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Ethylene-dependent regulation of oxidative stress in the leaves of fusaric acid-treated tomato plants

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ABSTRACT

The mycotoxin fusaric acid (FA) induces rapid oxidative burst leading to cell death in plants. At the same time, plant defence reactions are mediated by several phytohormones for instance ethylene (ET). However, previously conducted studies leave research gaps on how ET plays a regulatory role under mycotoxin exposure. Therefore, this study aims to the time-dependent effects of two FA concentrations (0.1 mM and 1 mM) were explored on the regulation of reactive oxygen species (ROS) in leaves of wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomatoes. FA induced superoxide and H_2O_2 accumulation in both genotypes in a mycotoxin dose- and exposure time-dependent pattern. 1 mM FA activated NADPH oxidase (+34% compared to the control) and *RBOH1* transcript levels in WT leaves. However, superoxide production was significantly higher in *Nr* with 62% which could contribute to higher lipid peroxidation in this genotype. In parallel, the antioxidative defence mechanisms were also activated. Both peroxidase and superoxide dismutase activities were lower in *Nr* but ascorbate peroxidase showed one-fold higher activity under 1 mM FA stress than in WT leaves. Interestingly, catalase (CAT) activity decreased upon FA in a time- and concentration-dependent manner and the encoding *CAT* genes were also downregulated, especially in *Nr* leaves at 20%. Ascorbate level was decreased and glutathione remained lower in *Nr* than WT plants under FA exposure. Conclusively, *Nr* genotype showed more sensitivity to FA-induced ROS suggesting that ET serves defence reactions of plants by activating several enzymatic and non-enzymatic antioxidants to detoxify excess ROS accumulation.

1. Introduction

Fusarium wilt is a well-known vascular disease of plants caused by *Fusarium oxysporum* infection and results in damage to the host plants' defence system (Ding et al., 2018; Hashem et al., 2021). The long-term occurrence of fusarium wilt results in the reduction of crop resistance and yield (Attia et al., 2022). Pathogenic *Fusarium* species can release various mycotoxins during plant-pathogen interactions causing plant infections and colonization leading to plant wilt and ultimately, death (Li et al., 2021). These mycotoxins can vary in their roles, mode of action, and structure as well as in their effect during the progression of disease (Sapko et al., 2011; Pushparaj et al., 2023). One of these harmful mycotoxins is the fusaric acid (FA) which is known for its phytotoxic effects and is produced by many *Fusarium* species like *F. oxysporum, F. heterosporum, F. proliferatum* (Iqbal et al., 2021; Fernandes and Ghag, 2022). FA causes disease symptoms in plants and leads to leaf wilting and necrosis affirming the role of FA in disease development (Ding et al., 2018). It has been reported in several crops for the disease development such as watermelons, bananas, cucumbers, and tomatoes (Ding et al., 2018; Singh et al., 2017; Wang et al., 2013a; Wu et al., 2008). During the pathogens' invasion, plants respond through a series of physiological and molecular changes as a part of their defence responses. FA exposure resulted in pathological changes in the host plants by interfering with plant metabolism, influencing the enzymatic activities in the leaves, and altering the membrane potential of FA-treated plants (Wu et al., 2008). However, the involvement of FA in pathogenesis and the mechanism of its action are still unexplored in detail. Moreover, effects of FA on redox signalling and anti-oxidative defence responses of plants remained mostly uninvestigated.

During the plant-pathogen interactions, the generation of reactive oxygen species (ROS) is one of the initial responses of plants at cellular level (Singh et al., 2017). Both the generation and scavenging of ROS

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under mycotoxin exposure determine the cell redox status and homeostasis (Iqbal et al., 2022). ROS production (e.g. H_2O_2, $O_2^{\cdot-}$, and $OH^{\cdot}$) can lead to three possible outcomes such as cell damage, signal transduction, or protection against pathogenic attacks (Arumugam et al., 2021; Noctor et al., 2018). Excessive ROS generation can cause protein and chlorophyll oxidation, lipid peroxidation, and DNA damage initiating programmed cell death (PCD) (Singh et al., 2017). Intriguingly, H_2O_2 plays a pivotal role in the defence progression due to its antimicrobial activity and it can induce local- and systemic responses in plants against pathogenic attacks depending on its concentration and the duration of its accumulation (Singh and Upadhyay, 2014). Different plant organelles are involved in this ROS generation such as mitochondria, chloroplasts, apoplastic peroxidases, or NADPH-oxidases in the plasma membrane (Chen et al., 2013; Czarnocka and Karpiński, 2018). The plant cells prevent excessive oxidative damage by activating effective antioxidant mechanisms upon mycotoxin exposure (Noctor et al., 2018).

