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Pseudomonas fluorescens mediated systemic resistance in black pepper (*Piper nigrum* L.) is driven through an elevated synthesis of defence enzymes

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Abstract
Efficient strains of *Pseudomonas fluorescens* identified were evaluated for their efficacy in inducing defence enzymes in black pepper. Increased levels of Peroxidase (PO), Catalase, Phenylalanine Ammonia Lyase (PAL) and Poly Phenol Oxidase (PPO) were induced in leaves apart from the roots of treated plants indicating the systemic protection offered to black pepper by the strains exploring the prevention of even foliar infection by the pathogen, *Phytophthora capsici*. The increase in production of defence enzymes upon challenge were higher in the non-bacterized plants compared to the bacterized plants, indicating the lesser requirement of defence enzymes in the bacterized plants upon encounter with the pathogen. Also found was a relatively higher quantity of lignification (30 – 100% over control) in the bacterized roots compared to the plants untreated which resulted in significant root rot suppression.

Keywords: *Pseudomonas fluorescens, Phytophthora capsici, PAL, PO, PPO, catalase*

Introduction

Pseudomonas spp. can induce systemic biochemical and ultra structural changes in the roots that lead to a greater ability of the host plant to defend itself against root-infecting pathogens. The use of Fluorescent pseudomonads for controlling soil-borne plant diseases has been well documented (Paulitz & Loper 1991, Sarma et al. 2000, Weller & Cook 1986).

Plants, even those possessing active defence mechanisms, become infected by a virulent pathogen because the pathogen avoids triggering or suppressing resistance reaction or evades the effect of activated defences. If defence mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease can be reduced (van Loon et al. 1998). Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens or by virulent pathogens under circumstance where infection is stalled owing to environmental conditions (Gorlach et al. 1996, Sticher et al.1997, Wei et al. 1991).

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Systemic resistance triggered in the plant by rhizobacteria is referred to as rhizobacterial mediated induced systemic resistance (ISR) (reviewed by van Loon et al. 1998). *Pseudomonas fluorescens* could act as strong elicitors of plant defence reaction (M'Piga et al. 1997). ISR is brought about by PGPRs (Plant Growth Promoting Rhizobacteria) through fortification of the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reactions of the host leading to the synthesis of defence chemicals against the challenge pathogen. It is well known that PGPRs induce cell wall structural modification in response to pathogenic attack. (Benhamou et al. 1996, 1998, M'Piga et al. 1997) Activation of defence gene production by prior application with PGPR against a challenging pathogen is a novel strategy in plant protection. The increased activity of the above substances in the PGPR-treated plants may play either a direct or an indirect role in the suppression of pathogen development in the host ultimately protecting the plants from pathogenic micro-organism. PGPR-mediated stimulation of defence-related biochemical compounds have been reported earlier (Albert & Anderson 1987, Chen et al. 1998, 2000, Maurhofer et al. 1994, M'Piga et al. 1997, Zdor and Anderson 1992).

A massive accumulation of phytoalexin (van Peer et al. 1991), phenolic compounds (M'Piga et al. 1997) increase in the activities of PR proteins (Maurhofer et al. 1994), peroxidase (Albert & Anderson 1987, Zdor & Anderson 1992) increase in the levels of mRNAs encoding phenyl alanine ammonia lyase (PAL) and enhanced lignification (Anderson & Guerra 1985) have been reported in plants following treatments with PGPR strains. However, there is not much study about the induction of various defence enzymes involved in the phenyl propanoid pathway due to *Pseudomonas fluorescens* treatment. The present study dealt with the induction of PGPR-mediated defence enzymes in black pepper viz., Phenyl alanine Ammonia Lyase, Peroxidases, Poly phenol oxidases and catalases. The rhizobacterial-induced addition of deposits of lignin was also studied.

Materials and methods

The strains 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 collected 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.

