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Induction of peroxidase, polyphenol oxidase and protease in Mung bean inoculated with *Macrophomina phaseolina* causing dry root rot

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Abstract
The induction of defense related enzymes i.e. PO, PPO and protease in root and shoot tissues of mung bean using four antagonists *viz.*, *T. atroviride* (Ta-JOB), *T. viride* (Tv-BKN), *T. harzianum* (Th-JU) and *P. fluorescens* (Pf-SIKR) were investigated at three different stages i.e. 15, 25 and 25 DAS under green house conditions. The antagonists *T. harzianum* (Th-JU) and *T. viride* (Tv-BKN), proved to be most effective in inducing the PO activity in both root and shoot tissues of mung bean genotype at all stages. The PPO activity was highest in *T. viride* (Tv-BKN) treatment followed by *T. harzianum* (Th-JU) in roots as well as in shoots at all stages of sampling in both *M. phaseolina* inoculated as well as uninoculated condition. Increase of protease activity was highest in *T. harzianum* (Th-JU) treatment followed by *P. fluorescens* (Pf-SIKR) treatment. The overall activity of enzymes in shoot samples was higher than root samples in both inoculated and uninoculated conditions.

Keywords: Peroxidase, poly phenol oxides, protease, *Macrophomina phaseolina*, induction, mung bean

Introduction
Mung bean [*Vigna radiata* (L.) Wilczek] is one of the most important pulse crop in india after chickpea and pigeonpea. Mung bean is an excellent source of high quality protein. Ascorbic acid (vitamin c) is synthesized in sprouted seeds of mung bean. The crop is affected by several fungal pathogens including root rot (*Macrophomina phaseolina*), web blight (*Thanatephorus cucumeris*), powdery mildew (*Erysiphe polygoni*), Cercospora leaf spot (*Cercospora canescens*) and anthracnose [*Colletotrichum dematium* and *C. lindemuthianum*). Root rot incited by *Macrophomina phaseolina* (Tassi) Goid has been rated as most devasting disease of mungbean. The pathogen attacks on all parts of plant i.e. root, stem, branches, petiols, leaves, pods and seeds. Induced resistance may provide an alternative approach to plant protection especially for problems not satisfactorily controlled by various fungicides [1]. Induced systemic resistance (ISR) is defined by the systemic protection of plants by the enhancement of the plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate inducing of infection by a pathogen. Induced systemic resistance (ISR) activates multiple defense mechanisms that include increased activity of pathogenesis related (PR) proteins like peroxidase (PO), polyphenol oxidase (PPO) and protease [2]. The defense enzymes include peroxidase (PO), polyphenol oxidase (PPO), which catalyses the formation of lignin. These enzymes have been correlated with defense against pathogens in several plants. The present study was aimed at induction of various defense related enzymes by bioagents in response to infection by the *M. phaseolina.*

2. Materials and Methods
2.1 Pot culture experiment
Changes in activity of host defense related enzymes and total soluble phenol and in mung bean (cv.RMG-62) in response to four test antagonists *viz.*, *T. atroviride T. harzianum, T. viride* and *P. fluorescens* were studied under green house conditions. The effect of bioagents on activity of three host defense related enzymes and i.e. peroxidase, polyphenol oxidase and protease was studied. Talc based formulation of antagonist's *viz.*, *T. atroviride*, *T. harzianum, T. viride*, and *P. fluorescens* were prepared as described in earlier sections. The two sets of experiments were laid out for root and shoot samples. In first set of experiment, mung bean seed treated with individual bioagent at 8 g kg^{-1} seed were sown in pathogen inoculated soil. For, soil application, each bioagent was applied to soil at 2 g kg^{-1} soil. The sand maize meal inoculum (20 g kg^{-1} soil) was used for inoculation 48 hours prior to sowing of mung bean seeds. In

second set of experiment, the bioagents were used as seed treatment and soil application but the soil was not inoculated with the pathogen. The seeds were sown in earthen pots of five kg capacity. Ten mung bean seeds were sown in each pot. The pots were irrigated usually on alternate day with uniform quantity of water. The mung bean plants were uprooted after 15, 25, 35 days of pathogen inoculation and the roots and shoots were thoroughly washed in tap water followed by washing in distilled water. These samples were stored in deep freeze at -50 to -40°C for determining enzyme activity.