Plants have evolved enzymatic antioxidant defence enzymes like superoxide dismutase (SOD), ascorbate peroxidases (APX), catalase (CAT), guaiacol-dependent peroxidase (POD) and non-enzymatic defence antioxidants, for example, glutathione (GSH), ascorbate (AsA), tocopherol to regulate surplus ROS generation (Xia et al., 2015). The dismutation of $O_2^{\cdot-}$ is catalyzed by SOD while CAT, APX, and POD can remove H_2O_2 (Pandey et al., 2015). SOD is considered as the first defence line of plants against pathogens protecting them from oxidative stress by regulating superoxide levels (Iqbal et al., 2022). CAT plays a crucial role in plant development and resistance against the aging processes and pathogen attacks by regulating high H_2O_2 concentrations (Pandey et al., 2015). Similarly, peroxidases can also scavenge ROS under stress conditions with a higher affinity to H_2O_2 as compared to CAT (Poór et al., 2018). Under FA stress, tobacco cell suspensions exhibited reduced levels of CAT and APX activities and enhanced ROS generation (Jiao et al., 2014). Besides antioxidant enzymes, non-enzymatic defence antioxidants like AsA and GSH are also involved in scavenging ROS to avoid plants from oxidative cellular damage (Czarnocka and Karpiński, 2018) and contribute to trigger the defence responses against xenobiotic toxins by their detoxification (Czékus et al., 2020). GSH-AsA cycle plays a vital role in scavenging H_2O_2, in which ascorbate is recycled to its reduced form (AsA) from its oxidized form (dehydroascorbate, DHA) by GSH acting as a reducing agent (Noctor et al., 2018). FA can activate defence responses in plants even at low concentrations (10^{-5} M) without phytotoxic effects and induce defence signalling during plant-pathogen interactions (Bouizgarne et al., 2006). Several mycotoxins have been known to provoke ROS production leading to the progression of disease symptoms (Liu et al., 2020). Nonetheless, it is important to investigate the interaction of the phytotoxic effects of mycotoxins (such as FA) on ROS production and mycotoxin-induced antioxidant defence mechanisms together with defence-related phytohormones.

Several phytohormones such as ethylene (ET) besides salicylic acid, jasmonic acid, or abscisic acid have been reported to trigger defence mechanisms of plants by regulating ROS generation (Czékus et al., 2022; Iqbal et al., 2022). It has been well-known that ROS accumulation provokes various ET-mediated responses in diverse plant species. Additionally, both ET emission and NADPH oxidase activity run parallel to modulate ROS generation under different abiotic stresses (Jiang et al., 2013). Further, the presence of ET insensitive 2 (EIN2) and ET receptor 1 (ETR1) is crucial for ROS accumulation causing stomatal closure and inducing defence responses in plants against pathogens (He et al., 2011). Additionally, ET is responsible for the regulation of ROS generation, hindrance of CTR1 (a negative regulator of ET), and induction of MAPK 3/6 signalling cascade (Xia et al., 2015). Moreover, MPK6 can phosphorylate ethylene response factor 6 (ERF6) and induce ROS-inducible genes. Both ET and accumulated ROS function jointly in an amplified forward arc that can lead to plant cell death under divergent environmental stress conditions (Wang et al., 2013b). In tomato, five out of seven ET receptors (SlETR1-7) showed the affinity to bind with ET (Iqbal et al., 2021). *Nr* plants are insensitive to ET in plant tissues but can produce ET under stress conditions confirming that ET biosynthesis is not impaired in mutants (Takács et al., 2021). Using wild-type and *Nr* mutant tomatoes, time- and concentration-dependent effects of ET were proved in the regulation of defence reactions of plants by modulating the photosynthetic efficiency upon mycotoxin exposure (Iqbal et al., 2021, 2022). Therefore, more accurate knowledge about the biological role of ET can be collected using these ET receptor mutant plants. At present, our knowledge about ET signalling is not enough to clearly explain the role of ET upon mycotoxin exposure and further research is required to understand the ET-regulated oxidative defence mechanisms under FA stress.

Numerous studies have been conducted on ROS production and detoxification by antioxidants but how ET is involved in PCD induction via ROS generation and/or the regulation of defence responses through activation of the antioxidant system has always been a less-studied field. The main hypothesis of this research is that ET plays a regulatory role under FA exposure in plants. Therefore, this research work investigates the role of ET in the tomato leaves of two genotypes wild-type (WT) as well as *Never ripe* (*Nr*) plants (ET receptor mutant) exposed to 0.1 mM and 1 mM of FA in the regulation of oxidative stress under mycotoxin treatments. Additionally, ET-dependent oxidative defence mechanisms triggered by FA treatments were also studied in both tomato genotypes.

2. Materials and methods

2.1. Plant material acquisition and greenhouse conditions

Tomato seeds (*Solanum lycopersicum* L.) wild-type (WT; Ailsa Craig) and its ET receptor mutant *Never ripe* (*Nr*) plants were germinated under dark conditions at 26 °C and then, seedlings were hydroponically grown in greenhouse under constantly controlled conditions such as 12 h light and 12 h dark period, 200 μmol m^{-2} s^{-1} (photon flux density), 55–60% relative humidity, and at 24 °C day/22 °C night for 28 days. Meanwhile, nutrient solution (pH 5.8) was provided to plants every second day. The electric conductivity of the nutrient solution was determined daily and distilled water or nutrient solution was applied to control the value (Poór et al., 2018). Thereafter, seven weeks old plants with more than five developed leaves were used for the experiments.

