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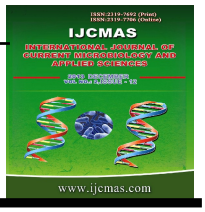


Original Research Article

Induction of defense-related enzymes in anthurium by application of fungal and bacterial biocontrol agents against *Colletotrichum gloeosporioides*

Thangeswari Selvaraj[1*] and Sankaralingam Ambalavanan[2]

[1]Department of Crop Protection, Vanavarayar Institute of Agriculture, Pollachi – 642 103, Tamilnadu, India
[2]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore – 641 003, Tamilnadu, India
Corresponding author

A B S T R A C T

Experiments were conducted in anthurium plants to study the induction of various defense enzymes and accumulation of phenol by the two isolates of three biocontrol agents *viz., Pseudomonas fluorecens, Bacillus subtilis* and *Trichoderma viride* in pot culture. The application of biocontrol agents triggered the activity of three defense related enzymes *viz.*, peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were induced and the accumulation of phenol was also noticed in anthurium upon challenge inoculation with *C. gloeosporioides* the causal agent for leaf anthracnose or spadix rot in anthurium. The activities of defense enzymes reached a peak at six days after inoculation (DAI) with the pathogen. Native PAGE analysis revealed the expression of an additional isoforms of PO and PPO were observed in biocontrol agents treated seedlings due to induced systemic resistance (ISR) induction.

Keywords

Bioagents; ISR; defense related enzymes; phenol accumulation.

Introduction

Anthurium is one of the top ten cutflower of the world. It is a genus of ornamental plant from the *Araceae* family. Diseases appear to be the major constraint to the production of anthurium. Among the diseases, anthracnose or spadix rot caused by *C. gloeosporioides* leads to massive losses in terms of quality and quantity. Anthracnose caused severe rotting incidence of anthurium resulting in 100 per cent death of plants in Alleppy district of Kerala (Santhakumari *et al.*, 2001). Severity of anthracnose in anthurium ranged from 21.67 to 54.89 per cent in Tamil Nadu (Nandinidevi, 2008). Induced resistance may provide an alternative approach to plant protection especially for problems not satisfactory controlled by various fungicides (Schoenbeck, 1996). Induced resistance is defined as an enhancement of the plant defensive capacity against a broad spectrum of

pathogens and pests that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by a pathogen is called ISR or SAR (Hammerschmidt and Kuc, 1995). Plant has endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al.,* 2001). Defense reaction occurs due to the accumulation of PR-proteins, phytoalexins, chalcone synthase, PAL, PO, PPO and phenolics. The objective of the present study is to unravel the induction of various defense related genes encoding proteins implicated in strengthening of plant cell walls by biocontrol agents treatments in response to infection by *C. gloeosporioides.*

Materials and Methods

Induction of systemic resistance in anthurium by biocontrol agents

The effective biocontrol agents viz., isolates of *Pseudomonas fluorescens* (Pf1 and CFP1), *Bacillus subtilis* (BsW2 and BsM3), *Trichoderma viride* (Tv1 and Tv2) selected based on *in vitro* and pot culture studies were formulated using talc as a carrier. Anthurium seedlings of variety Temptation were treated separately with the six formulated effective biocontrol agents and plant in pots containing rooting medium. Instead of soil, rooting medium was used for raising anthurium and the treatments were applied to the medium (coirpith: vermicompost @ of 5:3 v/v). Experiments were conducted in completely randomized design with three replications in each treatment. The biocontrol agents were sprayed in 30 day old plants and challenge inoculated with pathogen after two days. The treatments also included seedling treatment followed by foliar spray of biocontrol agents at 30 DAS without challenge inoculation. Leaf samples were collected at 0, 3, 6, 9 and 12 days after challenge inoculation with pathogen to assay the changes in activities of defense related enzymes viz., phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), b-1-3-glucanase and phenol. The plants inoculated only with pathogen and also healthy plants were maintained for comparison.

Phenylalanine ammonia lyase (PAL)

One g of anthurium leaf was homogenized in 2 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm (Dickerson *et al*., 1984). Sample extract of 0.4 ml was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 1 ml of 12 mM L-phenylalanine and incubated for 1 h at 30^{o}C. The reaction initiated by L-phenylalanine was stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine after the addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmol transcinnamic acid/min/g of fresh tissue.