2.2 Quantification of peroxidase activity

Peroxidase activity was determined by the method described by Thimmaiah [3]. One gram root or shoot samples were macerated in previously chilled mortar in 10 ml ice cold 0.1 M phosphate buffer, pH 6.0. The homogenate was strained through two folds of muslin cloth and centrifuged at 16,000 g for 20 minutes at 4°C. The supernatant was used as enzyme source. In order to assay the enzyme activity, 1 ml O–Dianisidine, 0.5 ml of H_2O_2, 1 ml of phosphate buffer and 2.4 ml distilled water were pipetted in test tubes. The blank was prepared by excluding H_2O_2 and adding additional volume of water in place of H_2O_2. The reaction was initiated by adding 0.1 ml of enzyme extract (supernatant) and incubating at 30°C for five minutes. The reaction was stopped by adding 1 ml of 2N H_2SO_4. The absorbance was measured at 430 nm against reagent blank. The unit of enzyme was defined as min^{-1} mg^{-1} protein.

2.3 Quantification of polyphenol oxidase activity

The polyphenol oxidase activity was determined by the method of Mayer *et al*. [4]. Two gram root or shoot samples were homogenized in 4 ml 0.1 M sodium phosphate buffer pH 6.5 and centrifuged at 16000 g for 15 minutes at 4°C. The supernatant was used as enzyme source. The reaction mixture was consisted of 1 ml enzyme extract supernatant and 3 ml of 0.1 M sodium phosphate buffer (pH 6.5). To initiate the reaction, 0.5 ml of 0.01 m catechol was added and the change in absorbance was recorded at 30 second interval up to 3 minutes at 410 nm. For blank enzyme extract replaced by distilled water. The activity of enzyme was expressed as absorbance min^{-1} mg^{-1} protein.

2.4 Quantification of protease activity

Protease activity was determined by modified kunitz caseinolytic assay [5]. One gram root or shoot samples ware macerated in chilled pestle and mortar with 10 ml 0.05 M sodium phosphate buffer for 30 minute. The homogenate was centrifuged at 14,000 g at 4°C for 10 minute. The supernatant was used as enzyme source. The substrate solution was prepared by suspending 0.5 g of casein in 100 ml Tris- acetate buffer, followed by heating the suspension in boiling water bath for 10 minutes. The volume of the samples was adjusted to 100 ml with buffer. The suspension was stirred gently to dissolve all the casein. The solution was cooled to room temperature before use. In order to assay the enzyme activity, 2 ml of casein solution which was pre-incubated at 37°C for 5 minutes was added to 100 µl enzyme preparation. The mixture was incubated for exactly 10 minutes and then 3 ml of 5% trichloroacetic acid was added. The blank was prepared by first precipitating 2 ml of the substrate solution with 5 ml of trichloroacetic acid solution and than adding the same amount of enzyme as used for the sample. The test tubes were kept for about 30 minutes at room temperature and filtered using Whatman filter paper No. 1. The enzyme activity was determined by using the difference in absorbance between the sample and blank. One unit was defined as the amount of enzyme which, after being inoculated with casein under the conditions defined above, liberates hydrolysis products that give the absorbance of 0.50 at 280 nm in 10 minutes at 37°C. The enzyme was expressed as absorbance min^{-1} mg^{-1} protein.

3. Results and Discussion

3.1 Peroxidase (PO) activity

The study revealed that peroxidase (PO) activity was significantly higher in all the four bioagent treatments in root as well as in shoot tissues in mung bean genotype RMG-62 at three stages of growth i.e. 15, 25 and 35 DAS under *M. phaseolina* inoculated as well as uninoculated conditions. The enzyme activity was highest in *T. harzianum* (Th-JU) treatment followed by *T. viride* (Tv-BKN), *P. fluorescens* (Pf-SIKR) and *Trichoderma atroviride* (Ta-JOB) treatments in root and shoot tissues in both inoculated and uninoculated conditions. It was also recorded that PO activity was higher in shoot as compared to root tissues in all the four respective bioagent treatments. Further, the PO activity in both root and shoot tissues in response to antagonist treatment was higher under *M. phaseolina* inoculated condition as compared to without *M. phaseolina* inoculation [Table 1(i) and table 1(ii)]. Peroxidases have been implicated in a number of physiological functions that may contribute to resistance including exudation of hydroxy-cinnamyl alcohol into free radical intermediates, phenol oxidation, polysaccharide cross linking, and lignification and also associated with deposition of phenolic compounds into plant cell walls during resistant interactions [6-11]. Two pathogenesis related peroxidases in green gram leaves induced by *M. phaseolina*. An elicitor was isolated from *M. phaseolina*, the root rot and leaf blight pathogen of greengram. When green gram leaves were inoculated with *M. phaseolina*, two new peroxidases appeared [12]. The efficacy of various *Pseudomonas fluorescens* isolates for the induction of systemic resistance against *M. phaseolina*. The plants treated with the strain *P. fluorescens* Pf4-99 enhanced the activity of PO in chickpea [13].