2.2. FA treatments

Tomatoes were exposed to 0.1 mM FA for the induction of defence responses of plants and 1 mM FA for provoking cell death induction prepared with the nutrient solution based on previous research works (Spss and Oliveira, 2009; Iqbal et al., 2021). During the treatment, plants were avoided from any kind of artificial injury or wound. The nutrient solution was provided to control plants without FA addition. All treatments started at 9 a.m. (3 h later from the end of the dark period). The sampling of fully expanded leaves was performed from the third or fourth branch from the upper side followed by 24 and 72 h from FA-exposed plants and controls. All oxidants and antioxidants were determined and analysed in three biological replicates. All applied chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Quantification of $O_2^{\cdot-}$ generation

Leaf samples (100 mg) were ground and homogenized with 1 mL of sodium phosphate buffer (100 mM, pH 7.2) having 1 mM sodium diethyldithiocarbamate trihydrate (SDDT). Then, the samples were centrifuged at 13,000 g and 4 °C for 15 min. Thereafter, the supernatant (300 μL) was poured to a reaction mixture containing 650 μL of 0.1 M sodium phosphate buffer (pH 7.2) and 50 μL of 12 mM nitro blue tetrazolium (NBT). The absorbance of all the samples was measured using a spectrophotometer at the 2nd (A_2) and 7th (A_7) min, respectively. The following formula was used to calculate the level of

superoxide production: ΔA= (A_7)-(A_2) and it was denoted as min^{-1} g $(FM)^{-1}$ (Iqbal et al., 2022).

2.4. Measurement of H_2O_2 levels

The H_2O_2 content of leaf samples was determined with some changes of the method described by Horváth et al. (2015). 200 mg of leaf samples was homogenized using 1 mL of 0.1% trichloroacetic acid (TCA), then samples were centrifuged at 4 °C, and 10,000 g for 20 min. Later, supernatant (250 μL) was added to the reaction mixture [250 μL of 10 mM phosphate buffer pH 7.0 and 500 μl of 1 M potassium iodide]. Then, the samples were placed under dark conditions for 10 min. The absorbance of all the samples was determined using a spectrophotometer (Kontron, Milano, Italy) at 390 nm wavelength and H_2O_2 levels were measured using different standards.

2.5. Detection of NADPH oxidase activity

The activity of NADPH oxidase was examined by omniPAGE electrophoresis system (Cleaver Scientific Ltd., Rugby, Warwickshire, UK) as explained by Poór et al. (2017). Leaf samples were crushed with liquid nitrogen and extraction buffer [50 mM sodium phosphate buffer (pH 6.8) containing 5% Triton X-100] was added with a 1:1 ratio and mixed thoroughly. Thereafter, the samples were centrifuged at 12,000 g and 4 °C for 20 min. Protein extracts of leaf samples were blended with 62.5 mM Tris-HCl buffer (pH 6.8). This buffer was comprised of ten percent glycerol and 0.025% bromophenol blue, and the same proportion of protein (30 μg) which was ladened on the gel. Thereafter, electrophoresis was carried out for 1–3 h at 4 °C and 120 V using running buffer [25 mM Tris and 192 mM glycine solution (pH 8.3)] on four percent stacking and ten percent separating polyacrylamide gels. For the detection of bands, gels were placed into a reaction buffer [0.5 mg ml^{-1} NBT in 50 mM Tris (pH 7.4) and 134 μM NADPH]. Diphenyleneiodonium chloride (DPI; 50 μM) was used as an inhibitor of NADPH oxidase (50 μM concentration). The protein content of the samples was determined in accordance with Bradford (1976). Comparative pixel intensity of bands was determined using ImageJ software.

2.6. Determination of antioxidant activities

Leaf samples (250 mg) were ground and mixed thoroughly with 1.25 mL of 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% polyvinyl-poly pyrrolidone (PVPP). Thereafter, the samples were centrifuged (12,000 g, 4 °C, 20 min) and the same supernatant was exploited to detect superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and guaiacol-dependent peroxidase (POD; EC 1.11.1.7) activities at 560, 240, and 470 nm, respectively using a spectrophotometer (KONTRON, Milano, Italy). SOD activity determines the ability of SOD to inhibit NBT reduction accompanied by riboflavin and light. 1 unit of SOD expresses the enzyme amount used to inhibit fifty percent NBT depletion in the presence of light and riboflavin. Similarly, CAT activity measures the decomposition of H_2O_2 at 24 °C in 3 min. One unit of CAT denotes the enzyme quantity required to consume 1 μmol min^{-1} H_2O_2. Likewise, POD activity shows an increase in absorbance due to guaiacol oxidation. One unit of POD expresses the enzyme amount used to produce 1 μmol min^{-1} of oxidized guaiacol (Horváth et al., 2015; Poór et al., 2017). Additionally, in the case of ascorbate peroxidase (APX; EC 1.11.1.11) activity, 250 mg leaf samples were ground with 750 μl extraction buffer [50 mM potassium-phosphate buffer (pH 7.0), 1 mM PMSF, 1 mM EDTA, 50 mM NaCl, and 1 mM ascorbate] and 1.875% (w:v) PVPP. Thereafter, the samples were centrifuged at 12,000 g and 4 °C for 20 min. The supernatant was used to measure APX activity at 290 nm spectrophotometrically. APX activity determines the decrease in AsA content at 25 °C in 3 min. 1 unit of APX corresponds to the enzyme quantity required for the oxidation of 1 μmol min^{-1} AsA (Czékus et al., 2020). The protein content of all the samples was calculated by using a standard of bovine serum albumin according to Bradford (1976).

