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Antagonistic mechanisms of fluorescent pseudomonads against *Phytophthora capsici* in black pepper (*Piper nigrum* L.)

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Abstract

Fluorescent pseudomonads were selectively isolated from black pepper (*Piper nigrum*) roots and screened for volatile and non-volatile metabolite production and inhibition in growth of *Phytophthora capsici*, the causal organism of foot rot disease. Among the isolates tested, the inhibition of *P. capsici* varied from 36.3% to 70.0% by non-volatile metabolites and from 2% to 23% by volatile-metabolites. Isolate IISR-51 caused maximum inhibition of *P. capsici* by production of non-volatile and volatile metabolites. Many of the isolates produced hydrogen cyanide, which limited the growth of *P. capsici*. The study indicated the involvement of siderophore-mediated antagonism in fluorescent pseudomonads. The fluorescent pseudomonads could systemically invade black pepper cuttings upon root bacterization.

Key words: biological control, black pepper, fluorescent pseudomonads, *Phytophthora capsici*, *Piper nigrum*.

Introduction

Foot rot disease of black pepper (*Piper nigrum*) caused by *Phytophthora capsici*, causes severe crop losses (Sarma 2003). Though chemical control measures are effective, biological control is a viable strategy for sustainable disease management, considering the cost of chemical pesticides and the environmental hazards involved. Further, the need for production of pesticide-free black pepper is important for export. Fluorescent pseudomonads have been widely tested against fungal pathogens because of their rapid growth rate and their ability to colonize rhizosphere to a large extent, besides their ability to suppress soil-borne pathogens (Fukui *et al*. 1994). Understanding the mechanisms of biocontrol of plant diseases is critical to the eventual improvement and effective use of biocontrol agents. In addition to competition for limited carbon sources in the rhizosphere, antagonism can be mainly attributed to the production of secondary metabolites like antibiotics (Ahl *et al*. 1986;

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Gardner *et al.* 1984; Stutz *et al.* 1986; Weller 1988), siderophores and cyanides (Kloepper *et al.*1980a, b; Kloepper & Schroth 1981). Introduced biocontrol bacteria that can reside within the plant system as endophytes provide added advantage (Hallmann *et al.* 1997). Mahaffee & Kloepper (1997) have shown that biological control by endophytic bacteria is possible and can involve induced resistance to soil-borne pathogens.

The present study was undertaken to understand the *in vitro* antagonistic action of fluorescent pseudomonads against *P. capsici*. Out of the 200 isolates of fluorescent pseudomonads isolated from rhizosphere soils collected from different agro-climatic regions in India, 12 isolates were short-listed based on inhibition of *P. capsici* in dual culture assay. These 12 isolates were tested for the production of volatile metabolites, hydrogen cyanide and siderophores. The endophytic nature of fluorescent pseudomonad isolate IISR-34 was also studied.

Materials and methods

The experiment was carried out at the Indian Institute of Spices Research, Calicut, Kerala.

Isolation and preservation of fluorescent pseudomonads

Root bits collected from healthy black pepper vines in diseased gardens from different agro-climatic regions of India were plated on King's B agar medium, supplemented with Ampicillin (40 mg ml^{-1}) + Cycloheximide (100 mg ml^{-1}) + Chloramphenicol (15 mg ml^{-1}) (Raaijmakers *et al.* 1999). Fluorescent pseudomonads were isolated and preserved at –80^{O} C using glycerol (20%) as cryoprotectant.

In vitro *assay for non-volatile metabolites*

A dual culture technique (Dennis & Webster 1971) was adopted for short listing the isolates based on inhibition of radial growth of *P. capsici*. The *P. capsici* isolate 99-101 used for the present study was obtained from the National Repository of *Phytophthora* maintained at the Indian Institute of Spices Research, Calicut. Nine mm disc of *P. capsici*, cut from the growing edge of the colony raised in carrot agar plate was placed at the center of a PDA plate. Streaks of the test bacteria were done, 1.5 cm away from both sides of the *P. capsici* disc and the plates were incubated at 25 ± 2^{0}C for 72 h. Radial growth inhibition of *P. capsici* was calculated using the formula:

$$\text{Radial growth inhibition} = \frac{Rc - Ri}{Rc} \times 100$$

where,

Rc=Radial growth in control plates; Ri=Radial growth in inoculated plates.

