Eur J Plant Pathol (2011) 131:121–134
DOI 10.1007/s10658-011-9792-4

Trichoderma harzianum- mediated reprogramming of oxidative stress response in root apoplast of sunflower enhances defence against *Rhizoctonia solani*

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Accepted: 7 April 2011 / Published online: 30 April 2011

Abstract *Trichoderma harzianum* is an effective biocontrol agent against the devastating plant pathogen *Rhizoctonia solani*. Despite its wide application in agriculture, the mechanisms of biocontrol are not yet fully understood. Mycoparasitism and antibiosis are suggested, but may not be sole cause of disease reduction. In the present study, we investigated the role of oxidant-antioxidant metabolites in the root apoplast of sunflower challenged by *R. solani* in the presence/absence of *T. harzianum* NBRI-1055. Analysis of oxidative stress response revealed a reduction in hydroxyl radical concentration ($^{\bullet}OH$; 3.6 times) at 9 days after pathogen inoculation (dapi), superoxide anion radical concentration ($O_2^{\bullet-}$; 4.1 times) at 8 dapi and hydrogen peroxide concentration (H_2O_2; 2.7 times) levels at 7 dapi in plants treated with spent maize-cob formulation of *T. harzianum* NBRI-1055 (MCFT), as compared to pathogen-inoculated plants. The protection afforded by the biocontrol agent was associated with the accumulation of the ROS gene network: the catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and ascorbate peroxidase (APx), maximum activity of CAT (11.0 times) was observed at 8 dapi, SOD (7.0 times) at 7 dapi, GPx (5.4 times) and APx (8.1 times) at 7 dapi in MCFT-treated plants challenged with the pathogen. This was further supported by the inhibition of lipid and protein oxidation in *Trichoderma*-inoculated plants. MCFT stimulated the accumulation of secondary metabolites of phenolic nature that increased up to five-fold and also exhibited strong antioxidant activity at 8 dapi, eventually leading to the systemic accumulation of phytoalexins. These results suggest that *T. harzianum*–mediated biocontrol may be related to alleviating *R. solani*-induced oxidative stress.

Keywords *Trichoderma harzianum* · *Rhizoctonia solani* · Oxidant-antioxidant metabolites · Sunflower · Phenolics

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Introduction

Plants contain a whole array of cellular mechanisms to defend themselves against invading pathogens such as fungi, bacteria and viruses (der Ent et al. 2009; Shoresh et al. 2010). One of the earliest defence responses is the generation of reactive oxygen species

(ROS) (Nanda et al. 2010), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) (Singh et al. 2010). This triggers many downstream processes leading to a dynamic defence response characterized by inhibition of the growth of invaders through phytoalexin formation, callose deposition, strengthening of cell walls, synthesis of secondary metabolites and pathogenesis related (PR) proteins (Jones an Dangl 2006; Shoresh et al. 2010; Xu et al. 2008; Vinale et al. 2008).

ROS are not only toxic by-products of aerobic metabolism, but when produced in a rapid and controlled manner, they are cellular requirements, being involved in signalling pathways and in the defence against stress (Nanda et al. 2010; Pitzschke and Hirt 2009). On the contrary, previous studies have clearly shown that ROS may also be a source of oxidative stress when plants encounter ionic toxicity, osmotic stress, drought, and an aggressive pathogen (Nanda et al. 2010; Xu et al. 2008). Uncontrolled overproduction of ROS can disrupt normal metabolism by oxidizing DNA, RNA, proteins, lipids, or carbohydrates, affecting the integrity of cell membranes and inactivating key cellular functions (Singh et al. 2009).

Prevention of ROS toxicity requires a large gene network, the so called "ROS gene network", which is composed of at least 150 genes in *Arabidopsis* (Mittler et al. 2004). Several families of proteins from plants are associated with the regulation of ROS levels. Among them, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), and glutathione peroxidase (GPx), can reduce ROS concentration (Singh et al. 2009). The specific group of phytomolecules such as phenolics are also members of the "ROS gene network" and have the interesting capacity to scavenge/or quench deleterious ROS (Singh et al. 2009, 2010). Several studies have shown that plant growth promoting fungi (PGPF) and rhizobacteria (PGPR) play important role in provoking plant resistance to various biotic or abiotic stresses (Mandal et al. 2008). These biocontrol agents (BCAs) also showed the capability in activating this "ROS gene network", which are associated with PGPR-mediated induced resistance and providing protection against diverse pathogens (Nanda et al. 2010).

In recent years, a number of BCAs have been reported to be effective in inhibiting root rot disease of plants (der Ent et al. 2009; Van Loon et al. 2008). An example of BCAs are *Trichoderma* spp., which have been shown to induce local and systemic defence responses in cucumber (Segarra et al. 2007; Viterbo et al. 2005; Viterbo et al. 2007; Yedidia et al. 1999, 2003) and other economically important agricultural crops, such as cotton, tobacco, lettuce, maize, chickpea and bell pepper (Ahmed et al. 2000; Chen et al. 2005; De Meyer et al. 1998; Howell et al. 2000; Singh et al. 2007; Wilson et al. 2008). Induced disease resistance is the phenomenon by which a plant mobilises its own defence mechanism to restrict disease development (Harman et al. 2004). Induced disease resistance, mediated by such nonpathogenic rhizosphere microorganisms, has been demonstrated in several plant species and shown to be effective against bacterial, viral, and fungal diseases (Harman et al. 2004; Shoresh and Harman 2008). The mechanisms of action of *Trichoderma* against fungal pathogens include secretion of antibiotics, competition for space and nutrients, production of lytic enzymes, and induction of host resistance (Harman et al. 2004). In particular, induced resistance is recognized as an important mode of biocontrol in vegetative tissues (Shoresh et al. 2010). In many previous studies, *Trichoderma* spp. were found to be effective in inducing activities of defence-related proteins and fungitoxic phenolic compounds in coconut (Karthikeyan et al. 2006) and cucumber (Yedidia et al. 2003). *T. harzianum* treatment induced accumulation of phenolic compounds in chickpea, resulting in a decrease in plant mortality caused by *Fusarium oxysporum* f.sp. *ciceri* and *Sclerotium rolfsii* (Singh et al. 2007). However, till now, most studies of *Trichoderma*-mediated disease control focused on the induction of defence related gene products including PR proteins, phytoalexins, phenols, and enzymes (Shoresh and Harman 2008). Little evidence is available demonstrating the role for antioxidant proteins in plants induced by these BCAs against pathogen-induced oxidative stress, especially in agricultural crops, for disease control. Recently, Chan et al. (2007) observed that the yeast *Pichia membranaefaciens*, used as a BCA, induced antioxidant enzymes and PR proteins in peach fruit by proteomic approach. Some studies have established the role of oxidant and antioxidant metabolites in the defence response of plants (e.g., Xu et al. 2008). Thus, the increased antioxidant enzymes induced by antagonistic BCA may act as a scavenger to reduce the pathogen-induced oxidative stress.

