Effect of mixture of *Trichoderma* isolates on biochemical parameters in leaf of *Macrophomina phaseolina* infected brinjal

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**Abstract**

A mixture of *Trichoderma harzianum* NBRI-1055 (Fx) and *T. harzianum* BHU-99 (Th) was evaluated for their efficiency to induce systemic resistance during three way interaction among brinjal-*Trichoderma-Macrophomina phaseolina*. Total phenol content (TPC), defence related enzymes Phenylalanine ammonia-lyase (PAL), Peroxidase (PO), Polyphenol oxidase (PPO) and PR proteins (PR-2 and PR-3) were recorded. Total phenolic content was recorded 12.82 times and 1.8 times higher in *Trichoderma* mixture treated-pathogen challenge (Fx-Th-Pth) treatment than in untreated healthy control and untreated pathogen challenged (Pth) plants respectively after 72 hr pathogen inoculation (hapi). Defence related enzymes PAL 4.54 times higher, 48hapi, PO, 3.96 times higher, 72hapi and PPO 8.1 times higher, 72hapi in Fx-Th-Pth treatment than untreated healthy control, and the PR-proteins such as PR-2, 2.15 times and PR-3, 2.16 times higher, 72hapi than untreated healthy control. The results showed that a mixture of *Trichoderma* (Fx+Th) performed better than single isolate.

**Key words**

Induction of defence proteins has been correlated with defense against pathogen invasion in cucumber (Rasmussen, 1991), green gram (Ramanathan et al., 2000), tobacco (Beaudoin–Eagan and Thorpe, 1985) and tomato (Bashan et al., 1985) and also with elicitors (Mandal, 2010) in brinjal. Root colonization by Trichoderma spp. frequently associated with the induction of both local and systemic resistance to pathogen attack (Shores et al., 2010). Indeed, induction of systemic resistance against fungal and bacterial pathogens in diverse dicot and monocot plants has been demonstrated (Harman et al., 2004; Yedidia et al., 2003). Extensive communication occurs between plants and Trichoderma during the early stages of their association. The main objective of the present study was to assess the effect of the mixture of two different Trichoderma isolates in comparison to single isolate on biochemical parameters such as total phenol content, defence related enzymes, PR proteins in leaf of Macrophomina phaseolina infected brinjal plants.

Materials and Methods

Isolation of pathogen and biocontrol agents (BCAs):

Pathogen (Pth) M. phaseolina, a causal agent of root rot was isolated from infected brinjal plant. Trichoderma harzianum NBRI-1055 (Fx) obtained from National Botanical Research Institute and T. harzianum BHU-99 (Th) isolated from B.H.U., Varanasi, were used in this study. All the fungi were grown and maintained on potato dextrose agar (PDA) and stored at 4°C.

Greenhouse experiment: Brinjal (Solanum melongena; variety-Sweekar-321) seeds were obtained from the market and grown under sterilized soil condition. Thirty five days old seedlings were treated with t alc based formulation of Fx and Th (2x10⁶CFU ml⁻¹). They were planted in a seedling tray containing sterile soil. Control seedlings were treated with talc powder. The experimental design was a randomized block design. There were eight treatments in each assay including a non-challenged healthy control, challenged pathogen control, BCAs (Fx and Th) treated alone and their mixture (1:1 ratio), challenged with and without pathogen. For pathogen inoculation, a bit of the fresh, pure culture containing active mycelium of M. phaseolina was cut with the help of 1 cm diameter cork borer and placed adjacent to plant at soil level. Leaves from each treatment were collected at different time intervals consisting of 24, 48, 72 and 96 hr after pathogen inoculation (hapi). The Samples were immediately transferred to ultra freezer (-80°C) and used for further biochemical analysis. Each treatment was replicated three times.