Table 1(1): Effect of antagonists on peroxidase (PO) activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* uninoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	11.36	19.85	22.64	34.51	15.70	24.26
T. viride (Tv – BKN)	15.09	23.86	28.09	38.92	19.36	30.48
T. harzianum (Th – JU)	16.07	26.85	31.64	42.66	21.71	34.00
P. fluorescens (Pf – SIKR)	15.04	21.54	25.68	37.96	17.21	27.32
Control (without bioagent)	8.08	14.27	14.22	21.62	9.94	18.69
Mean	13.12	21.27	24.45	35.13	16.78	26.95
S,Em.±	0.09	0.24	0.15	0.31	0.15	0.31
CD (5%)	0.30	0.75	0.47	0.97	0.46	0.97
CV (%)	1.25	1.95	1.06	1.52	1.52	1.98

DAS= Days after sowing

Table 1(2): Effect of antagonists on peroxidase (PO) activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* inoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	18.02	28.58	33.38	45.96	24.13	36.46
T. viride (Tv – BKN)	21.95	33.09	38.39	51.19	28.69	42.28
T. harzianum (Th – JU)	24.91	35.98	39.78	53.83	31.29	45.59
P. fluorescens (Pf – SIKR)	19.21	30.26	36.00	47.78	26.02	40.11
Control (without bioagent)	10.16	17.84	18.75	29.90	13.41	23.53
Mean	18.85	29.15	33.26	45.73	24.71	37.59
S,Em.±	0.16	0.37	0.42	0.57	0.20	0.29
CD (5%)	0.52	1.18	1.31	1.79	0.62	0.90
CV (%)	1.50	2.22	2.16	2.15	1.39	1.32

DAS= Days after sowing

3.2 Polyphenol oxidase activity

Polyphenol oxidase is a key enzyme involved in oxidation of phenolics to highly toxic quinines and involved in the terminal oxidation of diseased plant tissue, and hence plays important role in disease resistance [14, 15]. The activity of PPO was recorded in mung bean plants in response to antagonists treatments in green house. It was recorded that PPO activity was significantly higher in root and shoot tissues due to bioagent treatments at three stages of plant growth i.e. 15, 25 and 35 DAS. Among the bioagent treatments, PPO activity was highest in *T. viride* (Tv-BKN) treatment followed by *T. harzianum* (Th-JU) and *P. fluorescens* (Pf-SIKR) treatments in root as well as in shoot tissues at 15, 25 and 35 DAS in both *M. phaseolina* inoculated as well as uninoculated conditions. The overall PPO activity in shoot tissues was higher than root tissues. Further, the activity of this enzyme was higher in inoculated as compared to uninoculated conditions in all the bioagent treatments [Table 2(i) and table 2(ii)]. Increase in activity of PPO against pathogens has been reported by various workers [16-18] studied the induction of defense-related enzymes in response to isolate *P. fluorescens* Pf-I against *Macrophomina* root rot in mung bean. They observed the accumualation of PPO and PO due to application of *P. fluorescens* Pf-1 in mung bean plants inoculated with *M. phaseolina*. Jayalakshmi [19] also recorded induction of PPO due to treatment with *T. harzianum* L_1 in chickpea against soil borne pathogens.