2.7. Determination of glutathione (GSH) and ascorbate (AsA) contents

Leaf samples of 250 mg were ground and mixed with 1 mL of 5% (w/v) TCA. The samples were centrifuged at 12,000 g and 4 °C for 20 min. The same supernatant was exploited to determine both AsA and GSH. In the case of total AsA content, 10 mM dithiothreitol (DTT) was poured to reaction mixture containing 75 mM potassium phosphate buffer and sample, then 10 min later 0.5% N-ethylmaleimide (NEM) was poured into the mixture to remove surplus DTT. Thereafter, the total AsA content was determined in the samples by adding a reaction mixture [10% TCA, 43% H_3PO_4, 4% bipyridyl, 3% $FeCl_3$] and the absorbance of samples was computed spectrophotometrically at 525 nm. (Czékus et al., 2020). Similarly, total GSH was also measured from the same samples. The supernatant (20 μl) was poured to the mixture including 100 mM phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM NADPH, and 1 unit of glutathione reductase. The absorbance of samples was recorded at 412 nm spectrophotometrically (Czékus et al., 2020).

2.8. RNA extraction and detection of genetic expressions by qRT-PCR

Total RNA contents of the samples were extracted and their cDNAs were synthesized in accordance with Poór et al. (2017). Briefly, 100 mg of tomato leaf sample was ground using liquid nitrogen then TRI reagent containg 1.82 M guanidium isothiocyanate, 11.36 mM sodium citrate, 200 mM potassium acetate (pH 4.0), 0.73 mM N-lauryl sarcosine, 45.45% phenol was added to the samples followed by heating them at 65 °C for 3 min. Thereafter, 200 μl of chloroform was added and samples were centrifuged at 11180×*g*, 15 min, 4 °C. Then, the supernatant was pipetted into 375 μl of chloroform:isoamyl alcohol (24:1) and centrifuged at the earlier mentioned conditions followed by the addition of 500 μl of isopropanol to the supernatant which was then incubated at room temperature for 10 min and centrifuged. Later on, the pellet was cleaned with 500 μl of 70% cold ethanol, then the RNA in 30 μL of molecularly pure water (AccuGENE®, Lonza Group Ltd, Basel, Switzerland) was dissolved.

The samples were treated with DNase enzyme (Thermo Fisher Scientific, Waltham, MA USA) to remove the genomic DNA residues by preparing a reaction mixture containing 8 μL of DNase buffer, 34 μl of molecularly pure water and 0.4 μL of RNase inhibitor (Fermentas UAB, Vilnius, Lithuania). Thereafter, 15 μL of RNA sample and 8 μL of DNase enzyme were added into the reaction mixture. The samples were incubated at 37 °C for 30 min and then at 65 °C for 10 min. Proteins were removed using 300 μL of chloroform and 300 μL of phenol followed by centrifugation of the samples at 16090×*g*, 15 min, 4 °C. Further, the supernatant was purified with 400 μL of chloroform. After centrifugation was repeated under the same conditions, then the supernatant was added into a mixture of 550 μL of cold 96% ethanol and 20 μL of 3 M Na-acetate and left overnight at −20 °C. The next day, samples were centrifuged at 16090×*g*, 10 min, 4 °C, and the pellet was washed with 500 μL of 70% cold ethanol followed by dissolving it in 30 μl of molecularly pure water. Possible RNA degradation was checked by 1% agarose gel electrophoresis. The concentration of the isolated RNA was determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Washington, DC, USA). Thereafter, cDNA synthesis was conducted using 1 μL of reverse transcriptase (RT) enzyme (Thermo Fisher Scientific, Waltham, MA USA) and by preparing a reaction mixture containing 4 μL of RT reaction buffer, 0.5 μL of random hexamer primer, 1 μL of 25 mM dNTP mixture, 0.5 μL of RNase inhibitor and 13 μL of molecularly pure water. The reaction lasted for 1 h at 37 °C.

Quantitative real-time reverse transcription-PCR (qTOWER Real-Time qPCR System, Analytik Jena, Jena, Germany) was employed to uncover the expressions of the chosen genes of tomato plants obtained

from Sol Genomics Network (SGN; http://solgenomics.net/) and National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) databases (Horváth et al., 2015). NCBI and Primer 3 softwares were used to design primers as shown in the article of Poór et al. (2017). The PCR reaction mixture was containing 10 ng of cDNA template, 400 nM forward and 400 nM reverse primers, 5 μl of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA), and nuclease-free water (10 μl). PCR was performed by repeating steps at 95 °C for 7 min (15 s) (denaturation) and at 60 °C for 1 min (annealing extension). Thereafter, a melting arc examination was carried out to analyze the particularity of the assembled reaction by increasing temperature from 55 to 90 °C. The qTOWER 2.2 Software was used to perform data analysis. Moreover, *Elongation factor-1α* (*EF1α*) subunit genes were utilized as reference genes and $2^{(-\Delta\Delta Ct)}$ formula was used to analyze qRT-PCR data. Each assembled reaction consisted of three replicates and the data were presented with mean values.

