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Antagonistic effects of metabolites of *Pseudomonas fluorescens* strains on the different growth phases of *Phytophthora capsici,* foot rot pathogen of black pepper (*Piper nigrum* L.)

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Abstract
Foot rot of black pepper caused by *Phytophthora capsici* causes heavy crop losses. Ecofriendly biological control was given high priority in the Integrated Disease Management programs (IDM). Strains of *P. fluorescens* which were antagonistic to *P. capsici*, the foot rot pathogen in black pepper produced inhibitory metabolites. Up to 72% inhibition of mycelial growth of *P. capsici* was observed in dual culture experiments. The metabolites of *P. fluorescens* strain, IISR-6 completely inhibited the sporangial production the most explosive phase in *P. capsici*. The indirect germination of sporangia (release of zoospores) also was inhibited considerably. The percent inhibition varied from 89–98%. The germination of zoospores of *P. capsici* was negatively influenced by the metabolites of *P. fluorescens*. Up to 90% inhibition of germination was noticed. Partial characterization of the metabolites let to the detection of two antibiotics – Pyoluteorin and pyrrolnitrin. The volatile metabolites of *P. fluorescens* also inhibited the different phases in life cycle of *P. capsici*, particularly mycelial growth and production of sporangia. HCN was detected in the volatile metabolites of the bacterial strains in varying quantities. The results are discussed in relation to the mechanism of disease suppression.

Keywords: *Black pepper,* Phytophthora capsici, P. fluorescens, *antagonism, metabolites*

Introduction

Black pepper (*Piper nigrum* L.) is an important export-oriented spice crop. Foot rot of black pepper caused by *P. capsici* is a very serious disease and causes severe economic loss to farmers (Sarma 2003). Though chemical control measures are effective, considering the cost of chemical pesticides and the environmental hazard involved, biological control of plant diseases is a viable strategy for sustainable disease management. Fluorescent pseudomonads have been widely tested for biocontrol against fungal pathogens because of their rapid growth rate and their ability to colonize rhizosphere to a large extent besides their ability to suppress the soil borne pathogen in multiple modes of action (Fukui et al. 1994). Understanding the

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mechanisms through which the biocontrol of plant diseases occurs is critical to the eventual improvement and wider use of biocontrol methods. The susceptibility of the pathogen to inhibitory metabolites of biocontrol bacteria assumes greater relevance when the fungal pathogen is polyphasic, multicyclic and when all the different phases of life cycle of the pathogen are drastically affected by the inhibitory metabolites of these antagonists. The present study dealt with the antagonistic effect of metabolites produced by the biocontrol – *P. fluorescens* strains – on the different phases in the life cycle of *P. capsici.*

Materials and methods

The microorganisms used

The treatments included five strains of *P. fluorescens* viz. IISR-6, IISR-8, IISR-11, IISR-13 and IISR-51 that had been proved efficient in suppressing the root rot pathogen in black pepper, *P. capsici*. The strains were obtained from the repository of Plant Growth promoting Rhizobacteria (PGPR) maintained at the Indian Institute of Spices Research (IISR), Calicut, Kerala, India. Culture of the root rot pathogen *P. capsici* (99 – 101) was obtained from the National Repository of *Phytophthora* (NaRPh) at IISR, Calicut.

Effect of metabolites of P. fluorescens *on mycelial growth of* P. capsici

Dual culture technique (Dennis & Webster 1971) was adopted for testing the strains for their efficacy in inhibiting the mycelial phase of growth of the fungal pathogen based on the percent inhibition of radial growth of *P. capsici.*

Effect on sporangia production

Ten disks (3 mm) of *P. capsici* cut from the growing edge of a CA plate were placed in sterile micro petriplate (5 cm). The concentrated cell free culture filtrate (CFCF) (1 ml) was added to the plate and sealed with cling film. A plate with sterile water in it instead of CFCF served as the negative control. The plates were placed in fluorescent light for 48 h to induce sporulation (Ribeiro 1978). The plates were observed under an inverted microscope and the number of sporangia developed per disc of *P. capsici* were counted and compared with the negative and positive control.

Effect on release of zoospores

Disks of *P. capsici* were placed in sterile petriplate and 1 ml of sterile water was added and sealed with cling film. It was kept in fluorescent light for 48 h for sporulation. These sporulated discs were placed in micropetriplate with 1 ml of the concentrated CFCF of the bacterial strains and induced for release of the zoospores by giving cold shock for 15 min at 4°C. The opened and un-opened sporangia were counted separately per disc under an inverted microscope and compared with the negative and positive controls.