Peroxidase (PO)

Activity of PO was determined as detailed by Hammerschmidt *et al*. (1982). One g of leaf sample was homogenized in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was used to assay activities of PO and PPO. 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract were taken and added to cuvette. To

initiate the reaction 0.5 ml of 1% H_2O_2 was added. The change in absorbance was recorded at 420 nm at 30 sec interval for three min from zero second of incubation at room temperature. The results were expressed as change in absorbance/min/g of fresh tissue.

Polyphenol oxidase (PPO)

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 with 0.1 ml of enzyme extracts. To this 0.2 ml of 0.01 M catechol was added to initiate the reaction. The change in absorbance was recorded at 495 nm and the results were expressed as change in absorbance/min/g of fresh tissue (Mayer *et al*. 1965).

Phenol

Leaf samples were homogenized in 10 ml of 80% methanol and agitated for 15 min at 708C. To 1 ml of the extract 5 ml of distilled water and 250 ml of Folin-ciocalteau reagent (1 N) were added and incubated at 258C for 3 min. After that 1 ml of 20% sodium carbonate was added and mixed well. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was read at 750 nm and catechol was used as the standard. The total phenol content was expressed in mg of catechol/g of fresh tissue (Zieslin and Ben Zaken 1993).

Native-polyacralamide gel electrophoresis (PAGE) analysis

In spectrophotometric assay the maximum PO and PPO activities were noticed on 6th day after challenge inoculation of the pathogen in anthurium treated with biocontrol agents. Hence, leaf samples collected on 6th day were subjected to native-PAGE analysis to find out the expression of PO and PPO isoforms. Samples were homogenized with 1 ml of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4^{o}C. The protein content of the sample was determined by Bradford (1976) method. Samples (50 µg protein) were loaded onto 8% polyacrylamide gel. After electrophoresis, the gel was stained in 0.2 M acetate buffer at pH 4.2 containing 0.05% benzidine for 30 min in dark. Then drops of H_2O_2 (0.03%) were added slowly with constant shaking to visualize the PO isoforms. After staining the gel was washed with distilled water (Nadlony and Sequerira, 1980). For PPO, the gel was immersed in P-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer pH 7.0 for 30 min. Later 10 mM catechol was added and kept in a shaker with gentle shaking and observed for dark brown protein bands (Jayaraman *et al*., 1987).

Statistical analysis

All the analyses were repeated once with similar results. The data were statistically analysed by using the IRRISTAT package developed by International Rice Research Institute, Biometrics Unit, Philippines. The treatments means were compared by DMRT.

Results and Discussion

Induction of systemic resistance in anthurium by biocontrol agents

In the present study revealed enhanced activities of defense related enzymes PO, PPO and PAL and accumulation of phenol in anthurium plants treated with biocontrol agents and challenged with *C. gloeosporioides*. In isozyme analysis, additional PO and PPO isoforms with

greater intensity were induced with biocontrol agents that were absent in control. In general elevated levels of PAL, PO and PPO activity and accumulation of phenol were observed in plants treated with biocontrol agents when inoculated with the pathogen. PAL activity reached maximum at six days after inoculation (6 DAI) with the pathogen and declined thereafter in all the treatments. Plants that received only biocontrol agents as treatment also exhibited higher levels of PAL activity when compared with healthy control. However, activity of PAL was at its peak at 6 DAI in CFP1 inoculated with *C. gloeosporioides* (Fig. 1). At 6 DAI, PO activity was maximum in plants pretreated with CPF1 and challenged with the pathogen when compared to other biocontrol agents. Plants treated with biocontrol agents alone also showed enhanced activity as against healthy control (Fig. 2). The PPO activity was maximum at 6 DAI when the plants were pretreated with BsW2 and challenged with *C. gloeosporioides* (Fig. 3). Phenol accumulation increased from third day and attained peak on 6 DAI. Maximum accumulation of phenol was noticed in Pf1 challenged with *C. gloeosporioides* at 6 DAI when compared to plants inoculated with the pathogen alone (Fig. 4).

