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ORIGINAL ARTICLE

Induction of defensive enzymes in sunflower plants treated with agrochemicals against *Macrophomina phaseolina*

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Abstract

This study was carried out for the estimation of polyphenols (TP) and induction of oxidative enzymes polyphenol oxidase (PPO) and peroxidase (POD) in sunflower plants through seed immersion in agrochemicals of salicylic acid (SA) and water soluble chitosan (CH) in addition to a conidial suspension of *Trichoderma harzianum* and then analysis of plant content of carbohydrates and protein. The highest level of PPO 253.3 U · min^{-1} was detected in 50 ppm SA for 6 h. Next was *T. harzianum* when catalyzed PPO with 193.67 U · min^{-1}. Peroxidase was substantially catalyzed in accordance with the increment of inducers. Sunflower roots induced TP with up to 4.88 mg · g^{-1} in plants treated with SA at 50 ppm for 6 h and then declined with an increasing SA dose. The total carbohydrate content in leaves of 320 mg · 100 g^{-1} was found in treatments of CH at 50 ppm for 6 h. In roots, a carbohydrate content of 500 mg · 100 g^{-1} was observed using CH 75 ppm for 6 h. *Trichoderma harzianum* remarkably increased proteins in leaves and roots by up to 25% compared to 16.9% in the control. These results suggest that inducing the plants' own defense mechanism by applying salicylic acid and chitosan and bio-control of *T. harzianum* may offer alternative methods for controlling charcoal rot of sunflower due to the creation of defensive enzymes and could support plant vigor by enhancement of its protein and carbohydrate content.

Keywords: charcoal rot, chitosan, oxidative enzymes, salicylic acid, *Trichoderma harzianum*

Introduction

Macrophomina phaseolina is a plant-pathogenic fungus that causes charcoal rot in a wide variety of crops, including monocots like corn and sorghum and dicots like azuki bean and soybean (Gupta *et al.* 2012; Bandara *et al.* 2020).

The fungus causes necrotic lesions on stems, branches, and peduncles throughout production of phytotoxic metabolites including phaseolinon, botryodiplodin and patulin, which are believed to play a major role in the initial stages of infection, causing wilting of seedlings and charcoal rot (Abbas *et al.* 2020; Marquez *et al.* 2021). This increases the virulence of a pathogen and may explain the highly efficient mechanism to infect different hosts and tissues. The great adaptability of the fungus to a wide range of environmental stress also contributes to its ubiquitous distribution and infectivity of plants (Salvatore *et al.* 2020), and affects plants by secreting a group of cell wall degrading enzymes like pectinase, cellulase (Javaid and Saddique 2012). In addition, due to host tissue necrosis and the fragility of root tissues, the host is unable to absorb sufficient nutrients and water (Lodha and Mawar 2020). Depending on the pathogen, induced resistance can be classified as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Pieterse *et al.* 2014). After localized pathogen contact or treatment with synthetic or natural substances, SAR involves a unique defense signaling cascade that occurs systemically. It is also known as resistance to salicylic acid (SA) buildup and pathogenesis-related protein (PRP). In-

duced systemic resistance, on the other hand, was first described as a reaction triggered by plant growth-promoting rhizobacteria (PGPR), but it can also be triggered by antibiotics, surfactants, or chemical inducers (Gozzo and Faoro 2013). In contrast to SAR, ISR relies on the signaling pathways for jasmonic acid (JA) and ethylene rather than SA buildup. Induced systemic resistance can be found in both affected and non-infected areas of the plant (Llorens *et al.* 2017).

Foliar application or seed treatment of SA and chitosan (CH) increase plant resistance against diverse biotic and abiotic stresses. El-Hai *et al.* (2009) reported that SA and citric acid were used by seed soaking and foliar spray to combat seedling damping-off and charcoal rot *M. phaseolina* on sunflower plants. Salicylic acid is a phenolic endogenous growth regulator and a possible non-enzymatic antioxidant that regulates a variety of physiological processes in plants, including stomatal closure, photosynthesis, ion uptake, ethylene biosynthesis inhibition, transpiration, and stress tolerance (Arfan *et al.* 2007).

