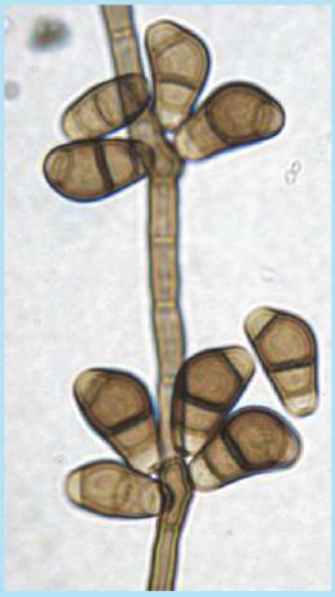
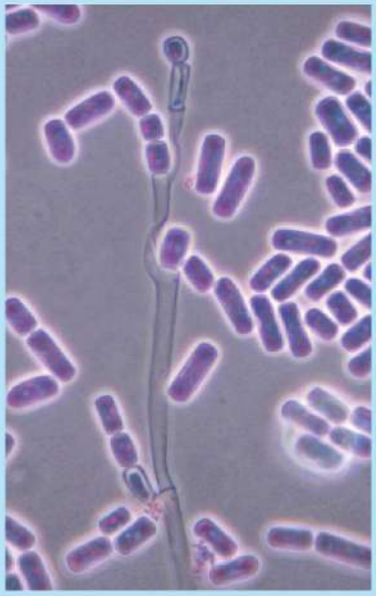
JBAM- EG

Journal of Basic and Applied Mycology (Egypt)





Volume 13 : 2022

ISSN (Print) : 2090 - 7583

ahamaumc@yahoo.com

ISSN (Online) : 1047 - 2357

http://www.aun.edu.eg/aumc/journal/index.php

Journal of Basic & Applied Mycology (Egypt) 13 (2022): 12-33 p-ISSN 2090-7583, e-ISSN 2357-1047 ©
2010 by The Society of Basic & Applied Mycology (EGYPT) http://www.aun.edu.eg/aumc/Journal/index.php

Production of terpenoid phytoalexins as a plant response against *Alternaria cerealis* AUMC 14484 controlled by *Trichoderma harzianum*

Ghada A. Mahmoud[1], Mohamed A. Abdel-Sater[1], Eshraq AL-Amery[1,2] , Nemmat A. Hussein[1]

[1]*Botany & Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt.*
[2]*Microbiology Department, Faculty of Applied Science, Taiz University, Taiz, Yemen.*
***Corresponding author e-mail:** nemmgoda@yahoo.com,

Received 1/7/2022,
Accepted 25/8/2022

Abstract: Terpenoid phytoalexins are secondary metabolites of plants act as defensive agents against plant pathogen attack. *Alternaria cerealis* AUMC 14484 (MT808477) is a phytopathogen of tomato that stimulates new types of phytoalexins especially during the biological control process. *Trichoderma harzianum* utilized as controlling agent of *A. cerealis* using the fungal filtrates or spore suspension in infected plants. Time course detection of terpenoid phytoalexins was 2, 24 and 48 h of infection and estimated with complete analysis of their types and concentrations using GC-MS analysis. The plant cells behavior during the biological control process was monitored by lipid peroxidation, hydrogen peroxide, and antioxidant enzymes (catalase and peroxidase) analyses. The results showed significant increase in total terpenoids, lipid peroxidation and hydrogen peroxide after 24 h in all treatments; however catalase and peroxidase increased in infected tomato plants but decreased during the biological control process which reflects decrease in the cell stress during the infection. Twelve terpenoid phytoalexins recorded in infected tomato leaves, seven of them are newly recorded in tomato plants including 5à-spirostan-23-ol, (22s,23r,25r); 2(1h)-naphthalenone, octahydro-1-methyl-1-(2-propenyl), (1à,4aá,8Aà); 2,2' (1h,1'h)-spirobi-s-indacene, ethanone.; Spiro[5hbenzocycloheptene 5,1'[2,5] cyclohexadiene] 4',9diol,6,7,8,9 tetrahydro2,3,3',4,5 'pentamethoxy-7,8-dimethyl-, 9-acetate; carvacrol; maslinic acid; Spirost8en11one,3 hydroxy, (3á,5à,14á,20á,22á,25R) and Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à). Rishitin derivatives (rishitinol and rishitinone) also recorded in infected tomato leaves. The application of *Trichoderma harzianum* as culture filtrate or spore suspension throughout the biological control procedure is critical for tomato plant resistance against *A. cerealis* leaf spot disease by enhancing redox buffer capacity, improving plant tolerance, and activating plant defense systems.

Keywords: *Alternaria cerealis*, *Trichoderma harzianum*, Terpenoid Phytoalexins, Antioxidants, Phytopathogens.

Introduction

Plants have evolved a complex defense mechanism that relies on the rapid perception and activation of secondary metabolites. These defensive metabolites, such as terpenoids, constitute a huge and diverse chemical substance (Li *et al.* 2015, Vaughan *et al.* 2015, Ashour *et al.* 2018, Erb and Kliebenstein 2020 and Divekar *et al.* 2022). Terpenoids act as phytoalexin compounds are low molecular weight compounds accumulate in response to biotic and abiotic factors (Singh and Sharma 2015 and Ashour *et al.* 2018). The biosynthesis of terpenes occurs in the cytoplasm through the mevalonate pathway or in the plastids through the methylerythritol phosphate pathway (Ashour *et al.* 2018 and Tiku 2020). Artificial injury or treatment with biological agents is usually sufficient to induce terpene synthase. The terpene synthesis induced by coniferous wounds has been well described (Martin *et al.* 2002 and Miller *et al.* 2005). Elicitors are signal molecules that motivate the plant to synthesize a phytoalexin compound. Elicitors are chemical compounds that can be derived from biotic or abiotic sources that can stimulate the response of stress in plants, improving the synthesis and accumulation of secondary metabolites or the induction of new secondary metabolites (Naik & Al-Khayri 2016).

Trichoderma has a strong antagonistic and fungal parasitic effect on plant pathogens that induce defense mechanisms in several plants and has previously been reported as an effective biological control agent against several soil-borne pathogens (Brotman *et al.* 2012, Kobori *et al.* 2015 and Daryaei *et al.* 2016). *T. harzianum* can greatly reduce the early and late blights of tomato plants (Abdel-Kader *et al.* 2012). This biocontrol activity is been probably attributed to the ability to produce antibiotic compounds and efficiently compete for niches and

nutrients (Zhao *et al.* 2013). *In vitro*, *T. harzianum* inhibited the mycelial growth of *Alternaria solani* and *Phytophthora infestans* in tomatoes. The inoculant induces the production of plant hormones (auxin and gibberellin) and increases the level of plant defense enzymes (Chowdappa *et al.* 2013). In addition, the use of *Trichoderma* species as biological agents can significantly increase enzymatic antioxidants such as peroxidase, catalase, polyphenol oxidase that play an important role in the defense mechanism of plants against pathogen infection (EL-Tanany *et al.* 2018).

The production of reactive oxygen species (ROS) including superoxide anion, hydroxyl radical and hydrogen peroxide is the main indicator of early plant defense responses encountered by fungal pathogens (Lehmann *et al.* 2015). One of the main functions of the ROS system is to control several biological and physiological processes, acting as a key intermediate signal to relieve biotic and/or abiotic stress, (Mittler *et al.* 2011 and Baxter *et al.* 2014). In protection signal transduction pathways, ROS are one of the first cell responses in invading pathogens associated with cell homeostasis damage, leading to the expression and activation of plant defense-related genes *via* phytoalexin formation, cell wall deposition, and protein-related pathogenesis (PR) (Xu *et al.* 2008 and Shoresh *et al.* 2010).

Some studies have found that susceptible and resistant tomato varieties have very different accumulations of phytoalexins after being infected with *Verticillium dahliae* or *Fusarium* wilt, indicating the accumulation of phytoalexin compounds as a possible mechanism of resistance as generation of reactive oxygen species (ROS) and induction of anti-microbial secondary metabolites (flavonoids, phenol and terpenoids) in plant resistance against fungal pathogens, where they are transported to the site of infection. Furthermore, they have been noticed that, phytoalexin compounds are merged with the cell walls of necrotic and adjacent plant cells (Hutson and Smith 1980 and Kiran *et al.* 2017). Several sesquiterpene and monoterpene phytoalexin compounds have been described, that referred as the mechanisms of plant defense strategies. Generally, Solanaceae plants produce sesquiterpenoid phytoalexin to defend against a variety of pathogens. These compounds are not only harmful to pathogens, but also harmful to plants, so after eliminating the threat, they need to be detoxified. The reactions performed may be oxidation, reduction, hydrolysis, or other mechanisms, depending on both fungal species and the chemical structure of phytoalexin (Pedras *et al.* 2011 and Camagna *et al.* 2020). Rishitin observes hypersensitivity reactions in the epidermal cells of resistant plants, thereby limiting the growth of pathogens (Shih *et al.* 1973). It accumulates in incised or injured tissues and biosynthesized *via* the acetate-mevalonate pathway (Shih *et al.* 1973).

The objectives of this study are to discover the comprehensive effects of different concentrations of fungal filtrate or spores suspension of *Trichoderma harzianum* on the terpenoid phytoalexin compounds of the infected tomato plants with *Alternaria cerealis* after different infection periods. Also, explaining the defense response of plant cell to induce other metabolic elements.

Materials and Methods

Tomato phytopathogen

Alternaria cerealis (AUMC 14484) was isolated from dark spot lesions on tomato leaves (*Solanum lycopersicum* L.) using PDA medium following Shabana (1987) method. The fungus examined and identified by conventional and molecular methods according to White *et al.* (1990) and Simmons (2007). Pathogenicity test was performed using four weeks tomato seedlings growing in soil contains clay 56.7%, silt 30.9%, sand 12.4% with pH 7.11 and EC 2.27, using 1×10^5 conidia/ml *A. cerealis* inoculum as described by Blagojević *et al.* (2020).

Antagonistic activity of *Trichoderma harzianum* against *Alternaria cerealis* (MT808477)

One week old *A. cerealis* conidia grown on PDA medium at 28±2°C was scratched, suspended in 0.1% Tween-80/ sterilized distilled water and diluted to 10^5 conidia/ml. One ml of aliquots conidia suspension of *A. cerealis* was transferred directly into empty sterilized plates, then about 15 ml sterilized PDA medium cooled to just above solidifying temperature were added to plate. The plates were rotated by hand in broad swirling motion in order to disperse of spore suspension in the medium. After solidification, *T. harzianum* was inoculated in the center of each plate and all plates were incubated at 28 ± 2°C for 7 days. The diameter of the inhibition zone was measured.

Biological control greenhouse experiment using two saprophytic inoculum types

Greenhouse experiment was performed according to Solino *et al.* (2017). Thirteen day old

tomato (*Solanum lycopersicum* L.) plants were utilized for this experiment. Plants were placed in black polyethylene bags with 30 cm diameter loaded with 1Kg autoclave sterilized soil and grown in environmental conditions.

Inoculum preparation of *Alternaria cerealis* AUMC (14484)

The strain of *Alternaria cerealis* (AUMC 14484) was re-cultured in Potato Dextrose Agar (PDA) medium at 28±2°C. Mycelial fragments were removed by filtering through sterile gauze. The conidial suspension was collected in sterile water containing trace amounts of tween-80 (0.1%) and adjusted to a concentration of 10^5conidia/ml using cell haemocytometer**.** The tested tomato plants were inoculated with the conidial suspension of the pathogen.

Preparation of *Trichoderma harzianum*

Both culture filtrates (10, 20, 30%) and spore suspensions (10^5 and 10^7) were applied. Fungal filtrates were obtained by transferring one disc (7 mm) of PDA medium covered with three days old mycelia of *Trichoderma harzianum* in 100 ml potato dextrose broth. The medium containing *T. harzianum* was incubated at 28±2°C for 15 days on rotatory incubator (200 rpm), then filtered on Whatman filter paper (No 1) (Solino *et al.* 2017).