2.9. Statistical analysis

There were more than three replicates in each treatment and the whole experiment was recurred thrice times. The entire acquired data were presented in mean values and standard deviation bars. Statistical analysis was conducted using Sigma Plot 11.0 software (SPSS Science Software, Erkrath, Germany). Moreover, analysis of variance (ANOVA) was performed to determine the difference among different treatments by Tukey test and the significant difference was found if $p \leq 0.05$.

3. Results

Firstly, the degree of oxidative stress induced by FA was determined by the detection of superoxide production and H_2O_2 levels in the tomato leaves of WT and *Nr* plants. The production of superoxide was found to be significantly higher in both WT (+95% compared to the control) and *Nr* (2.5-fold compared to the control) tomato plants exposed to 1 mM FA concentration following 72 h than in all other treatments (Fig. 1). In parallel, it was significantly more elevated in *Nr* leaves than WT plants followed by 72 h treatment exposed to 1 mM FA concentration (Fig. 1). However, neither of the tomato genotypes showed any significant difference in 0.1 mM FA-treated plants in superoxide content upon 0.1 mM FA treatment followed by respective 24 and 72 h time periods (Fig. 1).

The H_2O_2 production was also significantly elevated by FA. However, FA induced higher levels of H_2O_2 in a time- and FA concentration-dependent manner (Fig. 2). Both WT and *Nr* plants showed a significant rise in H_2O_2 generation particularly in 1 mM FA-exposed plants followed by 72 h. However, H_2O_2 content was significantly more increased in 1 mM FA-exposed WT (2.5-fold compared to the control) after 72 h as compared to *Nr* (+55% compared to the *Nr* control) leaves (Fig. 2). In addition, WT plants under 1 mM FA treatment after 24 h showed higher H_2O_2 levels as compared to control plants (+57%).

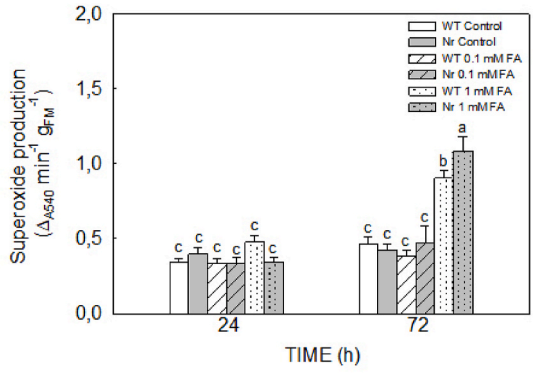

Fig. 1. Variations in superoxide production with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

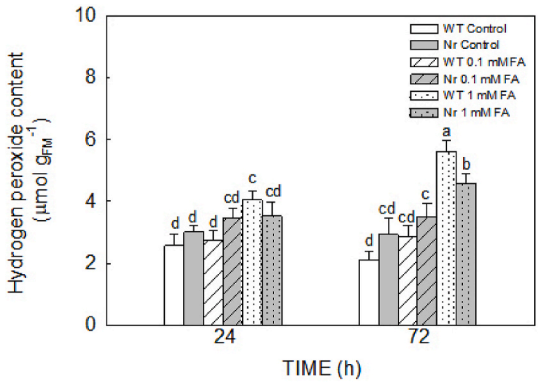

Fig. 2. Alterations in hydrogen peroxide content with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

In order to determine the generation of superoxide and H_2O_2 in the leaves of FA-exposed tomato genotypes, the presence of enzyme for the production of $O_2^{\cdot-}$ was also investigated. NADPH oxidase activity was enhanced in WT plants (+34% compared to the control) under 1 mM FA stress followed by 72 h treatment than *Nr* plants. However, 24 h treatment showed a significant difference neither in the treatments nor between WT and Nr plants (Fig. 3).

FA-mediated oxidative burst was followed by the activation of different antioxidants and triggered various enzymatic responses under FA exposure. The SOD activity, which correlates with the dismutation rate of superoxide into O_2 and H_2O_2, also showed a time- and FA concentration-dependent pattern in WT tomato leaves (Fig. 4). WT plants displayed the highest SOD activity under 1 mM FA concentration followed by 72 h (+33% compared to the control) than other treatments. Both FA treatments significantly elevated SOD activity already after 24 h in WT leaves (Fig. 4). Interestingly, SOD activity did not alter significantly in the tomato leaves of *Nr* plants upon FA treatments (Fig. 4).