Mode of treatment and sampling

Uniform cuttings of black pepper (*cv. Karimunda*) obtained from the nursery were planted in sterile coconut coir husk for rooting. Upon rooting and when the plants developed 3 – 4 leaves, the plants were bacterized with the five strains of *P. fluorescens* as separate treatments. Untreated control plants also were maintained. Bacterial cells were raised in nutrient broth by incubating for 48 h at 28 °C and the cells pelleted by centrifuging at 7000 rpm. The cells were re-suspended in 10mM MgSO4 and diluted to get 10^{10} cells/ml of the suspension. Bacterization was performed by drenching the bags with 25 ml of the cell suspension.

The study was conducted with two sets of all the treatments among which one set of plants were only bacterized and the other set was challenge-inoculated with *P. capsici* after seven days

of bacterization. Each set had six treatments including the un-treated control. The plants were irrigated regularly.

Destructive sampling was done on every second day of bacterization till the 14th day for analysis of the defence enzymes. A replicate of three plants were uprooted each time, the roots and leaves were severed and stored at − 80°C until processing.

Roots/leaves were washed in running tap water, homogenized in liquid nitrogen using pestle and mortar. It was extracted in 10 mM sodium phosphate buffer (pH 6.0), filtered through a 0.2 mm nylon filter and centrifuged at 12000 g for 20 min at 4°C. Supernatant was taken in 2 ml vials and stored at − 80°C. The concentration of protein in the samples was assayed using the procedure of Lowry et al. (1951) before estimation of defence enzymes.

Estimation of Phenyl alanine Ammonia Lyase (PAL)

The assay was carried out spectrophotometrically by the formation of trans-cinnamic acid. To 500 μl of the enzyme extract was added 500 μl of 0.5 M Tris HCl buffer. To this was added 500 μl of 0.15M L-phenyl alanine, and incubated at 37°C for 60 min. The reaction was stopped by adding 500 μl of 1M Trichloro acetic acid and incubated at 40°C for 5 min. It was centrifuged to remove any particles and the absorbance was read in the spectrophotometer at 270 nm. Blank was buffer and the control tube contained L-phenyl alanine added after TCA. The rate of the reaction was expressed as μg of trans cinnamic acid formed /h/g fresh weight.

Estimation of Peroxidase (PO)

50μl of the crude enzyme extract was taken in 1ml of 10mM sodium phosphate buffer and mixed with 1ml of100mM pyrogallol and 1ml of 1% H_2O_2. The initial rate of increase in absorbance was measured over 1 min at 470 nm. PO activity was expressed as units of PO/mg protein.

Estimation of Poly phenol Oxidase (PPO)

To 200 μl of the crude enzyme was added 700 μl of sodium phosphate buffer (pH 6.0). 100 μl of 0.2 M catechol was added and the absorbance was read at 420 nm (rate of increase in absorbance for 1 min) and compared with the standard. The enzyme activity was expressed as μg PPO/mg protein.

Estimation of catalase

50 μl of the enzyme extract was taken in sodium phosphate buffer (pH 7.0) (2 ml) and 500 μl of H_2O_2 was added. To this was added 1 ml of distilled water and the absorbance was read at 230 nm for 90 s with a time interval of 15 s. The lag time was 10 s.

Accumulation of lignin

A set of plants was maintained to study the relative lignification in rhizobacteria-treated black pepper. Black pepper cuttings were raised in sterile coconut coir pith and upon rooting were bacterized and after three months uprooted and the amount of lignin were quantified as per the protocol by Doster et al. (1988). The lignin accumulation in the stem was also observed under microscope after phloroglucinol staining as per the protocol of Vallet et al. (1996).

Root rot suppression

Another set of root-bacterized black pepper cuttings were challenge-inoculated after 15 days of bacterization with 10 ml of zoospore suspension of *P. capsici* (10^4 spores/ml) by drenching the bag. The plants were uprooted after seven days of challenge to observe the root rot index.