Protein oxidation (PO), lipid peroxidation (LPO) and generation rate of ROS are good indicators of oxidative damage and in plant disease control. These could be potential markers for studying the effects of antagonistic BCAs on pathogen-induced oxidative stress. In the present study, we investigated the effect of PO and LPO induced by ROS in the defence response of sunflower plants treated with *T. harzianum* with an objective to establish the role of alleviating oxidative stress in *T. harzianum*-mediated biocontrol of *R. solani*.

Materials and methods

Fungal strains and chemicals

Trichoderma harzianum (NBRI-1055) was isolated from the rhizosphere of sunflower plants (referred to here as 'T-1055'). This isolate was also found to be effective against *R. solani* in confrontation assay. T-1055 was grown and maintained on potato dextrose agar (PDA) medium (HiMedia M096). Analytical grade chemicals and solvents for all procedures were obtained from E. Merck, Mumbai, India. All authentic standards of enzymes were procured from Sigma–Aldrich, St. Louis, USA.

Mass multiplication and formulation preparation

For mass multiplication of T-1055, maize spent-cob (MSC; var. Malaviya-2) was used as the solid substrate. MSC was chopped into small pieces (approximately 5 cm diameter) and soaked in tap water for 2 h. One hundred g moist substrate (moisture 30% w/v) was filled in 2 kg capacity polypropylene bags and autoclaved twice at 15 lbs for 30 min. The bags were allowed to cool down to room temperature (RT) and inoculated with spore suspension of T-1055, prepared by harvesting the spores from 7d-old culture grown on PDA in sterile distilled water (SDW). Five ml of spore suspension (10^{10} colony forming units (CFU) ml^{-1}) was injected into autoclaved bags with the help of a sterile syringe. These bags were incubated at 25±2°C for 9 d and *Trichoderma*−colonized MSC was air-dried and ground to fine powder in a temperature controlled cool jacket grinder (Basco, India) at 20°C. The talc-based formulation for T-1055 was prepared by following the method described by Singh et al. (2007). Briefly, 1.0 kg of talc powder (Basco, India) was taken in a sterilized metal tray and its pH was adjusted to neutral by adding calcium carbonate ($CaCO_3$; HiMedia, RM397) @ 15 g kg^{-1}. Ten g of carboxymethyl cellulose (CMC; CDH 027929), was added to 1.0 kg of talc, mixed well and the mixture was autoclaved for 30 min on each of the two consecutive days. Spore powder (2%) was mixed with sterilized talc under aseptic conditions and the spore concentration was adjusted to 2×10^8 cfu g^{-1} of the product. The final product was named as maize spent cob-waste formulation of *Trichoderma* (referred to here as 'MCFT').

Biocontrol assay

Twenty four h before sowing, sunflower (cultivar, NFSH-9) seeds were surface sterilized with 0.2% mercuric chloride ($HgCl_2$; HiMedia, Mumbai) solution for 5 min and thoroughly washed many times with SDW to remove traces of $HgCl_2$. The moistened seeds were stored at RT (25±2°C) for 24 h to promote germination under aseptic conditions. Plastic pots (15×10 cm) were used for sterile soil assay (SSA). The pots were filled with 1.5 kg of potting mix containing sandy soil (available N, P, K, Ca and Mg contents of soil were 160, 18, 280, 0.28 and 0.19 kg ha^{-1}, respectively; pH 7.1); vermicompost and farm yard manure in 1:1:2 w/w/w. Potting mix was sterilized by autoclaving on three consecutive days. Seeds of sunflower were treated with 10% (w/v) gum arabic (HiMedia, RM682) as an adhesive and then uniformly coated with MCFT (2×10^8 CFU ml^{-1}) at 5 g kg^{-1} seed and air-dried for 2 h in a laminar flow hood.

After 1 week of inoculum application, sunflower seeds treated with MCFT were placed at about 3.0 cm depth in the potting soil and 50 g of the same soil mixture were placed above the seeds. Ten days after sowing (DAS), 500 sclerotia of *R. solani* (mass multiplied in sterile cornmeal sand −240 g of clean quartz sand, 6 g of yellow cornmeal, and 75 ml of SDW for 2 week at 25°C) were inoculated per pot. Carbendazim (Bavistin 50 WP) seed treatment at 2 g kg^{-1} seed and post-sowing soil application (2 g pot^{-1}) were included for comparison. Untreated seeds served as control. After sowing each pot received 250 ml of tap water. The seedlings were thinned to two per pot on the 7th DAS. Pots were maintained in a greenhouse at 21±2°C and watered daily for 30 days. All the pots were irrigated at one-day intervals until

harvest. Disease incidence and plant vigour was calculated by the formula:

$$\text{Per cent disease index} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

$$\text{Plant vigour} = [\text{shoot length (cm)} + \text{root length (cm)}] \times \text{germination(\%)}$$

Twelve pots constituted one replication and there were three replications per treatment. The pots were arranged in a randomized manner and the experiment was repeated twice.

Isolation of apoplastic fluids (AF)

About 2 g of sunflower roots were quickly washed in chilled SDW, placed in Petri dishes containing 10 mM sodium phosphate (pH 6.0) with 1.5% (w/v) polyvinylpolypyrrolidone (PVP), 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) The roots were subjected to vacuum (−60 kPa) for 5 min at 4 ^{0}C. Afterwards, the free water was removed with filter paper and placed in syringes taken in centrifugation tubes. The roots were centrifuged at 150 x *g* for 5 min, and the AF was recovered at the bottom of the tubes (Parra-Lobato et al. 2009). Cytosolic contamination of AF was monitored by assaying with glucose-6-phosphate dehydrogenase (G6PDH) activity as a marker.