Chitosan is a safe and biodegradable compound. It is a natural carbohydrate polymer composed of randomly distributed β-(1,4) D-glucosamine and N-acetyl-D- glucosamine, which is commonly obtained from shellfish byproducts. It has been the object of much interest for its wide application in biotechnology, food and agriculture. Chitosan is able to stimulate various enzymes relating to defense mechanisms such as phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase, superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs) activities (Cho *et al.* 2008; Gonzales *et al.* 2015; Katiyar *et al.* 2015). Chitosan not only has effects against pathogenic fungi, but also it generally has growth promoting effects that increase crop yield of sunflower (El-Hai *et al.* 2009), rice (Abdel-Mawgoud *et al.* 2010) and wheat (Zeng and Luo 2012).

The strongest oxidative enzymes, polyphenol oxidase (PPO) and POD, have a role in pathogen resistance by increasing the synthesis of phytoalexins and depositing lignin in the plant cell wall. In this context, various studies have discovered that when plants are exposed to biotic or non-biotic stress such as salinity, drought for different grain crops (Guan *et al.* 2009; Mahdavi *et al.* 2011), the concentration and activity of these two enzymes increases, and that the resistance of different plants is linked to their activity (Chavan 2007; Sreedevi *et al.* 2011). One of the earliest enzymes to respond and provide quick protection against plant diseases is peroxidase (Mydlarz and Harvell 2006).

Many biocontrol microorganisms such as fungi of *Trichoderma* spp. and bacteria like *Bacillus* and *Pseudomonas,* colonize the plant rhizosphere and establish endophytic symbionts. They are widely used for their ability to promote plant growth, nutrients and induce resistance in the plant against plant pathogens (Hidangmayum and Dwivedi 2018; Harman *et al.* 2019). Therefore physiological and chemical changes in the plant occur, including the stimulation of PRP and oxidative enzymes such as polyphenol oxidase, peroxidase, and catalase, as well as the accumulation of phenols, which are crucial for pathogen resistance (Ojha and Chatterjee 2012).

The current work was aimed to the induction of and estimation of phytoalexins of polyphenols in the sunflower plant and stimulation of the oxidant enzymes of polyphenol oxidase and peroxidase against charcoal root rot in addition to determining the effect of examined bio-agents on the plant content of proteins and carbohydrates.

Materials and Methods

Inoculum and inoculation

Diseased roots were washed thoroughly under tap water and small pieces of necrotic roots disinfested using 2% NaOCl for 2 min and cultured on potato dextrose agar (PDA) supplemented with chloramphenicol (250 mg · l^{-1}) to avoid bacterial contamination (Gaddeyya *et al.* 2012; Reddy *et al.* 2014). Plates were incubated at 28°C ± 2°C for a week. Each isolate culture was purified on PDA slants, and stored at 4°C for further studies.

According to Edmunds (1964) a substrate of millet seeds (*Pennisetum americanum*) in 250 ml flasks was inoculated with five discs (5 mm) of a pathogen before incubated at 28°C ± 2°C for 10 days until production of extensive microscleroria. Four weeks after sowing the sunflower seeds, when plants were 20–25 cm high, the pots of each treatment were inoculated by mixing 5 g of millet seeds with the potting substrate.

Plant material and seed pretreatment with agrochemicals to induce systemic resistance

Sunflower seeds were superficially disinfected using 1% NaOCl, and washed several times with distilled water. Agrochemical inducers of SAR, used as seed pretreatments, were SA (2-hydroxybenzoic (salicylic acid) provided by British Drug Houses Ltd. B.D.H. Laboratory, Chemicals Division Pool, England and Chitosan; chitosan hydrochloride (10–120 cps), fungal origin (Glentham Life Sciences, Ltd., Units 4 & 5 Ingoldmells Court, Corsham SN13 9XN, UK) at 0, 25, 50 and 75 mg · l^{-1} according to Bakhoum *et al.* (2020), in addition to the bio-agent of *Trichoderma harzianum* at 4 × 10^6 conidia · ml^{-1}

as described by Govindappa *et al.* (2010). The sterilized seeds were soaked in the various concentrations of SA, and CH for two periods (6 and 12 h) or in water to provide a control.