Results

PAL

All the bacterial strains induced significant quantities of PAL in the treated black pepper roots over the period of time, wherein the untreated plants did not show any change in the pattern of PAL production (see Figures 1a, 1b, 1c, 1d). IISR-51 induced maximum production of the enzyme in roots on the 4th day and for other strains it was on the 2nd day of bacterization itself. PAL induction was highest in IISR-8 & IISR-6 treated plants.

In the challenge-inoculated set of plants, the enzyme induction was highest on five days after challenge. Non-bacterized plants showed slightly higher activity of PAL upon challenge. The increase was significant compared to IISR-6, IISR-8 and IISR-11 on the 5th day of challenge.

There was a highly significant increase in induction of PAL in the bacterized black pepper leaves compared to the untreated. Even though the level started gradually declining after reaching a peak on the 6th day, there was significantly increased activity of the enzyme by all PGPR strains even on 14th day of bacterization. The non-bacterized plants produced only basal level of PAL. The increase and decline of PAL activity was found to be gradual in leaf unlike in roots where it was sudden. The highest quantity of PAL upon treatment with *P. capsici* was observed in the non- bacterized plants, which was significantly different than the bacterial treated plants. For all treatments, the peak was on the 5th day of challenge and then started diminishing drastically.

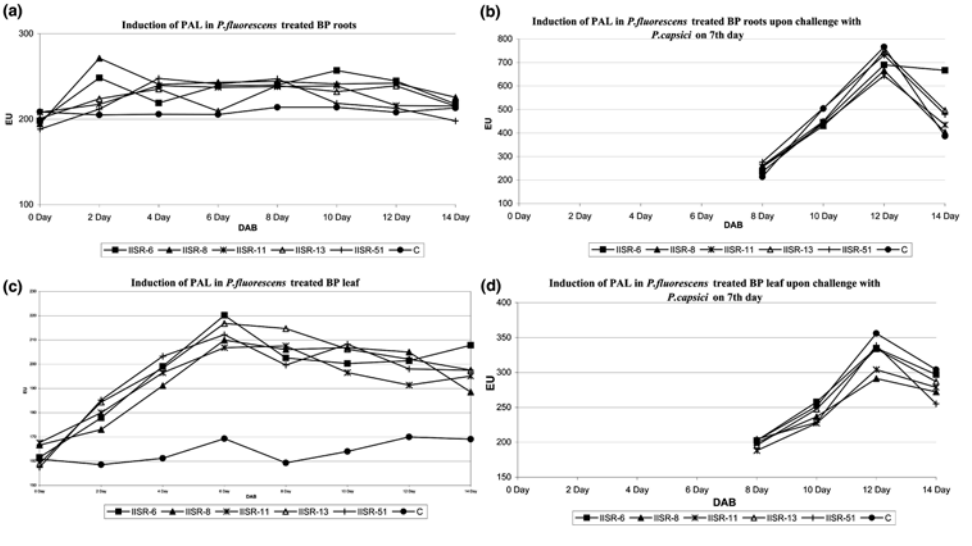

Figure 1

PO

All the strains increased the amount of peroxidase significantly over time upon root bacterization (see Figures 2a, 2b, 2c, 2d). Significantly higher quantities were observed only on the second day, except for IISR-8, for which it was on the 6th day of bacterization. Even though not significantly different after four days, there maintained a higher level of PO in bacterized roots than in the non-bacterized. PO activity remained constant in the untreated plants upon days after bacterization without any significant increase. Among the strains IISR-6 induced highest quantity of peroxidase in root, followed by IISR 13. In the challenge inoculated plants, pathogen elicitors increased the PO level in black pepper roots and a maximum level was reached on the 5th day of challenge. All the treatments showed the highest peak on the 5th day and there were no significant changes in the PO activity of bacterized and non-bacterized plants. The increase in enzyme induction was slightly more in the non-bacterized plants than in the bacterized plants, even though the difference was not significant. The PO level diminished uniformly in all treatments immediately after the 5th day of challenge.