Assessment of reactive oxygen species (ROS)

Hydroxyl ($^{\bullet}OH$) free radical

The $^{\bullet}$OH levels of sunflower root apoplastic fluid (AF) were measured by using NADPH-dependent method of Singh et al. (2009). Reaction mixture (3.0 ml) consists of 85 mM potassium phosphate buffer (pH 7.4), 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 0.5 mM $NADP^+$, 7 units of glucose-6-phosphate, and enzyme (5 mg protein). The addition of 0.1 mM EDTA augments the oxidation of ethanol, used as a substrate. Reactions were initiated by the addition of the NADPH and carried out at 37 ^{0}C in centre-well flasks containing 0.6 ml of 15 mM semicarbazide HCl in 180 mM potassium phosphate (pH 7.4). The sealed flasks were incubated overnight at room temperature. Aliquots of the centre-well contents, e.g., 0.2 ml, were diluted to 3.0 ml with DW and the absorbance was recorded at 224 nm. Results were expressed as nkat ml^{-1} AF.

Superoxide anion ($O_2^{\bullet-}$) radical production

Nitro blue tetrazolium (NBT) reducing method was used for measurement of $O_2^{\bullet-}$radical in sunflower root AF as described by Singh et al. (2010). Root AF (1.0 ml) was immersed in 3.0 ml 0.01 M potassium phosphate buffer (pH 7.8) containing 0.05% NBT and 10 mM NaN_3 for 1 h. The mixture was then heated at 85 ^{0}C for 15 min and cooled. The reducing activity of NBT by the AF of root was recorded the absorbance at 580 nm and expressed as μmol ml^{-1} AF.

Hydrogen peroxide generation

AF of the root (1.0 ml) was mixed with 2.0 ml of 1 M potassium iodide solution and incubated for 5 min before measuring of the oxidation product at A_{390} (Velikova 2000) using "Thermo Scientific" Vis-UV spectrophotometer. The amount of hydrogen peroxide (H_2O_2) formed was computed from the standard curve made earlier with known concentrations of H_2O_2 and expressed as μmol ml^{-1} AF.

Malondialdehyde determination

Lipid peroxidation (LPO) of root AF was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA, Sigma–Aldrich, St. Louis, USA) reaction. The assay was carried out according to Ohkawa et al. (1979). AF of the root (1.0 ml) was incubated with 4.0 ml of 20% trichloroacetic acid (TCA, w/v) containing 1% TBA (w/v) for 30 min at 95°C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000×*g* for 15 min. The reaction product was measured at A_{532}. The concentration of MDA was determined using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol ml^{-1} AF.

Protein carbonyl determination

Protein oxidation assay was carried out according to the method of Singh et al. (2010) and expressed

in terms of protein carbonyl (PCO) content. Briefly, AF of the root (30 μg protein; 1.0 ml) was mixed with 12% sodium dodecyl sulphate (SDS) (5.0 μl). Ten molar 2,4-dinitrophenylhydrazine (DNPH) (10 μl) dissolved in 10% trifluoroacetic acid (v/v) was added to the sample. The reaction mixture was vortexed and incubated for 15 min at RT. The solution was neutralized by addition of 2.0 M Tris (10 μl) and centrifuged for 5 min at 5,000×*g*. The protein pellet was washed three times with 2.0 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1.0 ml of guanidine hydrochloride (5 M, pH 2.5). The absorbance of the samples was recorded at 370 nm.

Antioxidant enzymatic activities

Enzymatic activities were assayed spectrophotometrically for AF obtained from sunflower roots. Reactions were developed at 25 ^{0}C for 5 min, with stirring, at a final volume of 3.0 ml containing 50–60 μg of protein.

Catalase

Catalase (CAT) activity was assayed by measuring the rate of disappearance of H_2O_2 at A_{240} as per the method of Singh et al. (2009). The reaction mixture consisted of 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.25 ml enzyme extract. One unit was defined as a change in absorbance of 0.1 under the conditions of the assay. Enzyme activity was expressed as nkat mg^{-1} protein.

Superoxide dismutase

Superoxide dismutase (SOD) was assayed following the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture contained 63 mM NBT, 13 mM L-methionine, 0.1 mM EDTA, 13 mM riboflavin, 0.05 M sodium carbonate and 0.5 ml enzyme sample (0.5 ml DW in case of control). It was kept under two 15 W fluorescent lamps for 15 min at 25°C, followed by transferring to dark for 15 min and then the absorbance was recorded at 560 nm. One unit of the SOD activity was defined as the amount of enzyme required to inhibit reduction of NBT by 50%. SOD activity was expressed as μkat mg^{-1} protein.

Guaiacol peroxidase

Guaiacol peroxidase (GPx) was assayed by measuring increase in absorbance at 470 nm due to oxidation of guaiacol to tetraguaiacol (Zheng and Van Huystee 1992). The reaction mixture contained 10 mM sodium phosphate (pH 6.0), 0.1 ml of 0.3% (v/v) H_2O_2, 0.1 ml of 1% (v/v) tetraguaiacol and the enzyme extract (0.3 ml). Reaction was initiated by the addition of H_2O_2. The linear portion of the activity curve was used to express enzyme activity and followed at A_{470} (expressed as μkat mg^{-1} protein). One unit of enzyme activity represented the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol min^{-1}.

Ascorbate peroxidase

Ascorbate peroxidase (APx) activity was measured by following the oxidation of ascorbic acid at A_{290} as per the method of Nakano and Asada (2001). The reaction mixture consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbic acid, 1.0 mM H_2O_2 and 0.2 ml enzyme extract. Decrease in absorbance was noticed 60 s after addition of enzyme extract. One unit was defined as a change in absorbance of 0.1 under the conditions of the assay. The enzyme activity was expressed as nkat mg^{-1} protein. The protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

Total phenolic content assay

Total phenolic content (TPC) was determined as per Singh et al. (2010). AF of sunflower root (1.0 ml) was mixed with 95% ethanol (1.0 ml) and SDW (5.0 ml). To this 0.5 ml of 1 N Folin-Ciocalteau regent was added. After 5 min, 5% sodium carbonate (1.0 ml) was added and the reaction mixture was allowed to stand for 60 min and the absorbance was recorded at 725 nm. Standard curves were prepared for each assay using various concentrations of gallic acid (GA) in 95% ethanol. Absorbance values were converted to mg GA equivalent (GAE) g^{-1} ml^{-1} AF.

Antioxidant activity

Antioxidant activity (AOA) was estimated as described by Singh et al. (2009) by monitoring the coupled autoxidation of β-carotene and linoleic acid (LiA). Two milligram β-carotene was dissolved in chloroform (20 ml). This solution (3.0 ml) was added to 40 mg of LiA and 400 mg of tween 40. Solvent was removed under a stream of nitrogen gas followed by the addition of water (100 ml) and the solution was mixed well. Aliquots (3.0 ml) of the β-carotene and LiA emulsion were mixed with 1.0 ml root AF and incubated in a water bath at 50 ^{0}C. Oxidation of this emulsified reaction mixture was monitored on a spectrophotometer by measuring absorbance at 470 nm at 15 min intervals for 60 min. Control contained methanol only in place of plant extract. AOA was expressed as per cent inhibition relative to control.

