



Effect of mixture of *Trichoderma* isolates on biochemical parameter in tomato fruits against *Sclerotinia sclerotiorum* rot of tomato plant

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Abstract

Experiments revealed that a mixture of *Trichoderma harzianum* isolates, BHU51 and BHU105 showed lowest mean disease rating (MDR) of 1.70 and 1.62 % and per cent disease reduction (PDR) by 41.00 and 44.84% during the year 2008-09 and 2009-10, respectively. Shoot length, chlorophyll content and yield was also recorded highest in the mixture of BHU51+ BHU105 treatment followed by single *Trichoderma* treatments while lowest was found in pathogen inoculated control. The nutritional quality such as lycopene content, protein and carbohydrate was recorded highest in BHU51+ BHU105 treatment. The antioxidant activity and free radical scavenging ability of tomato fruit extract was also recorded. The results indicated that maximum 1,1-diphenyl -2-picryl-hydrazyl (DPPH) radical scavenging activity (47.86%), ferrous ion chelation capacity (50.81%), hydroxyl radical scavenging ability (49.18%) and reducing power 0.203 O.D. at wavelength 700 nm was maximum for BHU51+ BHU105 treatment, followed by single *Trichoderma* treated treatments while these were recorded lowest in pathogen inoculated control.

Key words

Biocontrol, Nutritional quality, *Sclerotinia sclerotiorum*, Tomato, *Trichoderma*

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop in the world after potato, with annual production of around 16826000MT (FAOSTAT, 2011). Crops lost due to plant diseases were estimated 10% worldwide each year which can lead to considerable financial loss to the farmers (Strange and Scott, 2005). Many soil borne pathogens cause huge loss to the vegetable crops. *Sclerotinia sclerotiorum* a sclerotia forming soil borne pathogen is a major threat to production of tomato. It is one of the most devastating and cosmopolitan plant pathogen that infects over 400 species of the plants worldwide (Boland and Hall, 1994). Management of this pathogen is very difficult because of its ecological behaviour, extremely broad host range and high survival rate of sclerotia under various environmental conditions. An efficient strategy for the management of this pathogen is urgently needed. Biological control offers environmental friendly protection of plants from the plant pathogens (Harman, 2011; Singh *et al.*, 2011; Singh and Singh, 2012). Some *Trichoderma* strains can interact directly with

roots, increasing plant growth potential, resistance to disease and tolerance to abiotic stresses (Joshi *et al.* 2012; Hermosa *et al.*, 2012). *Trichoderma* species are worldwide in distribution and present in diverse habitats, acts upon pathogens by applying various mechanisms like mycoparasitism, nutrient competition and antibiosis (Dennis and Webster, 1971) etc. Numerous researchers have reported that using combination of two or more than two compatible microorganisms may provide improved disease control over single biocontrol application (Yobo *et al.*, 2009; Pandey and Maheshwari, 2006; Saman, 2009). It enhances the level and consistency of control by providing multiple mechanisms of action and it is also effective over a wide range of environmental conditions (Srivastava *et al.*, 2010). *Trichoderma* is one of the potent bioagents which is now used for the management of many soil borne diseases, including *S. sclerotiorum*. The use of consortium of compatible *Trichoderma* isolates may show increased biocontrol potential as compared to single isolate. *Trichoderma* spp. can improve food security by reducing the need of pesticides and can provide an economic advantage for farmers.

In light of the above, the objective of the present study was to investigate the potential of *Trichoderma* mixture on the growth, yield and management of *Sclerotinia sclerotiorum* rot of tomato as compared to single isolate under field condition. Effect of these treatments were also evaluated for nutritional quality and antioxidant activity of tomato fruits extract.

Materials and Methods

Trichoderma isolates, *Trichoderma harzianum* BHU51 (Gen Bank accession no. JN 618343.), *Trichoderma harzianum* BHU105 (Gen Bank accession no. JN 618344), and their consortium (BHU51+BHU105) and the pathogen *Sclerotinia sclerotiorum* were used in this study. The study was conducted in the agricultural field (4m² plots) of Banaras Hindu University, Varanasi during 2008-09 and 2009-10. Seeds of tomato (variety-Navodya) were purchased from the local market for study. In the present study, four treatments were used which were as follows: T1-Control, inoculated with pathogen (*Sclerotinia sclerotiorum*), T2-*Trichoderma* BHU51+ BHU105 treated seedlings and inoculated with pathogen (BHU51+BHU105+Pth), T3-*Trichoderma* BHU51 treated seedlings and inoculated with pathogen(BHU51+Pth) and T4- *Trichoderma* BHU105 treated seedlings and inoculated with pathogen(BHU105+Pth).

