's Manual of Determinative Bacteriology (10) and found to be a fluorescent *Pseudomonas* species.

The florescent *Pseudomonas* BRL-1 was subcultured and maintained on TSA medium for subsequent use. During *in vitro* antagonistic study, dual culture technique was performed on Peptone Glucose Agar (PGA) solid (23) and liquid (2) media. In the former experiment bacterial isolate was streaked on PGA plate in a circular / O shaped and semicircular / U shaped pattern (23). In the later case 50ml of PG broth was inoculated with 1ml the bacterial suspension (10^7 cfu ml^{-1}). Then the pathogen was subsequently challenged in the form of a single sclerotium 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 5 days old culture of *S. rolfsii.* Sole inoculation with pathogen in both solid and liquid media was served as control. Plates were incubated for 6 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 h in a rotary shaker and the mycelial dry weight were estimated. The liquid

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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.

Some biochemical studies regarding secondary metabolites were performed to evaluate the nature of antagonism of this strain. Production of volatile compound was assayed by 'inverted plate technique' (5) where two lids of separate petriplates were taken and poured with 20 ml of PGA. One was inoculated with a sclerotium and the other was streaked with a loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture. The petriplates were sealed mouth to mouth with parafilm. Control set consisted of only *S. rolfsii* inverted over uninoculated PGA plate. Plates were incubated at 30°C for 5 days as triplicate. After incubation period colony diameter of the fungal pathogen was measured and compared with the control set. Estimation of hydrogen cyanide production was done following the method of Wei *et al.* (24). Bacterial culture was inoculated in TSA medium supplemented with amino acid glycine (4.4g/l 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 brown indicates the production of HCN. Production of diffusible antibiotic was detected by an 'agar layer technique' (7). PGA plates covered with a cellophane membrane were inoculated in the center with 100 μl of the antagonist suspension (2×10^7 cfu/ml). After incubation for 48 h at 30°C, the membrane with the grown bacterial isolate was removed, and the plates were inoculated in the middle with a sclerotium of *S. rolfsii.* Controls were run with sterile water instead of bacterial suspension on the cellophane membrane. Plates were further incubated at 30°C for 4 days and the growth of the pathogen was measured and compared with the control set. Protease activity was performed by inoculating the antagonist on minimal media (5X: $(NH_4)_2SO_4$ -5g; KH_2PO_4-22.5g; K_2HPO_4-52.5g; Na-citrate-2.5g; distilled water-1L) containing 3% of skim milk and 1% agar; incubate at 30°C for 24-48 h and testing with 1% Tri Carboxylic Acid (TCA) solution. Presence of clear zone around the colonies indicated protease activity (8). To test the chitinololytic activity, the bacterium was inoculated on Luria Bertani (LB) medium (9) supplemented with 0.5% colloidal chitin and incubated at 30°C for three days. Formation of a clear halo region around the colonies indicated chitinase activity of the strain (7). IAA production was quantified spectrophotomertically growing the fluorescent *Pseudomonas* BRL-1 in 10 ml of minimal salt media supplemented with 100mg/ml of tryptophan, and incubated at 30°C under shaking for 48h. Broth culture was centrifuged at 7500 rpm for 10 min. To 1ml of culture supernatant, 2ml 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 (8). Chrome Azurol S (CAS) agar (blue agar medium) was used to detect siderophore production (21). Fluorescent *Pseudomonas* BRL-1 was inoculated at the center of the plate and incubated at 30°C for 72 h. Appearance of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

In dual culture, significant growth inhibition of *S. rolfsii* 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 *S. rolfsii* upto 6 days (Fig. 1F). Microscopic study of mycelia form interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching, and finally resulting into lysis (Fig. 1E). Co-culture of *S. rolfsii* and fluorescent *Pseudomonas* BRL-1 in liquid media resulted in 55 percent reduction in the mycelial dry weight. Among different biochemical tests performed to detect the cause of inhibitory effect of fluorescent *Pseudomonas* BRL-1, the isolate showed significant protease, chitinase, IAA and siderophore producing activity (Table 1). Lorito *et al.* (15) and Dunne *et al.* (6) reported earlier that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease can 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 Indoleacetic acid is universally accepted as a plant growth promoter. Chirst and Mosinger (1989) (4) reported that this