Native PAGE analysis for Isozyme induction PO and PPO

Native PAGE analysis showed three isoforms (PO1 to PO3) of peroxidase was observed in all treatments except healthy control. However, the intensity of the isoform was more in plants treated with biocontrol agents than those challenged with the pathogen (Plate 1). The treatments challenged with the *C. gloeosporioides* revealed the presence of four isoforms (PPO1 to PPO4) with more pronounced expression which was absent in healthy control (Plate 2).

Induced systemic resistance (ISR)

Induction of systemic resistance by PGPR against various diseases was considered as the most desirable approach in crop protection. There are major differences in ISR when compared to other mechanisms. First, the action of ISR is based on the defense machanim that is activated by inducing agents. Second, ISR expresses multiple potential defense mechanism that include increased activity of chitinase, β-1-3 glucanase and peroxidase (Maurhofer *et al.*, 1994; Xue *et al.*¸1998) and accumulation of antimicrobial low molecular substances-phytoalexins and formation of protective biopolymers *viz.*, lignin, callose and hydroxyproline rich glycoprotein (Hammerschmidt and Kuc, 1982). Third, an important aspect of ISR is the wide spectrum of pathogens that can be controlled by single inducting agent (Hoffland *et al*., 1996; Wei *et al*., 1996). In the present investigation revealed enhanced activities of defense related enzymes PO, PPO and PAL and accumulation of phenol in anthurium plants treated with biocontrol agents and challenged with *C. gloeosporioides.* In isozyme analysis, additional PO and PPO isoforms with greater intensity were induced with biocontrol agents that were absent in control.

Induction of PAL

Phenyl propanoid metabolism starts with the conversion of L-Phenylalanine into transcinnamic acid by PAL and supplies the precursors for flavanoid pigments, lignin and phytoalexins (Massala *et al*., 1980; Hahlbrock and Scheel, 1989). Increase in PAL activity subsequently increases the phenolic contents leading to disease resistance (Klessig and Malamy, 1994).

Figure.1 Induction of phenylalanine ammonia lyase in anthurium treated with biocontrol agents

