

Identification of Defense Related enzymes for the Management of Wilt Disease in Groundnut

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ABSTRACT

Groundnut seeds treated with the biocontrol agents *viz.*, *Trichoderma*, *Pseudomonas fluorescens* and *Bacillus subtilis* induced systemic resistance, activated defense related enzymes *viz.*, PAL, PO, PPO and accumulated phenol against challenge inoculated *Fusarium oxysporum*. The biocontrol agents increased the shoot and root length of groundnut seedlings. Plant growth enhancement was high in Tv₁, Pf₁ and Bs₁₀ combination followed by Tv1 and Bs10.

Keywords: Groundnut- Wilt - PAL- PO- PPO

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops in the world. In India it is one of the most important food and cash crops with valuable source of all nutrients. India ranks first in the world with regard to area (26.11 M ha) and second in production (7.54 Mt) (www.indiastat.com, 2011). Groundnut is susceptible to many foliar and soil-borne fungal diseases. Among the soil-borne diseases *viz.*, dry root rot, stem rot and wilt cause serious losses to the crop which is extensively grown under rainfed conditions (Mayee and Datar, 1988; Kannaiyan *et al.*, 1989).

In India, it is cultivated over an area of 5.31 M ha with the production of 6.93 Mt (Directorate of Economics and Statistics, Department of Agriculture and Cooperation, Ministry of Agriculture, Govt. of India, 2020). A large number of diseases attack groundnut in India (Mayee and Datar 1988). Management of the diseases through chemicals and the use of resistant varieties are possible to some extent. But, the hazardous impact of agrochemicals on the environment, development of resistant mutants, escalating cost of pesticides and frequent breakdown

of resistant varieties strongly demand a sustainable and an alternative management approach to disease. Biocontrol is an important component of integrated disease management (IDM) that provides disease control being harmless to humans, eco-friendly selective in mode of action and difficult for pathogens to develop resistance, unlike chemical control. Apart from biocontrol activity they improve soil health and sustainability of agriculture (Sheo Raj *et al.*, 2004). Kloepper (1992) documented induced triggered resistance mechanism in host due to biocontrol agent and introduction of antimicrobial compounds *viz.*, phenazines and phloroglucinals that contribute significantly for the control of several diseases.

MATERIAL AND METHODS

Formulation of biocontrol agents

Bacteria

The isolate of *P. fluorescens viz.*, Pf1 and the isolate of *B. subtilis viz.*, Bs10 which were found to be more effective *in vitro* were used to prepare talc based formulations. 400 ml of 72-h-old bacterial culture in their respective medium with a population

of 9×10^8 cfu/ml were mixed with 1 kg of talc containing 15 g of calcium carbonate and 10 g of CMC. Moisture content of the product was reduced to 20 per cent by shade drying and it was packed in polythene bags for further use (Vidhyasekaran and Muthamilan, 1995).

Trichoderma

The isolate of *T. viride* viz., Tv₁ was cultured in sterilized molasses yeast medium for 10 days. The fungal biomass and broth containing spore concentration of 1×10^7 cfu/ml were mixed with talc at 1:2 ratio. The formulation was air dried and packed in polythene covers and used for further study (Jeyarajan *et al.*, 1994).

Induction of systemic resistance in groundnut by biocontrol agents

Groundnut seeds were treated with talc-based formulations of effective biocontrol agents singly as., Tv₁, Pf₁, Bs₁₀ and in combinations and were sown in pots containing sterile potting medium. Biocontrol agents were applied to the pots at 30 DAS followed by challenge inoculation with pathogen five days later after treatment listed above. Leaf samples were collected at 0, 3, 6, 9 and 12 days after pathogen inoculation to assay the change in activities of defense related enzymes viz., PAL, PO, PPO and phenol.

Estimation of enzymes

Phenylalanine ammonia-lyase

One g of leaf sample 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°C. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion L-phenylalanine to trans-cinnamic 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 for 1 h at

30°C. The reaction was initiated by the addition of L-phenylalanine and stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine and later addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmol trans-cinnamic acid/min/g of fresh tissue.