In vitro *assay for volatile metabolites*

The centre of a carrot agar plate was inoculated with a 9 mm disc of *P. capsici* (isolate 99-101). The plate with *P. capsici* was placed up side down over another plate containing fluorescent pseudomonad culture in sterile King's B broth (25 ml). This assembly was sealed airtight with parafilm and after 72 h of incubation on a shaking platform, the radial growth of *P. capsici* was measured and compared with that of control.

Production of hydrogen cyanide

Production of hydrogen cyanide (HCN) by isolates of fluorescent pseudomonads was determined using the method of Kloepper *et al.* (1991) with modifications. Isolates of fluorescent pseudomonads were spread plated on to King's B agar supplemented with 4.4 g l^{-1} of glycine. Filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1 l of water) were placed on the lid of each plate. The dishes were sealed with parafilm and incubated for 72 h. Production of HCN was indicated by the change in colour of the filter paper strips from yellow to brown. The intensity of the colour was recorded visually.

Production of siderophores

Two isolates of fluorescent pseudomonads were tested for their iron dependent production of siderophores. 50 mM to 300 mM of $FeCl_3$ was incorporated to King's B broth and inoculated with fluorescent pseudomonads

and incubated for 72 h in an orbital shaking incubator at 150 rpm. Centrifuging at 7000 rpm for 15 min pelleted the cells out. The absorbance of cell-free culture filtrate measured at 366 nm indicated the concentration of siderophores released by the bacterial strains to the culture media (Kloepper *et al*. 1980b).

The iron dependent production of siderophores by fluorescent pseudomonads was studied by growing the bacteria in King's B agar with a range of iron concentrations [regulated by amending EDTA (100–1000 mg ml^{-1}) and $FeCl_3$ (20–80 mM)]. 10 ml of 12 h old bacterial culture was spot inoculated at the centre of the iron regulated King's B plates and incubated for 72h at 28^0C and the colonies were observed under UV light in an Alpha Imager-Multi Image Light Cabinet and compared the fluorescence produced by the bacteria.

Different concentrations of EDTA (100–1000 mg ml^{-1}) or $FeCl_3$ (20–80 mM) were incorporated to carrot agar to get a range of iron concentrations in the medium. Nine mm discs of *P. capsici* taken from the growing margins of a 48 h old culture was placed at the centre of the plates. The plates were incubated for 72 h and the diameter of the colony was measured. A graph was plotted with diameter of growth of *P. capsici* against the concentration of iron.

Endophytic nature of fluorescent pseudomonads

Fluorescent pseudomonad isolates, IISR-34 (Rif $^{100+}$) and IISR-51 (Kan $^{50+}$, Rif $^{100+}$, Nal $^{40+}$) were mass multiplied separately in nutrient broth and incubated for 24 h at 150 rpm. The cells were pelleted at 7000 rpm and re-suspended in 10 mM $MgSO_4$. Roots of black pepper (cv. Karimunda) were dipped in the bacterial suspension (10^8 cells ml^{-1}) for 30 min and the cuttings were planted in sterile potting mix. After 3 months, the plants were excavated and roots, leaves and stems were collected. The stems were cut to 2 cm pieces and weighed separately. Three leaves from the base of the plant along with the stem pieces and 1 g of root were surface sterilized by immersing the samples in 0.1% $HgCl_2$ for 10 min and then washed thrice in sterile water. The samples were then immersed in 70% ethanol for 10 min and washed five times in sterile water, macerated aseptically using sterile pestle and mortar in 10 ml of 10 mM $MgSO_4$. From this, aliquots were spread, plated on to King's B agar and nutrient agar and incubated at 26 ± 2^0 C for 48 h. Colonies of fluorescent pseudomonad isolates IISR-34 (Rif^{100+}) and IISR-51 (Kan $^{50+}$, Rif $^{100+}$, Nal $^{40+}$) that appeared in the antibiotic amended plates were enumerated. In order to make sure that the surface sterilization was complete, the samples, after surface sterilization were plated as such, without maceration on to nutrient agar.