The CAT activity, which catalyses the breakdown of H_2O_2 to O_2 and H_2O, revealed no significant differences in any FA-treated tomato genotypes after 24 and 72 h respectively. In general, CAT activity showed decreasing tendency in a FA dose- and time-dependent pattern in WT

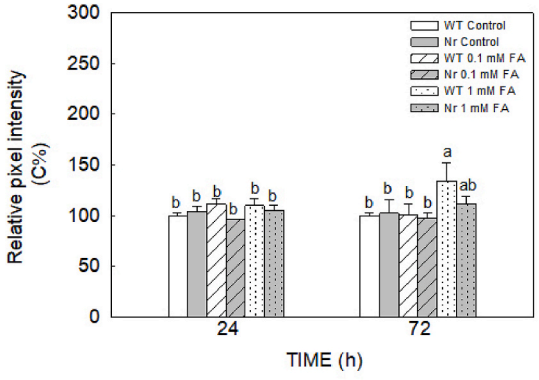

Fig. 3. Variations in the activity of NADPH oxidase enzyme. The relative pixel intensity determined on non-degraded gel with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

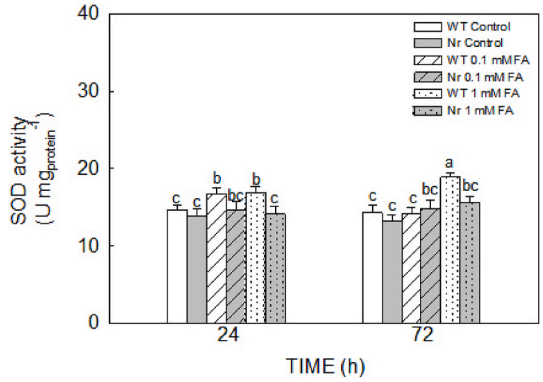

Fig. 4. Changes in superoxide dismutase (SOD) activity with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

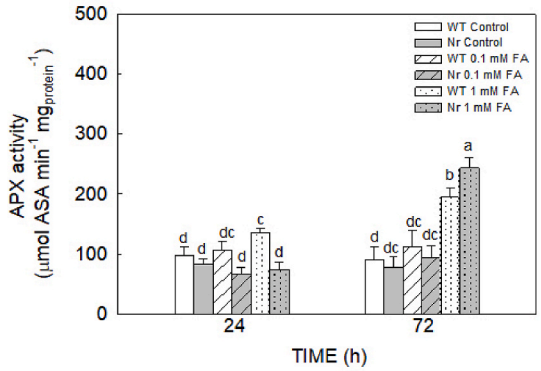

Fig. 6. Variations in ascorbate peroxidase (APX) activity with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

leaves (Fig. 5). Nevertheless, WT plants exhibited higher CAT activity as compared to *Nr* plants in the case of all treatments (Fig. 5). Both FA concentrations decreased CAT activity already after 24 h in *Nr* leaves (−20% compared to the control) but in WT plants, only 1 mM FA reduced it significantly to the same level as in *Nr* (Fig. 5).

The activity of APX, which catalyses H_2O_2 removal in the presence of a co-substrate (AsA), was found to be significantly higher under 1 mM FA concentration in WT leaves followed by 24 (+37%) and 72 h time period (2-fold compared to control), respectively (Fig. 6). However, *Nr* plants exhibited a more significant rise in APX activity than WT plants exposed to 1 mM FA concentration followed by 72 h (3-fold compared to the control). In contrast, WT plants displayed significantly higher APX activity (+81% compared to the control) than *Nr* plants under 1 mM FA exposure after 24 h. In addition, no significant difference was observed between 0.1 mM FA-exposed WT and *Nr* plants followed by 24 or 72 h time period, however, WT plants showed higher APX activity as compared to *Nr* mutants (Fig. 6).

FA exposure increased the POD activity in a dose- and time-dependent way, and POD enzyme plays a crucial role in H_2O_2 removal. Both tomato genotypes showed the highest POD activity under 1 mM FA exposure followed by respective 24 and 72 h treatments (Fig. 7). Nonetheless, WT plants exhibited significantly higher POD activity (9-fold compared to the WT control) as compared to *Nr* mutants (8-fold compared to the *Nr* control) treated with 1 mM FA concentration followed by 72 h. Except for 1 mM FA-exposed plants followed by 72 h time period, neither WT nor *Nr* plants did not display any significant

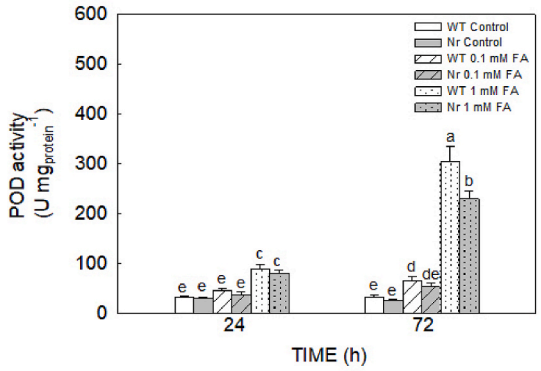

Fig. 7. Alterations in guaiacol-dependent peroxidase (POD) activity with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

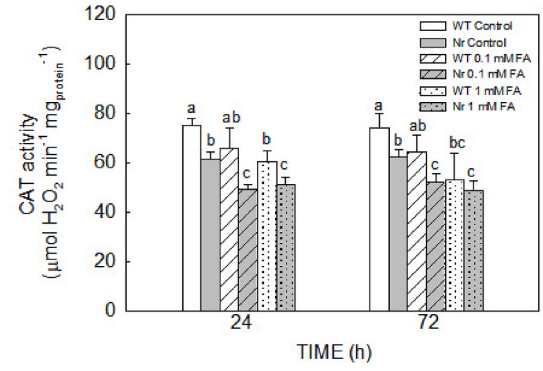

Fig. 5. Alterations in the activity of catalase (CAT) with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

difference within the treatments in their respective time periods (Fig. 7).

