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Upregulation of defense enzymes in groundnut (*Arachishypogaea* L.) by combined application of *Trichoderma* spp. and Mahua oil cake against *Sclerotium rolfsii*

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Abstract
Groundnut is one of the world's most popular edible legumes. Many soil-borne diseases were affected, among them, most notorious pathogen, *Sclerotium rolfsii*, which caused substantial constraints in groundnut production and productivity. Biological control offers wider treatment modalities than conventional chemical control. Under present study, induction of defense enzymes [PAL (phenylalanine ammonia lyase), PO (peroxidase), PPO (Polyphenol oxidase) and phenol] in groundnut treated with *Trichoderma* spp. and organic amendment (mahua oil cake) challenged with the *S. rolfsii* was studied. Highest PAL (0.298 changes in absorbance/minute/gram of leaf tissue), PO (0.291 changes in absorbance/minute/gram of leaf tissue /minute/gram), PPO (0.296µmole of transcinnamic acid/minute/gram) and Phenol (781µgram of catechol/g) activity was recorded in plants treated with sequential application of *Trichoderma* spp. and mahua oil cake. Inoculated control has shown minimum defense enzyme activity. Results suggest the role of *Trichoderma* spp. and mahua oil cake in inducing higher defense enzyme activity on groundnut against *S. rolfsii*.

Keywords: Groundnut, *Sclerotium rolfsii*, *Trichoderma* spp., Mahua oil cake, defense inducing enzymes

Introduction
Groundnut is an important oilseed crop that belongs to the family of Leguminosae. It is grown widely throughout the world under various agroclimatic conditions. It is a valuable source of all essential nutrients. In India, Gujarat ranks first in groundnut area (20 lakh ha) with the production of 26 lakh tonnes. The highest productivity of 1604 kg/ha was recorded in the State of Tamil Nadu, while the productivity was only about 1190 kg/ha in Gujarat. Groundnut is suffered from a number of diseases (Mayee and Datar, 1988) [18]. Among the many soil-borne diseases, stem rot or white mold was caused by *Sclerotium rolfsii* Sacc. During the absence of hosts, *S. rolfsii* survives as resting structures called sclerotia, which act as the primary source of inoculums (Aycock, 1966) [2]. Typical symptoms of stem rot are dark brown lesions on the stem just below the soil surface followed by drooping and wilting of the entire plant and white minute sclerotia showed on the collar region of the stem (Garren, 1959) [8]. Chemical-based management of *S. rolfsii* is not practicable owing to high cost besides causing environmental pollution and development of resistance to target fungus (Backman, 1997) [4]. Biological control is the best alternative, especially against soil-borne pathogens (Hibar *et al.*, 2007; Sreeramulu*et al.*, 2009) [13, 22]. Among the various antagonists, *Trichoderma* spp. Plays a vital role in reducing the population of *Sclerotium* spp. *Trichoderma* sp. exhibited different mode of actions such as competition, parasitism, antibiosis, lysis and Induced Systemic Resistance. Among them defense related enzymes plays a major role in boost the immunity of host plants and make the resistance to different plant parasitic pathogens. Biocontrol agents cause physical, physiological, and biochemical changes that result in the production of a wide range of defense-related compounds and secondary metabolites. Kloepper *et al.* (1992) [16] states that *PR* proteins such chitinase, 1-3 glucanes, phenolics, phenylalanine ammonia lyase, superoxide dismutase, peroxidase, and phytoalexins are among these defense chemicals. One of the most fundamental and important factors in the host-pathogen relationship is defense enzymes. The first enzyme produced in the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), leads to the biosynthesis of a diversity of phenols.

Plant disease resistance reactions begin with the activation of the phenylpropanoid metabolism, which leads to the creation of a variety of defense-related chemicals such as antimicrobial phytoalexins and lignin (Hahlbrock and Scheel, 1989) [9]. The current study was designed to investigate the induction of defense enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenyl alanine ammonia lyase (PAL), and phenols in groundnut plants treated with bioagents and challenged with stem rot.