Statistical analysis

Sampling of the plants was done in three independent experiments with three replicates. A minimum of six plants were evaluated for each replicate. Three plants constituted one sample. The data were analyzed by one-way analysis of variance (ANOVA). The treatment means were compared by Student's *t*-test, with level of significance $P<0.05$. The package used for analysis was SPSS version 11.

Results

Effect of MCFT on seedling blight disease and growth promotion

MCFT protected sunflower plants from *R. solani* infection (Fig. 1) with minimum plant mortality (10.54%) in comparison to pathogen treated (74.92%) and Carbendazim treated seeds (13.71%). This bioformulation also increased plant vigor by 1529 in comparison to untreated control (951) and Carbendazim (1024).

Effect of MCFT on ROS production in AF of sunflower roots

Plants treated with the pathogen alone showed increased $^{\bullet}$OH concentration, started at 5 days after pathogen inoculation (dapi) and reached the maximum observed at 9 dapi (Fig. 2a). AF obtained in our experiments showed only slight contamination from the cytosol, as deduced from the controls made with G6PDH activity, a marker for cytosolic contamination (data not shown). On the contrary, MCFT-treated plants challenged with the pathogen (or MCFT-co-treated) recorded significantly decreased $^{\bullet}$OH level which was 3.6 times lower at 9 dapi when compared to pathogen inoculated control (49.4 nkat g^{-1} FW). The increased levels were observed for 5–7 dapi but later declined drastically. The plants treated with MCFT alone also had higher $^{\bullet}$OH concentration compared to untreated control, but level was low compared to MCFT-co-treated plants. The levels of $O_2^{\bullet-}$ were also found to be significantly higher in the pathogen inoculated plants and reached maximum at 8 dapi, thereafter decreased marginally till the end of the experiment (Fig. 2b). MCFT-co-treated plants showed significant reduction in $O_2^{\bullet-}$ production; the increased level was observed for 5–7 dapi but later declined drastically. Similarly, the concentration of H_2O_2 was 2.7 times lower in MCFT-co-treated plants at 7 dapi compared to pathogen inoculated plants (66.4 μmol g^{-1} FW) (Fig. 2c). The level of H_2O_2 increased till 7 dapi, but later alleviated drastically

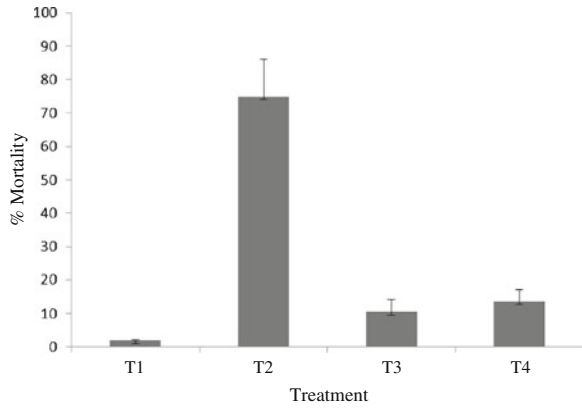

Fig. 1 Effect of treatment MCFT on *R. solani*-infected sunflower plants on their mortality grown in greenhouse conditions for 30 days. Key to the treatments: T_1, Plants grown without *R. solani* and MCFT inoculation were used as an control; T_2, Plants inoculated with *R. solani*; T_3, Plants coinoculated with *R. solani* and MCFT; T_4, Plants coinoculated with *R. solani* and fungicide Carbendazim, used as a positive control. Bars represent standard error (SE) of the mean. Treatments with different letters are significantly different ($P\leq0.05$) according to the least significant difference test

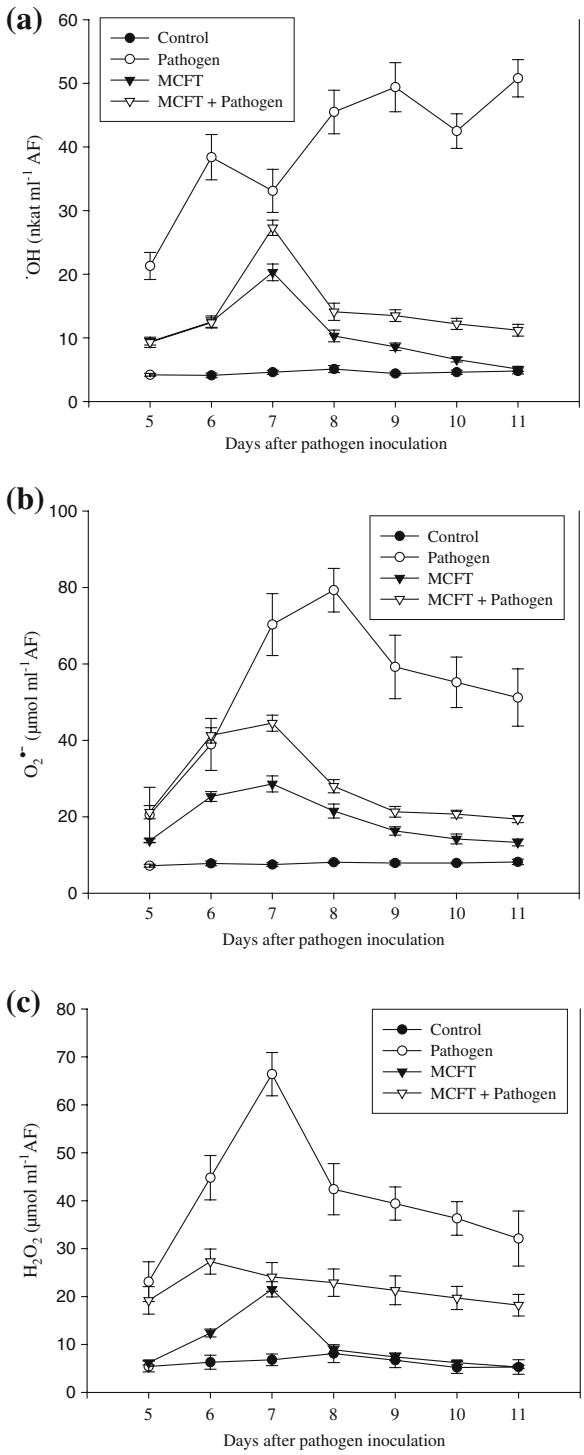

Fig. 2 Inhibitory effect of MCFT on reactive oxygen species (ROS) formations in healthy and or *R. solani*-infected sunflower root apoplastic fluid (AF). (**a**) hydroxyl radical ($^{\bullet}OH$; nmol ml^{-1} AF), (**b**) superoxide anion radical ($O_2^{\bullet-}$; µmol PCO ml^{-1} AF) and (**c**) hydrogen peroxide (H_2O_2; µmol ml^{-1} AF). Vertical bars indicate SE of six repeated analysis in one experiment