Effect on germination of zoospores

Zoospore suspension of *P. capsici* was prepared and centrifuged at 500 rpm for 5 min in a cooling centrifuge (10°C) to get the zoospores concentrated in 1 ml of sterile water. 50 μl

of the CFCF was taken in a sterile cavity slide and 10 μl of the zoospore suspension was incorporated to it. The cavity slides were kept in a moisture chamber prepared using a petriplate. The slides were observed after 24 h under microscope for germination of the zoospores. The CFCF-slides were compared with the negative and positive controls.

Detection of the antibiotics by Thin Layer Chromatography (TLC)

Pyoluteorin (Plt) and Pyrrolnitrin (Prn). The selected five strains of *Pseudomonas* spp. were tested for the production of antibiotics with Pyoluteorin (Plt) and Pyrrolnitrin (Prn) (Howel & Stipanovic 1980) by thin layer chromatography (Kraus & Loper 1992).

Bioassay carried out with other CFCF fractions. The other fluorescing bands in the TLC plates were eluted with acetone. Also the plates were kept in iodine vapour chamber for 60 min for development, the bands were marked, eluted out, dissolved in acetone and concentrated to 100 μl at room temperature (RT). The silica was removed from the suspension by centrifugation at 1000 rpm for 5 min in a cooling centrifuge at 10°C. The metabolite fraction (50 μl) was spotted on to sterile filter paper discs. Bioassay was carried out against *P. capsici* as given below.

Nine millimetre discs of *P. capsici* cut from the growing edges of a 48-h-old culture was placed in the centre of a sterile PDA plate. The filter paper disks containing the different fractions of CFCF of *Pseudomonas* were placed aseptically on the PDA plate, 2 cm away from the *P. capsici* disk. The plates were incubated for 48 h and the inhibition of the *P. capsici* mycelium was recorded.

Action of volatiles of Pseudomonas *spp. on* P. capsici

The effect of volatiles produced by the selected five strains of bacteria against growth of *P. capsici* was studied using a sandwich plate assembly as mentioned below:

Effect on the mycelial phase of P. capsici

The centre of a carrot agar plate was inoculated with a 9 mm disk of *P. capsici*, 99 – 101. The plate with *P. capsici* was placed upside down over another plate where the *Pseudomonas* strain was inoculated in sterile King's B broth (25 ml). This assembly was sealed airtight with cling film so that the volatiles produced by the bacteria did not escape. This plate assembly was incubated for 72 h in a shaking platform (GENEI-Rocker-100). The radial growth of *P. capsici* was measured and compared with that of the control.

Effect on the sporangial production phase of P. capsici

P. capsici that has been exposed to the volatiles produced by the *Pseudomonas* strains were cut using a 4 mm cork borer and induced for sporulation. The number of sporangia produced were counted in an inverted microscope and compared with that of the control.

Production of HCN

Production of hydrogen cyanide by the selected strains *Pseudomonas* spp. was determined using the method reported by Kloepper et al. (1991).

Results and discussion

Efforts worldwide, which are focused on the mechanism of action of fluorescent pseudomonads against plant pathogenic fungi, stress the involvement of the production of antifungal metabolites (Loper et al. 1994). Based on the reduction in the colony diameter of *P. capsici*, the metabolites of all the strains inhibited the mycelial phase of *P. capsici* to varying degrees (60.93 – 71.87%). IISR-51 inhibited *P. capsici* to the highest percentage followed by IISR-6 and IISR-13 (Table I). The CFCF of IISR-6 completely inhibited (100%) the sporangia production in *P. capsici*. The sterile water control and the positive control plates had a mean value of 209 and 200 sporangia produced respectively per disc. The sporangia production was inhibited by the CFCF in varying degrees (92.3 – 99.5%) (Table I). The CFCF of all the *Pseudomonas* spp. strains studied, inhibited the indirect germination of sporangia considerably (Table I). When 102 and 93 numbers of sporangia per disk liberated zoospores in the sterile water control and in the positive control plates respectively, only 2 – 16 sporangia released their zoospores in the CFCF-treated plates. The percent inhibition of release of zoospores varied from 89.22 – 97.70%. Only a few zoospores germinated due to the inhibitory action of metabolites from the bacteria in the treated sets (2 – 8 numbers in a microscopic field) while there were 33 and 27 zoospores germinated in the sterile water control and positive control respectively. The percent inhibition of germination of zoospores noticed in the CFCF-treated zoospore suspension was 75 – 90%, the highest inhibition being with the CFCF of IISR-6 (Table I).