Ten homogeneous healthy soaked seeds were sown in a greenhouse in pots 25 cm diam. containing an inoculated substrate of clay and sand soil (2 : 1 v/v).

Induction of oxidative enzymes

Preparation of sunflower root extract

After 2 months, sunflower fresh roots (0.5 gm) were washed and squashed using 10 ml of potassium phosphate buffer (0.1 mol) pH 7 in a sterilized ceramic mortar under cooling conditions. The root extracts were filtered using filter paper, and centrifuged at 4,000 rpm for 10 min. The filtrates were kept under freezing conditions for estimation of enzymatic activity (Pitotti *et al.* 1994).

Estimating the activity of polyphenol oxidase (PPO) (mg · l^{-1})

The polyphenol oxidase enzyme was estimated according to the method of Shi *et al.* (2002).

Estimating the activity peroxidase enzyme (POD) (mg · l^{-1})

The peroxidase enzyme was estimated according to the procedure of Muftugil (1985).

Determination of total phenols (TP) (mg · g^{-1} fresh weight)

The total phenols were determined using Folin-Ciocalteu Phenol Reagent according to Jain *et al.* (2017).

Statistical analysis

The data for all trials was analyzed using ANOVA and the difference between means was calculated with DMRT at ≤ 0.05 using SPSS version 14.0 software.

Results and Discussion

The seed treatments with examined agrochemicals and *T. harzianum* led to considerable activation of such defense enzymes as PPO, POD, and total phenols with noticeable variation based on each treatment concentration and duration of seed immersion. The highest activity of PPO by 253.33 U · min^{-1} resulted when using SA at 50 ppm for 6 h followed by 193.67 and 171.33 U · min^{-1} in the seeds immersed in a spore suspension of *T. harzianum* and CH at 75 ppm for 6 h. In contrast, increasing the SA to 75 ppm for immersion of seeds for 12 h assigned the enzyme energy to 60.33 U · min^{-1} (Table 1). In this aspect, the literature confirms that at reasonable concentrations, polyphenol

Table 1. *In vivo*: Induction of polyphenols and oxidative enzymes of polyphenol oxidase and peroxidase in sunflower roots after applied salicylic acid (SA), chitosan (CH), and *Trichoderma harzianum* (T.h.)

Inducers	Duration [h]	Concentration [ppm]	PPO activity (A420) nm [U · min^{-1} · g^{-1} fw]	POD activity (A420) nm [U · min^{-1} · g^{-1} fw]	Total phenols [mg · g^{-1} fw]
Inoculated (control)	–	0	49.00 g	109.67 h	3.86 ij
SA	6	25	161.00 bcd	202.33 efg	4.26 c
		50	253.33 a	204.33 defg	4.88 a
		75	123.67 def	295.33 bc	3.94 hi
	12	25	106.33 f	246.00 cde	3.97 ghi
		50	128.67 def	257.33 cd	4.22 cd
		75	60.33 g	355.67 ab	3.82 j
CH	6	25	117.33 ef	181.67 fg	4.10 ef
		50	148.00 cdef	159.67 g	4.07 efg
		75	171.33 bc	357.00 a	3.98 gh
	12	25	120.00 def	224.00 def	4.02 fgh
		50	155.00 bcde	188.33 fg	4.15 ed
		75	127.33 def	214.33 defg	4.41 b
T.h.	6	4×10^6	126.33 def	212.83 defg	3.90 hij
	12		193.67 b	193.67 efg	3.98 gh

PPO – polyphenol oxidase; POD – peroxidase
Means followed by the same letter(s) in each column are not significantly different at ≤ 0.05

oxidase is a crucial factor in disease resistance due to its ablate ability to catalyze the oxidation of phenolic compounds to quinones and the biosynthesis of lignin (Kavitha and Umesha 2008; Inayati *et al.* 2020).