and the level remained low. The lowest level was observed on 6 dapi and was steady throughout the experimental period. MCFT treated plants showed increased concentration of H_2O_2 for 5–7 dapi and later declined drastically but compared to untreated control, the level was little higher. In case of MCFT treated plants, the increased ROS level was observed for 5–7 dapi but later declined drastically and the levels remained marginally higher compared to untreated controls.

Effect of MCFT on MDA and PCO contents in AF of sunflower roots

Lipid peroxidation (LPO) and protein oxidation (PO) were measured in terms of MDA and PCO contents, respectively. The effect of MCFT on LPO and PO in the AF of sunflower roots is shown in Fig. 3a, b. The results revealed that the plants inoculated with the pathogen alone exhibited maximum MDA and PCO contents, but started on the 5 dapi and sharply declined after 7 dapi. The content of MDA and PCO reached maximum levels at 7 dapi after which there was sharp decline. On the contrary, MCFT-co-treated plants had significantly lower MDA and PCO contents in AF of sunflower roots, which started on the 6 dapi and drastically declined after 7 dapi till the end of experiment, and which were lower compared to plants inoculated with the pathogen control. MDA and PCO contents in sunflower roots treated with MCFT alone remained unaltered during the experimental period but compared to control, the levels were higher.

Effect of MCFT on antioxidant enzymes in AF of sunflower roots

Investigations on induction of antioxidant enzymes revealed that higher level of CAT activity was observed in MCFT-co-treated plants (Fig. 4a). Induction of CAT activity started at 5 dapi and reached maximum on the 8 dapi which was 11.2 times higher when compared to corresponding untreated control (0.5 nkat mg^{-1} protein). Similarly, sunflower plants inoculated with the pathogen alone recorded increased activity of CAT but the induction was observed for 5–6 dapi, thereafter declined drastically, but, compared to MCFT-co-treated plants, the level was lower. Activity of SOD also increased in MCFT-co-treated

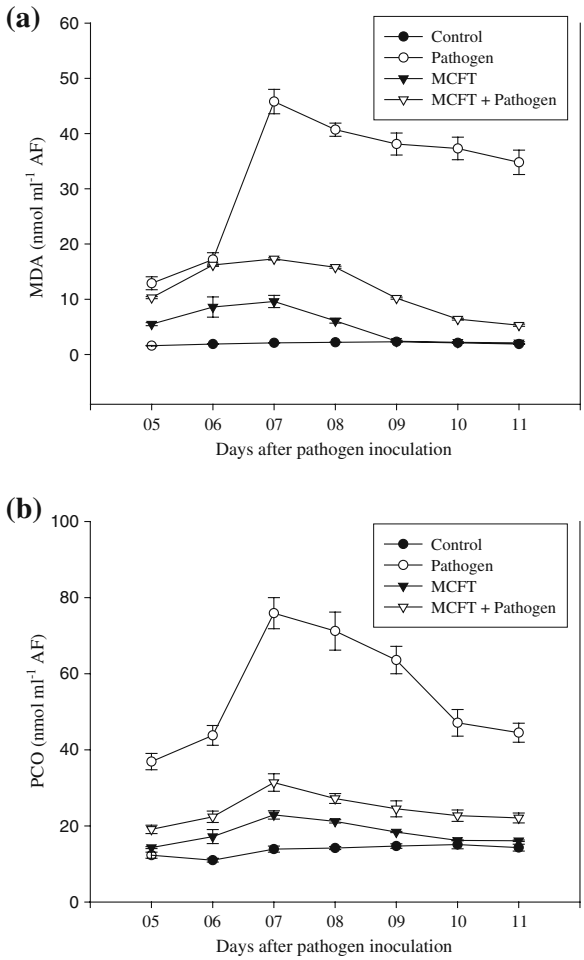

Fig. 3 Inhibitory effect of MCFT on oxidative stress-related damages in healthy and or *R. solani*-infected sunflower root apoplastic fluid (AF). (**a**) lipid peroxidation (LPO; expressed as nmol malondialdehyde (MDA) content ml^{-1} AF) and (**b**) protein oxidation (PO; expressed as nmol protein carbonyl (PCO) content ml^{-1} AF) in healthy and or *R. solani*-infected sunflower roots. Vertical bars indicate SE of six repeated analysis in one experiment

plants (Fig. 4b). The maximum activity was observed on the 7 dapi which was 7 times higher than their respective untreated control (9.8 μkat mg^{-1} protein). The activity maintained at the higher levels throughout the experimental period. Plants inoculated with the pathogen alone had comparatively less SOD activity. A similar pattern of increased activity of GPx and APx was observed in the MCFT-co-treated roots (Figs. 4c and d). The activity of GPx and APx reached maxima on 7 dapi, which were 5.4 and 8.1 times higher when compared to corresponding untreated controls (45.2 & 6.8 nkat mg^{-1} protein) respectively. In plants inoculated with the pathogen alone, increased enzymatic activities were recorded on 7 dapi, which then declined drastically till the end of experiment at 11 dapi. Generally, MCFT treated plants also had significantly higher activity of all the antioxidant enzymes recorded for 5–8 dapi, thereafter declined drastically but the level was higher compared to untreated control plants.

Effect of MCFT on accumulation of TPC and AOA in AF of sunflower roots

Studies on induction of defence-related chemicals revealed that a higher accumulation of TPC was observed in MCFT-treated plant roots challenged inoculated with *R. solani*. Accumulation of TPC started on the 5 dapi (Fig. 5). The maximum accumulation was observed on 8 dapi which was 4.9 times higher when compared to corresponding untreated control (1.1 mg GAE g^{-1} FW). Plants inoculated with the pathogen alone also recorded increased accumulation of TPC, but accumulation started on 6 dapi and then drastically declined 7 dapi. Moreover, accumulation of TPC was less compared to MCFT-co-treated plants. Increased accumulation of TPC was recorded in MCFT treated plants, but accumulation started on 5 dapi and drastically declined after 7 dapi, which was higher compared to untreated control. It has been well known that phenolic compounds possess strong AOA due to their ability to scavenge FRs by simple and complex mechanisms. The maximum AOA was observed on 8 dapi which was 5.4 times higher compared to corresponding untreated control (12%). Plants inoculated with the pathogen also exhibited higher AOA but activity started on 5 dapi and declined drastically at 6 dapi, which was less compared to MCFT-co-treated plants (Fig. 6). MCFT alone treated plants showed higher AOA than untreated control and the activity maintained at the higher levels throughout the experimental period.