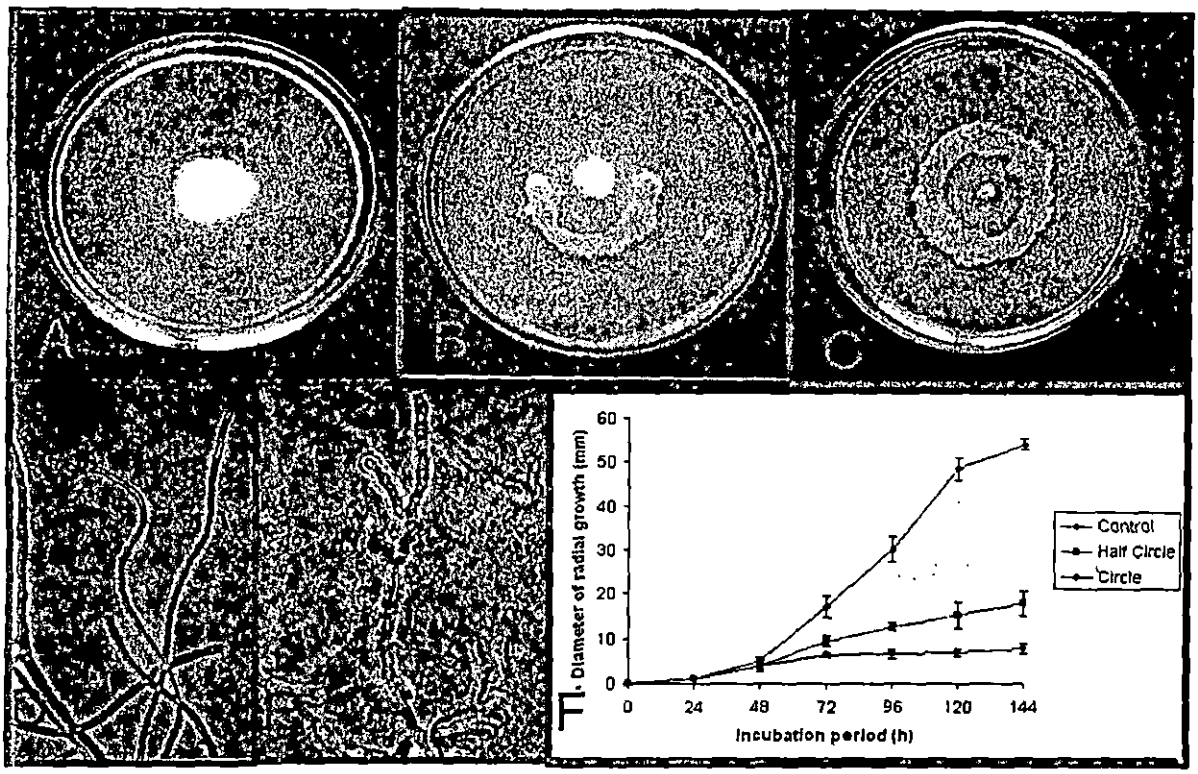

Fig. 1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *S. rolfsii*. (A) Control; *S. rolfsii* colony in pure culture showing more rapid growth at 30°C; (B) Colony of *S. rolfsii* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist and (C) Colony of *S. rolfsii* 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

phytohormone could also induce resistance through PR protein production; Sharaf and Farrag (2004) (22) 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 soil pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1. 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. Siderophore have been shown to play a role in increased growth response of certain plants to treat the planting material with fluorescent pseudomonads (1, 14, 19). The response is thought to involve suppression of deleterious rhizospheric microorganism (14, 19, 20).