Table 2(1): Effect of antagonists on polyphenol oxidase (PPO) activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* uninoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	1.57	2.34	3.03	4.52	2.32	3.04
T. viride (Tv – BKN)	1.78	2.71	3.43	5.21	2.66	3.45
T. harzianum (Th – JU)	1.72	2.57	3.30	4.94	2.57	3.41
P. fluorescens (Pf – SIKR)	1.66	2.49	3.13	4.76	2.46	3.21
Control (without bioagent)	1.37	2.12	2.50	3.60	1.85	2.65
Mean	1.62	2.45	3.08	4.61	2.37	3.15
S,Em.±	0.01	0.02	0.02	0.02	0.02	0.03
CD (5%)	0.04	0.06	0.07	0.06	0.06	0.10
CV (%)	1.38	1.33	1.33	0.76	1.50	1.67

DAS= Days after sowing

Table 2(2): Effect of antagonists on polyphenol oxidase (PPO) activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* inoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	1.94	2.99	3.65	5.79	2.80	3.74
T. viride (Tv – BKN)	2.16	3.15	4.28	6.31	3.24	4.15
T. harzianum (Th – JU)	2.10	3.05	4.05	6.18	3.11	4.04
P. fluorescens (Pf – SIKR)	2.02	3.00	3.78	5.97	2.94	3.90
Control (without bioagent)	1.49	2.28	2.88	4.06	2.08	2.88
Mean	1.94	2.89	3.73	5.66	2.83	3.74
S,Em.±	0.04	0.03	0.03	0.05	0.03	0.03
CD (5%)	0.12	0.11	0.10	0.14	0.11	0.10
CV (%)	3.47	2.06	1.52	1.38	2.08	1.42

DAS= Days after sowing

3.3 Protease activity

The protease activity was significantly higher in root and shoot tissues of mung bean plants in response to bioagent treatments at all three stages under *M. phaseolina* inoculated and uninoculated conditions. It was observed that the protease activity was highest in *T. harzianum* (Th-JU) treatment followed by *P. fluorescens* (Pf-SIKR) treatment in root as well as in shoot tissues [Table 3(i) and table 3(ii)]. Proteases are involved in defense mechanism of host plants. These are also associated in complex process of signal transduction [20, 21]. Proteinase inhibitors (PR 6) in plants are able to suppress enzymatic activities of phytopathogenic microorganisms [22]. Several reports have shown that proteinase inhibitors are associated with resistance. More observations are required for understanding the role of protease in defence mechanism during pathogenesis.

Table 3(1): Effect of antagonists on protease activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* uninoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	1.33	1.80	1.83	2.20	1.55	1.97
T. viride (Tv – BKN)	1.36	1.88	1.91	2.43	1.65	2.05
T. harzianum (Th – JU)	1.47	2.00	2.07	2.76	1.80	2.25
P. fluorescens (Pf – SIKR)	1.38	1.94	1.99	2.58	1.74	2.15
Control (without bioagent)	1.12	1.44	1.37	1.78	1.27	1.61
Mean	1.33	1.81	1.83	2.35	1.60	2.01
S,Em.±	0.01	0.01	0.01	0.01	0.02	0.02
CD (5%)	0.03	0.04	0.04	0.05	0.05	0.05
CV (%)	1.38	1.33	1.06	1.08	1.85	1.37

DAS= Days after sowing

Table 3(2): Effect of antagonists on protease activity (min^{-1} mg^{-1} protein) in mung bean genotype RMG-62 under *M. phaseolina* inoculated condition

Antagonists	15 DAS		25 DAS		35 DAS	
	Root	Shoot	Root	Shoot	Root	Shoot
Trichoderma atroviride (Ta – JOB)	1.64	2.12	2.22	3.02	1.95	2.44
T. viride (Tv – BKN)	1.71	2.21	2.26	3.06	2.02	2.57
T. harzianum (Th – JU)	1.85	2.42	2.42	3.30	2.19	2.78
P. fluorescens (Pf – SIKR)	1.81	2.31	2.35	3.14	2.10	2.66
Control (without bioagent)	1.22	1.67	1.61	2.00	1.41	1.86
Mean	1.65	2.15	2.17	2.90	1.93	2.46
S,Em.±	0.01	0.02	0.02	0.05	0.03	0.02
CD (5%)	0.03	0.08	0.05	0.17	0.09	0.05
CV (%)	1.02	1.92	1.37	3.15	2.59	1.19

DAS= Days after sowing

4. Conclusions

The data in this investigation have revealed a significantly enhanced the activity of Peroxidase, polyphenol oxidase and protease in root and shoot tissues of mung bean genotype when plant inoculated with four antagonists *viz.*, *T. atroviride* (Ta-JOB) *T. harzianum* (Th-JU) *T. viride* (Tv-BKN) and *P. fluorescens* (Pf-SIKR) againt *Macrophomina phaseolina.*