Results and discussion

In vitro *assay for non-volatile and volatile inhibitory metabolites*

The short-listed isolates of fluorescent pseudomonads were found to suppress *P. capsici* through different modes namely, production of volatile and non-volatile inhibitory metabolites including HCN, and siderophore mediated antagonism. In the *in vitro* assay performed for non-volatile inhibitory metabolites, the isolates showed varying degrees of inhibition of *P. capsici* in dual culture which ranged from 36.3% to 72.0% (Figs. 1 and 2). All the 12 isolates tested for production of volatile metabolites proved positive and the best was isolate IISR-51 (Figs. 1 and 3). Efforts focused worldwide, on the mechanism of action of fluorescent pseudomonads against plant pathogenic fungi, stress on the involvement of production of antifungal metabolites by fluorescent pseudomonads (Loper *et al.* 1994). These anti-fungal metabolites produced by fluorescent pseudomonads may be 2, 4, diacetyl phloroglucinol (Raaijmakers *et al.* 1999; Keel *et al*. 1992; Vincent *et al*. 1991), oomycin A and phenazine (Thomashow & Weller 1995).

In pseudomonad species, HCN is released by the decarboxylation of glycine (Wissing 1974). As described by Kloepper *et al.* (1991), the HCN formed a brownish red compound with sodium picrate and the intensity of the colour

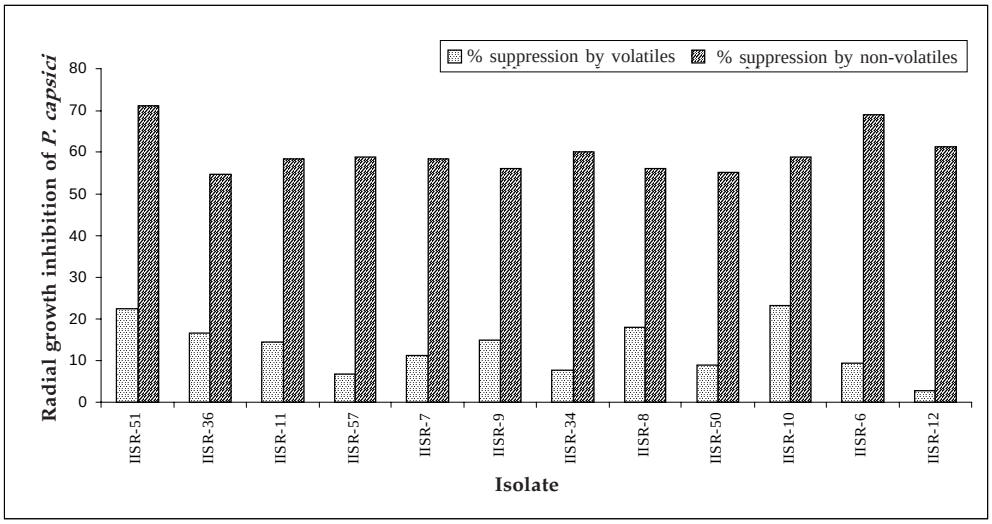
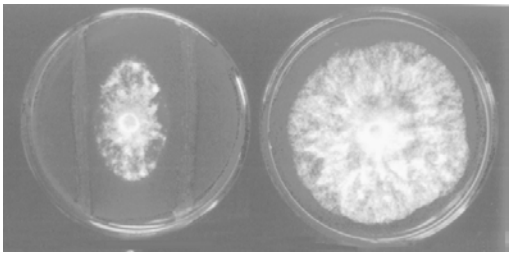


Fig. 1. Suppression of *Phytophthora capsici* by volatile and non-volatile metabolites of fluorescent pseudomonads *in vitro*



a b

Fig. 2. *In vitro* antagonism by fluorescent pseudomonads on *Phytophthora capsici*
(a) *Phytophthora capsici* x fluorescent pseudomonad IISR-6 (b) *Phytophthora capsici* alone