Materials and Methods

Colorimetric Assay

Trichoderma spp. was applied as seed treatment and soil application and mahua oil cake was applied as soil amendment. Carbendazim was used as chemical check both seed and soil. Two untreated controls were maintained as inoculation and uninoculation of *Sclerotium rolfsii* Similarly, ten treatments were applied as per schedule given in Table.1. The biochemical constituents of peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase and phenols was determined through colorimetric assay. It was determined in groundnut plants which were challenge inoculated with *S. rolfsii* followed by application of *Trichoderma* spp., mahua oil cake and chemical fungicide. *Trichoderma* spp. *viz.*, *Trichoderma longibrachiatum* T(SP)-20, *Trichoderma asperellum* T(AR)-10 and mahua oil cake used against the virulent isolate of *S. rolfsii*

Peroxidase assay

In a pre-cooled pestle and mortar, one gram of fresh leaf tissue was mashed using one ml of 0.1M phosphate buffer at pH 7.0. The homogenate was centrifuged at 15000 rpm at 4°C for 15 minutes. The supernatant was served as a source of enzyme. About 1.5 ml 0.05M pyrogallol, 0.5 ml of one per cent H_2O_2 and 0.1 ml of enzyme extract was used for preparation reaction mixture. The reaction mixture absorbance was measured at 420 nm for every 30 seconds up to 3 minutes at room temperature (28 ±2°C). The boiled enzyme preparation served as blank. The enzyme activity was measured as a change in the reaction mixture's absorbance $min^{-1}g^{-1}$ of the leaf (Hammerschmidt *et al.*, 1982) [11]

Polyphenol oxidase Assay

One gram of fresh leaf sample was ground in one ml of 0.1M sodium phosphate buffer, (ph 6.5). The homogenate was centrifuged at 15000 rpm in 4 °C for 15 minutes, and the supernatant was employed as an enzyme source. About 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.1 ml of enzyme extract was used to made up the reaction mixture. After adding 0.2 ml of catechol (0.01 M) to the mixture, the reaction was started. The activity was measured as a change in absorbance at 495 nm from every 30 second intervals for three minutes, and measured the enzyme activities in terms of the change in absorbance per minute per gram of leaf (Harel *et al.*, 1965) [12].

Phenylalanine ammonia-lyase Assay (PAL)

For phenylalanine ammonia lyase assay (PAL),about five ml of cold 25mM Borate HCL buffer (pH 8.8) containing 5mM mercaptoethanol (0.4 ml per litre)was used to homogenize the500 mg of plant leaves. The homogenate was centrifuged at 15000 rpm for 15 minutes and the supernatant served as an enzyme source. About 0.2 ml of enzyme extract, 1.3 ml of water and 0.5 ml of borate buffer used to make up the reaction mixture. About one ml of 12Mm L-Phenylalanine was added and initiated the reaction. The reaction mixture was incubated for one hour at 32℃. The reaction was stopped with the addition of 0.5 ml of 2N HCL. The 2N HCL and phenylalanine was added after the blank run. The absorbance was measured at 290 nm. The enzyme activities were measured in µ mol of cinnamic acid per minute per gram of leaf (Dickerson *et al.*, 1984) [6].

Phenol Assay

Phenolic content present in clusters bean leaf was estimated by the procedure given by (Anand *et al.*, 2010) [1]. After adding 10 ml of 80 percent methanol to 1g of groundnut leaf sample from each treatment, it was grounded well in a sterile pestle and mortar. The twenty minutes homogenate was centrifuged at 10000 rpm. Each residue was dissolved in 5 ml of sterile distilled water and the supernatant was collected separately. From this, 0.2 ml was withdrawn and the volume was increased to 3 ml with sterile distilled water by adding 0.25 ml of (1N) Folin-ciicaltteau reagent followed by 1 ml of sodium carbonate (20%) was added after 3 minutes, and mixed properly. The tubes were boiled for 1 minute and then cooled. The absorbance of each mixtures was measured at 725nm against a reagent blank. The phenolic content of the leaves was measured in mg of catechol per gram of leaf.

Results and Discussion

Thus, the production of defense related enzymes *viz.*, peroxidase (PO), polyphenoloxidase (PPO), Phenylalanine ammonia lyase (PAL), and phenol in *Trichoderma* spp. and mahua oil cake treatment were estimated (Fig.1).

Induction of defense related enzymes by biocontrol agents in groundnut Induced resistance is a state of enhanced defensive capacity against broad spectrum of pests and pathogens developed by a plant when appropriately stimulated (Van Loon*et al.*, 1998)[23]. Generally, the induced systemic resistance (ISR) mechanism of plant is effective against several disease causing plant pathogens including viruses and are associated with the production of pathogenesis related (PR) proteins through a salicylic acid dependent processes (Hammerschmidt, 1999)[10]. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. ISR mediated through bio-control agent's resulted on lignification and with increased activities of defense gene products that synthesized via phenyl propanoid pathway (Boller and Mauch, 1988; Kloepper*et al.*, 2004) [5, 15].