The inoculum of *T. harzianum* was grown on PDA plates incubated at 28 ± 2°C for 7 days on static condition. The growing fungal spores were suspended in 0.1% tween-80 in sterilized distilled water. The inoculum size was adjusted to 10^5 and 10^7 spores/ml by microscopic enumeration with a cell-counting hematocytometer.

The infected tomato plants were sprayed with either culture filtrates (10, 20, 30%) or spore suspensions (10^5 and 10^7) to evaluating the phytoalexin compounds induced in the infected tomato plants. Three pots were used as replications for each treatment, in addition to the control plants injected with *T. harzianum* only (Solino *et al.* 2017). Plant leaves were examined after 2, 24 and 48 h for testing the physiological traits including terpenoids, MDA, hydrogen peroxide analysis and evaluating activities of tested enzymes (CAT and POD) (de Oliveira *et al.* 2021).

Extraction and estimation of terpenoid phytoalexins

Phytoalexins from tomato leaves (1 g) were extracted with 10 ml methanol in liquid nitrogen. The extracts were filtered through Whatman filter paper (No 1), then the filtrate was evaporated to dryness at 40°C and finally, the dried sample was dissolved in 10 ml methanol for Gas Chromatography-Mass Spectrometry analysis (Price *et al.* 1976). Quantitative determination of terpenoid phytoalexins were carried out using Trace GC-ISQ mass Spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (film thickness 30 mx 0.25 mm x 0.25 µm) and splitless sample injector. The unknown component spectrum was identified by comparing the mass-to-charge ratios and the abundance of each compound detected against a standard mass chromatogram in the National Institute of Standards and Technology (NIST) database and the Wiley registry of the mass spectral library. The identification of the obtained compounds was achieved using PubChem software.

Determination of total terpenoids content

Determination of total terpenoid contents were carried out according to Fan and He (2006). 10 mg of leaves were dissolved in 1ml dichloromethane. The extract was mixed with vanillin-glacial acetic acid solution (150 µl, 5% w/v), heated for 45 min at 60°C, then cooled in an ice-water bath to the ambient temperature. After the addition of glacial acetic acid (2.25 ml), terpenoid contents were determined at 548 nm and calculated from calibration curve of pure ursolic acid (0.025–0.5 mg/ml in methanol). Total terpenoid was expressed as milligram ursolic acid equivalents (mg ursolic acid/g extract).

Determination of lipid peroxidation

The leaf material (0.5 g) was homogenized in 5 ml of ethanol, and centrifuged at 10000 rpm for 5 min. One ml of the supernatant was added to 3 ml of 20% trichloroacetic acid (TCA) containing 0.65% of thiobarbituric acid (TBA) was added. The mixture was heated at 95 ºC for 30 minutes and cooled quickly on an ice bath. The mixture was then centrifuged at 10,000 rpm for 15 minutes, and the absorbance of the supernatant was measured at 532 nm. The level of lipid peroxidation was expressed as nmol g^{-1} FW (Fresh Weight) of Malondialdehyde formed using an extinction coefficient of 155 mM^{-1} cm^{-1}(Hodges *et al.* 1999).

Determination of hydrogen peroxide

The leaf material (0.3 g) was homogenized in an ice bath with 5 ml of 0.1% TCA. The homogenate was centrifuged at 10,000 rpm for 15 mins. The supernatant (0.5 ml) was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. After 20 min, the absorbance of the supernatant was read at 390 nm. A calibration curve using H_2O_2 was constructed, and the concentration expressed as mg g−1 FW (Velikova *et al.* 2000).

Determination of catalase (CAT) and peroxidase (POD) antioxidant enzymes

Frozen leaf segments (1g) was ground to a fine powder in liquid nitrogen, mixed in 10 ml of 100 mM potassium phosphate buffer (pH 7.8), containing, 0.1 Na_2-EDTA and 0.1 g polyvinylpyrrolidone (PVP). The mixture was centrifuged at 15000 rpm for 15 min at 4ºC and the supernatant collected and used for evaluating activities of all tested enzymes (Abdelhamid *et al.* 2020). Protein content was assessed based on Lowry *et al.* (1951) method, using bovine serum albumin (BSA) as the standard curve. Catalase (CAT) activity was determined by the reaction medium (3 ml volume) contained 50 mM potassium phosphate buffer (pH 7), 100 µl H_2O_2 (10 mM) and 20 µl of enzyme extract and measuring the decrease in absorbance at 240 nm (µg protein^{-1} min^{-1}) (Havir and McHale 1987). The activity of peroxidase (POD) was evaluated by adding 100 µl of enzyme extract to 2.5 ml of a solution containing 30 mM potassium phosphate buffer (pH 7), 6.5 mM H_2O_2, and 1.5 mM guaiacol. The absorbance was measured at 470 nm. The results were expressed as µg protein^{-1} min^{-1} (Hammerschmidt *et al.* 1982).

Statistical analysis

All the obtained results were analyzed statistically using one- way analysis of variance (ANOVA). The significance of mean differences was determined at P ≤0.05 through SPSS software version 25 (Duncan's multiple tests).

Results

Biological control of *Alternaria cerealis*

Alternaria cerealis AUMC 14484 was isolated from dark spot lesions on tomato leaves, the pathogenicity was confirmed at the beginning of infection by *A. cerealis* symptoms appeared as yellow haloes with small brown spots, but in the later stages, the spots gradually increased in size, forming dark brown concentric circles. The diseased plants died after 15 days of fungal inoculation. It was found that, the pathogen infected all the tested plants with 100% prevalence. *Alternaria cerealis* AUMC 14484 was recorded in the current study for the first time as a phytopathogen to tomato plants. Therefore, it was identified morphologically and genetically using ITS 1 & 4 primer set as *Alternaria cerealis* (MT808477). Saprophytic *Trichoderma harzianum* showed high antagonistic potentiality against *A. cerealis* in PDA plates in the 5th day of incubation (Figure 1).

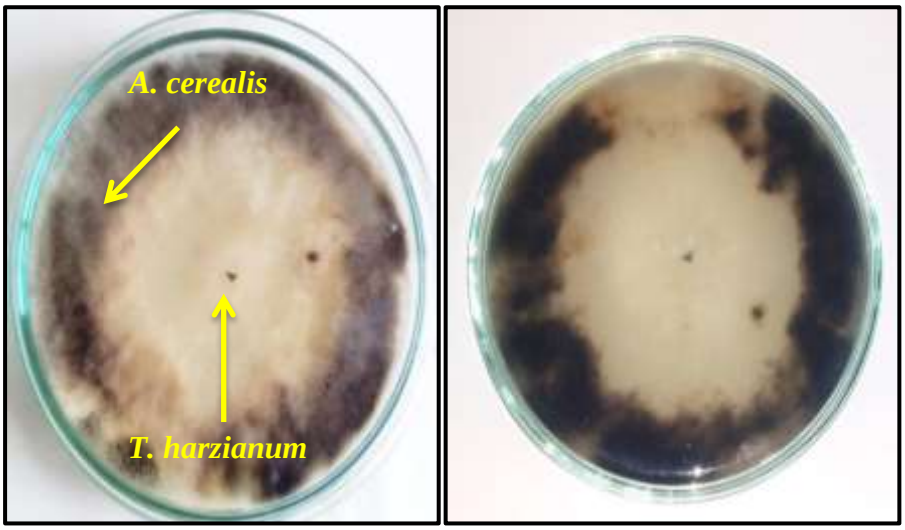


Figure 1: Antagonistic activity of *Trichoderma harzianum* against *A. cerealis* AUMC14484

Effect of time-course biological control of tomato plant infected with *Alternaria cerealis* (MT808477) by *T. harzianum* on physiological traits of the plant

Total terpenoids

Time-course total terpenoids were determined after 2, 24 and 48 h of the application of biological control process against *Alternaria cerealis* (MT808477) by *T. harzianum* using culture filtrates (10%, 20 and 30%) and spores suspensions (10^5 and 10^7 spores/ml). Data obtained in figures (2&3) clarify that the total terpenoid content in tomato plants increased significantly with time achieving the highest values after 48 h in both culture filtrates (10%, 20 and 30%) and spore suspensions (10^5 and 10^7 spores/ml). Also, statistical analysis showed strong positive correlation between the two types of application of *T. harzianum* and terpenoid content in tomato leaves. Application of biological control on tomato plants infected with *A. cerealis* stimulates the terpenoid phytoalexins production, comparing with the pathogen only or the saprophytic fungus only in both inoculum types. Fungal filtrate (20%) recorded the highest terpenoids content (47.46±0.99 mg/g FW), followed by 30% (37.83±1.2 mg/g FW) and 10% (37.5±0.91 mg/g FW). Generally, using spore suspension of *T. harzianum* recorded higher terpenoid content than the filtrates, especially in the higher spores concentration 46.13±0.6 mg/g FW after 48 h (10^7spores/ml). Also, statistical analysis showed a strong positive correlation between different inducers (filtrates or inoculum concentrations of *T. harzianum*) and terpenoids content in tomato leaves (0.327** and 0.516** respectively).

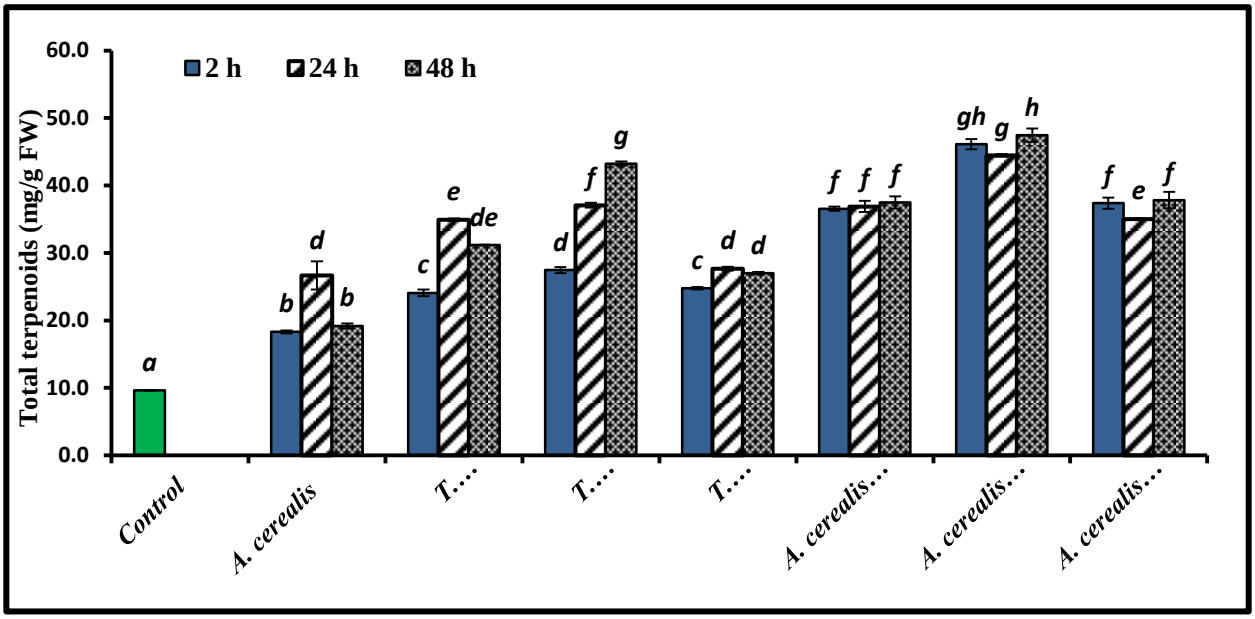


Figure 2: Total terpenoids of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different concentrations (10%, 20% and 30%) of *T. harzianum* filtrates and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$**).**