The expression patterns of the key antioxidant enzyme-coding genes in tomato leaves differed depending on the time-period of FA treatments (Fig. 8). The genetic expression of the *RBOH1* was enhanced in WT tomato plants in both FA-exposed treatments, especially 0.1 mM FA. Concurrently, transcript levels of *RBOH1* were elevated only 72 h after FA treatments in *Nr* leaves. Interestingly, no significant increase was found for *SOD-Fe* expression in FA-treated tomato leaves. Conversely, the expression level of *SOD-CuZn* was significantly enhanced in both WT and *Nr* plants under both FA concentrations, particularly after 72 h treatments. Similar tendency was observed in the case of *SOD-Mn*, which was expressed after 72 h-long-FA exposure in both tomato genotypes. The expression of *CAT1* showed a similar tendency to *RBOH1*. Nonetheless, *CAT2* and *CAT3* transcript levels were increased significantly under 72 h FA treatments, especially 0.1 mM FA stress. Furthermore, *CAT3* exhibited the maximum expression exposed to 1 mM FA concentration followed by 72 h in WT as well as *Nr* tomato plants. In contrast to WT plants, the expressions of *CATs* were reduced in *Nr* leaves in the first 24 h. In the case of *APX1* and *APX2*, their expression levels were elevated followed by 72 h of FA stress in WT plants, however, *Nr* plants displayed a significant increase of both *APX1* and *APX2* expression in FA-exposed leaves only under 72 h (Fig. 8).

FA treatments induced significant genotype-dependent changes in the non-enzymatic antioxidants levels. Especially, 1 mM FA reduced AsA content (−47% compared to the control) followed by 72 h in the tomato

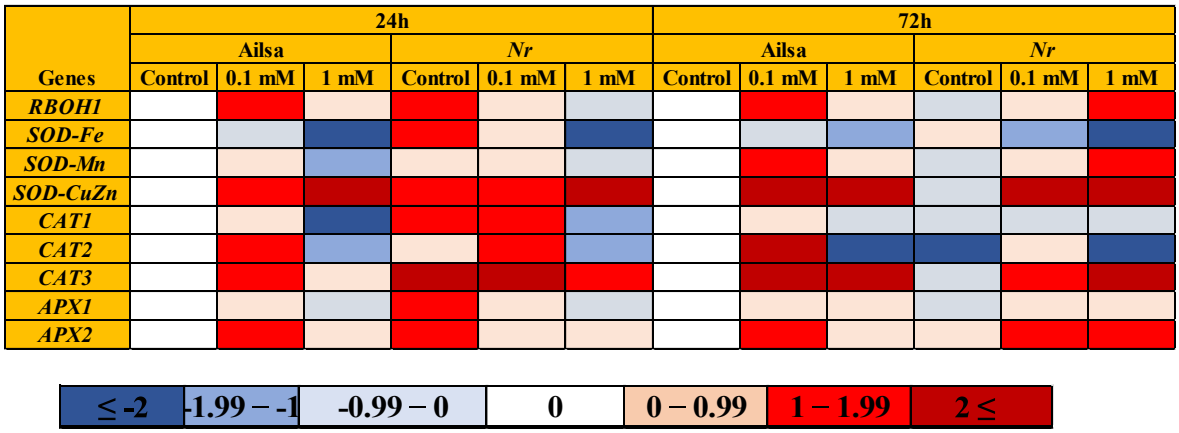

Fig. 8. Variations in the expression pattern of the key antioxidant enzyme-coding genes with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means while different colours of the heat map show the significant difference of 1.

leaves of *Nr* plants. In parallel, AsA contents did not alter in WT plants (Fig. 9A).

On the other hand, GSH content increased after FA treatments in both tomato genotypes. WT plants showed a significantly higher GSH level (3-fold compared to the WT control) than *Nr* plants (2-fold compared to *Nr* control) exposed to 1 mM FA followed by 72 h. Furthermore, WT plants displayed enhanced GSH accumulation in an exposed time- and FA dose-dependent pattern. Nevertheless, in the case of 0.1 mM FA-treated plants, no significant difference was observed between the examined tomato genotypes nor between 24 h and 72 h time periods (Fig. 9B).