Peroxidase (PO)

The treatment with soil application of ST with (T(SP)-20 +T(AR)-10) @ 4 g/kg+ SA of mahua oil cake @ 100g/pot + SA of(T(SP)-20 +T(AR)-10) @50 g/pot(B.S)+ T(SP)-20 +T(AR)-10) @ 50g/pot on 30 DAS challenge inoculated with *S. rolfsii* recorded the maximum peroxidase activity(0.291 changes in absorbance/minute/gram of leaf tissue) (Table. 2; Fig.2). Gajera *et al.* (2015) [7] reported the similar induction of pathogenesis related defense response to *Trichoderma viride* application against rot infection in groundnut. Selim *et al.* (2017) [21] reported that the consortia of antagonistic biocontrol agents produced maximum peroxidase activity. Thus, enhanced induction of peroxidases in biocontrol agents treated plants might have been a part of ISR induction which eventually reduced the pathogen infection caused by *S. rolfsii* in groundnut.

Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) enzymes use molecular oxygen to catalyze the oxidation of monophenolic and orthodiphenolic compounds. PPO is a copper containing enzyme that oxidizes phenolics to highly toxic quinines and involved in the terminal oxidation of diseased plant tissue and role in disease resistance. In the present study, treatment with ST with (T(SP)-20 +T(AR)-10) @ 4 g/kg+ SA of mahua oil cake @ 100g/pot + SA of(T(SP)-20 +T(AR)-10) @50 g/pot(B.S)+ T(SP)-20 +T(AR)-10) @ 50g/pot on 30 DAS challenge inoculated with *S. rolfsii* recorded the maximum polyphenol oxidase activity (0.298 changes in absorbance/minute/gram of leaf tissue) (Table. 2; Fig.2). Kumar *et al.* (2015) [17] also reported the higher accumulation of enzymes PPO in tomato seedlings treated with *T. harzianum* strain OTPB3+*B. subtilis* strain OTPB1 consortium followed by *P. putida* foliar spray after challenge inoculation with *P. infestans*. Similarly, (Azadi*et al.*, 2016) [3] studied the mechanism of *Beauveria bassiana* against *Rhizoctonia* disease of tomato and enunciated that the production of polyphenol oxidase significantly reduced the incidence of disease.

Phenylalanine ammonia lyase (PAL)

In the present study, a significant difference of phenylalanine ammonia lyase activity was observed in various treatments. Gradual increase in phenylalanine ammonia lyase activity was observed in all the treatments. The treatment ST with (T(SP)-20 +T(AR)-10) @ 4 g/kg+ SA of mahua oil cake @ 100g/pot + SA of(T(SP)-20 +T(AR)-10) @50 g/pot(B.S)+ T(SP)-20 +T(AR)-10) @ 50g/pot on 30 DAS recorded the maximum activity of phenylalanine ammonia lyase (0.296 micro mole of transcinnamic acid/minute/gram of leaf tissue) (Table. 2; Fig.2). This is in corroboration with the work of Radjacommare *et al.* (2004) [19] they reported that seedling dip with talc based formulation of *P. fluorescens* induced the activity of PAL in finger millet leaves against blast disease. Kamalakannan (2004) [14] reported the increased activity of PAL, PO, PPO and total phenolics in the bioagents (*Trichoderma viride*, *Pseudomonas fluorescens* and *Bacillus subtilis*) pretreated peppermint plants challenged with *R. solani*. Yasin *et al.* (2017) [24] reported the production of PAL and suppression of leaf spot disease caused by *Alternaria brassicae*. Thus, enhanced induction of PAL in bacterial bioformulation treated plants might have been a part of ISR which eventually reduced the pathogen infection caused by *S. rolfsii* upon artificial inoculation under pot culture and field applications.

Phenol

This study revealed that the treatment T_7 performed best in stimulating phenol activity (781 microgram of catechol/gram of leaf tissue) and it was followed up with the treatments T_7 and T_5 (Table. 2; Fig.2). Ram *et al.* (2019)[20]reported a comparable rise of total phenolic content to 3.10 times after the consortium application of *Trichoderma harzianum* TNHU27 and *Pseudomonas aeruginosa* PJHU15 upon challenge inoculation with collar rot pathogen in cauliflower.