5. References

1. Schoenbeck F. Induced resistance: Mechanisms and evaluation. In: Lyr H, Russel P, Sisler HD, editors. Modern fungicides and antifungal compounds. Andover, UK: Intercept Ltd, 1996, 447-450.
2. Maurhofer M, Hase C, Meuwly P, Metraux JP, Defago G. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHAO: Influence of the gacA gene and of pyoverdine production. Phytopath, 1994; 84:139-146.
3. Thimmaiah SR. Standard Methods of Biochemical Analysis, Kalyani Publishers, New Delhi, 1999, 230-23.
4. Mayer AM, Harel E, Shoul RB. Assay of catechol oxidase, a critical comparison of methods. Phytochem. 1965; 5:783-789.
5. Belew M, Porath J. Extracellular proteinase from *Penicillium notantum.* In: Perimann GE, Lorond L, eds. Methods in Enzymology. New York, USA: Academic Press, 1970; 19: 576-581.
6. Gross GG. The biochemistry of lignification. Adv Bot Res. 1980; 8:25-63.
7. Schmidt PS, Feucht W. Tissue specific oxidation browning of polyphenols by peroxidase in cherry shoots. Gartenbauwissenschaft. 1980; 45:68-73.
8. Fry SC. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann Rev Plant Physiol. 1986; 37:165-186.
9. Everdeen KE, Kiefer S, Willard JJ, Muldoon EP, Dey PM, Li XB *et al.* Enzymic cross linkage of monomeric extensin precursors *in vitro*. Plant Physiol. 1988; 87:616-621.
10. Walter MH. Regulation of lignification in defense. In: Genes Involved in Plant Defences. Boller T, Meins F, (edis), New York, Springer-Verlag, 1992, 327-352.
11. Graham MY, Graham TL. Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytopthora megasperma* f. sp. *glycinea* wall glucan. Plant Physiol. 1991; 97:1445-1455.
12. Ramanathan A, Vidhyasekaran P, Samiyappan R. Two pathogenesis-related peroxidases in green gram (*Vigna radiata* (L.) wilczek) leaves and cultured cells induced by *Macrophomina phaseolina* (Tassi.) Goid. and its elicitor. Microbiol Res. 2001; 156:139-144.
13. Kumar V, Kumar A, Verma VC, Gond SK, Kharwar RN. Induction of defense enzymes in *Pseudomonas fluorescens* treated chickpea roots against *Macrophomina phaseolina.* Indian Phytopath. 2007; 60:289-295.
14. Chen C, Bélanger RR, Benhamou N, Paulitz TC. Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol Mol Plant Pathol 2000; 56:13-23.
15. Sujatha N, Ammani K. The activities of the pathogenesis related proteins in *Vigna mungo* grown in pathogen amended soil as influenced by *Pseudomonas fluorescens*. Drug Invention Today. 2011; 3:86-90.
16. Srivastava SK. Peroxidase and polyphenol oxidase in Brassica juncea plants infected with *Macrophomina phaseolina* (Tassi.) Goid. and their implication in disease resistance. J Phytopathol. 1987; 120:249-254.
17. Shivakumar G, Sharma RC. Induced biochemical changes due to seed bacterization by *Pseudomonas*

fluorescens in maize plants. Indian Phytopath. 2003; 56:134-137.
18. Saravanakumar D, Harish S, Loganathan M, Vivekananthan R, Rajendran L, Raguchander T, *et al.* Arch Phytopathol Plant Prot. 2007; 40:323-337.
19. Jayalakshmi SK, Raju S, Usha Rani S, Benagi VI. Sreeramulu K. *Trichoderma harzianum* L1 as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum* f. sp. *ciceri*. Australian J Crop Sci. 2009; 3:44-52.
20. Schaller A, Ryan CA. Molecular cloning of a tomato leaf cDNA encoding an aspartic protease, a systemic wound response protein. Plant Mol. Biol. 1996; 31:1073-1077.
21. Tornero P, Conejero V, Vera P. Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants. Similarity of functional domains to subtilisin-like endoproteases. Proceedings Nat Acad Sci, USA. 1996; 93:6332-37.
22. Ferreira RB, Monterio S, Freitas R, Santos CN, Chen Z, Batista LM *et al.* The role of plant defence proteins in fungal pathogenesis. Mol Plant Pathol. 2007; 5:677-700.