Total phenol content (TPC): Approximately 100 mg of fresh leaf samples were placed in 5.0 ml of 95% ethanol. Each leaf sample was then homogenized and centrifuged at 13000 x g for 10 min. One milliliter of the supernatant was removed and mixed with 1.0 ml of 95% ethanol and 5.0 ml of SDW. To this 0.5 ml of 1N Folin-Ciocalteau reagent was added. After 5 min, 1.0 ml of 5% Na₂CO₃ was added, the reaction mixture was allowed to stand for 60 min and the absorbance at 725nm was recorded using “Varian Cary 50” Vis-UV spectrophotometer (Zeng and Shetty, 2000). Absorbance values were converted to mg gallic acid equivalent (GAE) g⁻¹ fresh weight (FW).

Phenylalanine ammonia-lyase (PAL): Plant samples (0.5 g) were homogenized in ice cold 0.1M sodium borate buffer (5.0 ml; pH 7.0) containing mercurpentoethanol and centrifuged at 16000 x g at 4°C for 15 min. Reaction mixture containing the enzyme extract (0.2 ml) was incubated with 0.2M borate buffer (0.5 ml; pH 8.7), distilled water (1.3ml) and 0.1M L-phenylalanine (0.5 ml) were added to the same buffer and then incubated for 30 min at 32°C. The reaction stopped by addition of 1M trichloroacetic acid (0.5 ml) as described by Brueske (1980). The absorbance was recorded at 290nm and the amount of cinnamic acid formed was calculated and was expressed as µM trans-cinnamic acid mg⁻¹ FW.

Peroxidase (PO): For PO activity, plant samples (0.5 g) were homogenized in 0.1 M phosphate buffer (5.0 ml; pH 7.0), at 4°C. The homogenate was centrifuged at 16000 x g at 4°C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 0.05 M pyrogallol (0.5 ml) enzyme extract and 1% H₂O₂ (0.5 ml). The changes in absorbance at 420nm were recorded at 30 sec intervals for 3 min (Hammerschmidt et al., 1982). The enzyme activity was expressed as changes in the O.D. min⁻¹ mg⁻¹ protein.

Polyphenol oxidase (PPO): Plant samples (0.5g) were homogenized in 0.1M phosphate buffer (5.0 ml; pH 6.5). The homogenate was centrifuged at 16000 x g for 30 min at 4°C. The supernatant was used directly in the enzyme assay. The reaction mixture contained 0.01M catechol (0.4 ml) in 0.1M sodium phosphate buffer (3.0 ml; pH 6.5) and enzyme extract (0.4 ml). Change in absorbance at 495nm was recorded at 30 sec interval for 3 min. (Gauillard et al., 1993). PPO activity was expressed as a change in O.D. min⁻¹ mg⁻¹ protein.

PR-2 and PR-3 proteins: For PR-2 protein (β-1,3 glucanase), plant samples (0.1 g) were homogenized in 0.05 M sodium acetate buffer (2.0 ml, pH 5.0) and centrifuged at 16000 x g for 15 min at 4°C. The reaction mixture consisted of dialyzed enzyme solution (0.25 ml), 1 M sodium acetate buffer (0.3 ml; pH 5.3) and 4% laminarin (0.5 ml). The reaction was carried out at 40°C for 60 min and stopped by adding dinitro salicyclic (DNS) acid (0.375 ml), then heated for 5 min in boiling water bath, vortexed and finally absorbance was recorded at 500 nm (Pan et al., 1991).
enzyme activity was expressed as µg glucose released min⁻¹ mg⁻¹ protein.

For PR-3 protein (chitinase activity), colloidal chitin was prepared by acetylation of glycol chitosan following the method of Trudel and Asselin (1989). Plant samples (0.1 g) were homogenized in 2.0 ml sodium citrate buffer (pH 5.0) and centrifuged at 16000 x g for 15 min at 4°C and the supernatant was used for the study. N-acetylglucosamine (GlcNAc) was used as standard and enzyme activity was expressed as nmols GlcNAc equivalent min⁻¹ mg⁻¹ protein. The protein content was measured by the method of Lowry et al. (1951).