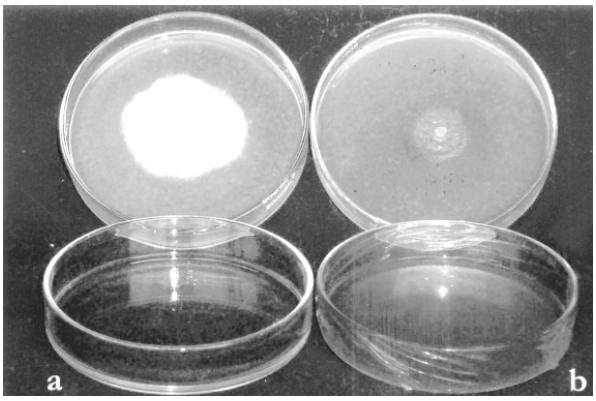


Fig. 3. Reduced growth of *Phytophthora capsici*, exposed to volatiles of fluorescent pseudomonads
(a) Unexposed (b) Exposed

increased with the amount of HCN. In the present study, different isolates produced different intensities of colour indicating different amounts of HCN produced, the highest being produced by the isolate, IISR-51 (Table 1). Among the isolates tested, three isolates (IISR-50, IISR-10 and IISR-12) did not produce HCN. Defago *et al.* (1990) demonstrated by mutational analysis and complementation that production of HCN by *Pseudomonas fluorescens* strain, CHAO, accounted for about 60% of the biocontrol activity. They suggested that since CHAO also colonized the root cortex, the strain may produce a stress effect in the plant leading to cyanide resistant respiration and possible modification of tobacco metabolism resulting in enhanced host resistance mechanisms.

Production of siderophores

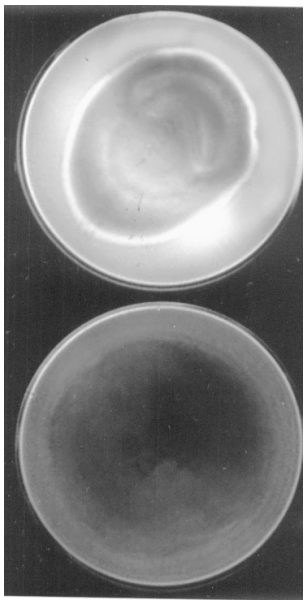
The fluorescent pseudomonads were characterized by their production of yellow-green pigments that fluoresce under UV irradiation and function as siderophores termed pyoverdins and pseudobactins (Meyer & Abdallah 1978). The iron dependent production of siderophores by the fluorescent pseudomonad isolates was found to be in agreement with the findings of Kloepper *et al.* (1980b). As the concentration of iron in-

Table 1. Production of hydrogen cyanide by fluorescent pseudomonads

Isolate	Intensity of colour
IISR-51	+++
IISR-11	++
IISR-8	++
IISR-6	++
IISR-36	+
IISR-34	+
IISR-50	–
IISR-10	–
IISR-12	–

+++=Dark brown; ++=Brown; +=Yellowish brown; – =Yellow

creased, the production of siderophores by the isolates decreased (Fig. 4). A similar trend was also observed in the King's B agar plate wherein iron chelating EDTA (1000 mg ml^{-1}) amended plates of fluorescent pseudomonad showed higher fluorescence under UV light indicating higher level of production of siderophores whereas $FeCl_3$ (80 mM) amended plates showed no fluorescence (Fig. 5).



a

b

Fig. 5. Iron dependant production of siderophores by fluorescent pseudomonad IISR-57
(a) King's B Plate amended with EDTA (1000 mg ml^{-1}); (b) King's B Plate amended with $FeCl_3$ (80 mM)