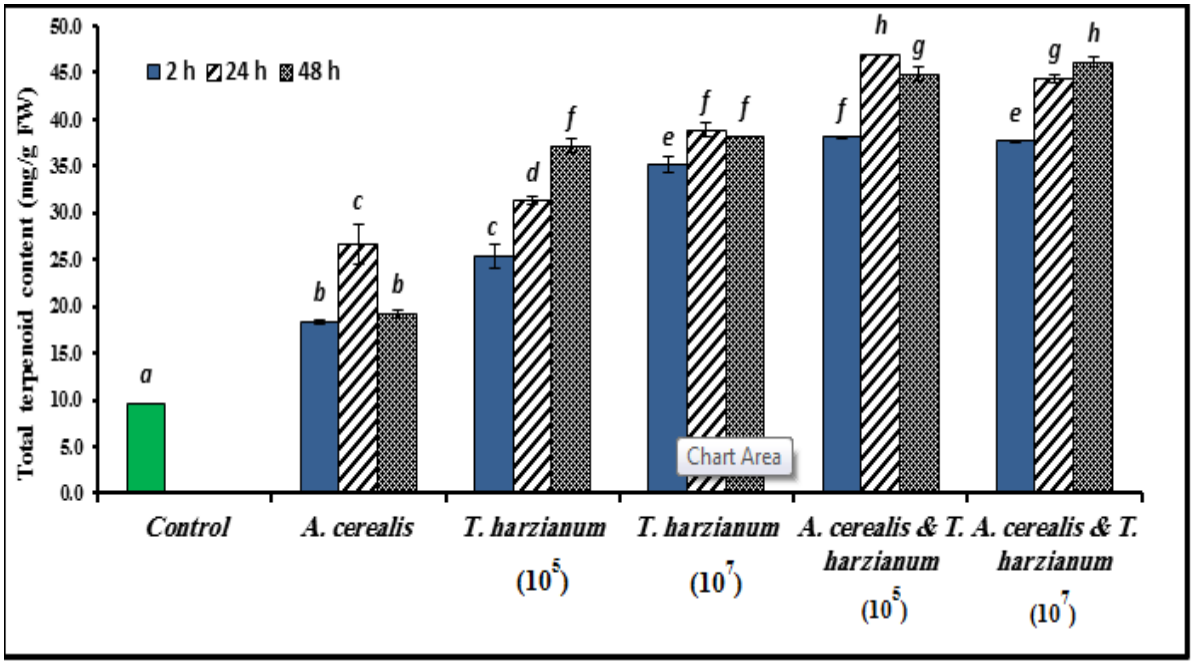


Figure 3: Total terpenoid content of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different inoculum volumes of *T. harzianum* and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$).

Lipid peroxidation and hydrogen peroxide production

Oxygen free radicals play a role in the pathogenesis of tissue damage in many pathological conditions *via* the peroxidation of membrane phospholipids. The content of malondialdehyde (MDA) is determined in infected tomato plants by *Alternaria cerealis* (MT808477) and controlled by

T. harzianum using two types of inoculua culture filtrates (10%, 20 and 30%) and spore suspensions (10^5 and 10^7 spores/ml) with time-course 2, 24 and 48 h of the treatments. The MDA content was significantly higher in plant infected with *A. cerealis* after 48 h (351.5±2.56 mM/g FW), and decreased in the biological control treatment by increasing the filtrate concentration (199.04±6.56 mM/g FW at 30% filtrate) than the pathogen control (Figure 4). The increase of free radicals will lead to the overproduction of malondialdehyde (MDA) that referred to as a marker of oxidative stress and antioxidant status in membrane damage in plants. Increasing *T. harzianum* spore concentration stimulated MDA production, especially after 48 h. by 407.47±5.79 and 392.4±9.87 mM/g FW for 10^7 & 10^5 spores/ml respectively as illustrated in figure (5). Further, results appeared that the MDA content had strong positive correlation with all treatments with filtrates and inoculum suspension at different concentrations with infected or non-infected plants. Hydrogen peroxide production indicated significantly high concentration at 2 h then decreased after 48 h in all treatments. The highest production of H_2O_2 reflect high stress on the plant was observed by fungal filtrate (30%) (3.7±0.01 mg/g FW) followed by 20% (3.37±0.07 mg/g FW) and 10% (2.79±0.05 mg/g FW) after 2 h (Figure 6). Using spore suspension recorded higher stimulation of H_2O_2 than the filtrates, also increasing the spores concentration increase the H_2O_2 content by 4.59±0.08 (10^7 spores/ml) and 4.12±0.03 (10^5 spores/ml) mg/g FW (Figure 7). It is worth to mention that correlation between terpenoid and H_2O_2 contents was highly positive (0.396**) in the filtration treatments.

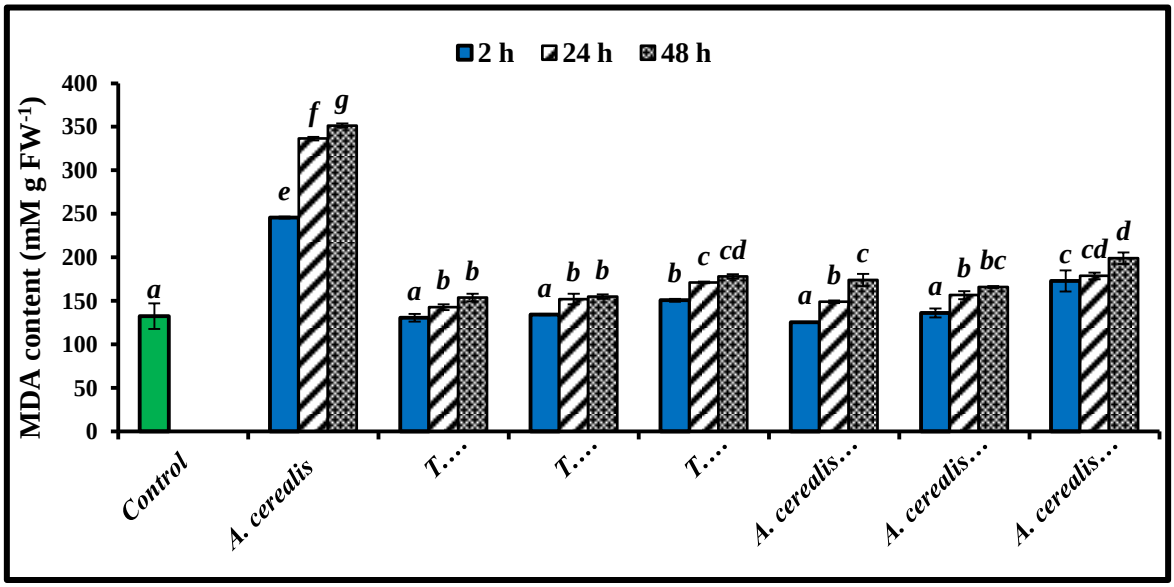
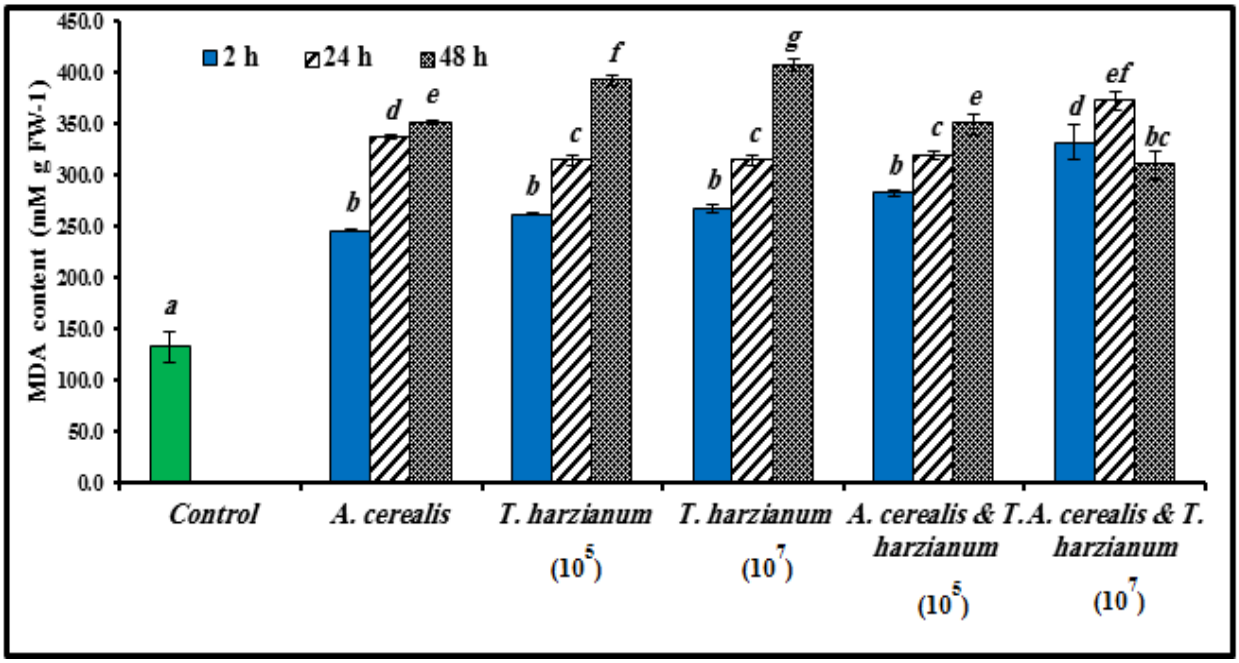


Figure 4: MDA content of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different concentrations of *T. harzianum* filtrates and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$)



.

Figure 5: MDA content of healthy (control) and infected tomato leaves L.) by *A. cerealis* (MT808477) under different inoculum volumes of *T. harzianum* and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$).

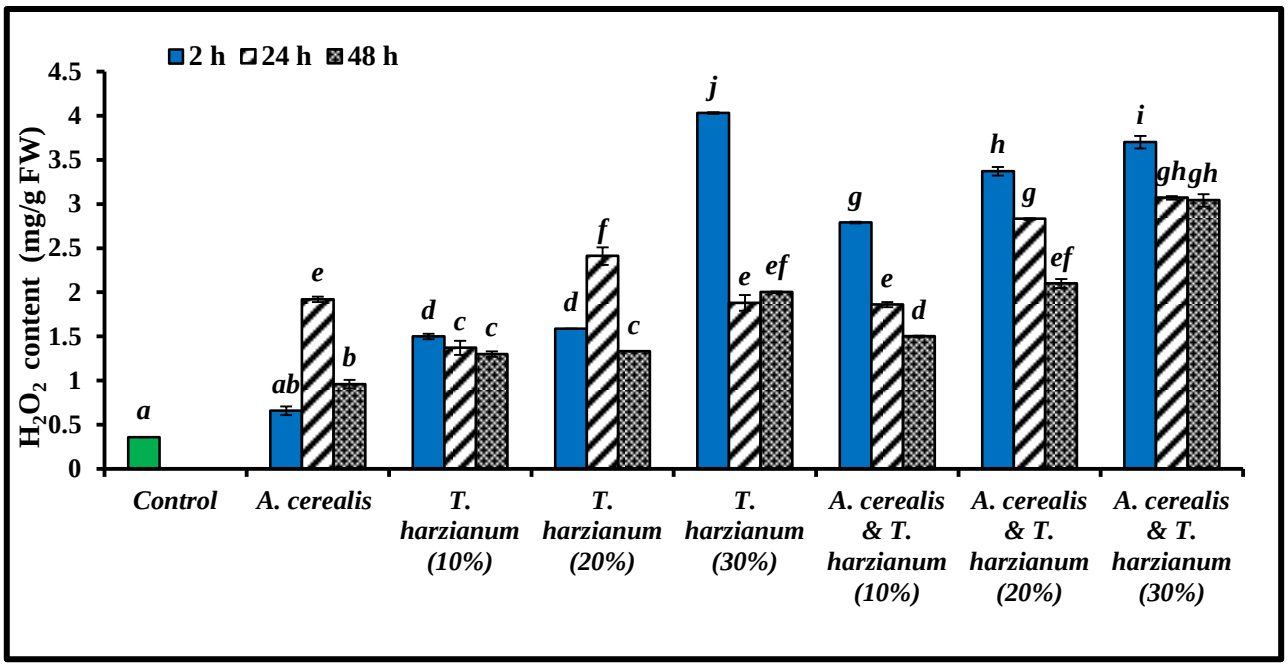


Figure 6: H_2O_2 content of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different concentrations of *T. harzianum* filtrates and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$)

.