Peroxidase

Activity of peroxidase 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°C. The supernatant was used to assess activity of PO and PPO. 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract were taken and added to a cuvette. To initiate the reaction, 0.5 ml of 1% H₂O₂ was added. The change in absorbance was recorded at 420 nm at 30 sec interval for three min from zero sec of incubation at room temperature. The results were expressed as change in absorbance/min/g of fresh tissue.

Polyphenol oxidase

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer of pH 6.5 with 0.1 ml of enzyme extract. 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 per cent methanol and agitated for 15 min. at 70°C. To 1 ml of the extract, 5 ml of distilled water and 250 µl of Folin-ciocalteu reagent (1 N) were added and incubated at 25°C for three 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 μg of catechol/g of fresh tissue (Zieslin and Benzaken, 1993).

Effect of formulated products on plant vigour

Seeds of groundnut were surface sterilized with 2% sodium hypochlorite for 30 sec, rinsed in sterile distilled water and soaked overnight in sterile distilled water. The seeds were treated with formulated products and the seedling vigour index was assessed by the standard sand tray method (ISTA, 1999). The vigour index was calculated using the formula of Abdul Baki and Anderson (1973).

Vigour index = seedling length x germination %

RESULTS AND DISCUSSION

Induced systemic resistance

P. fluorescens could act as strong elicitor of plant defense reaction (M'piga *et al.*, 1997). The induced resistance of *P. fluorescens* is associated with accumulation of pathogenesis related proteins (Viswanathan and Samiyappan, 1999). Application of fluorescent pseudomonads strengthen the cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou *et al.*, 2000; Chen *et al.*, 2000). Saravanan *et al.* (2004) reported that increased level of PO and PAL in roots treated with *P. fluorescens* against Fusarium wilt of banana. In the present investigation enhanced activities of defense related enzymes were observed in groundnut plants in response to the application of biocontrol agents against *F. oxysporum* indicating the induction of systemic resistance.

Phenols

The accumulation of phenol increased from the third day and attained a peak on six DAI and thereafter slowly declined. Groundnut plants treated

with consortial formulation of Pf1 + Bs₁₀ and challenged with the pathogen recorded maximum total phenol content on 12 DAI (638 μg /g tissue) followed by Tv1 + Bs10 and pathogen combinations (601 μg /g tissue). (Table1 and Fig 2).

Phenylalanine ammonia lyase

The present study revealed that all the biocontrol agents induced synthesis of PAL in plants. The maximum PAL activity was observed on 12 day in plants treated with Tv₁ + BS₁₀ and challenge inoculated with the pathogen. (Fig 3) The product of PAL activity is transcinnamic acid which is an immediate precursor for the biosynthesis of SA, a signal molecule in SAR (Klessing and Malany, 1994). PAL activity could be induced during plant-pathogen interactions (Ramanathan *et al.*, 2000; Bharathi *et al.*, 2004; Kandan *et al.*, 2002; Harish, 2005). Induction of PAL by fluorescent pseudomonads was reported in cucumber against *P. aphanidermatum* (Chen *et al.*, 2000). Turmeric plants when sprayed with *P. fluorescens* was reported with enhanced PAL activity (Kavitha, 2004).

Peroxidase

Enhanced PO activity was noticed in plants treated with bioagents. The results revealed that additional increase in PO activity was higher in plants inoculated with the pathogen. The PO activity reached its peak at sixth day in plants treated with bioagents compared to healthy control. In pathogen inoculated plants also the activity of PO attained its peak at 6 DAI and declined steeply at 12 DAI.

Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989). Increased activity of PO has been elicited by fluorescent pseudomonads as reported against rice sheath blight (Nandakumar *et al.*, 2001; Radjacommar *et al.*, 2002), blackgram root rot (Karthikeyan *et al.*, 2003) and groundnut