There was a significantly increased synthesis of peroxidase in the leaf of bacterized plants. IISR 6 as was in the case of root which also induced the highest quantity of peroxidase in leaf. The induction was not sudden as for roots, but gradual, reaching a peak at 4–8 days and gradually diminishing or maintaining. All the strains maintained the significantly increased level of PO activity even up to the 8th day compared to the non-bacterized control. PO activity increased in leaf upon challenge in bacterized plants earlier than in the non-bacterized plants. But in the non-bacterized plants PO level rose even higher on the 12th day. There was no significant difference in the PO activity between treatments.

PPO

Unlike the other enzymes studied, the highest peak in PPO level upon bacterization of the black pepper roots occurred only in a later stage, i.e., eight days after bacterization (see

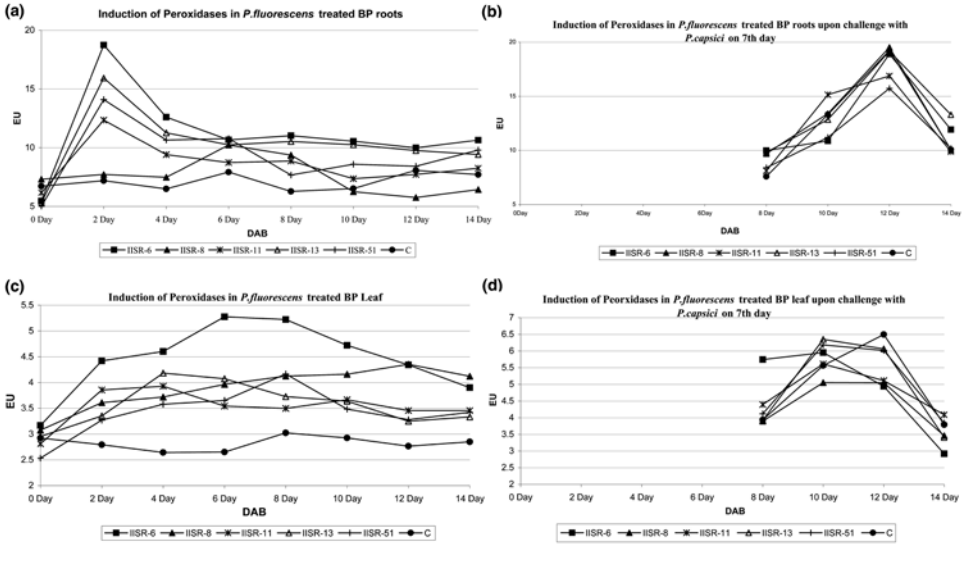

Figure 2

Figures 3a, 3b, 3c, 3d). Untreated plants did not show any significant fluctuations in PPO activity in the study period. IISR-6 and IISR-51 maintained the PPO activity in a significantly higher level throughout. Even in the plants which were challenge-inoculated after bacterization, there was an early hike in the PPO level in roots with all treatments compared to the other defence enzymes studied. For PPO, the peak appeared on the 3rd day of challenge and remained almost static until the 5th day and diminished gradually, but in the control plants the fall in enzyme activity was sudden. The increase in the quantity of enzyme was more in the control plants than in the bacterized plants, but significantly higher levels were seen only to IISR-11 and IISR-51.

All the strains of *P. fluorescens* increased the PPO levels in the leaves of the treated black pepper plants significantly over the period of study. This increase was maintained throughout by the strains. The control plants did not show any increase in the pattern of PPO in leaf. Upon challenge-inoculation with the pathogen, PPO response was highest after five days. Even though the control plants produced more PPO in the leaf than that of the bacterized plants, the decline in the PPO level was faster in the control plants.