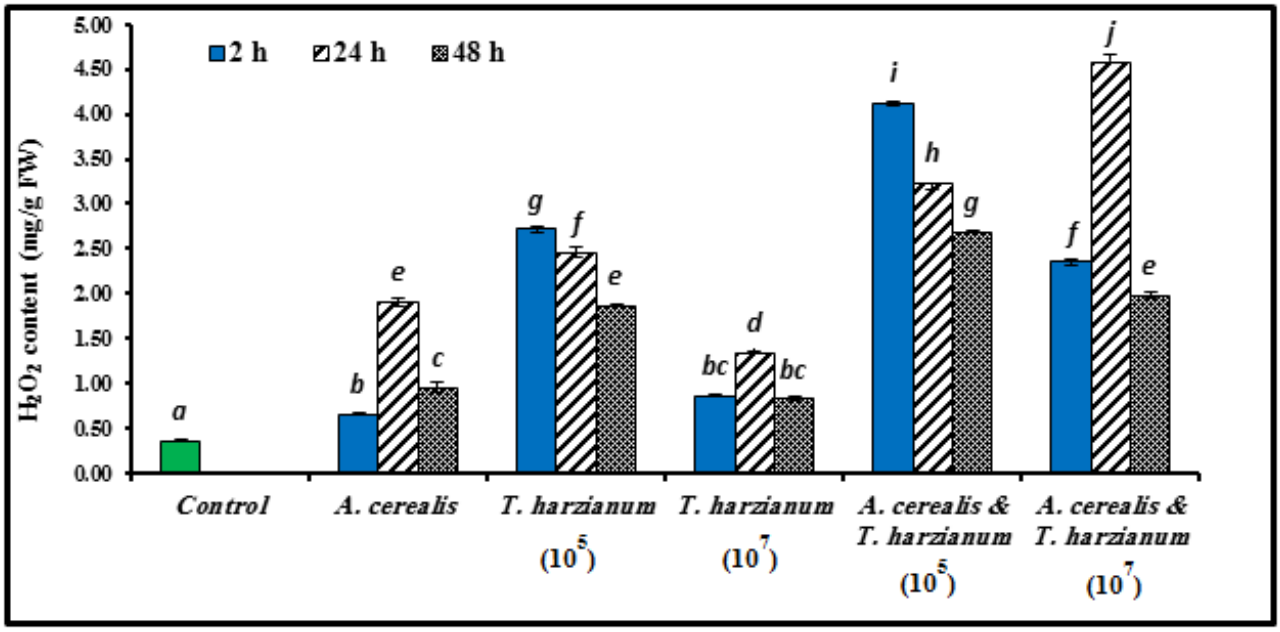


Figure 7: H_2O_2 content of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different inoculum volumes of *T. harzianum* and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$).

Antioxidant enzymes (Catalase and Peroxidase)

Enzymatic antioxidants are the first line of defense against fungal pathogens. Catalase (CAT) catalyzes the decomposition of H_2O_2 into water and oxygen to decrease the toxicity effect of the stress. In our study, CAT activity reached its peak after 2 h then decrease after 24 h in either different filtrates or spores inoculum of *T. harzianum* (Figures 8&9). In biological control treatment (*Alternaria cerealis* & *T. harzianum*), 20% fungal filtrate recorded higher CAT specific activity (0.06±0.005 µg protein^{-1} min^{-1}) followed by 10% (0.03±0.006 µg protein^{-1} min^{-1}) and 30% (0.01±0.003 µg protein^{-1} min^{-1}), however the concentration of filtrates or spores inoculum of *T. harzianum* could decrease CAT specific activity compared with the infected plants. Generally *T. harzianum* as biotic inducer exhibited strong negative correlations between the CAT activity and H_2O_2 (–0.265* and -0.496**, respectively).The obtained data is clearly demonstrated that tomato plants that treated with fungal filtrates (10%, 20 and 30%) or spore inoculums (10^5 and 10^7 spores/ml) adversely affected the peroxidase enzyme (POD) activity as shown by the current study (Figures 10 &11). The highest activity was observed after 48 h in plant samples that infected by *A. cerealis*. However, the changes were found significant ($P \leq 0.05$) when the tomato leaves treated with *Trichoderma* filtrates or spore inoculums in

infected or non-infected plants. In addition, spore suspension of *T. harzianum* showed little variance in POD activity by 0.67±0.06 µg protein^{-1} min^{-1} (10^5spores/ml) and 0.64±0.012 µg protein^{-1} min^{-1} (10^7spores/ml). While all *Trichoderma* treatments was exhibited negative correlations between POD activity and H_2O_2 (-0.291* and -0.376*, respectively).

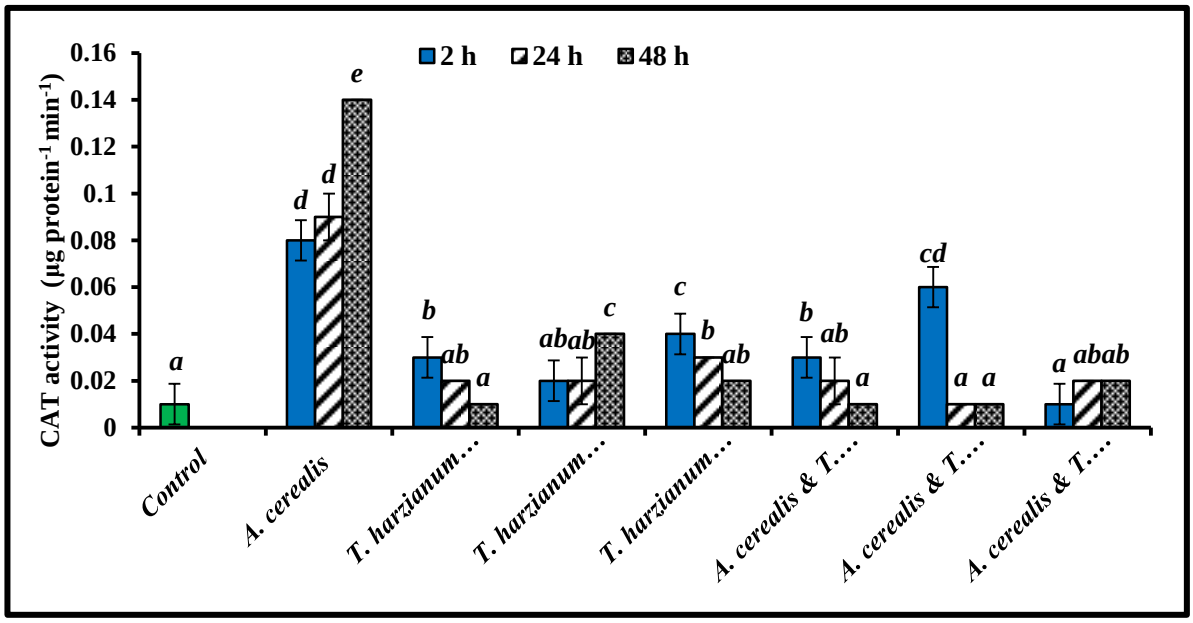


Figure 8: CAT activity of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different concentrations of *T. harzianum* filtrates and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$).

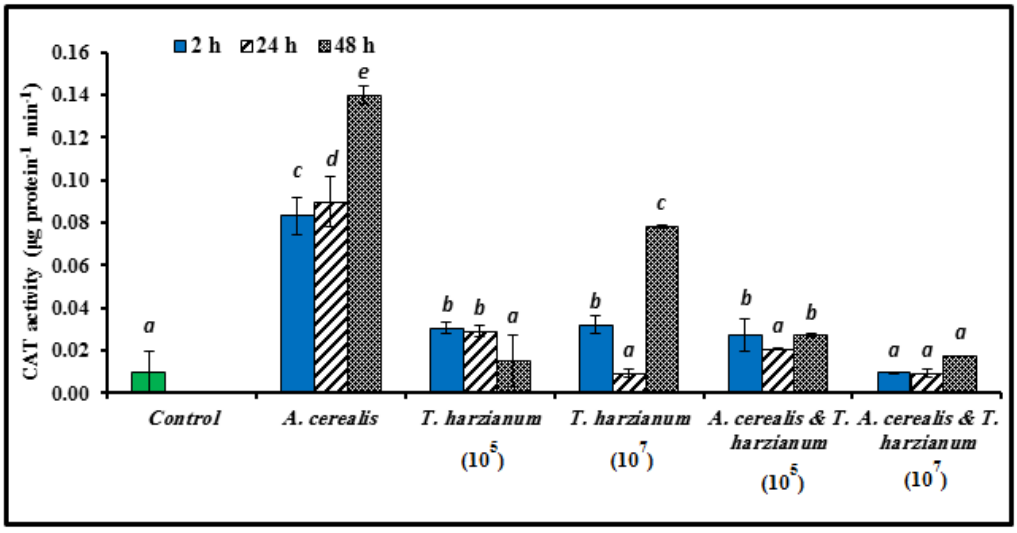


Figure 9: CAT activity of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different inoculum volumes of *T. harzianum* and periods of fungal infection, data are means + SD (n = 3), with statistically significant differences ($P \leq 0.05$).

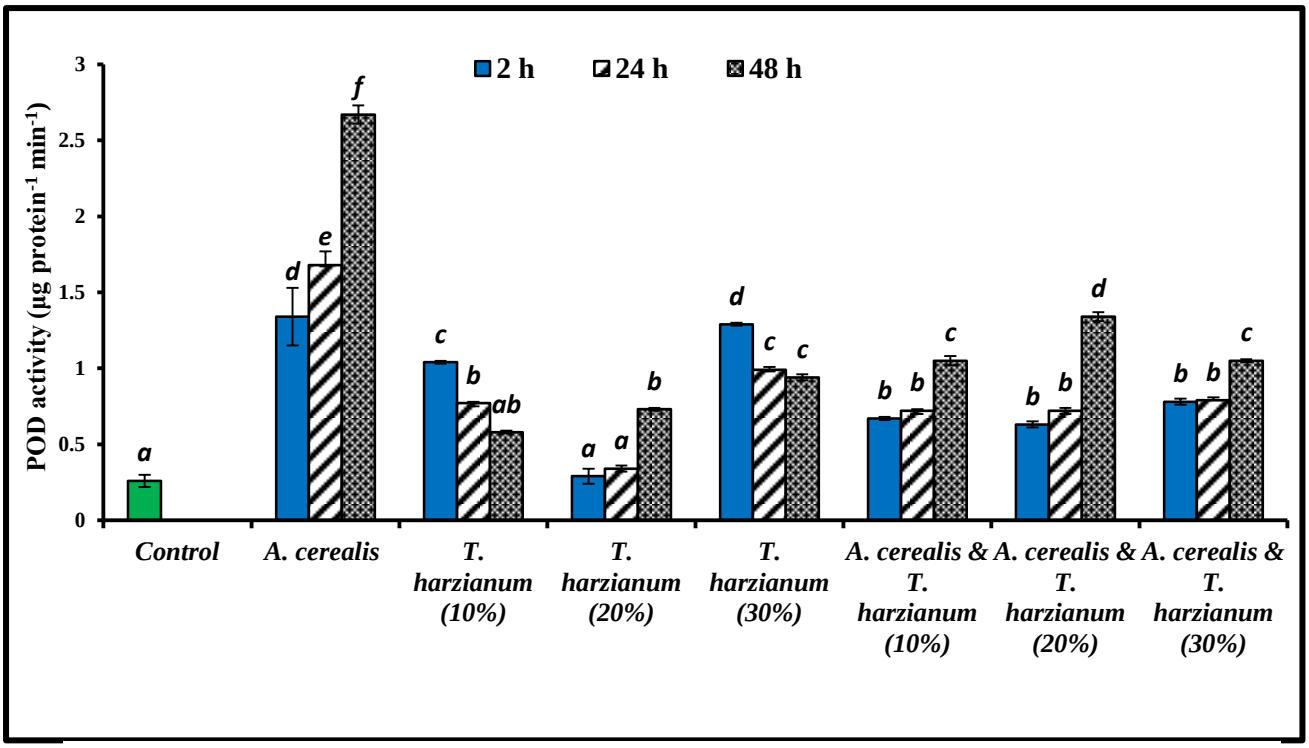


Figure 10: POD activity of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different concentrations of *T. harzianum* filtrates and periods of fungal infection, data are means ± SD (n = 3), with statistically significant differences ($P \leq 0.05$).