Fig 1: Induction of defense enzymes under pot culture conditions

Table 1: Effect of *Trichoderma* spp., mahua oil cake and chemicalson the induction of defense related enzymes activity of against stem rot of groundnut

T. No.	Treatments	Mean of 0, 3,5,7 and 9 Days after last application(DALA) Changes in absorbance/min/gram of leaf tissue *			
		PO	PPO	PAL	PHENOL
T_1	Seed treatment (ST) with *Trichoderma longibrachiatum* T(SP)-20@ 4 g/kg of groundnut seed+ Soil application(SA)of *Trichoderma longibrachiatum* T(SP)-20@50g/pot before sowing (B.S)	0.205	0.220	0.226	562
T_2	ST with *T. asperellum* T(AR)-10 @ 4 g/kg+ SA of *T. asperellum* T(AR)-10@ 50g/pot (B.S)	0.198	0.226	0.215	543
T_3	Soil amendment(basal) with Mahua oil cake @ 100g/pot	0.237	0.217	0.202	625
T_4	ST with (T(SP)-20 +T(AR)-10)@ 4 g/kg+ SA of (T(SP)-20 +T(AR)-10) @ 50g/pot (1:1) ratio(B.S)	0.201	0.265	0.252	654
T_5	ST with T(SP)-20@ 4 g/kg+S Amahua oil cake @ 100g/pot + SA of T(SP)-20 @ 50g/pot (B.S)+SA of T(SP)-20 @ 50g/pot on 30 DAS	0.256	0.285	0.283	753
T_6	ST with T(AR)-10 @ 4 g/kg+ SA of mahua oil cake @ 100g/pot + SA of T(AR)-10@50g/pot (B.S)+SA of T(AR)-10@ 50g/pot on 30 DAS	0.251	0.254	0.272	735
T_7	ST with (T(SP)-20 +T(AR)-10) @ 4 g/kg+ SA of mahua oil cake @ 100g/pot + SA of(T(SP)-20 +T(AR)-10) @50 g/pot(B.S)+ T(SP)-20 +T(AR)-10) @ 50g/pot on 30 DAS	0.291	0.296	0.298	781
T_8	ST with Carbendazim at 2g /kg +Soil drenching with Carbendazim @0.2% (Chemical check)	0.189	0.211	0.215	556
T_9	Inoculated control	0.125	0.165	0.123	203
T_{10}	Healthy control	0.140	0.180	0.182	407
CD(*P*=0.05)		0.014	0.012	0.008	22.739

*Mean of three replication

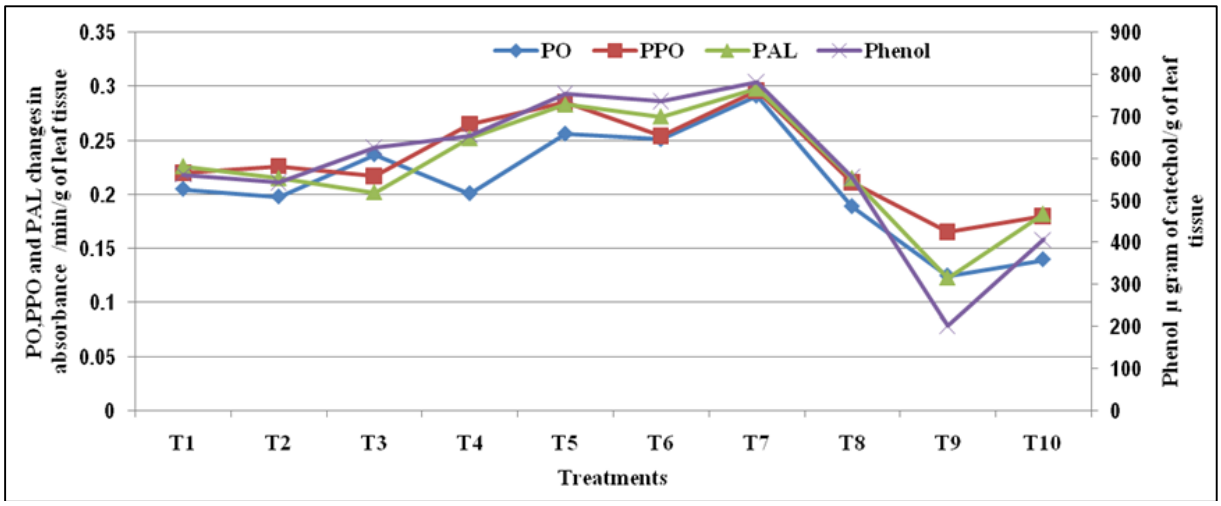

Fig 2: Effect of *Trichoderma* spp., mahua oil cake and fungicide on PO, PPO, PAL and Phenol content in groundnut

Conclusion

The combined application of *Trichoderma* spp. [*Trichoderma longibrachiatum* + *Trichoderma asperellum*] and organic amendment (mahua oil cake) significantly induced and upregulated the defense enzymes *viz.*, PO, PPO, PAL and Phenol on *Arachishypogaea* plants. Which prominent enzymes boost the host immune and fight against the *S. rolfsii* pathogen. These positive treatment will forward to the field condition to overcome stem rot incidence of groundnut.

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