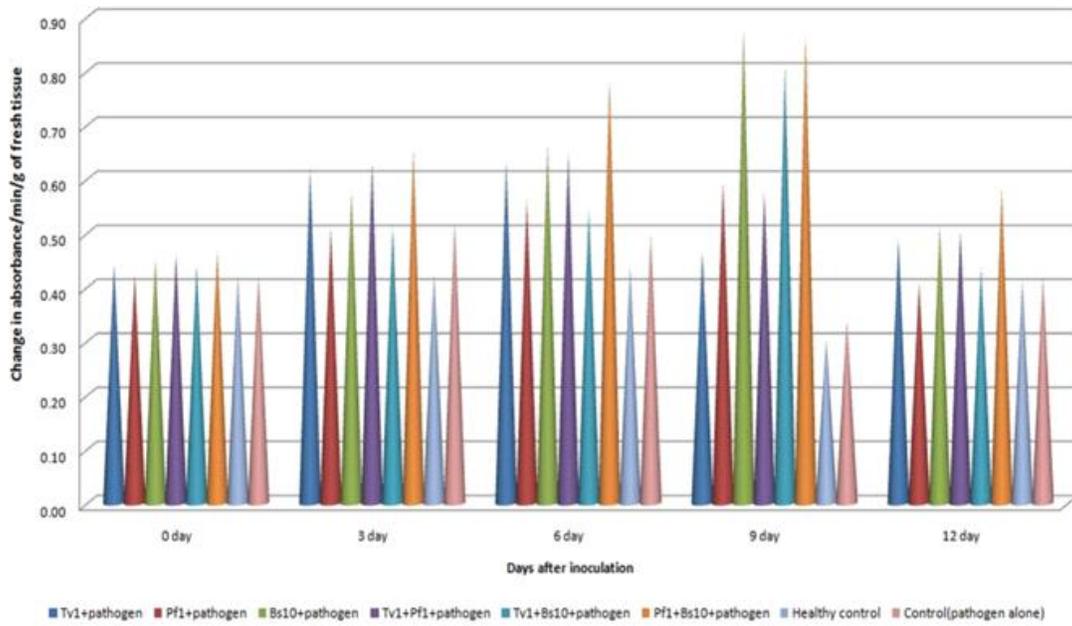


Fig1. Induction of Poly phenoloxidase in groundnut treated with biocontrol Agents