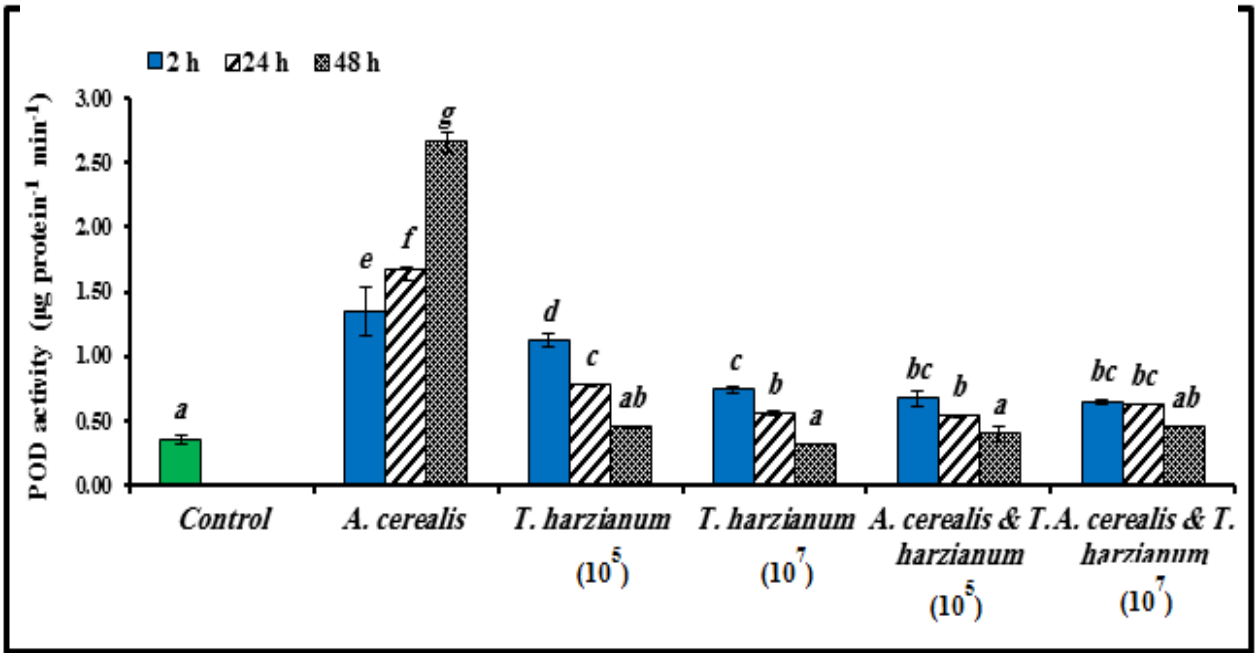


Figure 11: POD activity of healthy (control) and infected tomato leaves by *A. cerealis* (MT808477) under different inoculum volumes of *T. harzianum* and periods of fungal infection, data are means + SD (n = 3), with statistically significant differences ($P \leq 0.05$).

Gas chromatography-mass spectrometry (GC-MS) analysis of terpenoid phytoalexins

GC-MS as used to determine the terpenoid phytotoxins in healthy and infected tomato leaves after different *A. cerealis* infection periods (0, 2, 24, and 48 hours). The results exhibited 12 terpenoid compounds in infected tomato samples with different structures and concentrations (Table 1). From these compounds, rishitin derivatives (rishitinone and rishitinol) and the sesquiterpene solavetivone were detected. Whereas the control plants exhibited only two different diterpenoids, phytol (83.58%) and neophytadiene (16.42%) that naturally found in the tomato plants**.** Time plays a very significant role in induction of phytoalexins, after 48 h; the concentration of terpenoid phytoalexin in tomato leaves was the highest, representing 13.74% of the total compounds. Interesting, seven newly terpenoid phytoalexins were recorded here for the first time. Those are named: 5à-spirostan-23-ol,(22s,23r,25r); 2,2'(1h,1'h)- spirobi-s-indacene, ethanone derive; Spiro [5 hbenzocycloheptene 5,1' [2,5] cyclohexadiene] 4', 9 diol, 6, 7, 8, 9 tetrahydro 2, 3, 3', 4, 5' pentamethoxy-7,8-dimethyl-, 9-acetate; Maslinic acid; Carvacrol; Spirost8en11one,3 hydroxy, (3á,5à,14á,20á,22á,25R); and Olean-12-ene-3,15,16, 21, 22, 28 - hexol, (3á,15à,16à, 21á, 22à).

Methanol extract of infected tomato leaves recorded five terpenoid phytoalexin compounds after 2 h. Among these, two were the most prevailing compounds in tomato plant; 2(1h)-naphthalenone, octahydro-1-methyl-1-(2-propenyl)-, (1à,4aá,8Aà) (Rishitinone) (85.07%) and 1,2,3,4 tetrahydro5,6,7,8 tetramethoxy1,4 methanonaphthalene (Rishitinol) (1.49%). In the current study, three new terpenoid phytoalexin compounds have been identified for the first time from the tomato plant extract after 2 h, following infection by *A. cerealis*. These compounds are named: Olean-12-ene-3,15,16, 21, 22, 28-hexol, (3á,15à, 16à, 21á, 22à); Spirost8en11one, 3 hydroxy, (3á, 5à, 14á, 20á, 22á, 25R) and Carvacrol. When tomato seedlings were treated with spore suspension of *A. cerealis* and analyzed after 24 h using GC-MS, it was observed that new terpenoid phytoalexins were mainly produced to halt or hinder fungal infection. Our results indicated maslinic acid (24.18 %) as a new record in the tomato plant. Spiro [5hbenzocycloheptene5,1' [2,5] cyclohexadiene] 4', 9 diol, 6,7,8,9 tetrahydro 2,3,3',4,5' pentamethoxy -7,8-dimethyl-, 9-acetate (12.42%); 5à-spirostan-23-ol,(22s, 23r, 25r) (26.14%), 1, 2, 3, 4 tetrahydro 5, 6, 7,8 tetramethoxy-1,4-methanonaphthale ne (10.46%) and 2,2'(1h,1'h)-spirobi-s-indacene, ethanone deriv. (26.80%) were observed in methanol extract of tomato after 24 h (Figure 12). One of the important obtained compounds is Rishitinol, it is formed with high concentration in methanol extract after 24 h (10.46%). After 48 h of fungal infection of tomato plants, GC-MS analysis of the samples recorded three terpenoid phytoalexin compounds in methanol extract**.** On the other hand, the presence of some compounds such as 1,2,3,4-tetrahydro-5,6,7,8-tetramethoxy-1,4-methanonaphthalene and Spirost8en11one,3 hydroxy, (3á,5à,14á,20á,22á,25R) has declined after 48 h.

Table 1: Terpenoid phytoalexins identified in methanol extracts of healthy and infected tomato plants by *Alternaria cerealis* (MT808477) after 2, 24 and 48 h of infection using GC-MS analysis.

No.	Name of the compound	RT (min)	Control		After 2 h		After 24 h		After 48 h		Molecular Formula	Molecular Weight
			Area %	Con.%	Area %	Con.%	Area %	Con.%	Area %	Con.%		
1	Neophytadiene	10.00	2.1	16.42	-	-	-	-	-	-	$C_{20}H_{38}$	278
2	5à-spirostan-23-ol,(22s,23r,25r)	10.07	-	-	-	-	0.4	26.14	-	-	$C_{27}H_{44}O_3$	416
3	2(1h)-naphthalenone, octahydro-1-methyl-1-(2-propenyl)-, (1à,4aá,8Aà) (Rishitinone)	11.25	-	-	3.42	85.07	-	-	13.49	98.18	$C_{14}H_{22}O$	206
4	Phytol	12.89	10.69	83.58	-		-	-	-	-	$C_{20}H_{40}O$	296
5	1,2,3,4tetrahydro5,6,7,8tetramethoxy-1,4-methanonaphthalene (Rishitinol)	13.16	-	-	-	-	0.16	10.46	-	-	$C_{15}H_{14}C_{l4}O_6$	430
6	2,2'(1h,1'h)-spirobi-s-indacene, ethanone deriv.	13.24	-	-		-	0.41	26.80	-	-	$C_{26}H_{28}O_3$	388
7	Spiro[5hbenzocycloheptene5,1'[2,5]cyclohexadiene]4',9diol,6,7,8,9tetrahydro2,3,3',4,5'pentamethoxy-7,8-dimethyl-, 9-acetate	16.74	-	-	-	-	0.19	12.42	-	-	$C_{25}H_{34}O8$	462
8	Carvacrol	17.15	-	-	0.03	0.75	-		-	-	$C_{13}H_{22}OSi$	222
9	1,2,3,4-tetrahydro-5,6,7,8-tetramethoxy-1,4-methanonaphthalene (Rishitinol)	18.14	-	-	0.06	1.49	-	-	-	-	$C_{15}H_{14}C_{l4}O_6$	430
10	1,2,3,4-tetrahydro-5,6,7,8-tetramethoxy-1,4-methanonaphthalene	18.19	-	-	-	-	-	-	0.2	1.46	$C_{15}H_{14}C_{l4}O_7$	431
11	Maslinic acid	20.64	-	-	-		0.37	24.18	-	-	$C_{30}H_{48}O_4$	472
12	Spirost8en11one,3hydroxy(3á,5à,14á,20á,22á,25R)	21.66	-	-	-	-	-	-	0.05	0.36	$C_{27}H_{40}O_4$	428
13	Spirost8en11one,3 hydroxy,(3á,5à,14á,20á,22á,25R)	21.77	-	-	0.18	4.48	-	-	-	-	$C_{27}H_{40}O_5$	428
14	Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à)-	21.91	-	-	0.33	8.21	-	-	-	-	$C_{30}H_{50}O_6$	506
	Total		12.79		4.02		1.53		13.74			