Discussion

Plants have a very well organized and coordinated defence network, which is inducible in response to appropriate stimuli/or signals (Bradley et al. 1992; Jones, and Dangl, 2006). Inducing the plant's own defence mechanisms by prior application of BCAs

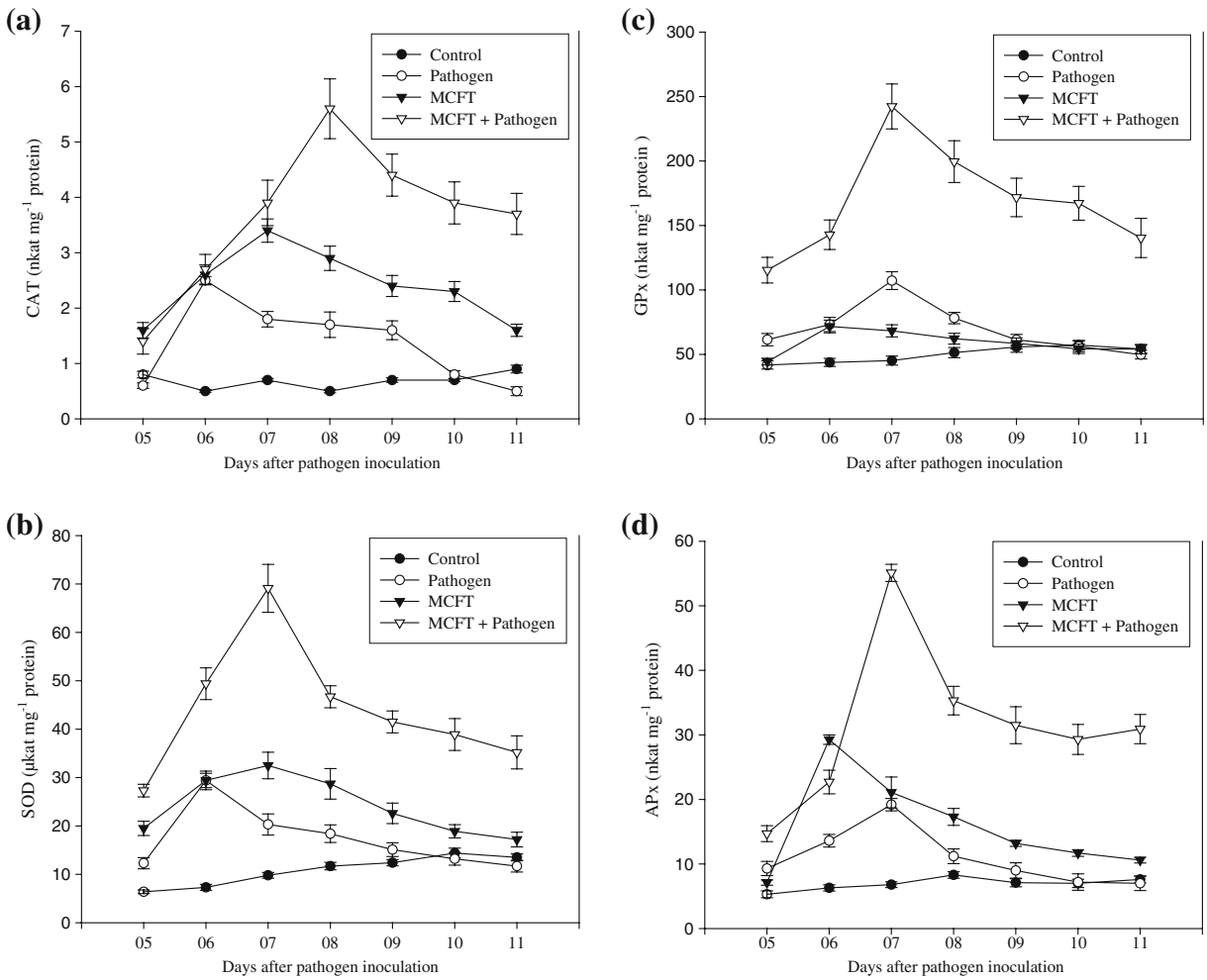

Fig. 4 Effect of treatment with MCFT on induction of ROS gene network in healthy and or *R. solani*-infected sunflower root apoplastic fluid (AF). (**a**) catalase (CAT, nkat mg^{-1} protein); (**b**) superoxide dismutase (SOD, μkat mg^{-1} protein); (**c**) guaiacol peroxidase (GPx, nkat mg^{-1} protein); (**d**) ascorbate peroxidase (APx, nkat mg^{-1} protein). Vertical bars indicate SE of six repeated analysis in one experiment

such as fungi and bacteria are thought to be a novel plant protection strategy (der Ent et al. 2009; Shoresh and Harman 2008; Van Loon et al. 2008). Application of *T. harzianum* could act as a strong elicitor of plant defence reactions against plenty of soilborne phytopathogens (Harman et al. 2004; Shoresh et al. 2010; Vinale et al. 2008). However, there is no information available on BCAs enhancing defence reactions against the pathogen invasion by modulating oxidant and antioxidant metabolites in the plants. The present study clearly indicates that the MCFT (a formulation of *T. harzianum*-NBRI-1055) induced disease resistance against *R. solani* by reprogramming oxidant and antioxidant metabolites in AF of sunflower roots (Fig. 7).