Assessment of disease incidence, growth and yield : *S. sclerotiorum* inoculums grown on sand maize media were inoculated in the field according to the treatments. The symptom of rot and wilting caused by *S. sclerotiorum* was recorded at regular interval and the severity index of plants grown in the field was calculated at 0-4 scale as described by Grau *et al.* (1982) and the mean disease rating (MDR) and per cent disease reduction (PDR) was calculated by the formula given by Pal *et al.* (2001). Shoot length was measured 60 days after transplanting (DAT); yield was recorded at regular intervals and expressed in kg per plot (4m²). Chlorophyll content was recorded at 60 DAT by the method described by Arnon (1949) and was expressed in mg g⁻¹ f.wt.

Assessment of nutritional quality and antioxidant activity : Nutritional quality and antioxidant activity of tomato fruits were assessed from the 2nd year field trial (2009-2010) only. The lycopene content was measured by the method of Fish *et al.* (2002) and was expressed in mg kg⁻¹ f.wt. and ascorbic acid was determined by the method described by Klein and Perry (1982) and was expressed in mg100g⁻¹ f.wt. Total carbohydrate content in tomato fruits was determined by anthrone method (Hedge and Hofreiter, 1962) and total phenolic content (TPC) was measured by using Folin-Ciocalteu's phenol reagent (Ragazzi and Veronese, 1973) and values were expressed in mg gallic acid equivalent (GAE) g⁻¹ d.wt., while protein content was determined by Lowery method (Lowry *et al.*, 1951) and was expressed in g100g⁻¹ f.wt.

Five gram dry powdered tomato fruit was extracted with 50 ml 50% methanol: water. The filtrate was evaporated to

dryness under vacuum in a rotary evaporator. All the extracted solid material was stored at -20°C and was used to study the antioxidant activities.

Free and hydroxyl radical scavenging activity : Free radical scavenging activity or hydrogen donating ability was measured by 1,1-diphenyl -2-picryl-hydrazyl (DPPH) radical scavenging method described by Brand-Williams *et al.* (1995). DPPH is a stable radical which is reduced in the presence of antioxidant active substances. A 0.1 ml methanolic solution of tomato extract (1 mgml⁻¹) was added to 2.9 ml of methanolic solution of DPPH. Reduction of DPPH radical was measured by monitoring the decrease of absorbance at 515 nm for 60 min and was expressed in percent radical scavenging ability. Iron chelation effect was determined according to the method of Dinis *et al.* (1994). Briefly, 100µl tomato extract solution (5 mgml⁻¹) was taken and made upto volume of 1ml, then mixed with 3.7 ml of methanol and 0.1 ml 2 mM l⁻¹ ferrous chlorides, and reaction was initiated by addition of 0.2 ml, 5 mM l⁻¹ ferrozine. After 10 min of incubation at room temperature, the absorbance was taken at 562 nm. Chelation ability was calculated by the following equation:

$$\text{Chelation capacity (\%)} = \left\{ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right\} \times 100$$

Hydroxyl radical scavenging activity was determined by deoxyribose assay that determines the constant rate for reaction between antioxidants and hydroxyl radicals. Non-site specific scavenging assay was estimated following by the method of Halliwell *et al.* (1987). Briefly, 0.5ml tomato fruit extract was mixed with 2.5ml of reaction buffer (100 µM FeCl₃, 104 µM EDTA, 1.5 mM H₂O₂, 2.5 mM deoxyribose and 100 µM L-ascorbic acid, pH7.4) and incubated for 1hr at 37 °C. One ml of 2-thiobarbituric acid (1%) in 50 mM NaOH and 1ml of 2.8% trichloroacetic acid were added and heated at 80 °C for 30 min. The mixture was cooled on ice and the absorbance was measured at 532 nm, using UV Vis spectrophotometer. Reducing power of the tomato extract was determined by the method described by Oyaizu (1986). A stock solution was made (5 mg ml⁻¹). Briefly 500 µl from this solution was used for the study and sodium phosphate buffer, potassium ferricyanide, trichloroacetic acid and ferric chloride were used for the reaction. The absorbance was read at 700 nm, greater absorbance indicated higher reducing power.

Experiments were designed as randomized block design, values from different experiments shown in tables were mean ± standard deviation (SD) of at least three replications and analyzed by analysis of variance (ANOVA). The data were presented as mean of two year experiments. The treatment means were compared with level of significance at *p* = 0.05 (Gomez and Gomez, 1984) and Duncan multiple range test (DMRT) by using SPSS.