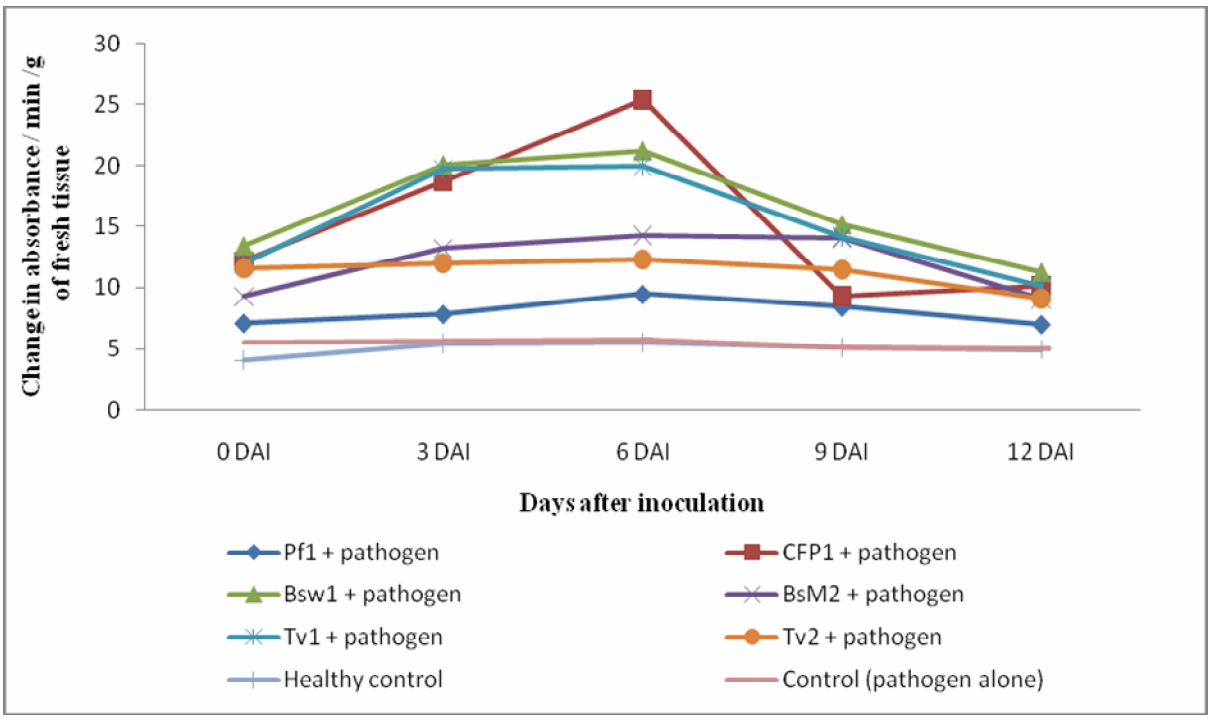


Figure.2 Induction of peroxidase in anthurium treated with biocontrol agents

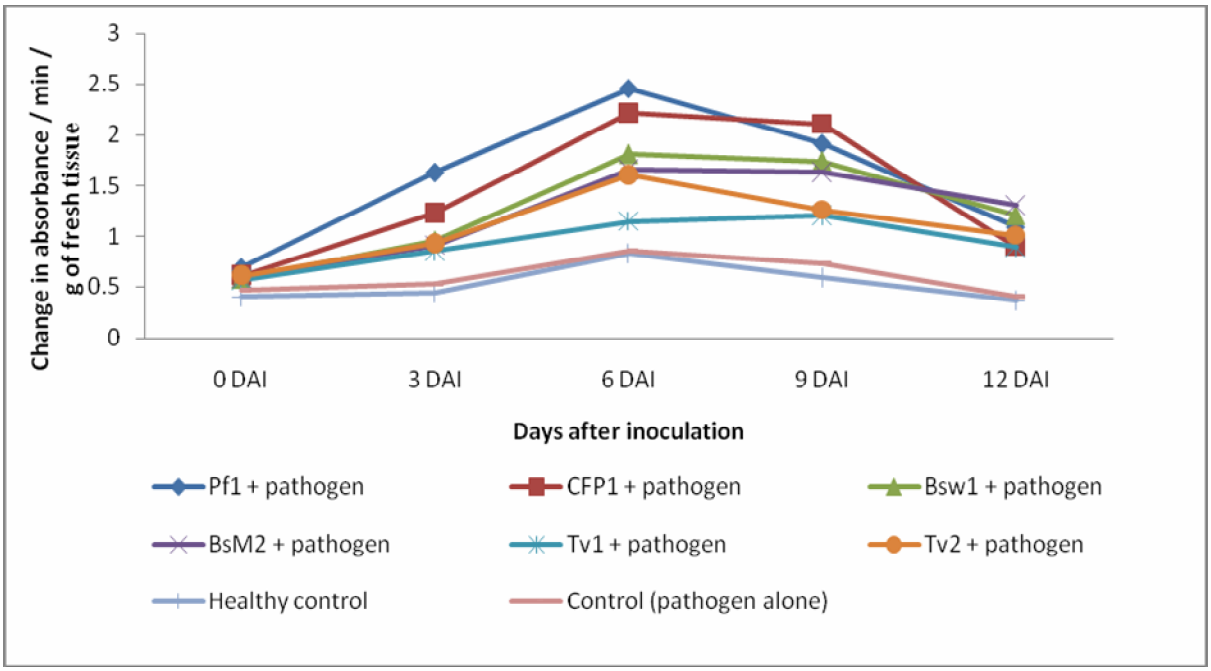


Figure.3 Induction of polyphenol oxidase in anthurium treated with biocontrol agents