Peroxidase was also strongly catalyzed with increasing SA and CH concentrations regardless of the seed immersion periods. Thus, SA increased peroxidase activity by 355.0 U · min^{-1} when applied at 75 ppm for 12 h whereas 75 ppm of CH for 6 h raised the enzyme catalysis to 357.0 U · min^{-1}.

These results were consistent with the association of plant resistance and peroxidase. The latter is one of the first enzymes to respond to plant pathogens by increasing their activity as a resistance-specific response. Physiological peroxidase activity was previously associated with the oxidation of certain phenolic acids to oxidize antifungal agents, or the direct suppression of fungi (Caruso *et al.* 2001). Furthermore, the decline in peroxidase activity described with plant ageing (Regnier and Macheix 1996), which is consistent with the findings of our study, could explain why sunflowers are more susceptible to *M. phaseolina* in the mature plant stage than in the early plant stage (Mydlarz and Harvell 2006). Peroxidase enzymes play a role in plant resistance by catalyzing indol acetic acid, lignin biosynthesis, cell wall suberization, and serve as an H_2O_2 detoxification system in plant cells (Golubenko *et al.* 2006).

The results in Table 1 also showed that sunflower root content of total phenols induced the highest levels of 4.88 mg · g^{-1} in plants treated with SA at 50 ppm for 6 h whereas the lowest content of 3.82 mg · g^{-1} was observed when plants were treated with a high dose of SA at 75 ppm for 12 h.

Polyphenol oxidase was catalyzed effectively when plants were treated with agrochemicals of SA and CH; the highest activity of this enzyme, 315.5 and 285.67 U · min^{-1}, was recorded with a high concentration of 75 ppm for both inducers followed by 224.17 and 230.83 U · min^{-1} which resulted when using SA at 25 ppm and 50 ppm, respectively (Fig. 1).

The lowest activity of PPO 202.83 and 174 U · min^{-1} appeared when seeds were immersed in CH at 25 and 50 ppm, respectively. Several authors confirmed that chemical antioxidants increase the activity of enzymes, including PPO (Ahmed 2016) which catalyzes the phenolic substances used in the synthesis of lignin, strengthening the cell wall structure and preventing the invasion of pathogens (Li and Steffens 2002; Li and Zhu 2013).

Data submitted (Fig. 2) confirmed that SA at 50 ppm activated the action of POD with 191 U · min^{-1} followed by 160 and 151.5 U · min^{-1} in the treatments of *T. harzianum* and CH 50 ppm, respectively, compared to 105.5 U · min^{-1} in the control. In this aspect Soliman *et al.* (2015) have also found that greater CH

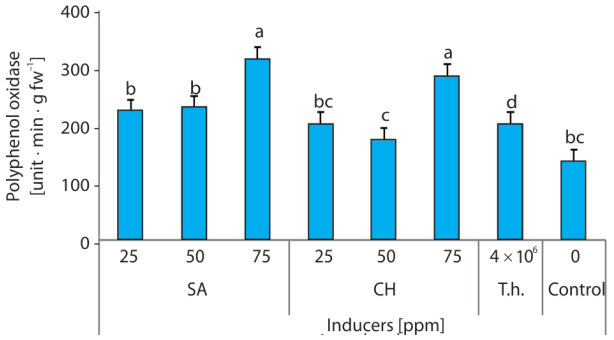

Fig. 1. Effect of inducer concentrations and *Trichoderma harzianum* (T.h.) on induction of polyphenol oxidase in sunflower roots. SA – salicylic acid, CH – chitosan