Results and Discussion

In field experiments, the seedlings treated with *Trichoderma* showed significantly higher shoot length, chlorophyll content and yield than the untreated pathogen inoculated control (Table 1) during both the year 2008-09 and 2009-10. Results revealed that the maximum shoot length (80.67 cm and 78.67 cm at 60 DAT) was recorded in the consortium (BHU51 + BHU105) treated treatment followed by single *Trichoderma* treated treatments and the yield of consortium treatment (11.428 kg plot⁻¹ and 11.665 kg plot⁻¹) was found significantly higher than other treatments (Table 1). It may be due to the synergistic effect of the mixture of *Trichoderma* isolates. Variation in yield was due to the effect of *Trichoderma* and pathogen. Srivastava *et al.* (2010) also reported that consortium of compatible microorganisms enhanced the growth parameters and yield of tomato and legumes in the field experiments that confirms the present findings. Chlorophyll content was also recorded maximum in the consortium (BHU51 + BHU105) treated plants (1.037 mg g⁻¹ f.wt.) followed by individual *Trichoderma*, BHU105 (0.986 mg g⁻¹ f.wt.) and BHU-051 (0.980 mg g⁻¹ f.wt.) treated plants respectively, while it was found lowest (0.757 mg g⁻¹ f.wt.) in the untreated pathogen inoculated control plants during the year 2008-09. During 2009-10 experiment, chlorophyll content was also found significantly higher in *Trichoderma* treatments than control. Low amount of chlorophyll content in pathogen inoculated control might be due to reduced supply of essential component from the soil (Table 1). Abou *et al.* (2011) also reported that application of *Paenibacillus polymyxa* and *Bacillus megaterium* increased the photosynthetic pigments and consequently plant growth and production of tomato that supported our findings. Table 1 clearly shows that maximum MDR was observed in *S. sclerotiorum* inoculated, untreated control (2.99 and 3.23) while minimum (1.70 and 1.85) was recorded in

consortium treated treatment during the year 2008-09 and 2009-10 field experiment. In single *Trichoderma* isolate BHU51 and BHU105 treatment, MDR was recorded 1.95, 1.85% and 2.01, 1.96% during both the year (Table 1). The findings of this study clearly showed that *Trichoderma* treatments considerably reduced the disease incidence under soil infestation condition by *S. sclerotiorum*. Using *Trichoderma* mixture for seedling treatment found to be more effective, as a significant reduction in disease was noted (41.00%), than using single *Trichoderma* BHU51 and BHU105 treatment during the year 2008-09. A similar trend was also observed in the second field experiment (2009-10), in which 44.84, 40.83 and 38.72% reduction in disease was recorded in BHU51 + BHU105, BHU51 and BHU105 treated plants (Table 1). Increased survivability of plants and reduction in disease caused by soil borne pathogens, by using mixture of compatible microbes and individual *Trichoderma* have also been reported by Singh and Singh (2012), Pal *et al.* (2001) and Pandey and Maheshwari (2006).

In all the *Trichoderma* treatment significantly high vitamin C and lycopene content in tomato fruits was observed as compared to pathogen inoculated control (Table 2). Significantly higher vitamin C (41.80 mg 100g⁻¹ f. wt.) and lycopene content (21.04 mg kg⁻¹ f.wt.) was recorded in the treatment in which seedlings were transplanted after treating with *Trichoderma* mixture than single *Trichoderma* treatment. While minimum vitamin C (26.11 mg 100g⁻¹ f. wt) and lycopene content (9.896 mg kg⁻¹ f. wt) was recorded in untreated pathogen inoculated control. Nzanza *et al.* (2012) reported increased vitamin C content in *Trichoderma* treated tomato plants. Difference among the four treatments, in the term of carbohydrate and protein content showed that treatments having *Trichoderma* isolates had more carbohydrate and protein content in comparison to pathogen inoculated and *Trichoderma* untreated control. Maximum

Table 1 : Effect of treatment with different *Trichoderma* isolates on shoot length, chlorophyll content, disease incidence and yield of tomato under pathogen *Sclerotinia sclerotiorum* inoculated field conditions