Table 1. Showing different secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore	+++
Antibiotic Substances	-
HCN	-
Volatile substances	-
Chitinase	+
Portease	++
IAA	+++

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

Thus the present study concluded that the fluorescent *Pseudomonas* BRL-1 showed significant 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 (18). These observations and further study might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential biocontrol agent against *S. rolfsii.*

REFERENCES

1. **Ahl, P., Voisard, C.** and **Defago, G.** (1986). *Phytopathol.* **116:** 121-134.
2. **Basha, S.** and **Ulagnathan, K.** (2002). *Curr. Sci.* **82:** 1457-1463.
3. **Bilgrami, K.S., Jamaluddin, S.** and **Rizwi, M.A.** (1991) *Fungi of India List and References.* Todays and Tomorrow's Printing Publishers. New Delhi. pp. 454-456.
4. **Christ, U.** and **Mosinger, E.** (1989). *Physiol. Mol. Plant Pathol.* **35:** 53-65.
5. **Dennis, C.** and **Webster, J.** (1971). *Trans. Br. myco. Soc.* **82:** 41-48.
6. **Dunne, C., Crowley, J.J. Moenne-Loccoz, Y., Dowling, D.N., de Brunijn, F.J.** and **O'Gara, F.** (1997). *Microbiology.* **143:** 3921-3931.
7. **Frandberg, E.** and **Schnurer, J.** (1998). *Can. J. Microbiol.* **44:** 121-127.
8. **Gaur, R., Shani, N., Kawaljeet, Johri, B.N., Rossi, P.** and **Aragno, M.** (2004). *Curr. Sci.* **86:** 453-457.
9. **Gunasekaaran, P.** (1995). *Laboratory Manual in Microbiology.* New Age International (P) Limited, New Delhi. pp. 125.
10. **Holt, G.J., Krieg, R.N., Sneath, P.H.A., Staley, J.T.** and **Williams, S.T.** (1994). *Bergy's Manual of Determinative Bacteriology.* Willims & Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp.151-168.
11. **Huang, H.C.** and **Hoes, J.A.C.** (1976). *Can. J. Bot.* **54:** 406-410.
12. **Keel, C. U., Schnider, M., Maurhofer, C., Voisard, J., Laville, U., Burger, P., Wirthner, D.** and **Haas, G.** (1992). *Mol. Plant-Microbe Interact.* **5:** 4-10.
13. **King, E.O., Ward, M.K.** and **Raney, D.E.** (1954). *J. Lab Clin. Med.* **44:** 301-307.
14. **Kloepper, J.W.** and **Schroth, M.N.** (1981). *Phyotopathology* **71:** 1020-1024.
15. **Lorito, M., Peterbauer, C., Hayes, C.K.** and **Harman, G.E.** (1994). *Microbiology.* **140:** 623-629.
16. **Neilands, J.B.** (1981) *Annl. Rev. Biochem.* **50:** 715-731.
17. **O'Sullivan, M, Stephens, P.M.** and **O'Gara, F.** (1991). *Soil Biol. Biochem.* **23:** 623-627.
18. **Rameshkumar, N., Thirumalai Arasu, V.** and **Gunasekaran, P.** (2002). *Curr. Sci.* **82:** 1463-1466.
19. **Scher, F.M.** and **Bakker, R.** (1982). *Phytopathology* **72:** 1567-1573.
20. **Schippers, B., Bakker, A.W.** and **Bakker, P.A.H.M.** (1987). *Annu. Rev. Phytopath.* **25:** 339-358.
21. **Schwyn, B.** and **Neilands, J.B.** (1987). *Annal. Bichem.* **160:** 47-56.
22. **Sharaf, E.F.** and **Farrag, A.A.** (2004). *Pol. J. Microbiol.* **53:** 111-116.
23. **Skidmore, A.M.** and **Dikinson, C.H.** (1976). *Trans. Br. mycol. Soc.* **66:** 57-64.
24. **Wei, G., Kloepper, J.W.** and **Sadik, T.** (1991). *Phytopathology.* **81:** 1508-1512.

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