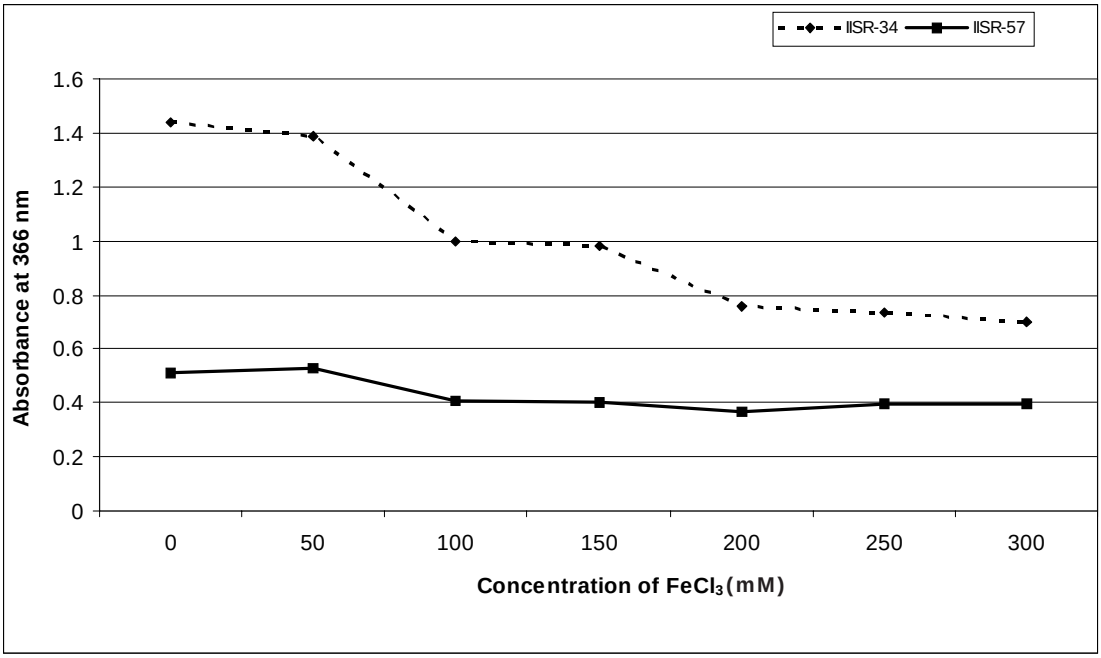


Fig. 4. Iron regulated production of siderophores by two strains of fluorescent pseudomonads
For isolate IISR-57, Y=0.509-0.00046 X, r=-0.788; For isolate IISR-34, Y=1.424-0.0027 X, r=-0.937

Studies on the effect of iron chelation on growth of *P. capsici* revealed that at 1000 mg ml^{-1} of EDTA, the growth was completely inhibited (Figs. 6 and 7) due to deficiency of available iron. The increased production of siderophores by fluorescent pseudomonads as well as the limited growth of *P. capsici* at lower concentrations of iron in the medium indicated the involvement of siderophores in fluorescent pseudomonad-*P. capsici* antagonistic system. Weller (1988) suggested that siderophore, produced by the fluorescent pseudomonad strain 2-79 supplies essential iron needed for the production of phenazine, an antifungal metabolite, indicating the iron regulated production of antibiotics by the fluorescent pseudomonads.

Endophytic nature of fluorescent pseudomonads

Experiments on the endophytic nature of fluorescent pseudomonads showed that it could reside within the internal tissues of the plant. The population of strains IISR-51and IISR-34 in the root ranged from 10^3 to 10^4 cells g^{-1} of the root tissue. The shoot tissue contained up to 10^3 cells g^{-1} of the introduced fluorescent pseudomonad cells. There were only 10^1 to 10^2 cells g^{-1} of the isolate IISR-51 of the leaf tissue as endophytes, whereas in the case of isolate IISR-34, no cells were detected in the leaf tissue (Table 2). The population of the bacteria inside the stem decreased with height of the plant and the region close to the collar sustained a population of 10^4 cells g^{-1} of shoot tissue. According to McInRoy & Kloepper (1995), the population density of bacterial endophytes is highest in the root and lower in the stem and decreases acropetally and the common populations vary from 10^3 to 10^6 cells. Systemic spread of the endophytic bacteria has been demonstrated for *Erwinia* spp., which was recovered from cotton roots, stems and unopened flowers (Misaghi & Donndelinger 1990). Mahaffee & Kloepper (1997) described that although both bacterial endophytes, *Pseudomonas fluorescens* and *Endobacter asburiae* colonized the cortical and vascular root tissues of common bean, only *E. asburiae* colonized the stem tissue following seed application. The main entry for endophytic bacteria may be through wounds that naturally occur