Statistical analysis: Figures from different experiments showed mean and bars indicating standard deviation (SD) of three determinations. Collected plant material was randomly divided into three parts and analyzed by analysis of variance (ANOVA) (Gomez and Gomez, 1984).

Results and Discussion

The Total phenol content (TPC) was recorded significantly higher in all the treatments when treated with Trichoderma isolates as compared with the untreated control (Fig. 1A). Seeding treated with NBRI-1055 + BHU-99, challenged with the pathogen (Fx-Th-Pth) showed maximum TPC, which was 1.8 times higher than the corresponding pathogen challenged control, while it was 12.8 times higher than untreated unchallenged control at 72 hapi. Higher concentration of TPC was recorded in treatments where Fx and Th were treated independently as well as along with pathogen challenged (Fx-Pth and Th-Pth) treatment. Furthermore, suppression of root rot of brinjal caused by M. phaseolina might be due to higher accumulation of TPC. Increase in phenolic compounds can be correlated with deposition of lignin by Trichoderma-treated plants. These observations resembled eminently with the findings in maize roots elicited with T. harzianum strain T-22 (Bigrimiana et al., 1997). Treatment with bioagents and their effector molecules elevates the level of flux through phenylpropanoid pathway, thereby supplying carbon skeletons for phenolics, which are precursor molecules of lignin (De Ascensao and Dubery, 2000).

In Fig. 1 (B and C) it can be clearly seen that Fx and Th treated seedlings unchallenged with pathogen did not lead to much increase in PAL and PPO activities and also BCAs untreated pathogen challenged and healthy control respectively at 72 hapi. The rise in PO activities under the influence of BCAs treatments was transient and showed a gradual decreasing trend after 72 hapi as compared to Pth treatment (pathogen treated), in which the activity decreased drastically after that. These activities in leaves of Trichoderma treated plants alone remained unchanged during the experimental period but as compared with the healthy control, the activity was higher. In another study, gene Pal1 was found to be upregulated by Trichoderma, which encodes for PAL (Shoresh and Harman, 2008). Furthermore, Lamba et al. (2008) and Singh et al. (2011) demonstrated that sunflower inoculated with Trichoderma also increased PAL activity. Viterbo et al. (2007) showed that the 18-residue peptibols induced expression of phenylalanine ammonia lyase (pal), hydroperoxide lyase (hp) and peroxidase, which are involved in the production of antimicrobial compounds and concomitantly increase in systemic increase in antimicrobial compound content in the plants. In the present study, rapid and transient increase in PAL level in the treated roots is in agreement with earlier reports (Harman et al., 2004, Karthikeyan et al., 2006). Plants inoculated with pathogen alone, the PAL activity declined drastically 72 hapi whereas in case of Trichoderma treated treatment increased PAL activity might have prevented the fungal invasion, and thus, the activity was maintained at higher levels than the control during the experimental period. Increased activity of cell wall bounded PO has been elicited in different plants such as tomato (Ramamoorthy et al., 2002), cucumber (Chen et al., 2000) and rice (Nandakumar et al., 2001) due to pathogen infection. In bean, rhizosphere colonization by various biocontrol agents induced PO activity (Zdor and Anderson, 1992). In addition to its plant growth promoting and antifungal activities, T. harzianum has the potential for inducing defence-related enzymes and phytoalexins (Harman et al., 2004, Karthikeyan et al., 2006; Yedidia et al., 1999). A recent report indicated that the mixed tado-based formulation of T. viride and P. fluorescens containing chitin when applied as soil treatment in coconut palm induced significant increase in the PO and PPO activities and proved antifungal against Ganoderma (Karthikeyan et al., 2006). The results obtained in this study on PO and PPO activities indicate that the brinjal plants responded actively to Trichoderma treatment through rapid induction or activation of specific isoforms. Maximum activities of these enzymes were induced in brinjal upon treatment with Fx and Th-pathogen, which might have contributed to induce resistance against M. phaseolina invasion.