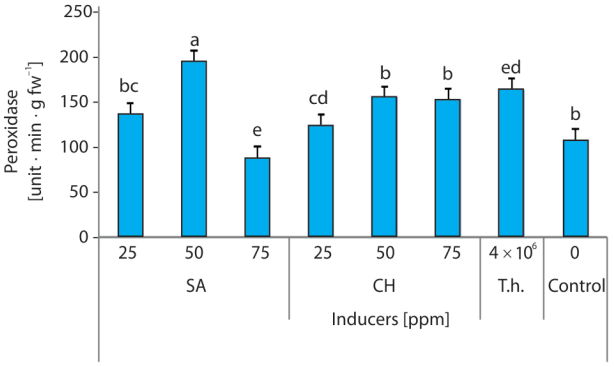

Fig. 2. Effect of inducer concentration and *Trichoderma harzianum* (T.h.) on induction peroxidase in sunflower roots. SA – salicylic acid, CH – chitosan

concentration was more effective for induction of the peroxidase enzyme in cucumbers. The high dose of 75 ppm of SA resulted in the lowest induction 86.33 U · min^{-1} of POD and was not different from the control.

Peroxidase and β-1,3-glucanase play a significant role in initiating the plant defense response against various pathogens through production of highly toxic phenolic compounds and higher production of reactive oxygen species or establishment of structural barriers such as lignin accumulation (Yusnawan *et al.* 2019; Inayati *et al.* 2020). Khaledi and Taheri (2016) reported a comparable increment in peroxidase activity and phenolics in soybean roots when seeds were sown after inoculation with *T. harzianum* isolates.

Abiotic and biotic factors induced systemic resistance in different plants that was associated with increased efficacy of peroxidase and PAL enzymes (Chen *et al.* 2009; Umamheswari *et al.* 2009). However, plants treated with resistance-inducing chemical antioxidant compounds, including CH and SA, led to an increase in the peroxidase and PAL enzymes and the accumulation of phenolic compounds (Bui *et al.* 2019).

The results in Figure 3 show noticeable variation in the total phenol content of sunflower roots when treated with agrochemicals and the bioagent of *T. harzianum*; 50 ppm of SA significantly improved total phenols to 4.55 mg · g^{-1}, followed by 4.19 mg · g^{-1} when applied CH at 75 ppm. In contrast SA at 75 ppm exhibited the lowest total phenol content by 3.88 mg · g^{-1}.

Plant phenols have led to various discoveries related to plant defense against various pathogens (Treutter 2006). Hence, induction of systemic resistance is associated with phenol accumulation that increases the activity of PRP, and augmented physical and mechanical resistance of the cell wall structure. Viacava and Roura (2015) also reported that exogenous daily spraying of chitosan induced biosynthesis of phytochemicals, including phenolic compounds and flavonoids, from lettuce buds.

Data in Table 2 revealed that soaking sunflower seeds in agrochemicals and *T. harzianum* caused significant increases in carbohydrate and protein content in roots and leaves compared to the control. It is obvious that total carbohydrates in leaves increased (to 320 mg ·100 g^{-1}) when seeds were treated with CH 50 ppm for 6 h. Salicylic acid at 25 ppm for 12 h also improved carbohydrate content (to 310 mg · 100 g^{-1}), whereas the lowest content of 140 mg · 100 g^{-1} was recorded when seeds were immersed in SA 25 ppm for 6 h. The highest total carbohydrate content in roots,

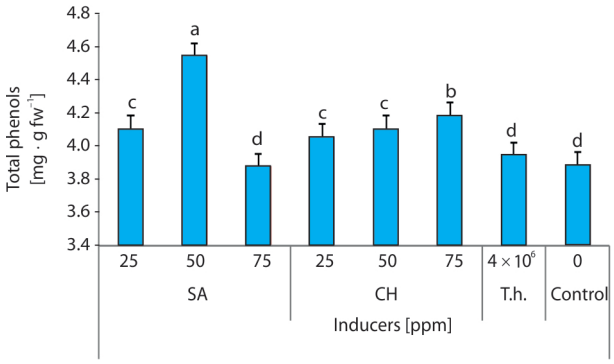

Fig. 3. Effect of inducer concentration and *Trichoderma harzianum* (T.h.) on induction of total phenols in sunflower roots. SA – salicylic acid, CH – chitosan

500 mg · 100 g^{-1}, resulted when using CH 75 ppm for 6 h. Similarly, SA at 50 and 75 ppm for 12 h also coincided in carbohydrate increases by 490 mg · 100 g^{-1} for both treatments. The lowest content of carbohydrate (220 mg · 100 g^{-1}) was detected in roots when using SA at 50 ppm for 6 h.