Catalase

The catalase level in the roots upon bacterization increased significantly on the 2nd day except for IISR 8 for which the peak appeared on day 6 (see Figures 4a, 4b, 4c, 4d). Even though gradually diminished, the activity still maintained higher throughout with respect to the plants untreated. Upon challenge with *P. capsici*, catalase activity showed a highest peak after three days and started declining. There was no significant difference between the bacterized and non-bacterized plants even though the non-bacterized plants synthesized more catalase than the bacterized plants.

A significant level of catalase was induced in leaf by only IISR-6 and IISR-11 over time even though all the strains induced a higher quantity of catalase in leaf compared to the untreated. There was a slight increase in the level of catalase induction upon challenge in the

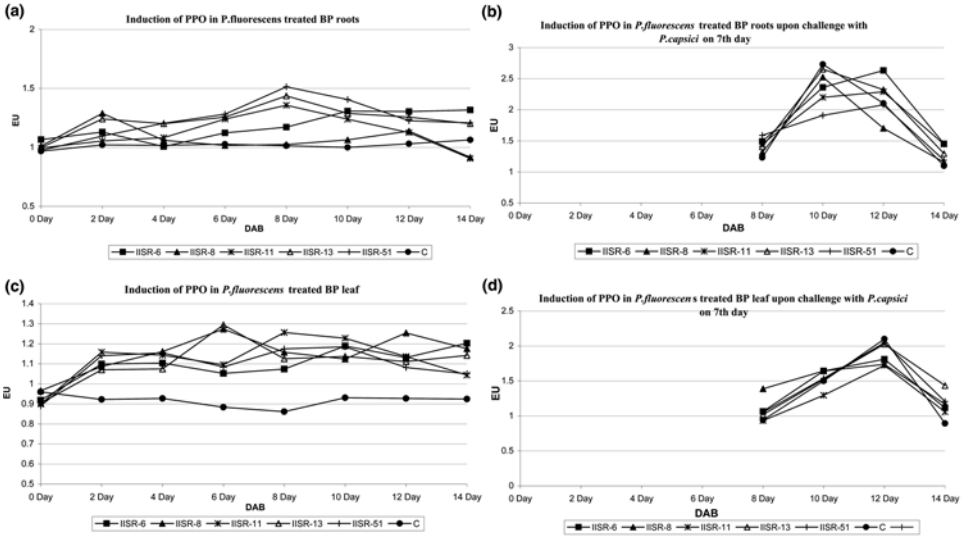

Figure 3

leaf of the control plants compared to the bacterized plants even though there was no significant difference. The peak appeared three days after challenge and the level gradually diminished.

Accumulation of lignin

There was a 30 – 100% increase in the accumulation of lignin in the vascular region of the bacterized plants. (see Figure 5). The highest accumulation was with the strain, IISR-6. The sections of stem of the root-bacterized black pepper cuttings had higher cell wall thickening due to accumulation of lignin as evidenced by a strong red staining reaction in the vascular region. Non-induced control plants had only slight lignifications.

Discussion

Systemic acquired resistance (SAR) is induced upon contact by a pathogen to distant non-infected tissue in a plant system conferring an elevated level of protection (reviewed by Ryals