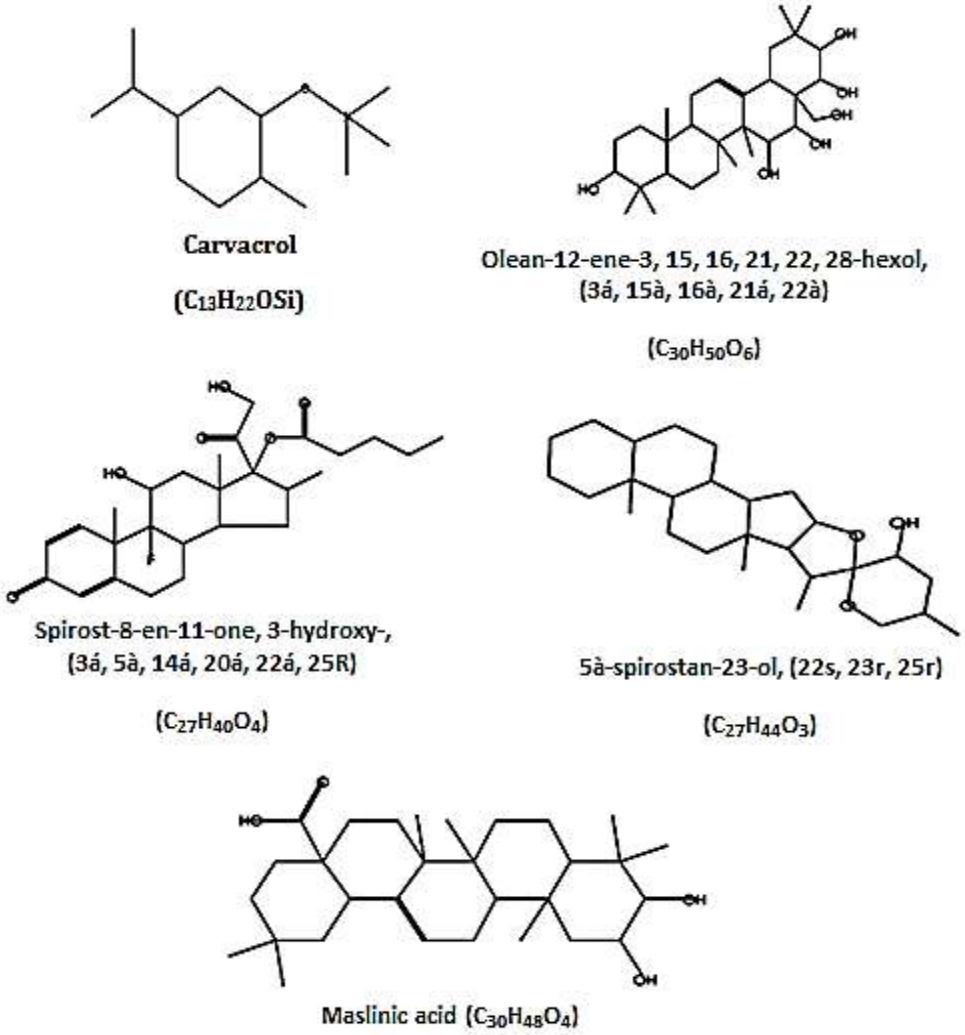


Figure 12: Some of terpenoid phytoalexins recorded in infected tomato leaves (*S. lycopersicum* L.) under the influence of different periods of fungal infection by *A. cerealis* (MT808477).

Discussion

During the plant infection, phytopathogens represents a huge stress factor on the plant tissue that urge the plant cell defense mechanism by secreting signaling oligopeptides, proteins, oligosaccharides, and toxins (Shoresh *et al.* 2010 and Islam *et al.* 2021). Biological control aims to decrease the destruction occurred by the phytopathogens using biotic agents, this agent control the oxidative stress in infected plants by up-regulation of cell metabolites and antioxidant enzymes such as peroxidase, catalase and superoxide dismutase (Chowdappa *et al.* 2013 and Pandit *et al.* 2022). Phytoalexins accumulated during the phytopathogen infection as a good defense mechanism of the plant cells; however the responses of phytoalexins under the biological control conditions still was not clear. Therefore, this work tested the effect of using a saprophytic fungus, *Trichoderma harzianum* at different infection periods (2, 24, and 48 h) as biological control against *Alternaria cerealis* (MT808477) which recorded as a new tomato phytopathogen on the terpenoid phytoalexins. The effect of inoculum type including different *Trichoderma harzianum* filtrate (10%, 20 and 30%) and their spore inoculum concentrations (10^5 and 10^7 spores/ml) on terpenoid phytoalexins and explaining the plant cell behavior during the experiment by measuring total terpenoids, malondialdehyde (MDA), hydrogen peroxide analysis and enzymatic activities (CAT and POD) was performed.

Terpenoid phytoalexins are low molecular weight antimicrobial active compounds that accumulate inside plant cell in response to biological and abiotic factors (Ashour *et al.* 2018) and act as antioxidants to protect cells from oxidative damage (Kim *et al*. 2020). The accumulation of terpenoids in tomato plants significantly increased through the biological control process after 48 h of infection comparing with non-infected or infected plant and also increased by increasing the spore's inoculum concentration. Elgersma (1980) observed the accumulation of terpenoid phytoalexin rishitin, in susceptible and resistant tomato plants after inoculation with *Verticillium albo-atrum*. Also, EL-Tanany *et al.* (2018) studied the effect of antagonistic fungi against *Alternaria solani* in tomato plants and reported increasing in total flavonoids and phenols compared to the control. It can be assumed that, to optimize the output of phytoalexin compounds, there is an optimum exposure period. The behavior of phytoalexin production inside tissue during time of infection was also studied. Induction of ajmalicin by *Catharanthus roseus* was higher in the first 48 h (Namdeo *et al.* 2002).

Free fatty acids produced after the degradation of membrane lipids act as lipoxygenase (LOX) substrates, leading to oxidative degradation and membrane peroxidation (Farmer and Mueller 2013). Through the antagonistic effect of biological inducer (*T. harzianum*) and the pathogen (*A. cerealis*), the content of MDA in tomato leaves was measured to prove the degree of oxidative stress. According to our results, the nature and concentration of inoculum effect highly in MDA production, the degree of change in the MDA content gradually increase by increasing the inoculum concentrations. In this respect, Zehra *et al.* (2017) observed that the MDA content gradually increased in tomato plants that infected by *Fusarium oxysporum* f. sp. *lycopersici*. The results showed that in the infected plants, the H_2O_2 content was significantly high after 2 h and decreased gradually in all treatments after 48 h. H_2O_2 is a key metabolite in oxidative stress (Sies *et al.* 2017). Similarly, Zehra *et al.* (2017) reported the same result for treatment of tomato leaves with salicylic acid especially when applied with pathogen markedly increased reactive oxygen species (ROS) production. Pathogen stress, salicylic acid, methyl jasmonate and *T. harzianum* treatment caused increase in H_2O_2 content after 24 and 48 h. Zhou *et al*. (2019) found that drought and comprehensive stress caused oxidative damage in tomato plants after 6 days, thus increase in H_2O_2 content in tomato plants. In our results show that a higher percentage of H_2O_2 content is found in plants pretreated with a combination of *Trichoderma harzianum* and pathogen. This indicates that the biological inducer has a protective effect, involves different mechanisms of action, and produces a variety of biologically active metabolites, which leads to a competition for space and nutrients (Nagórska *et al.* 2007).

The capacity of bio-control agents to mitigate the impact of oxidative bursts has been reported in several studies (Kumar *et al.* 2009, Yang *et al.* 2009 and Lahlali *et al.* 2022). In response to oxidative damage, plants have effective ROS detoxification mechanisms to protect themselves, including the use of enzymes and non-enzymatic components to trigger antioxidant defense mechanisms. The enzymes (CAT and POD) are located in different subcellular compartments and constitute antioxidant mechanisms include CAT and

POX (Zehra *et al.* 2017). Peroxidase enzyme has the ability to use a variety of organic and inorganic compounds to detoxify H_2O_2 (Zhou *et al.* 2019). Therefore, plants can activate antioxidant enzymes to prevent the harmful effects of ROS (Hasanuzzaman *et al.* 2012). Reactive oxygen species (ROS) overproduction, however, negatively impacts the mechanism of cells by oxidising proteins, carbohydrates, lipids, DNA and RNA. In order to avoid ROS accumulation and cell toxicity, fortunately, plants have an effective ROS detoxification mechanism to protect themselves from toxic effects. The activation of antioxidant enzymes such as peroxidase (POD) and catalase (CAT) is one significant mechanism involved in ROS metabolism (Shimizu *et al.* 2006, Zehra *et al* 2017 and Tavanti *et al* 2021).

In the present study, the increase in CAT and POD activities in tomato plants infected with *A. cerealis* in all infection periods was observed. Tomato plants treated by different filtrates (10%, 20 and 30%) or inoculum (10^5 and 10^7 spores/ml) concentrations after infection or non-infection by pathogen showed a decline in the activity of CAT and POD in all studied accessions. The decrease in enzyme activity confirmed the earlier report of García-López *et al.* (2018), who concluded that the balance of antioxidant defense systems in plants can be delayed or altered to eliminate oxidative stress. In addition, Siddiqi and Husen (2017) reported that controlling the phytopathogen by ZnO NPs at a concentration more than 50 μgml^{-1} significantly inhibited the activity of POD. Using salicylic acid as disease control agent could inhibit catalase activity in tomato and many other plant species, such as Arabidopsis, tobacco, cucumber plant due to the over accumulation of ROS (Sanchez-Casas and Klessig 1994 and Conrath *et al.* 1995).

The detection of phytoalexin compounds is an important method in the study of events of resistance induction. The phytoalexins produced in plants were extremely variable based on the physiological state of the plant, resistance of the plant, time of infection, microbe elicitation, and genetic background of the cultivar (Darvill and Albersheim 1984 and Kaur *et al.* 2022). In our study, 12 terpenoids were recorded in infected tomato samples with different structures and concentrations. The results showed various terpenoid phytoalexins in leaf plants, such as rishitin derivatives (rishitinone and rishitinol) and sesquiterpene solavetivone. Whereas the control plants exhibited only two types of diterpenoids, phytol (83.58%) and neophytadiene (16.42%) that naturally found in the tomato plant. Regarding to this, Cáceres *et al.* (2015) investigated the sources of phytol and neophytadiene with the highest insecticidal activity and biologically active compounds, which can be further produced for the use of biological pesticides. Kostik and Bauer (2017) showed that eggplants, tomatoes, potatoes, carrots and beans have lower levels of phytol.

Methanol extract of tomato sampling recorded five terpenoid phytoalexin compounds after 2 h. Among these, two were the most prevailing phytoalexins in tomato plant, Rishitinone (85.07%) and Rishitinol (1.49%). In harmony with the current results, some previous studies have reported terpenoid phytoalexin compounds from tomatoes and other plants infected by different fungi, namely esquiterpenes rishitin, rishitinol, solavetivone and rishitinone (Elgersma and Liem 1989, Kúc 1995 and Mostafa 2018). Rishitinol is another terpenoid, hydroxyoctanol, which, after dehydration and acetylation, may create acetyl dehydrorishitinol. The rishitinol and rishitinone are known to be present in potatoes (Jadhav ***et*** *al.* 1991). Interesting studies by Elgersma (1980) showed that on the 2nd until the 4th day after inoculation, rishitin accumulated in the lower and upper parts of the resistant and susceptible varieties of tomato. After week, the accumulation was lasted more slowly than susceptible varieties. In the present results indicated three new terpenoid phytoalexin compounds for the first time after 2 h, following infection by *A. cerealis*. These compounds are named: Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à); Spirost8en11one, 3 hydroxy, (3á,5à,14á,20á,22á,25R) and Carvacrol. Carvacrol was stated in several studies a strong antifungal against phytopathogenic fungi, such as *Botrytis cinerea*, *Fusarium moniliforme*, *Sclerotinium sclerotiorum*, *Alternaria tomatophila*, *Phytophthora capsici* and others (Camele *et al.* 2012, Marchese *et al.* 2018 and Liu *et al.* 2019). Carvacrol is a phenolic monoterpene compound present in essential oils such as oregano, thyme, pepper, wild bergamot and other plants. Carvacrol has a wide range of biological activities and may be useful for therapeutic applications such as antimicrobial, antioxidant and anticancer function (Sharifi-Rad *et al.* 2018). In many studies, the mode of action of carvacrol on various bacteria and fungi, especially plant pathogens and related mechanisms have been studied (Marchese *et al.* 2018 and Liu *et al.* 2019).