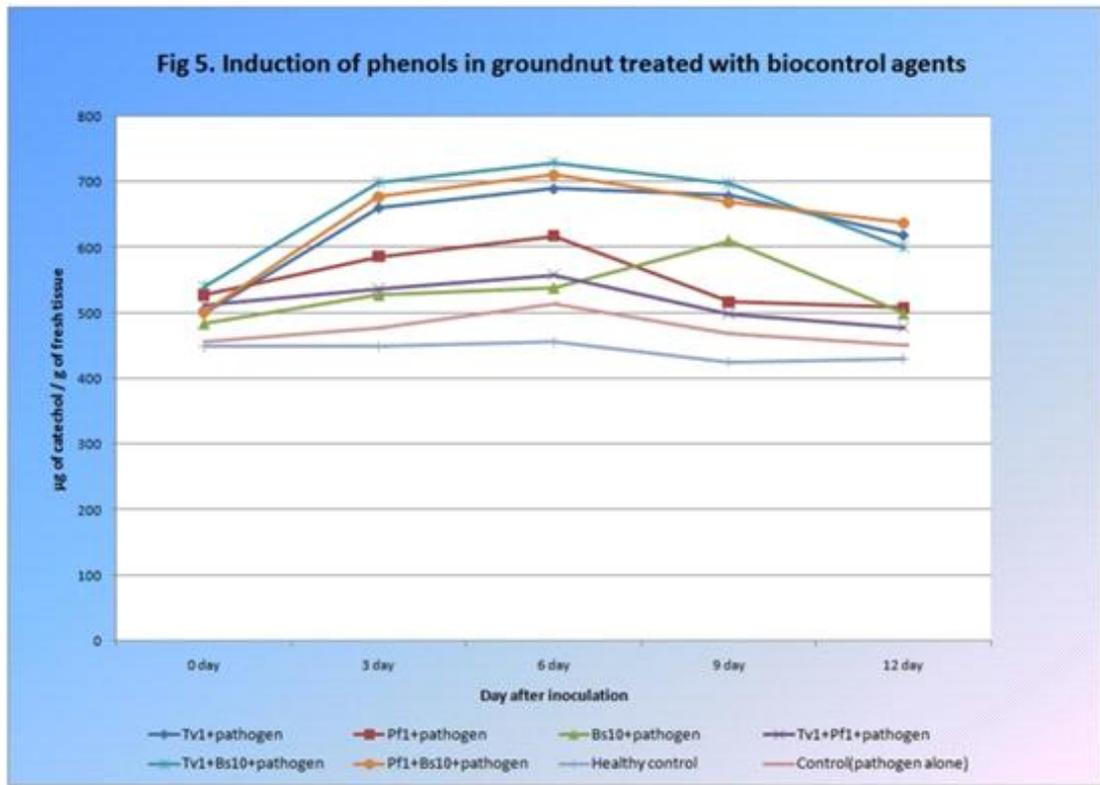


Fig.2. Induction of Phenols in groundnut treated with biocontrol agents

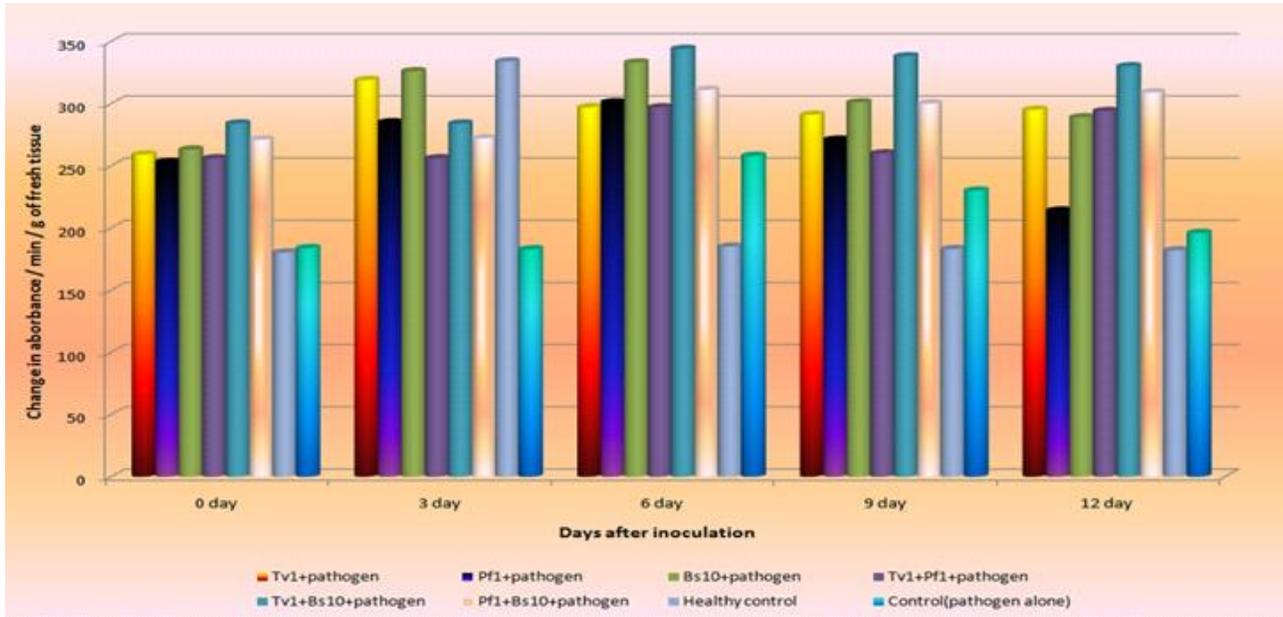


Fig 3. Induction of Phenyl alanine ammonia lyase in groundnut treated with biocontrol agents

Table 1. Induction of defense enzymes in groundnut treated with biocontrol agents

S. No.	Treatments	PO	PPO	PAL	Phenol
		Absorbance /min/g of fresh tissue	Absorbance /min/g of fresh tissue	Moles of transcinamic acid/min/g	µg of catechol/g of fresh tissue
1	Tv1	0.432g	0.330h	225f	475ef
2	Tv1+ Pathogen	0.429g	0.489c	253c	619b
3	Pf1	0.552f	0.339h	214g	444i
4	Pf1 + Pathogen	0.557e	0.408ef	225f	509d
5	Bs10	0.797d	0.377g	246e	460gh
6	Bs10 + Pathogen	0.825c	0.513b	289c	499d
7	Tv1 + Pf 1	0.553df	0.400f	223f	452gh
8	Tv1 + Pf1 + pathogen	0.572ef	0.504b	260c	478de
9	Tv1 + Bs10	0.786b	0.399f	280d	462fg
10	Tv1 + Bs10 + pathogen	0.835bc	0.584a	330a	601c
11	Pf1 + Bs10	0.855ab	0.428d	239e	453gh
12	Pf1 + Bs10 + pathogen	0.871c	0.437d	309b	638a
13	Healthy control	0.290h	0.413ef	182i	431j
14	Control (Pathogen alone)	0.292h	0.419e	196h	452hi