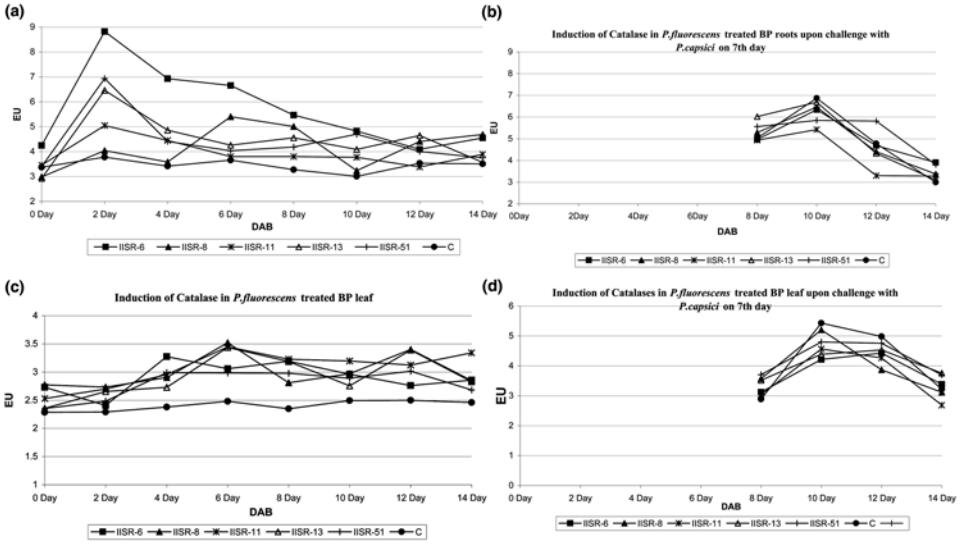

Figure 4

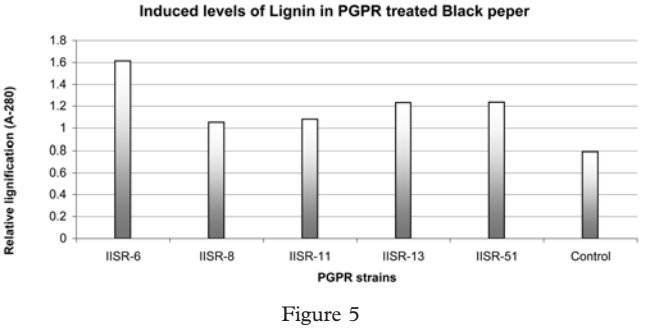

Figure 5

et al. 1996). Such a rapid defence reaction at sites of fungal entry delays the infection process and allows sufficient time for the host to build up other defence reaction to restrict pathogen growth to the outermost layers of root tissue.

In the current study, all the five strains of *P. fluorescens* induced defence enzymes in roots and leaves of black pepper when the plants were root-bacterized, which in turn reflected in the reduced root rot when the plants were challenge-inoculated with the root rot pathogen. The synthesis of the defence enzymes even in the distant end of the plant upon root treatment shows the systemic nature of protection the *P. fluorescens* strains offered. To our knowledge, this is the first report of the rhizobacteria-mediated induction of defence enzymes in black pepper – *P. capsici* pathosystem.

P. fluorescens has been demonstrated to induce systemic resistance to a variety of diseases including wilt diseases, anthracnose, bacterial and viral diseases (Wei et al. 1991, Liu et al. 1995, Maurhofer et al. 1994). A number of metabolites of bacteria are under consideration as triggers of ISR; among others these include liposacharides (Newman et al. 1995) enzyme (Palva et al. 1993) and siderophores (Leeman et al. 1996), and also salicylic acid (Meyer & Höfte 1997). Black pepper roots and leaves showed increased synthesis of PAL, PO, PPO and catalase. Similar results of elevated levels of PAL, PO and PAL have been reported in cucumber plants treated with PGPR strains, which peaked 2–4 days after root treatment (Chen et al. 2000).

Increase in the PAL activity was observed in the roots of plants treated with strains of *P. fluorescens* within two days of treatment. An early induction of PAL is very important as the biosynthesis of lignin originate from L-phenyl alanine. General phenyl propanoid metabolism is defined as the sequence of reactions involved in the conversion of L-phenyl alanine to activated cinnamic acids (Hahlbrock & Grisebach 1975). The first enzyme of this pathway is PAL, that catalyzes the trans-elimination of ammonia from L-phenyl alanine to form trans cinnamic acid which in turn enter different biosynthetic pathways leading to lignin. Studies in cucumber revealed that PAL is a key enzyme in the production of Phenolic and phytoalexin (Daayf et al. 1997). Systemic increase in the PAL activity upon treatment with PGPRs has been reported in several crops including pigeon pea and rice. (Podile & Lami 1998, Meena et al. 1999).