Maslinic acid is a triterpene found in various natural resources, used in traditional Asian medicine for edible vegetables and fruits. It has a range of biological activities in recent years, including anti-diabetic, antioxidant, cardioprotective, neuroprotective and antiparasitic effects (Parra *et al.* 2011, Lozano-Mena *et al.* 2014 and Pavel *et al.* 2016). The two newly identified triterpenoids from the extract of tomato leaves (olean-12-ene-3,15,16,21,22,28-hexol,(3á,15à,16à,21á,22à) and 22,22-dideutero-lanosta-8,24 -dien-3-yl acetate) were recorded previously from other plants, such as frangipani (*Plumeria rubra*), olive fruit (*Olea europaea*) and cipó-una (*Arrabidaea brachypoda*) (Siddiqui *et al.* 2004, Stiti and Hartmann 2012 and Rocha *et al.* 2015).

Non-sterol triterpenoids can respond to biotic and abiotic stress and contribute to plant protection (Van der Heijden *et al.* 1989). Stiti and Hartmann (2012) discovered 20 sterols and 29 non-steroidal tetracyclic and pentacyclic triterpenoids from olives (*Olea europaea*), which belong to the carbon skeleton (olein, ursane, Lupan, taraxerane, taraxastane, euphane and lanostan). When the growth phase cell suspension culture of *Tabernaemontana divaricata* was treated with a *Candida albicans* inducer, the growth and monoterpene indole alkaloid were inhibited (Van der Heijden *et al.* 1989). Treatment of tomato seedlings with spore suspension of *A. cerealis* observed new terpenoid phytoalexins after 24 h to halt or hinder fungal infection. The results revealed that maslinic acid (24.18 %) was recorded for the first time in the tomato plant, although it was found in the hawthorn (*Crataegus oxyacantha* L.) plant, and could be used in traditional anti-diabetic, anti-tumor and antioxidant drugs (Lu *et al*. 2009 and Juan and Joana 2016). In accordance with our results, Elgersma (1980) noticed the accumulation of rishitin in the lower stems of susceptible and resistant tomato varieties. Also, solavetivone, rishitin and desacetylphytuberin were produced in the entire potato tubers of all cultivars infected with *Phytophthora infestans* (Price *et al.* 1976). Thus, some metabolic activities within the plant maybe change their behavior during the time of the treatment.

Moreno *et al.* (1993) reported that *Catharanthus roseus* cultures that exposed to fungal elicitors for a longer period of time have adversely affected alkaloid synthesis.

Also, fungal plant pathogens can resist plant defense mechanisms by synthesizing enzymes with a variety of catalytic activities. Some fungi effectively detoxify metabolites including phytoalexins through the action of detoxification enzymes, and these enzymes will exhaust these beneficial defenses (VanEtten *et al.* 1995, Pedras *et al.* 2017, Jeandet 2018 and Westrick *et al.* 2021).

In general, during the first 3 days, terpenoid compounds were continuously synthesized within the plant, whereas most of them (for examples: rishitinone, rishitinol, sesquiterpene solavetivone, carvacrol and maslinic acid) were disappeared after 48 h. The biotic and abiotic elicitors stimulate the biosynthesis of phytoalexins. This means that the fungus has destroyed the terpenoid phytoalexin that appeared in the first two days (2, 24, and 48 h) by releasing enzymes that detoxifies the phytoalexin. The ability of *Alternaria brassicicola*, *Botrytis cinerea*, *Phoma lingam*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and other virulent strains of phytopathogenic fungi have been studied to make cruciferous plants resistant to toxins through different mechanism reactions (such as: oxidation, reduction or hydrolysis). The ability to detoxify phytoalexin compound depends on fungal strain and the chemical structure of the target compound (Pedras *et al.* 2011 and 2017). There are also certain mechanisms in plant cells that limit the accumulation of these molecules. These may include inhibition of biosynthesis, induction, transformation, and degradation of metabolites (Ahmed and Kovinich 2021). Phytoalexins are biosynthesized in relatively low quantities mainly when induced. Whether this is to restrict any possible self-toxicity or to ensure adequate cellular toxicity is not clear (Ahmed and Kovinich 2021). The present results noticed the production of sesquiterpenoid phytoalexin rubemin, rishitin and solavetivone in tomato which also reported in potato cell suspension cultures after infection (Brindle *et al.* 1983). Furthermore, in eggplant injected with *Verticillium dahliae* at 24, 48 and 72 hours, the accumulation of solaretivone phytoalexin was observed using callus culture technology, under *in vitro* conditions after 72 hours, which was higher than that of susceptible species in callus cultures of resistant species (Kiran *et al.* 2017).

Conclusion

Several plants expose to fungal infection, but few of them could half or prevent the disease development naturally through production of

metabolic compounds including terpenoid phytoalexin compounds. This depends mainly on the degree of plant tolerance. Susceptible plants need external intervention; *Trichoderma* species are good biological control to perform this purpose. Explanation and interpretation of the interaction and behaviour of both pathogen and host (plant) need further studied for upscaling plant resistant against phytopathogenic fungi.

References

Abdelhamid H. N., Mahmoud G. A. E. and Sharmouk W. (2020): A cerium-based MOFzyme with multi-enzyme-like activity for the disruption and inhibition of fungal recolonization. Journal of Materials Chemistry B, 8(33): 7548-7556.

Abdel-Kader M. M., El-Mougy N. S., Aly M. D. E. and Lashin, S. M. (2012): Integration of biological and fungicidal alternatives for controlling foliar diseases of vegetables under greenhouse conditions. International Journal of Agriculture and Forestry, 2(2): 38-48.

Ahmed S. and Kovinich N. (2021): Regulation of phytoalexin biosynthesis for agriculture and human health. Phytochemistry Reviews, 20: 483-505.

Ashour M., Wink M. and Gershenzon J. (2018): Biochemistry of terpenoids: Monoterpenes, sesquiterpenes and diterpenes. Annual Plant Reviews Online, Second Edition, 40: 258-303.

Baxter A., Mittler R. and Suzuki N. (2014): ROS as key players in plant stress signalling. Journal of Experimental Botany, 65(5): 1229-1240.

Blagojević J. D., Vukojević J. B. and Ivanović Ž. S. (2020): Occurrence and characterization of *Alternaria* species associated with leaf spot disease in rapeseed in Serbia. Plant Pathology, 69(5): 883-900.

Brindle P. A., Kuhn P. J. and Threlfall D. R. (1983): Accumulation of phytoalexins in potato-cell suspension cultures. Phytochemistry 22(12): 2719-2721.

Brotman Y., Lisec J., Méret M., Chet I., Willmitzer L. and Viterbo A. (2012): Transcript and metabolite analysis of the *Trichoderma*-induced systemic resistance response to *Pseudomonas syringae* in *Arabidopsis thaliana*. Microbiology, 158(1): 139-146.

Cáceres L. A., McGarvey B. D., Briens C., Berruti F., Yeung K. K. C. and Scott I. M. (2015): Insecticidal properties of pyrolysis bio-oil from greenhouse tomato residue biomass. Journal of Analytical and Applied Pyrolysis, 112, 333-340.

Camagna M., Ojika M. and Takemoto D. (2020): Detoxification of the solanaceous phytoalexins rishitin, lubimin, oxylubimin and solavetivone *via* a cytochrome P450 oxygenase. Plant Signaling and Behavior, 15(2): 1-9.

Camele I., Altieri L., De Martino L., De Feo V., Mancini E. and Rana G. L. (2012): *In vitro* control of post-harvest fruit rot fungi by some plant essential oil components. International Journal of Molecular Sciences 13(2): 2290-2300.

Chowdappa P., Kumar S. M., Lakshmi M. J. and Upreti K. K. (2013): Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. Biological Control, 65(1): 109-117.

Conrath U., Chen Z., Ricigliano J. R. and Klessig, D. F. (1995): Two inducers of plant defense responses, 2, 6-dichloroisonicotinec acid and salicylic acid, inhibit catalase activity in tobacco. Proceedings of the National Academy of Sciences, 92(16): 7143-7147.

Darvill A. G. and Albersheim P. (1984): Phytoalexins and their elicitors-a defense against microbial infection in plants. Annual Review of Plant Physiology, 35(1): 243-275.

Daryaei A., Jones E. E., Glare T. R. and Falloon R. E. (2016): PH and water activity in culture media affect biological control activity of *Trichoderma atroviride* against *Rhizoctonia solani*. Biological Control, 92: 24-30.

de Oliveira C.M., Almeidab N.O., Cortesc M.V., Juniorc M.L., da Rochab M.R., and Ulhoa C.J. (2021): Biological control of *Pratylenchus brachyurus* with isolates of *Trichoderma* spp. on soybean. Biological Control, 152:10-25.

Divekar P. A., Narayana S., Divekar B. A., Kumar R., Gadratagi B. G., Ray A. and Behera T. K. (2022). Plant secondary metabolites as defense tools against herbivores for sustainable crop protection. International Journal of Molecular Sciences, 23(5): 1-58.

Elgersma D. M. (1980): Accumulation of rishitin in susceptible and resistant tomato plants after inoculation with *Verticillium albo-atrum*. Physiological Plant Pathology 16(2): 149-153.

Elgersma D. M. and Liem J. I. (1989): Accumulation of phytoalexins in susceptible and resistant near-isogenic lines of tomato infected with *Verticillium albo-atrum* or *Fusarium oxysporum* f. sp. *lycopersici*. Physiological and Molecular Plant Pathology 34(6): 545-555.

EL-Tanany M. M., Hafez M. A., Ahmed G. A. and El-Mageed M. A. (2018): Efficiency of biotic and abiotic inducers for controlling tomato early blight. Middle East Journal, 7(2): 650-670.

Erb M., and Kliebenstein D. J. (2020): Plant secondary metabolites as defenses, regulators, and primary metabolites: the blurred functional trichotomy. Plant Physiology, 184(1): 39-52.

Fan J. P. and He C. H. (2006): Simultaneous quantification of three major bioactive triterpene acids in the leaves of *Diospyros kaki* by high-performance liquid chromatography method. Journal of Pharmaceutical and Biomedical Analysis, 41(3): 950-956.

Farmer E. E. and Mueller M. J. (2013): ROS-mediated lipid peroxidation and RES-activated signaling. Annual Review of Plant Biology, 64, 429-450.

García-López J. I., Zavala-García F., Olivares-Sáenz E., Lira-Saldívar R. H., Díaz Barriga-Castro E., Ruiz-Torres N. A. and Niño-Medina G. (2018): Zinc oxide nanoparticles boosts phenolic compounds and antioxidant activity of *Capsicum annuum* L. during germination. Agronomy, 8(10): 1-13.

Hammerschmidt R., Nuckles E. M., and Kuć J. (1982): Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiological Plant Pathology, 20(1), 73-82.

Hasanuzzaman M., Hossain M., da Silva J. and Fujita M. (2012): Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor. In crop stress and its management: Perspectives and Strategies, 261-315 pp.

Havir E. A., and McHale N. A. (1987): Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiology, 84(2): 450-455.

Hodges D. M., DeLong J. M., Forney C. F. and Prange R. K. (1999): Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta, 207(4): 604-611.

Hutson R. A., and Smith I. M. (1980): Phytoalexins and tyloses in tomato cultivars infected with *Fusarium oxysporum* f. sp. *lycopersici* or *Verticillium albo-atrum.* Physiological Plant Pathology, 17(3): 245-257.

Islam M. S., Mohamed G., Polash S. A., Hasan M. A., Sultana R., Saiara N. & Dong W. (2021): Antimicrobial peptides from plants: A cDNA-Library Based isolation, purification, characterization approach and elucidating their modes of action. International Journal of Molecular Sciences, 22(16): 8712.

Jadhav S. J., Mazza G. and Salunkhe D. K. (1991): Terpenoid phytoalexins in potatoes: A review. Food Chemistry, 41(2): 195-217.

Jeandet P. (2018): Structure, chemical analysis, biosynthesis, metabolism, molecular engineering, and biological functions of phytoalexins, Molecules, 23(61): 1-4.

Juan M. E., and Joana M. P. (2016): Bioavailability and metabolism of maslinic acid, a natural pentacyclic triterpene. Recent Adv Pharm, 6, 131-145.