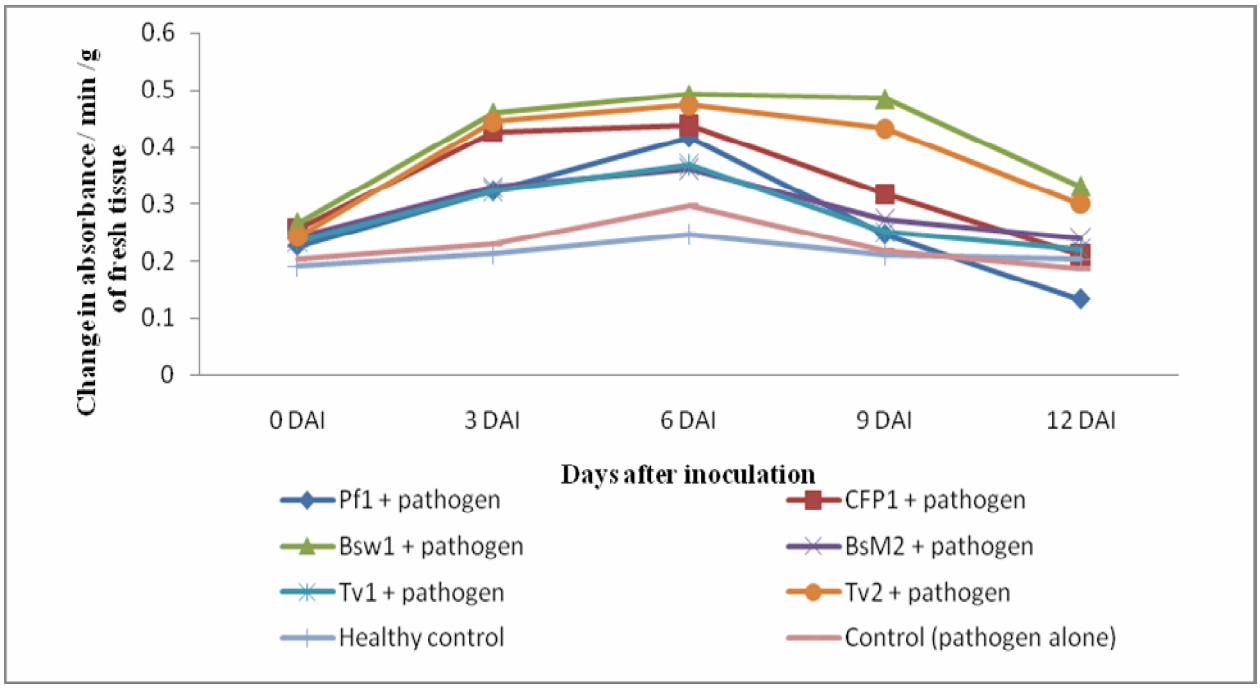


Figure.4 Accumulation of phenol in anthurium treated with biocontrol agents

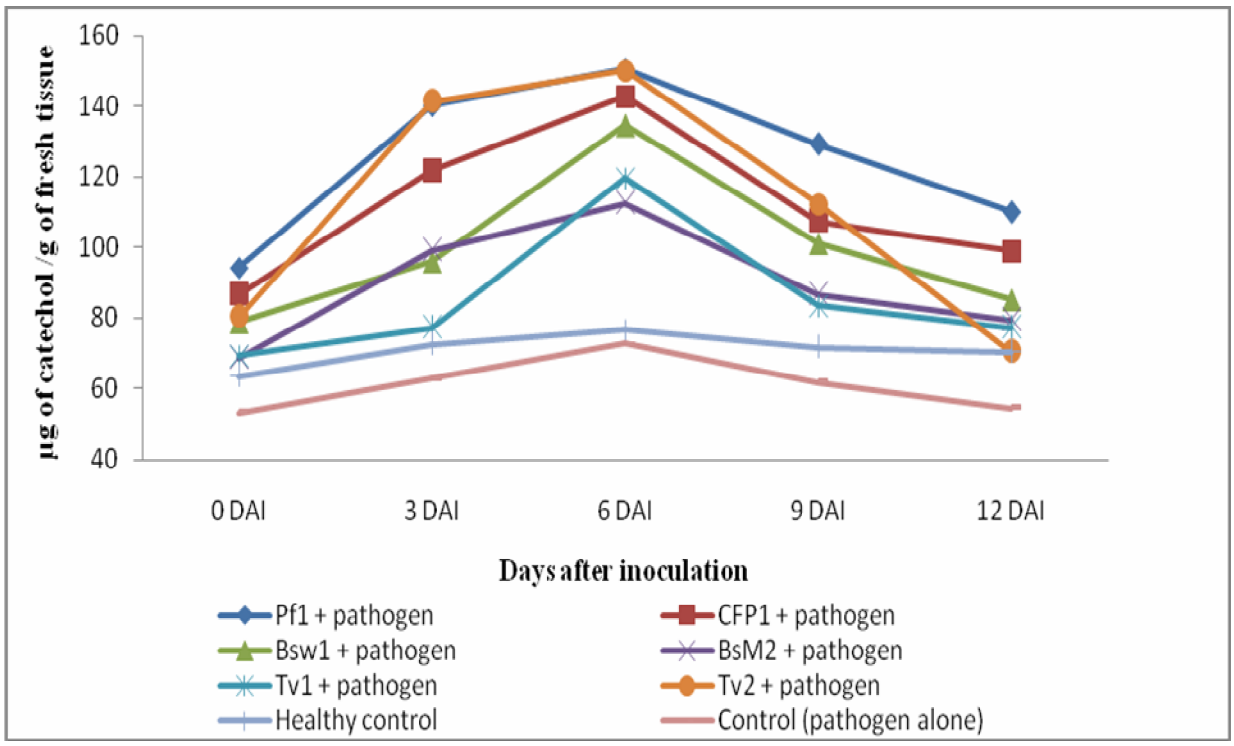
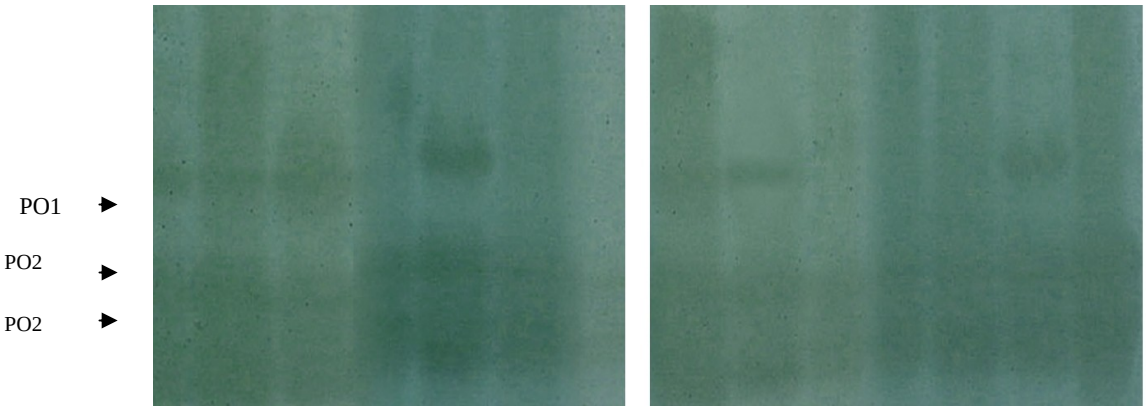


Plate.1 Induction of isoforms of peroxidase in anthurium treated with biocontrol agents against *C. gloeosporioides*



1. Pf1
2. CFP1
3. BsW2
4. BsM3
5. Tv1
6. Tv2
7. Healthy control
8. Pf1 + Pathogen
9. CFP1 + Pathogen
10. BsW2 + Pathogen
11. BsM3 + Pathogen
12. Tv1 + Pathogen
13. Tv2 + Pathogen
14. Control (Pathogen alone)

Plate.2 Induction of isoforms of polyphenoloxidase in anthurium treated with biocontrol agents against *C. gloeosporioides*

1. Pf1
2. CFP1
3. BsW2
4. BsM3
5. Tv1
6. Tv2
7. Healthy control
8. Pf1 + Pathogen
9. CFP1 + Pathogen
10. BsW2 + Pathogen
11. BsM3 + Pathogen
12. Tv1 + Pathogen
13. Tv2 + Pathogen
14. Control (Pathogen alone)

Activity of PAL in plants pre-treated with biocontrol agents was induced upon challenge inoculation with *C. gloeosporioides.* The PAL activity reached maximum at 6 days after inoculation (6 DAI) with the pathogen and declined thereafter in all the treatments with biocontrol agents. Plants that received only biocontrol agents as treatment also exhibited higher levels of PAL activity when compared with healthy control. Activity of PAL was at its peak at 6 DAI in CFP1 strain of *Pseudomonas fluorescens* inoculated with *C. gloeosporioides* (Figure 1). Induction of PAL by fluorescent pseudomonads was reported in mango and noni against *C. gloeosporioides* (Vivekananthan *et al*., 2004 and Manjunath, 2009).