All the strains of *Pseudomonas* spp. viz. IISR-6, IISR-8, IISR-11, IISR-13 and IISR-51 produced pyrrolnitrin (Prn) (Rf: 0.86). Even though pyrrolnitrin (Plt) was not detected in the CFCF of IISR-8, it was observed in the cell extract of the same. The fluorescent band for pyrrolnitrin appeared in the cell extraction of IISR-6 and IISR-8. The band at Rf: 0.36 (Pyoluteorin) appeared in the CFCF of only IISR-11 and IISR-51. Pyoluteorin was detected in the cell extractions of all the strains except IISR-13. So it was found that all the strains produced plt while IISR-13 produced only prn. Varying degrees of inhibition of radial growth of *P. capsici* was observed with the metabolite fractions of different Rf values. The fraction with Rf value 0.22 of the strain, IISR-6 inhibited the mycelial growth of *P. capsici* considerably. Pyrrolnitrin and pyoluteorin are broad-spectrum antibiotics produced by several strains of *Pseudomonas* and *Burkholderia* species. Both antibiotics play important roles

Table I. Inhibition of different phases in the life cycle of *P. capsici* by metabolites produced by *P. fluorescens*.

Strains	1	2	3	4	5	6	7
IISR-6	64.06	100	92.15	90.90	40.32	100	+++
IISR-8	60.93	97.6	90.2	84.84	24.19	100	+++
IISR-11	62.50	97.6	89.22	84.84	24.19	100	++
IISR-13	64.06	99.5	97.70	93.03	30.65	100	+
IISR-51	71.87	92.3	89.22	75.75	24.19	100	+
LSD	0.129	1.895	8.549	3.204	1.832		

1. % inhibition of mycelial growth of *P. capsici*.
2. % inhibition of sporangia production by CFCF.
3. % inhibition of zoospore-release by the CFCF.
4. % inhibition of germination of zoospores with CFCF of *Pseudomonas* spp.
5. % inhibition of mycelial growth of *P. capsici* with volatiles of *Pseudomonas* spp.
6. % inhibition of sporangia production in mycelium of *P. capsici*, exposed to the volatiles of *Pseudomonas* spp.
7. Relative HCN Production (+, low; ++, medium; +++, high).

in the suppression of multiple plant pathogenic fungi. (Jorge et al. 2003). All the strains under the present study were found producing Prn. IISR-6 and IISR-8 produced Plt. The antifungal activity of Plt and Prn has been proved in many cropping systems in the field wherein the negative mutants failed to protect the crop from the soil borne fungal pathogen. Genetic and molecular analysis has demonstrated that production of various antifungal compounds is a primary mechanism of biocontrol for many strains, accounting for as much as 90% of the disease-suppressing activity (Thomashow & Weller 1995).

The volatile metabolites of the antagonistic bacteria inhibited the mycelial growth of *P. capsici* in the range of 24 – 40 %. IISR-6 and IISR-13 inhibited the fungi the highest percentage (Table I). When the *P. capsici* in the control plates produced up to 200 sporangia, the *P. capsici* mycelium exposed to the volatiles of *Pseudomonas* spp. did not produce any sporangia (Table I). Studies are scanty on the volatile metabolites produced by beneficial rhizobacteria. Many researchers suggested that cyanogenic antagonists could be potential biocontrol agents (Laville et al. 1998). All the five strains under study were found to produce HCN. Among the strains, IISR-6 and IISR-8 were found to be the highest producers of HCN (Table I). Defago et al. (1990) demonstrated by mutational analysis and complementation that production of HCN by *Pseudomonas fluorescens* strain, CHAO accounted about 60% of the biocontrol activity. They suggested that, since CHAO also was found to colonize 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. With regard to bacterial determinants that trigger growth promotion and ISR in plants, the role of volatile emissions from bacteria needs further in-depth investigation.

The inhibitory volatile and non-volatile metabolites produced by the *Pseudomonas* spp. strains inhibited the different stages in the lifecycle of *P. capsici* in terms of mycelial growth, sporangial formation, release of zoospores, and germination of zoospores. This clearly shows the potency of the strains to antagonize all the reproductive phases of the pathogen thus effectively preventing its growth and proliferation. This attribute of the *Pseudomonas* strains makes them effective candidates in suppressing *P. capsici* in all seasons of plant growth and especially in the rainy season to take care of the zoospore-mediated spread of the pathogen to the adjacent vines. However, the population of *Pseudomonas* in the rainy season and the level of inhibitory mechanisms in the soil need in-depth study. Similar observations have been reported by Parke et al. (1991) wherein *Burkholderia cepacia* AMMD and the rif-resistant derivative strain AMMDR1 effectively controlled *Pythium* damping-off and *Aphanomyces* root rot of peas grown in fields and the antibiotics produced displayed biocontrol effects during at least two separate stages of the pathogens' life cycle, i.e., on zoospore lysing and inhibited cyst germination up to 100%.

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