Physiologically, soluble carbohydrates have been shown to improve membrane stabilization by acting as a reactive oxygen species (ROS) that was produced as a normal product of plant cellular metabolism including respiration and photosynthesis (Hosseini *et al.* 2014). Salicylic acid can activate

Table 2. Effect of inducers on carbohydrate and protein content of sunflower leaves and roots

Inducers	Duration [h]	Concentration [ppm]	Leaf carbohydrate [mg · 100 g^{-1}]	Root carbohydrate [mg · 100 g^{-1}]	% Leaf protein	% Root protein
Inoculated (control)	–	0	60 f	160 d	21.21 d	16.88 g
SA	6	25	150 de	430 ab	23.63 c	17.79 g
		–	–	–	–	–
		50	180 bcde	220 cd	23.44 c	18.59 fg
		75	140 ef	420 ab	25.11 bc	21.03 def
	12	25	310 a	350 abc	24.94 bc	22.63 bcd
		50	270 abc	490 a	23.96 c	24.04 bc
		75	250 abcd	490 a	24.07 c	18.99 fg
CH	6	25	300 a	410 ab	24.21 c	21.77 cde
		50	320 a	280 bcd	24.79 c	20.42 ef
		75	250 abcd	500 a	24.07 c	21.07 def
	12	25	190 abcd	270 bcd	24.07 c	21.67 cde
		50	250 abcd	390 ab	24.50 c	23.33 cde
		75	170 cde	230 cd	25.09 bc	23.79 bc
T. h.	6	4 × 10^6	150 de	370 abc	26.54 ab	26.81 a
	12		270 ab	340 abc	27.68 a	25.51 ab

T.h. — *Trichoderma harzianum*, SA – salicylic acid, CH – chitosan
Means followed by the same letter(s) in each column are not significantly different at ≤ 0.05

the metabolic consumption of soluble sugars for the formation of new cellular components to stimulate the growth of sunflower plants. It could also be assumed that treatment of SA inhibits the polysaccharide hydrolase system and/or accelerates the incorporation of soluble sugars into polysaccharides. Our hypothesis may be supported by the finding that SA activates the consumption of soluble sugar metabolism by increasing polysaccharide levels and increasing osmotic pressure (Zahra *et al.* 2010)

Protein content in sunflower leaves and roots increased considerably when seeds were soaked in various concentrations of inducers and *T. harzianum* (Table 2). The highest protein in leaves (26.54 and 27.68%) was found in plants treated with *T. harzianum* spore suspension for 6 and 12 h. In roots, the protein percentage also exceeded 26 and 25.5% for the same treatments compared to 16.9% in the control.

Trichoderma spp. possess a high ability to detoxify poisonous substances in the soil and accelerate the decomposition of organic materials (Amira *et al.* 2011; Sharma *et al.* 2012; Zafra *et al.* 2015). Notably, the success of *Trichoderma* spp. as a natural decomposer in the soil ecosystem is due to its ability to activate plant growth and to modify the structure of the rhizosphere, as well as its availability and absorption of nutrients. Furthermore, it can tolerate adverse environments and has a strong destructive capacity against pathogens (Harman 2006). However, the biological control by *Trichoderma* spp. against pathogens might be due to various modes such as PR protein production, mycoparasitism and competition for nutrients (Karmel Reetha *et al.* 2014; Inayati *et al.* 2020) in addition to induction of systemic resistance in various plant species and pathogens (Angel *et al.* 2016; Małolepsza *et al.* 2017).

Figure 4 shows that the roots of treated plants contained more carbohydrates than the leaves; the highest content in roots, 460 mg · 100 g^{-1}, was recorded with SA at 75 ppm, while the highest leaf content of carbohydrate (290 mg · 100 g^{-1}) resulted when seeds were immersed in CH at 50 ppm followed by 250 mg · 100 g^{-1} for CH utilization at 25 ppm compared to 60 mg · 100 g^{-1} in the control.