β-1, 3-glucanase (PR-2) and chitinase (PR-3) activities has been related with plant defence mechanism against pathogen infection through their ability to degrade fungal cell walls. Fig. 1 (E and F) clearly indicate that treatment Fx-Th-Pth seedlings showed significantly increased level of β-1, 3-glucanase and chitinase respectively, followed by Fx-Pth treatment over control. Induction of these PR proteins was
observed after 24 hapi but maximum activity was recorded at 72 hapi. In case of BCAs untreated pathogen challenged treatments, the activities decreased sharply at 72 hapi, while in Trichoderma treated treatments challenged with the pathogen gradual decrease in the activities was recorded. PR-proteins are host coded proteins induced only in pathological or related situations (Harman et al., 2004). More recent reports have shown the induction of PR proteins as a result of colonization by non-pathogenic/beneficial fungi (Karthikeyan et al., 2006). β-1,3-glucanase and chitinase are two important PR-proteins which play major role in plant protection against pathogen ingress. Maize seedlings from *T. harzianum* T22 treated seeds had elevated levels of proteins and increased activity levels of chitinase and β-1, 3-glucanase while these activities were further increased when the plants were coinfected with *Pythium ultimum* (Harman et al., 2004). It is also common for some PR proteins such as PR-2 and PR-3 to display synergism. In the present investigation, PR-2 and PR-3 levels significantly increased in Trichoderma treated brinjal plants and the highest activity was observed in the challenged plant treated with a mixture of

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**Fig.1:** Effect of *Trichoderma* isolates and their combination on induction of defense-related compounds in brinjal leaves against *Macrophomina phaseolina* on a time course after pathogen inoculation, bars indicated SD. (A) Total phenol content, (B) Phenylalanine ammonia-lyase, (C) Polyphenol oxidase, (D) Peroxidases, (E) β-1,3-glucanase and (F) Chitinase.
Combined effect of Trichoderma isolates on M. phaseolina

Trichoderma (Fx+Th) treatment. Colonization of cucumber roots by T. harzianum strain T-203 was correlated with induction of PR proteins resulting in induced systemic disease resistance against broad range of pathogens (Yedidia et al., 2003).

Trichoderma spp. as potential BCAs have recently led to the proposal that besides their recognized antifungal properties, such organisms could also act as elicitors of plant defence reactions, thereby promoting the expression of plant defence related metabolomes (Yedidia et al., 2003, Segarra et al., 2007). Biological control of M. phaseolina with T. harzianum on several plants has been reported in many studies over the last few years. In the present study, the effect of a mixture of T. harzianum (Fx+Th) on biochemical parameters in leaves of M. phaseolina infected brinjal plants was studied. It is crucial to understand the mechanism of bioprotection conferred by Trichoderma and to develop innovative strategies to control root rot caused by M. phaseolina. Increased protection against M. phaseolina infection has been observed in plants grown in the presence of Trichoderma isolates and their combination. These results are of particular relevance since they highlight the properties of Trichoderma isolates, which is capable of induced systemic resistance (ISR) against M. phaseolina. From the present study it can be postulated that seedling dip treatment of brinjal plants with mixture of Trichoderma isolates, resulted in rapid and higher accumulation of phenolics, defence-related enzymes and PR-proteins, which showed synergistic effects. Enhanced defence related proteins and enzymes in the mixture of Fx and Th could explain greater protection than single isolates against pathogen. These enzymatic activities offer an alternative mechanism of defence which was seen during plant-microbe interaction. Future efforts should focus on characterizing the chemically complex nature of these compounds and proteins.

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References


Segarra, G., E. Casanova, D. Bellido, M.A. Odena, E. Oliveira and I.