Means in columns followed by the same letter are not significantly different ($P < 0.05$) according to DMRT.

Table 2. Effect of antagonists on growth parameters of groundnut seedling

S. No.	Treatments	Germination	Shoot length	Root length	Vigour
		(%)	(cm)	(cm)	index
1	Tv1	90.0	11.7	7.3	1710.0
2	Pf 1	90.6	11.0	7.5	1676.1
3	Bs 10	95.3	12.2	11.3	2239.6
4	Tv1 + Pf 1	90.0	12.0	10.0	2205.0
5	Tv1 + Bs 10	95.3	14.5	18.9	2944.8
6	Pf 1+ Bs 10	87.5	10.7	9.0	1723.8
7	Tv1 + Pf 1+ Bs 10	95.3	18.5	21.9	3850.1
8	Control	85.6	9.5	6.0	1326.8
CD		5.2	0.7	0.8	140.0

late leaf spot (Meena *et al.*, 2000) pathogens. In the present study, groundnut plants treated with the combination of Tv₁ and BS₁₀ when challenged with the pathogen showed increased activity of PO.

Accumulation of PO has been correlated with ISR in several crops (Ramamoorthy and Samiyappan, 2001). Isolates of *Pseudomonas* systemically induced resistance against Fusarium wilt of chickpea and suppressed the disease by 34.45 per cent when compared to control (Saikia *et al.*, 2005). Mandal (2009) reported that exogenous application of SA could induce resistance against *F. o. f. sp. lycopersici* in tomato. (Table 1).

Polyphenoloxidase

The increasing trend of PPO activity was observed similar to that of PO in all the treatments tested. The PPO activity reached its maximum at 12 DAI in treatment Tv₁ + Bs₁₀ when challenged with *F. oxysporum*. (Fig.1)

PPO is a copper containing enzyme which usually accumulates on wounding in plants. Induction of PPO activity has been correlated with a resistance response (Velazhahan and Vidhyasekaran, 1994). Expression of new PPO isoform was observed in *P. fluorescens* Pf₁ treated tomato plants challenged with

F.o.f.sp. Lycopersici (Ramamoorthy *et al.*, 2002). Harish (2005) reported higher induction of PPO enzymes in plant growth promoting bacteria treated banana plants.

Ramamorothy and Samiyappan (2001) observed accelerated PPO activity in chilli plants treated with *P. fluorescens* when challenge inoculated with *C. capsici*. Kavitha (2004) and Kamalakannan (2004) observed that application of *P. fluorescens* and its combination with *B. subtilis* significantly increased PPO against *P. aphanidermatum* and *M. phaseolina* respectively (Table 1 and Fig 1).

Plant growth promotion

In the present investigation, application of biocontrol agents increased the shoot and root lengths of groundnut in sand tray method. Plant growth enhancement was more in Tv₁, Pf₁ and Bs₁₀ combination followed by Tv₁ and Bs₁₀. Similar results have been documented in many crops by earlier workers (Table 2).

Application of *B. subtilis* F2B24 increased the growth and yield in peanut (Backmann *et al.*, 1994). Manjula and Podile (2001) reported that use of *B. subtilis* Af₁ promoted seed germination and biomass of groundnut and pigeonpea even at high

pathogen pressure. Bharathi *et al.* (2004) reported that *P. fluorescens* (Pf-) and *B. subtilis* increased the seed germination and seedling vigour of chillies. Similarly, promotion of plant growth by *P. fluorescens*, *Bacillus* spp. and *Trichoderma* spp. has been documented by various workers (Chang *et al.*, 1986; Schippers *et al.*, 1987; Rabindran *et al.*, 2005). (Table 2).

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