In Figure 5 the protein content in leaves was more than in roots with the same treatments; the highest protein in leaves (27.11%) and roots (26.16%) was recorded when applied with *T. harzianum* compared to 21.21 and 16.88% in the control. The lowest percentage of protein was recorded when seeds were immersed in SA at 50 ppm by 22.69% in leaves, while in roots it was recorded when seeds were soaked in SA 75 ppm by 20.01% and roots. The effect of inducer on the contents of protein in both roots and leaves of other oil crops infected with *M. phaseolina* was clarified by Sharma (2011) and Doley and Jite

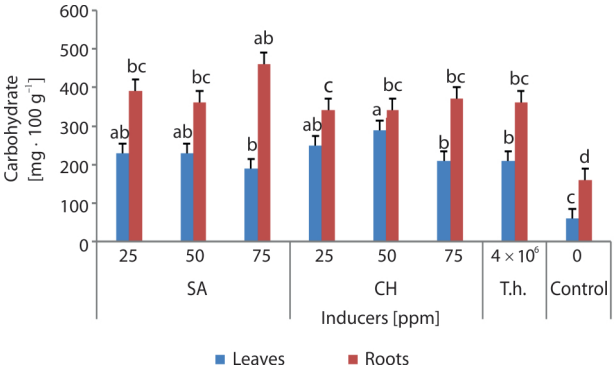

Fig. 4. Effect of inducer concentration and *Trichoderma harzianum* (T.h.) on carbohydrate content of sunflower leaves and roots. SA – salicylic acid, CH – chitosan

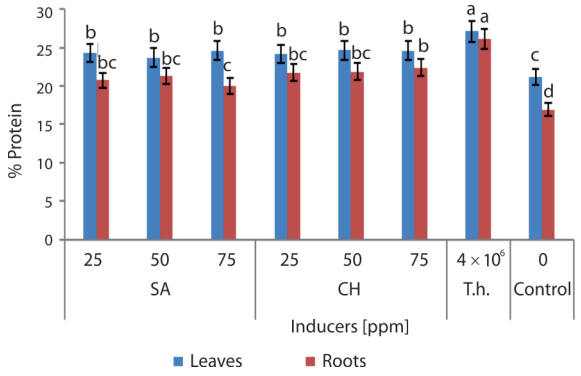

Fig. 5. Effect of inducer concentration and *Trichoderma harzianum* (T.h.) on protein content of sunflower leaves and roots. SA – salicylic acid, CH – chitosan

(2013), who reported that an increase in all defense-relevant proteins in sesame at varying degrees of *M. phaseolina* infection and protein content was higher in peanut plants inoculated with *M. phaseolina* than in control plants.

The positive correlation between polyphenol content and charcoal rot resistance was also found in other crops such as sorghum cultivars (Kumari *et al.* 2015), and groundnut plants (Doley and Jite 2013) and sesame cultivars (Sharma 2011).

Conclusions

In this study, it seems that all plants are enriched with defense genes. These genes are quiescent in nature and require appropriate stimulation signals including agrochemicals or bio-agents for activation for cratering and activating SAR. Thus, we conclude that application of CH, SA and the bio-agent of *T. harzianum* offered a clear increment in activity of such oxidative enzymes

as POD and PPO in plant cells when seeds were treated with CH at 75 ppm, particularly when seeds were immersed for 6 h.

Agrochemicals also led to augmentation of carbohydrate and protein content of sunflower leaves and roots, some of these carbohydrates are elicitors of plant defenses, while others act as signaling molecules in a manner similar to phytohormones against diseases. Many of these interactions are recognized in particular lectins which play a major role in the immune system. Apparently, utilizing *T. harzianum* significantly enhanced the protein content of leaves and roots that resulted in a comparable improvement of plant vigor. Therefore, plant defense inducers are able to induce broad disease resistance that proffer additional options for farmers to complement genetic disease resistance.

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