Peroxidase is involved in lignification leading to disease resistance (Lagrimini et al. 1987). Polymerization of cinnamyl alcohols to lignin is catabolized by PO (Harkin & Obst 1973). For lignification, specific cell wall peroxidases are thought to be required to generate H_2O_2 and monolignol radicals (van Huystee 1987). The present study revealed over threefold increase in the peroxidase level within two days of bacterial treatment and continues to be synthesized throughout the study period. The increased peroxidase activity in the present study was correlated with increased lignification in the treated plants. ISR in rice has been correlated with a twofold increase in activity of pathogenesis related peroxidase and chitinase proteins in PGPR treated plants inoculated with the rice sheath pathogens, *Rhizoctonia solani* (Nandakumar et al. 2001).

The induction of poly phenol oxidases was found to be gradual in the bacterized plants unlike the other defence enzymes studied. The accumulation of PPO occurs usually upon wounding in plants. Constabel and Ryan (1998) proved that PO could be induced upon induction by jasmonic acid. A one to twofold increase in the catalase activity was observed in the roots of the bacterized plants.

PO and PAL showed a peak of maximum enzyme production on the 3rd day and for PPO and Catalase the peak appeared only on the 5th day of challenge-inoculation. The increase in production of defence enzymes upon challenge with the pathogen were higher in the non-bacterized plants compared to the bacterized plants, indicating the lesser requirement of

defence enzymes in the bacterized plants upon encounter with the pathogen. The level of induced resistance can be enhanced further when ISR and SAR are activated simultaneously, indicating that ISR and SAR are additive. The defence proteins induced while treatment with PGPR reinforces the cell wall structure by lignification and accumulates the phenolic compounds including phytoalexin in the phenyl propanoid pathway. The study found a relatively higher quantity of lignification (30 – 100% over control) in the bacterized roots compared to the untreated plants. Anderson and Guerra (1985) observed lignification of cell walls in beans upon seed treatment with PGPRs. *Pseudomonas fluorescens*, WCS 417r-mediated ISR, in carnation was reported to be associated with Potentiation of phytoalexin accumulation resulting in higher phytoalexin levels after infection by the fungal pathogen *Fusarium oxysporum* f. sp. dianthi. (van Peer et al. 1991). The defence enzymes induced in plants were reported to be of different pathways. The bacteria and the pathogens induced one acidic peroxidase Isozyme in the roots. Two other isozymes induced by the pathogen were not found to be induced by PGPRs (Chen et al. 2000). The quantity of the defence enzymes produced was higher in the bacterized plants compared to the non-bacterized throughout the study period even though the increase was not significant. Studies by van Wees et al. (1997) on defence-related gene expression showed that ISR was triggered within the first seven days after bacterization of the roots with WCS 417r and lasted until at least 21 days after bacterization. This needs in-depth study in order to find out the exact duration of persistence of ISR in black pepper.

The induction of defence-related enzymes by PGPR treatment was in turn correlated with the percentage root rot suppression in the bacterized plants upon challenge inoculation with the fungal pathogen, *P. capsici*. The bacterized plants showed up to 85% protection on the roots from root rot compared to the non-bacterized plants. The bacterial determinants involved in the induction of systemic resistance in the current study can be lipopolysaccharides present in the outer membrane of bacterial cells or siderophore (van Loon et al. 1998) as the strains were siderophores positive (Diby Paul et al. 2001). In conclusion *Pseudomonas fluorescens* strains are capable of inducing higher levels of four defence enzymes viz., PAL, PO, PPO and Catalase in black pepper and it could be speculated that induced enzyme activities by *Pseudomonas fluorescens* may be associated with the bio-synthesis of phenolic compounds and lignin that have been considered as major determinants in inducing systemic resistance against the root rot disease. Further studies have to be performed on the duration of ISR by PGPRs and to find out the exact determinants or signaling molecules involved in rhizobacteria mediated ISR in black pepper.

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