Treatments	Shoot length (cm)	Chlorophyll content (mg g ⁻¹ f.wt.)	Mean disease rating	Disease reduction (%)	Yield (kg plot ⁻¹)
2008-09					
Control with pathogen (Pth)	61.33±3.51b	0.757±0.059b	2.99±0.12a	-	6.01±0.97c
BHU 51 + BHU 105+ Pth	80.67±3.06a	1.037±0.007a	1.70±0.14c	41.00±2.92a	11.428±1.21a
BHU 51 + Pth	75.33±3.06a	0.980±0.055a	1.95±0.09b	35.77±2.27b	9.208±0.95b
BHU 105 + Pth	74.33±4.16a	0.986±0.034a	2.01±0.16b	34.89±1.97b	8.307±0.50b
LSD (P=0.05)	7.04	0.06	0.28	4.43	1.39
2009-10					
Control with pathogen (Pth)	58.00±2.65c	0.774±0.023d	3.23±0.06a	-	5.72±0.64c
BHU 51 + BHU 105 +Pth	78.67±2.52a	1.012±0.011a	1.62±0.11c	44.84±0.91a	11.665±0.97a
BHU 51 + Pth	70.00±2.00b	0.982±0.007b	1.85±0.09b	40.83±2.16b	9.957±0.83b
BHU 105 + Pth	75.67±2.52a	0.955±0.005c	1.96±0.09b	38.72±1.93b	8.560±0.69b
LSD (P=0.05)	4.17	0.03	0.20	3.04	1.72

All the values are mean of three replications and the values represent ± Standard Deviation. Different letters denote a statistically significant difference (p=0.05), Pth = Pathogen (*Sclerotinia sclerotiorum*)

Table 2 : Effect of treatment with different *Trichoderma* isolates on nutritional quality of tomato inoculated with pathogen *Sclerotinia sclerotiorum*

Treatments	Lycopene (mg kg ⁻¹ f.wt.)	Carbohydrate (mg g ⁻¹ f.wt.)	Protein (g 100g ⁻¹ f.wt.)	Vitamin C (mg 100g ⁻¹ f.wt.)	Reducing power (OD)
Control with pathogen (Pth)	9.896±0.79c	19.071±1.23d	0.964±0.07b	26.113±1.32c	0.107±0.009c
BHU 51+BHU 105+Pth	21.040±1.49a	29.030±1.09a	1.951±0.30a	41.800±1.74a	0.203±0.013a
BHU 51+Pth	15.520±1.26b	25.420±1.21b	1.633±0.19a	37.893±1.01b	0.143±0.008b
BHU 105+Pth	17.849±1.43b	22.461±1.94c	1.561±0.31a	38.607±1.90b	0.188±0.013a
LSD (P=0.05)	1.71	3.24	0.25	3.48	0.02

All the values are mean of three replications and the values represent ± Standard Deviation. Different letters denote a statistically significant difference (p=0.05), Pth = Pathogen (*Sclerotinia sclerotiorum*).

carbohydrate (29.03 mg g⁻¹ fw) and protein (1.95 g 100g⁻¹ fw) content was recorded in plants treated with *Trichoderma* mixture (Table 2).

Fig. 1 clearly shows that the total phenol content in fruits of consortium of *Trichoderma* treated plant (2.201 mgGAEg⁻¹ d. wt.) was 1.54 times higher than pathogen inoculated control (1.428 mgGAEg⁻¹ d.wt.) fruits, while in the fruits of individual *Trichoderma* BHU-051 and BHU-105 treated plants it was 1.30 times and 1.39 times higher than control fruits. High total phenol content in tomato fruit in *Trichoderma* treated treatments, as observed in this study, is consistent with the earlier finding in *Trichoderma* treated plants (Singh *et al.*, 2013). Since polyphenolic compounds have hydroxyl group that have significant radical scavenging ability, all the treatment had different antioxidant capacity. *Trichoderma* treated fruits showed greater antioxidant activity than untreated pathogen inoculated control. The highest percent DPPH (47.86 %) and hydroxyl radical (49.18 %) scavenging properties were recorded in consortium treated plant fruits and ferrous ion chelating (50.81 %) also found significantly higher in the consortium treatment (Fig. 2). It is clear that three treatments T2 (BHU51+BHU105+Pth), T3(BHU51+Pth) and T4 (BHU105+Pth) had no significant difference in DPPH radical scavenging activity among them but were higher than the control (Fig. 2). High amount of TPC and DPPH recorded in *Trichoderma* treatments, in comparison to control, might be responsible for high scavenging ability. Similar was observations was earlier made by Mc Cue and Shetty (2005) and Kroyer (2004). While ferrous ion chelating and hydroxyl radical scavenging activity, consortium treatment showed significant difference among individual *Trichoderma* treated treatments. All the three *Trichoderma* treated treatment showed higher reducing power than control. Reducing power (absorbance at 700 nm) was recorded maximum in consortium treatment (0.203) followed by single *Trichoderma* BHU105 (0.188) and *Trichoderma* BHU51 (0.143) treatment, while minimum reducing power was recorded in pathogen inoculated control (0.107). Results (Table 2) of the present study postulate that the pathogen inoculated control caused destruction of ferrous ion chelation, reducing components, and other antioxidant compounds, whereas *Trichoderma* treated plants counteracted the effect of pathogen.