ROS are often detected in plant–pathogen interactions and are associated with disease development. In this investigation, we found that the MCFT was effective at controlling seedling blight caused by *R. solani* in sunflower roots. In order to verify the influence of T-1055, pathogen-induced accumulation of ROS were evaluated. These ROS are extremely reactive oxidizing species of FRs that are produced *in vivo* as a result of plant-pathogen interaction. ROS have been suspected to play a role in many defence processes including direct antimicrobial action, lignin formation, phytoalexin production, the hypersensitive response and triggering of systemic acquired resistance (Harman et al. 2004; Shoresh et al. 2010; Xu et

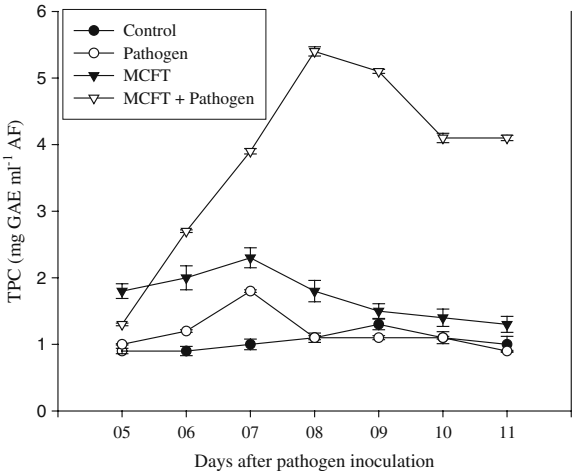

Fig. 5 Effect of treatment with MCFT on accumulation of total phenolic content (TPC; mg gallic acid equivalent (GAE) ml^{-1} apoplastic fluid (AF)) in healthy or *R. solani*-infected sunflower roots. Vertical bars indicate SE of six repeated analysis in one experiment

al. 2008). However, overproduction of ROS causes oxidative damage, leads to LPO and damages macromolecules such as pigments, proteins, nucleic acids and carbohydrates (Singh et al. 2009, 2010). The impact of ROS on macromolecules is well documented in animals and humans, where it leads to a number of different diseases and may contribute to ageing (Singh et al. 2009). It is often expressed by the hypersensitive response of challenged plant cells and is characterized by induced rapid and localized death of plant tissues at the site of infection (Nanda et al. 2010; Singh et al. 2009). Such damage is also likely to occur in plant mitochondria, but this area has received little attention to date. The pathogen-inoculated sunflower plants showed multi-fold increase in the levels of $HO^{\bullet}$ and $O_2^{\bullet-}$ which are reduced in the challenged plants treated with MCFT, indicating prevention of increased ROS production might be due to accumulation of more antioxidant proteins and antioxidant compounds (i.e. phenolics).

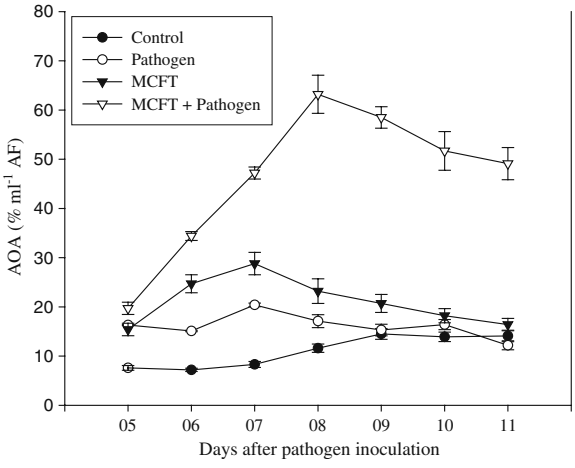

Fig. 6 Effect of treatment with MCFT on antioxidant activity (AOA,% ml^{-1} apoplastic fluid (AF)) in healthy or *R. solani*-infected sunflower roots. Vertical bars indicate SE of six repeated analysis in one experiment

The generation of H_2O_2 during plant-pathogen interaction is involved in the integration of cellular processes and the adaptation to environmental stimuli (Chamnongpol et al. 1998; Nanda et al. 2010). It leads to rapid cell wall reinforcement because it is involved in oxidative cross-linking, insolubilization of Hyp-rich proteins (Bradley et al. 1992), release of calcium into the cellular matrix, which may be central to the signal transduction process (Price et al. 1994), altering concentrations and redox status of intracellular anti-oxidants (Nanda et al. 2010). We observed that the MCFT-pathogen-coinoculated plants showed higher H_2O_2 concentrations than untreated control plants. Furthermore, Zhang et al. (2006) reported that H_2O_2 induced the activation of a 46-kD mitogen-activated protein kinase (MAPK) and enhanced the expression of antioxidant genes. Induction of non-radical H_2O_2 concentration in MCFT-treated plants was associated with higher accumulation of antioxidants which might be due to activation of defence-related pathways. These results suggest that H_2O_2 plays an important role in the regulation of antioxidant metabolites. In order to further verify whether MCFT influenced FRs-dependent oxidative damage in sunflower roots, the contents of lipid and protein oxidation significantly decreased as compared to pathogen inoculated plants. The results were expressed in the terms of MDA and PCO contents, respectively. The results showed that the MDA and PCO contents in MCFT-pathogen-co-treated plants were significantly lower as compared to pathogen inoculated plants, implying that the T-1055 had potent efficacy in alleviating pathogen-induced oxidative damages in sunflower roots.

The mechanism by which MCFT mitigates lipid and protein oxidation or pathogen-induced oxidative damage is complicated. Generally, an appropriate intracellular balance between ROS generation and scavenging exists in all the cells. This redox homeo-stasis requires efficient coordination of reactions in different cell compartments and is governed by