Peroxidase

Peroxidase (PO) is a component of an early response in plants to pathogen infection and plays a major role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West, 1989). The products of the enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity (VanLoon and Callow, 1983). PO is one of the key enzyme involved in phenyl propanoid pathway and it is associated with disease resistance in plants (Hammerschmidt *et al.*, 1982). At 6 DAI the enhanced activity of PO was observed in anthurium treated with *P. fluorescens* isolate CFP1 upon challenge inoculation with *C. gloeosporioides.* Plants treated with biocontrol agent alone also showed enhanced activity as against healthy control (Figure 2). Bradley *et al*. (1992) reported that increased PO activity has been correlated with resistance in many species including barley, cucurbits, cotton, tobacco, wheat and rice and these enzymes are involved in the polymerization of proteins and lignin or suberin precursor into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls or movement through vessels. Native PAGE analysis showed the presence of the three isoforms (PO1 to PO3) of peroxidase in all treatments except healthy control. However the intensity of the isoform was more in plants treated with biocontrol agents than those challenged with *C. gloeosporioides* (Plate 1). Vivekananthan (2003) reported four isoforms *viz.*, PO1 to PO4 in mango fruits pre-treated with FP7 strain of *P. fluorescens* followed by challenge inoculation with *C. gloeosporioides*.

Polyphenol oxidase

Polyphenol oxidase (PPO) is enzymes which use molecular oxygen to catalyze the oxidation of monophenolic and orthophenolic compounds. In the present study, the trend of increasing PPO activity was similar to that of PO in all the treatments. The enzyme activity was maximum at 6 DAI when the plants were pre-treated with BsW2 strain of *Bacillus subtilis* challenged with the pathogen (Figure 3). Native PAGE revealed the inductions of four isoforms of PPO (PPO1 to PPO4) of higher intensity were observed in anthurium plants treated with biocontrol agents followed by challenge inoculation of *C. gloeosporioides* which was absent in healthy control.

Phenol

Phenolics are fungitoxic in nature and increase the physical and mechanical strength of the host cell wall. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi thereby offering resistance against a wide range of pathogens (Cahill and Mccomb, 1992). In our study a trend on phenol

accumulation was noticed in all plants treated with the biocontrol agents had a profound effect on the accumulation of phenols in plants upon challenge inoculation with *C. gloeosporioides*. Its accumulation increased from third day and attained peak on 6 DAI. Maximum accumulation of phenol was noticed in Pf1 strain of *P. fluorescens* challenged with *C. gloeosporioides* at 6 DAI when compared to plants inoculated with the pathogen alone. Some phenolics may act as a signal molecules or antioxidants and thus induce resistance (Malamy *et al.*, 1990).

In conclusion, prior treatment of anthurium seedlings with biocontrol agents triggered the plant defense mechanism in response to infection by *C. gloeosporioides*. Hence, it is speculated that among the various direct antagonistic tools, ISR is also the one indirect tool by which the tested biocontrol agents afforded resistance to anthurium against the pathogen.

References

Bostock, R.M., Karban, R., Thaler, J.S., Weyman, P.D. and Gilchrist, D. 2001. Signal interactions in induced resistance to pathogens and insect herbivores. *Eur. J. Plant Pathol.,* 107: 103-111.

Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.*, 72: 248-254.

Cahill, D.M. and McComb, J.A. 1992. A comparison of changes in PAL activity, lignin and phenolic synthesis in the roots of *Eucalyptus caryophylla* and *E. marginata* when infected with *Phytophthora cinnamomi*. *Physiol. Mol. Pl. Pathol.*, 41: 307-316.

Dickerson, D.P., Pascholati, S.F., Hagerman, A.E., Butler, L.G. and Niholson, R.L. 1984. Phenylalanine ammonia lyase and hydroxyl cinnamate : CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiol. Plant Pathol.,* 25: 111-123.