Trichoderma is one of the important bioagent that is now being used worldwide for the management of diseases. It not only reduces the severity of disease causing agents but also improves the plant growth activities, yield and nutritional quality (Singh and Singh, 2012). The results of this experiment indicated that use of compatible isolates of *Trichoderma* as consortium showed better plant growth, chlorophyll content and plant yield than individual

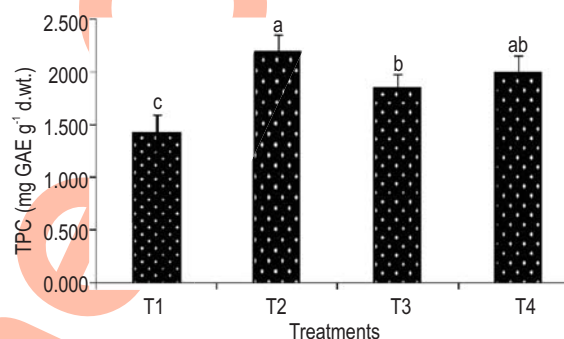


Fig. 1 : Total phenolic content expressed as gallic acid equivalents (TPC, mg GAEg⁻¹ dry weight) in tomato fruits of different treatments. Different letters denote a statistically significant difference (p=0.05), and bars indicated SD; T1 (Control with Pathogen); T2 (BHU51+ BHU105+Pth); T3 (BHU51+Pth) and T4(BHU105+Pth)

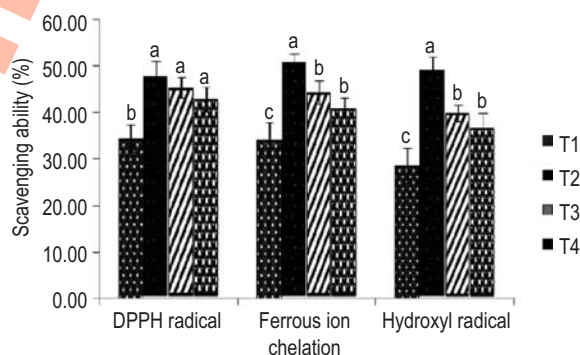


Fig. 2 : Radical scavenging ability (%) and ferrous ion chelation capacity (%) of methanolic extracts of tomato fruits of different treatments. Different letters denote a statistically significant difference (p=0.05), and bars indicated SD; T1 (Control with Pathogen); T2 (BHU51+ BHU105+Pth); T3 (BHU51+Pth) and T4(BHU105+Pth)

use of *Trichoderma* isolates. Srivastava *et al.* (2010) reported that the use of consortium of bioagents *Trichoderma* and fluorescent *Pseudomonas* were more effective than single isolates treatments, as it increase seed germination and reduced the incidence of diseases, which may be due to the ability of *Trichoderma* isolates to survive and colonize in root and rhizosphere. Combined application of biocontrol agent, *Trichoderma virens* with bacterial biocontrol agents *Bacillus cepacia*, *Serratia marcescens* etc. significantly improved suppression of *Rhizoctonia solani* in cucumber, over individual application (Robert *et al.*, 2005). *Trichoderma* sp. colonizes root and release lot of metabolites in the rhizosphere that are responsible for antagonistic and plant growth promotion activity (Vinale *et al.*, 2008) and has ability to increase photosynthetic efficiency (Vargas *et al.*, 2009). Similar results was also observed in the present study. *Trichoderma* release hydrolytic enzymes and proteins, Chitinase, β -1, 3-glucanase, peroxidase, polyphenol oxidase etc., that are responsible for reducing the incidence of pathogens, by stimulating plant defense mechanism and also increasing the nutrient status (Harman, 2011; Singh *et al.*, 2011). Jain *et al.* (2013) reported that triple consortium of *Pseudomonas aeruginosa*, *Trichoderma harzianum* and *Bacillus subtilis* showed better protection against *Sclerotinia sclerotiorum* rot in pea by increasing antioxidant enzymes and other defence related compounds than single treatment.

Findings of the present study clearly showed that use of mixture of *Trichoderma* isolates was more beneficial over single application, which might be due to synergistic effect of both the isolates. Mixture of *Trichoderma* reduced the incidence of pathogen and promoted plant growth and yield, greater than the single isolate. Apart from these qualities, it also improved the nutritional quality and antioxidant activity of the produce; by providing protection to plants from pathogens and thereby increasing the nutrient uptake from soil.

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