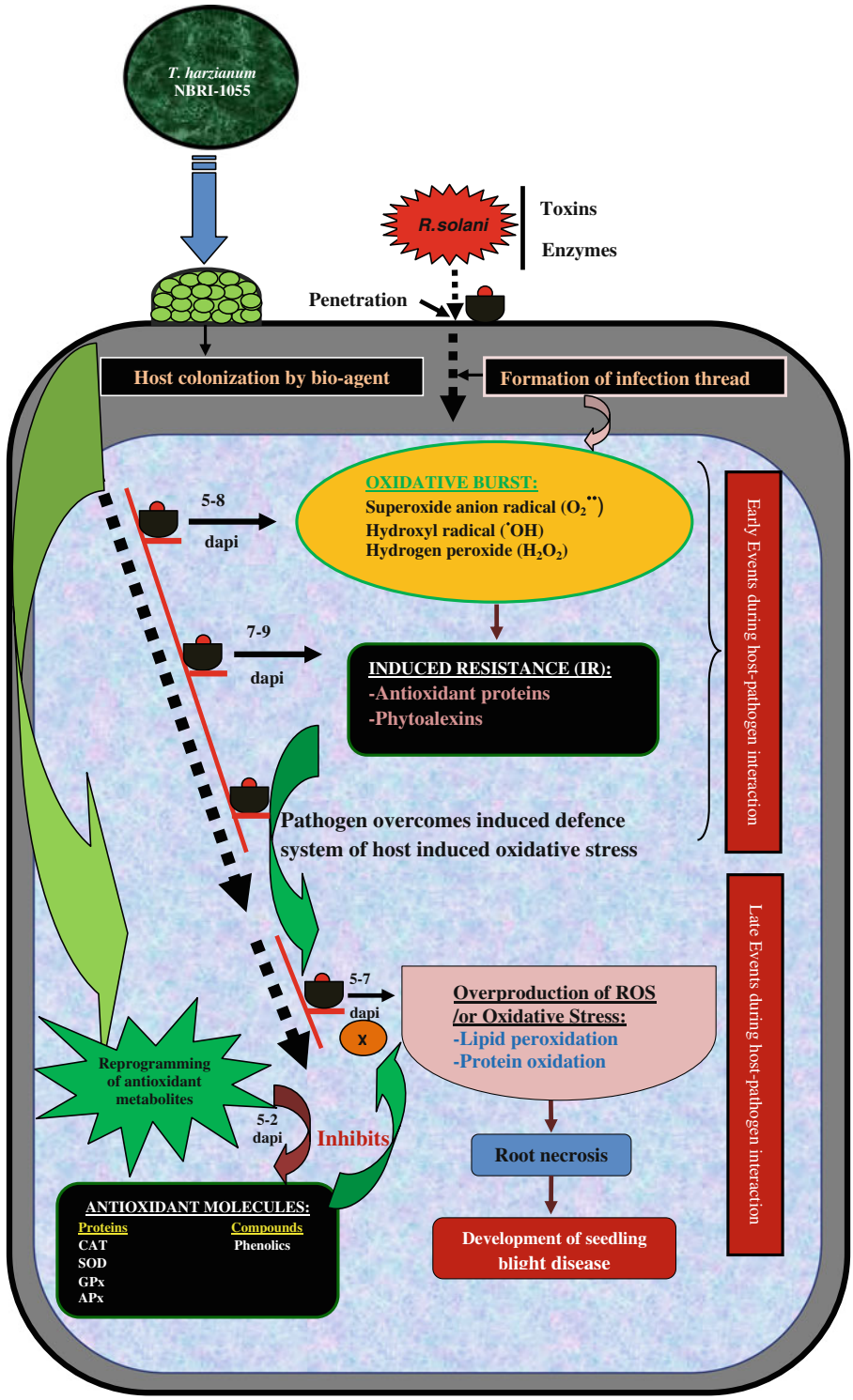

Fig. 7 Mechanism of regulation of oxidant-antioxidant metabolites by *T. harzianum* NBRI-1055 against *R. solani*. APx, ascorbate peroxidase CAT, catalase; dapi, days after pathogen inoculation; GPx; guaiacol peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species

complex signal transduction pathways. Plants possess an array of antioxidant weapons that can protect the cells from oxidative damage by scavenging ROS. The antioxidant enzymes include SOD and CAT, which work together with other enzymes of the ascorbate–glutathione cycle (e.g. APx and GPx) to promote the

scavenging of ROS (Singh et al. 2009, 2010). SOD is thought to be the key enzyme which catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Singh et al. 2009). CAT is present in the peroxisomes of nearly all aerobic cells. It can protect the cell from FRs by catalyzing its decomposition into stable products (Nanda et al. 2010). In this study, MCFT significantly stimulated the activities of antioxidant enzymes CAT, SOD, GPx and APx. These results, in agreement with earlier findings with many BCAs (Wang et al. 2004; Chan et al. 2007), indicate that the high levels of the antioxidant proteins elicited by the MCFT, played important roles in reducing oxidative damages such as LPO and protein oxidation induced by the pathogen, which partially account for the observed delay in disease development in MCFT-treated plants.

As well as the induction of antioxidant proteins, the accumulation of phenolic compounds is also associated with oxidative stress in plant defence responses (Nanda et al. 2010; Xu et al. 2008). The present study show that MCFT-pathogen-co-treatment significantly accumulated phenolics in sunflower roots. Phenolics are known to be antimicrobial, and play a variety of roles in plant defence such as production of phytoanticipins, phytoalexins, structural barriers, modulators of pathogenecity, and activators of plant defence genes (Shoresh and Harman 2008). Yedidia et al. (2003) further explained the role of *T. asperellum* (T-203) as a resistance inducer in cucumber plants to infection by the *Pseudomonas syringae* pv. *lachrymans* and the relationship of the induced resistance to phenol metabolism. The increased accumulation of phenolic compounds is important in retarding fungal growth and decreasing plant mortality caused by *R. solani*. Moreover, antioxidant activity (AOA) is important in amelioration of the deleterious effects of FRs in biological systems. Excessive formation of FRs accelerates oxidation of macromolecules thereby increasing the risk of disease development (Singh et al. 2009). Phenolic compounds contribute greatly to the AOA through simple and complex mechanisms (Singh et al. 2010). Positive correlations were found between AOA and TPC of *Acacia* bark (Singh et al. 2009), *Moringa*, onion (Prakash et al. 2007), and fermented soybean (Singh et al. 2010). Hence, AOA is greatly influenced by the phenolic content which accounted for high AOA in MCFT-pathogen-co-treated plants. This indicates that elevated accumulation of phenolic compounds in MCFT-pathogen-co-treated plants may play a role as electron and hydrogen donors thereby protecting the root tissues against ROS-mediated oxidative stress produced during *Rhizoctonia* pathogenecity. So it is clear that accumulation of more phenolic compounds had a high negative correlation with plant mortality of sunflower plants. In many previous studies, *Trichoderma* spp. were found to be effective as mycoparasitic fungi in controlling *R. solani* (Harman et al., 2004; Howell et al. 2000; Wilson et al. 2008). Mycoparasitic activity of T-1055 controlled growth of the pathogen and reduced plant mortality.

Taken together, the novel findings of this study included: (i) Maize spent-cob-based *Trichoderma harzianum* NBRI-1055 formulation (MCFT) could alleviate the level of ROS production, lipid and protein oxidation in response to oxidative stress induced by *R. solani*; (ii) the products of ROS gene network such as antioxidant defence proteins are involved in the mechanisms of biocontrol against fungal pathogens and play an important role in mitigating pathogen-induced oxidative stress to sunflower plants These findings add new information for the pathway of induced resistance by antagonistic fungi.

Acknowledgements Brahma N. Singh is thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for award of Senior Research Fellowship and Akanksha Singh to University Grants Commission (UGC) for award of Junior Research Fellowship. This research was also supported by a grant from the Ministry of Agriculture & Cooperation, Govt. of India under Macromode Project.

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