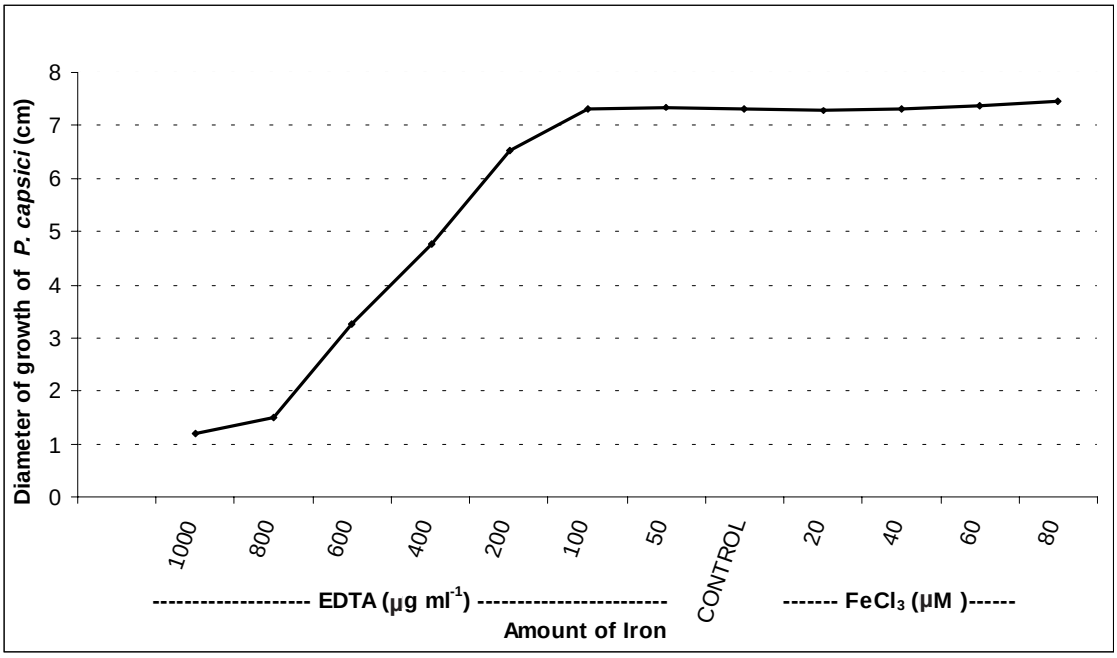
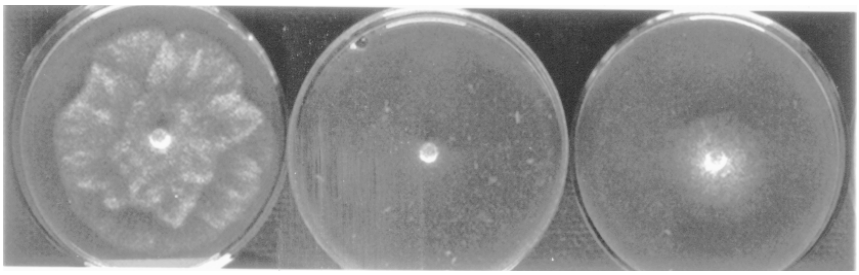


Fig. 6. Role of iron on the growth of *Phytophthora capsici in vitro*



a b c

Fig. 7. Reduced growth of *Phytophthora capsici* under iron limited conditions (a) Control (b) 800 mg ml^{-1} EDTA (c) 400 mg ml^{-1} EDTA

Table 2. Endophytic nature of beneficial root colonizing fluorescent pseudomonads in black pepper

Strain	Bacterial population (cfu g^{-1})		
	Root	Shoot	Leaf
IISR-34	$26x10^4$	$1.34x10^3$	0
IISR-51	$12x10^3$	$2x10^2$	$9x10^1$

as a result of plant growth or through root hairs and at epidermal conjunctions (Sprent & Faria 1998). Chen *et al*. (1994) suggested that biocontrol effect of endophytic strains within plant tissues mainly result from enhanced host defence rather than from bacterial metabolites.

The multifarious action of antagonism by fluorescent pseudomonads, namely, production of volatile and non-volatile inhibitory compounds, HCN and siderophores and its endophytic nature makes it an efficient biocontrol agent of *P. capsici* of black pepper.

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