Hahlbrock, K. and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* 40: 347-369.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber of *Colletotrichum lagenarium*. *Physiol. Plant Pathol.,* 20: 73-82.

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Jayaraman, K.S., Ramanuja, M.N., Vijayaraghavan, P.K. and Vaisyanathan, C.S. 1987. Oxidative enzyme in pearl millet. *Food Chem.*, 24: 203.

Klessig, D.F. and Malamy, J. 1994. The salicylic acid signals in plants. *Plant Mol. Biol.,* 26: 1439-1458.

Malamy, J., Carr, J.P., Klersig, D.F. and Raskin, I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science,* 250: 1002-1004.

Manjunath, H. 2009. Morphological and molecular characterization of *Alternaria alternata* and *Colletotrichum gloeosporioides* incitants of leaf blight and anthracnose diseases of noni and their management. *M.Sc. (Agri.) Thesis*,

Tamil Nadu Agricultural University, Coimbatore.

Massala, R., Legrand, M. and Fritig, B. 1980. Effect of amino oxyacetate, a competitive inhibitor of phenylalanine ammonia lyase, on the hypersensitive resistance of tobacco to tobacco mosaic virus. *Physiol. Plant Pathol.*, 16: 213-226.

Mayer, A.M., Harel, E. and Shaul, R.B. 1965 Assay of catechol oxidase a critical comparison of methods. *Phytochemistry*, 5: 783-789.

Nadlony, L. and Sequeira, I. 1980. Increase in peroxidae activities are not directly involved in induced resistance in tobacco. *Physiol. Plant Pathol*., 16: 1-8.

Nandinidevi, S. 2008. Studies on the foliar diseases of anthurium (*Anthurium andreanum* lind. Ex andre). *M.Sc.(Agri.) Thesis*, Tamil Nadu Agricultural University, Coimbatore.

Santhakumari, P., Mary, C.A. and Dhanya, M.K. 2001. Occurrence of rotting disease in anthurium. *J. Tropical Agri.*, 39: 79.

VanLoon, L.C. and Callow, J.A. 1983. Transcription and translation in the diseased plant *In: Biochemical Plant Pathology*, Callow, J.A. (Eds.) John wiley and Sons, Chichester, UK.

Vivekananthan, R., Ravi, M., Ramanathan, A. and Samiyappan, R. 2004. Lytic enzymes induced by *Pseudomonas fluorescens* and other biocontrol organisms mediate defense against the anthracnose pathogen in mango. *World J. Microbiol. Biotech.*, 20: 235-244.

Vivekanathan, R. 2003. Biotechnology based approaches for early detection and management of anthracnose disease of mango. *M.Sc. (Agri.) Thesis*, Tamil Nadu Agricultural University, Coimbatore, 204pp.

Zieslin, N. and Ben-Zaken, R. 1993. Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. *Plant Physiol. Biochem*., 31: 333-339.

Schoenbeck, F. 1996. Induced resistance: mechanisms and evaluation. In: Lyr, H., Russel, P. and Sisler, H.D. (Eds.). modern fungicides and antifungal compounds. Andover, UK: Intercept Ltd., pp. 447-450.

Maurhofer, M., Hase, C., Meuwly, P., Metraux, J.P. and Defago, G. 1994. Induction of systemic resistance of tabocco to *Tabocco necrosis virus* by the root-colonizing *Pseudomonas fluorescens* strain CHAO: influence of the gacA gene and of pyoverdine production. *Phytopathology,* 84: 139-146.

Xue, L., Charest, P.M. and Jabaji-Hare, S. 1998. Systemic induction of peroxidases, β-1, 3-glucanses, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. *Phytopathology*, 88: 359-365.

Hammerschmidt, R. and Kuc, J.A. 1982. Lignification as a mechanism for induced systemic resistance in cucumber. *Physiol. Plant Pathol.*, 20: 61-71.

Hoffland, E., Kakulinem, J. and Van Pelt, J.A. 1996. Comparison of systemic resistance induced by avirulent and non-pathogenic *Pseudomonas* species. *Phytopathology*, 86: 757-762.

Wei, G., Kloepper, J.W. and Tuzun, S. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth promoting rhizobacteria under field conditions. *Phytopathology*, 86: 221-224.