Kaur S., Samota M. K., Choudhary M., Choudhary M., Pandey A. K., Sharma A., and Thakur J. (2022): How do plants defend themselves against pathogens-biochemical mechanisms and genetic

interventions. Physiology and Molecular Biology of Plants, 28(2): 485-504.

Kim T., Song B., Cho K. S. and Lee I. S. (2020): Therapeutic potential of volatile terpenes and terpenoids from forests for inflammatory diseases. International Journal of Molecular Sciences, 21(6): 2-32.

Kiran S., Ellialtioğlu Ş. Ş., Üstün A. S., Mehmetoğlu Ü., Bayraktar H., and Dolar F. S. (2017): Phytoalexin accumulations in the callus culture of two eggplant genotypes by using *Verticillium dahliae* Kleb. elicitor. International Journal of Forestry and Horticulture, 3(3): 1- 8.

Kobori N. N., Mascarin G. M., Jackson M. A. and Schisler D. A. (2015): Liquid culture production of microsclerotia and submerged conidia by *Trichoderma harzianum* active against damping-off disease caused by *Rhizoctonia solani*. Fungal Biology, 119(4): 179-190.

Kostik V. and Bauer B. (2017): Determination of phytol content in some vegetables and fruits. The 3th International Mediterranean Symposium on Medicinal and Aromatic Plants 3, 13-16.

Kúc J. (1995): Phytoalexins, stress metabolism, and disease resistance in plants. Annual Review of Phytopathology, 33(1): 275-297.

Kumar V., Parkhi V., Kenerley C. M. and Rathore K. S. (2009): Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from *Trichoderma virens* in response to interaction with *Rhizoctonia solani*. Planta, 230(2): 277-291.

Lahlali R., Ezrari S., Radouane N., Kenfaoui J., Esmaeel Q., El Hamss H and Barka, E. A. (2022): Biological control of plant pathogens: A global Perspective. Microorganisms, 10(3): 596.

Lehmann S., Serrano M., L'Haridon F., Tjamos S. E. and Metraux J. P. (2015): Reactive oxygen species and plant resistance to fungal pathogens. Phytochemistry, 112: 54-62.

Li R., Tee C. S., Jiang Y. L., Jiang X. Y., Venkatesh P. N., Sarojam R. and Ye J. (2015): A terpenoid phytoalexin plays a role in basal defense of *Nicotiana benthamiana* against Potato virus X. Scientific Reports, 5(1): 1-6.

Liu Q., Qiao K. and Zhang S. (2019): Potential of a small molecule carvacrol in management of vegetable diseases. Molecules, 24(10): 1-8.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951): Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193: 265-275.

Lozano-Mena G., Sánchez-González M., Juan M. E. and Planas J. M. (2014): Maslinic acid, a natural phytoalexin-type triterpene from olives a promising nutraceutical. Molecules, 19(8): 11538-11559.

Lu H., Xi C., Chen J. and Li W. (2009): Determination of triterpenoid acids in leaves of *Eriobotrya japonica* collected at in different seasons. China Journal of Chinese Materia Medica 34(18), 2353-2355.

Marchese A., Arciola C. R., Coppo E., Barbieri R., Barreca D., Chebaibi S. and Daglia M. (2018): The natural plant compound carvacrol as an antimicrobial and anti-biofilm agent: Mechanisms, synergies and bio-inspired anti-infective materials. Biofouling, 34(6): 630-656.

Martin D., Tholl D., Gershenzon J. and Bohlmann J. (2002): Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiology, 129(3): 1003-1018.

Miller B., Madilao L. L., Ralph S. and Bohlmann J. (2005): Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in *Sitka spruce*. Plant Physiology, 137(1): 369-382.

Mittler R., Vanderauwera S., Suzuki N., Miller G. A. D., Tognetti V. B., Vandepoele K. and Van Breusegem F. (2011): ROS signaling: The new wave?. Trends in Plant Science, 16(6): 300-309.

Moreno P. R. H., Van der Heijden R. and Verpoorte R. (1993): Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus*. Plant Cell Reports, 12(12): 702-705.

Mostafa M. H. (2018): Elicitation of phytoalexin rishitin in potato tuber slices infected by *Fusarium* spp., does it consider a factor of pathogenicity. International Journal of Phytopathology, 7(2): 53-61.

Nagórska K., Bikowski M., and Obuchowski M. (2007): Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochimica Polonica, 54(3): 495-508.

Naik P. M. and Al-Khayri J. M. (2016): Abiotic and biotic elicitors–role in secondary metabolites production through *in vitro* culture of medicinal plants. Abiotic and biotic stress in plants-Recent, Advances and Future Perspectives, 247-277pp.

Namdeo A., Patil S. and Fulzele D. P. (2002): Influence of fungal elicitors on production of ajmalicine by cell cultures of *Catharanthus roseus*. Biotechnology Progress, 18(1): 159-162.

Pandit M. A., Kumar J., Gulati S., Bhandari N., Mehta P., Katyal R. and Kaur J. (2022): Major Biological control strategies for plant pathogens. Pathogens, 11(2), 273.

Parra A., Rivas F., Martin-Fonseca S., Garcia-Granados A. and Martinez A. (2011). Maslinic acid derivatives induce significant apoptosis in b16f10 murine melanoma cells. European Journal of Medicinal Chemistry, 46(12): 5991-6001.

Pavel I. Z., Danciu C., Oprean C., Dehelean C. A., Muntean D., Csuk R., and Muntean D. M. (2016). *In vitro* evaluation of the antimicrobial ability and cytotoxicity on two melanoma cell lines of a benzylamide derivative of maslinic acid. Analytical Cellular Pathology, 1-6pp.

Pedras M. S. C., Abdoli A. and Sarma-Mamillapalle V. K. (2017): Inhibitors of the detoxifying enzyme of the phytoalexin brassinin based on quinoline and isoquinoline scaffolds. Molecules, 22(8): 1-15.

Pedras M. S. C., Yaya E. E. and Glawischnig E. (2011): The phytoalexins from cultivated and wild crucifers: chemistry and biology. Natural Product Reports, 28(8): 1381-1405.

Price K. R., Howard B. and Coxon D. T. (1976): Stress metabolite production in potato tubers infected by *Phytophthora infestans*, *Fusarium avenaceum* and *Phoma exigua*. Physiological Plant Pathology, 9(2): 189-197.

Rocha C. Q. D., Vilela F. C., Santa-Cecília F. V., Cavalcante G. P., Vilegas W., Giusti-Paiva A. and Santos M. H. D. (2015): Oleanane-type triterpenoid: An anti-inflammatory compound of the roots *Arrabidaea Brachypoda*. Revista Brasileira de Farmacognosia 25(3): 228-232.

Sanchez-Casas P., and Klessig D. F. (1994): A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. Plant Physiology, 106(4): 1675-1679.

Shabana Y. M. N. E. (1987): Biological control of water weeds by using plant pathogens. Mansoura University, El-Mansoura, Egypt, 78pp.

Sharifi-Rad M., Varoni E. M., Iriti M., Martorell M., Setzer W. N., del Mar Contreras M. and Sharifi-Rad J. (2018): Carvacrol and human health: A comprehensive review. Phytotherapy Research, 32(9): 1675-1687.

Shih M., Kuć J. and Williams E. B. (1973): Suppression of steroid glycoalkaloid accumulation as related. Phytopathology, 63: 821-826.

Shimizu N., Hosogi N., Hyon G. S., Jiang S., Inoue K. and Park P. (2006): Reactive oxygen species (ROS) generation and ROS-induced lipid peroxidation are associated with plasma membrane modifications in host cells in response to AK-toxin I from *Alternaria alternata* Japanese pear pathotype. Journal of General Plant Pathology, 72(1): 6-15.

Shoresh M., Harman G. E. and Mastouri F. (2010): Induced systemic resistance and plant responses to fungal biocontrol agents. Annual Review of Phytopathology, 48: 21-43.

Siddiqi K. S. and Husen A. (2017): Plant response to engineered metal oxide nanoparticles. Nanoscale Research Letters, 12(1): 1-57.

Siddiqui B. S., Ilyas F., Rasheed M. & Begum S. (2004). Chemical constituents of leaves and stem bark of *Plumeria obtusa*. Phytochemistry 65(14), 2077-2084.

Sies H., Berndt C. and Jones D. P. (2017): Oxidative stress. Annual Review of Biochemistry, 86: 715-748.

Simmons E. G. (2007): *Alternaria*, an identification manual. CBS Biodiversity series 6, Netherlands, 444-445 pp

Singh B. and Sharma R. A. (2015): Plant terpenes: Defense responses, phylogenetic analysis, regulation and clinical applications. 3 Biotech, 5(2): 129-151.

Solino A. J. D. S., Schwan-Estrada K. R. F., Oliveira J. S. B., Ribeiro L., and Saab M. F. (2017): Accumulation of phytoalexins in beans, soybeans and sorghum by fungal filtrates. Revista Caatinga, 30(4): 1073-1078.

Stiti N. and Hartmann M. A. (2012): Nonsterol triterpenoids as major constituents of *Olea europaea*. Journal of Lipids. 1-13pp.

Tavanti T. R., de Melo A. A. R., Moreira L. D. K., Sanchez D. E. J., dos Santos Silva R., da Silva R. M., and Dos Reis A. R. (2021): Micronutrient fertilization enhances ROS scavenging system for alleviation of abiotic stresses in plants. Plant Physiology and Biochemistry, 160: 386-396.

Tiku A. R. (2020): Antimicrobial compounds (Phytoanticipins and Phytoalexins) and their role in plant defense. Co-Evolution of Secondary Metabolites, 845-868 pp.

Van der Heijden R., Threlfall D. R., Verpoorte R., Whitehead I. M. (1989): Regulation and enzymology of pentacyclic triterpenoid phytoalexin biosynthesis in cell suspension cultures of *Tabernaemontana divaricata*. Phytochemistry 28(11): 2981-2988.

VanEtten H. D., Sandrock R. W., Wasmann C. C., Soby S. D., McCluskey K. and Wang P. (1995): Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. Canadian Journal of Botany 73(1), 518-525.

Vaughan M. M., Christensen S., Schmelz E. A., Huffaker A., Mcauslane H. J., Alborn H. T. and Teal P. E. (2015): Accumulation of terpenoid phytoalexins in maize roots is associated with drought tolerance. Plant, Cell and Environment, 38(11): 2195-2207.

Velikova V., Yordanov I. and Edreva A. (2000): Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Science, 151(1): 59-66.

Westrick N. M., Smith D. L., and Kabbage M. (2021): Disarming the host: detoxification of plant defense compounds during fungal necrotrophy. Frontiers in Plant Science, 12: 1-46.

White T. J., Bruns T., Lee S. J. W. T. and Taylor J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: A guide to methods and applications, 18(1): 315-322.

Xu X., Qin G., and Tian S. (2008): Effect of microbial biocontrol agents on alleviating oxidative damage of peach fruit subjected to fungal pathogen. International Journal of Food Microbiology, 126(1-2): 153-158.

Yang H. H., Yang S. L., Peng K. C., Lo C. T. and Liu S. Y. (2009): Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. Mycological Research, 113(9): 924-932.

Zehra A., Meena M., Dubey M. K., Aamir M. and Upadhyay R. S. (2017): Synergistic effects of plant defense elicitors and *Trichoderma harzianum* on enhanced induction of antioxidant defense system in tomato against *Fusarium* wilt disease. Botanical Studies, 58(1): 1-14.

Zhao Q., Ran W., Wang H., Li X., Shen Q., Shen S. and Xu Y. (2013): Biocontrol of *Fusarium* wilt disease in muskmelon with *Bacillus subtilis* Y-IVI. BioControl, 58(2): 283-292.

Zhou R., Kong L., Yu X., Ottosen C. O., Zhao T. Jiang F. and Wu Z. (2019): Oxidative damage and antioxidant mechanism in tomatoes responding to

drought and heat stress. Acta Physiologiae Plantarum, 41(2): 1-20.