4. Discussion

Many research reports have documented the link of mycotoxins-evoked ROS generation and plant cell death (Ding et al., 2018) however, the sublethal effects of ROS and ROS-induced defence mechanisms in plants remained unexplored. *F. oxysporum* responsible for FA production, leads to wilt disease symptoms in plants due to ROS-mediated necrosis (Singh and Upadhyay, 2014). In our former study, effects of FA toxicity due to ROS induction on the photosynthetic activity of tomato plants have been investigated on both photosystem PSII and PSI and we found that plants deficient in ET signalling exhibited more sensitivity to FA confirming the role of ET in the initiation of defence responses of tomato (Iqbal et al., 2021). In this work, we further analysed the FA-induced ROS generation and ET-mediated antioxidant defence mechanisms after FA exposure at biochemical and molecular levels. It is well known that ROS metabolism, PCD or defensive mechanisms in plants are governed by several phytohormones such as ET under mycotoxin exposure (Xing et al., 2013). Therefore, the pivotal role of ET was studied in this research work by using *Nr* plants (ET receptor mutant). However, the concentration of mycotoxins and their exposure time also determine the disease outbreak (Singh et al., 2017). Hence, FA was added to the rooting medium in two different concentrations (Wang et al., 2013a) and phytotoxic effects of FA were observed 24 and 72 h after the mycotoxin treatments. In our previous study, an elevated level of ET production under FA exposure was found in both tomato genotypes than untreated plants followed by respective 24 and 72 h periods (Iqbal et al., 2021). It is well-established that ET can initiate PCD or activate plant defence responses in a time- and FA dose-dependent pattern (Iqbal et al., 2021). Therefore, ET plays a vital role in plant defence signalling under environmental stress conditions, however, the pivotal part of ET in response to mycotoxin exposure and ET-regulated defence mechanisms remained only partially investigated.

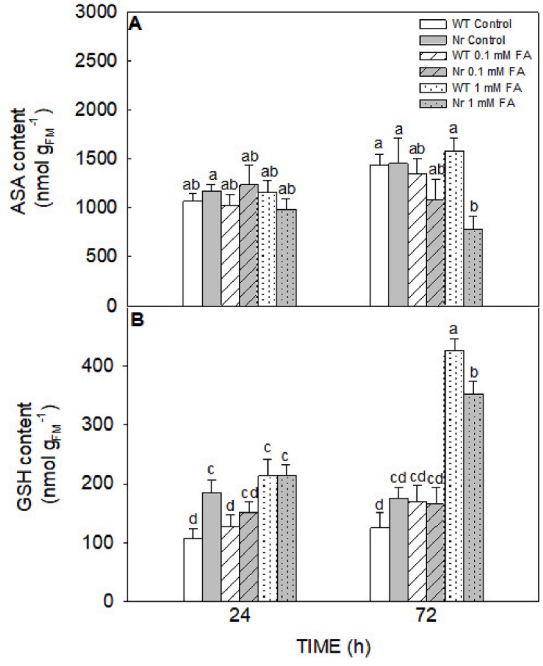

Fig. 9. Alterations in ascorbate (AsA) and glutathione (GSH) contents with respect to time in wholly developed leaves of both genotypes wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to 0.1 mM and 1 mM FA. Data were presented in means with standard error bars while small letters show the significant difference using Tukey test at $P \leq 0.05$.

Rapid and high ROS accumulation in tomato plants is a major cause of PCD under different stress conditions (Noctor et al., 2018). Here we were focusing on the changes in the levels of $O_2^{\cdot-}$ and H_2O_2 and we found significantly higher ROS production in both genotypes under the lethal, 1 mM FA concentration after 72 h. However, superoxide ($O_2^{\cdot-}$) production was significantly higher in *Nr* while H_2O_2 was higher in WT leaves upon FA exposure which suggested the ET-dependent ROS generation in the leaves of tomato. Singh and Upadhyay (2014) also found that FA resulted in higher levels of $O_2^{\cdot-}$ production after 72 h based on NBT reduction in the leaves of tomato plants. FA-treated tomato cell cultures and leaves also exhibited higher levels of $O_2^{\cdot-}$ production (Kuźniak, 2001). Moreover, FA-induced higher H_2O_2 levels were also documented in tomato leaves (Singh and Upadhyay, 2014; Singh et al., 2017). FA exposure enhanced H_2O_2 production in tomato and potato cell cultures after 48 h, respectively (Kuźniak, 2001; Sapko et al., 2011). Therefore, higher production of ROS, especially the more reactive $O_2^{\cdot-}$ could cause

irreversible damage to cells and cell organelles including lipid peroxidation and deprived membrane stability (Singh et al., 2017). At the same time, H_2O_2 in lower concentrations can act as a signalling molecule whereas poses harmful effects at higher levels (Iqbal et al., 2022). Earlier we found also that higher concentration of FA induced significant lipid peroxidation and loss of membrane integrity in leaves of tomato, but *Nr* leaves were more sensitive to FA (Iqbal et al., 2021). These findings indicate that FA-induced ET accumulation plays an essential role in the ROS production and the activation of anti-oxidative defence mechanisms in plants.

In order to investigate the production of ROS in tomato leaves of FA-treated plants, the superoxide anion radicals using enzymatic approach were analysed. NADPH oxidase is also involved in the production of these superoxide radicals (Bouizgarne et al., 2006). Based on our results, higher NADPH oxidase activity was observable particularly in 1 mM FA exposed plants followed by 72 h. In contrast, expression levels of *RBOH1* were elevated already in 24 h treatment exposed to both FA concentrations in WT plants, but *Nr* tomato leaves showed only in 72 h treatment. At the same time, the high superoxide accumulation can originate from the inhibition of photosynthetic electron transfer upon FA in *Nr* plants (Iqbal et al., 2021). Earlier it was found that the H_2O_2 production was prevented in *Arabidopsis* and saffron cells under FA stress upon treatment with diphenyl iodonium salt (DPI; an inhibitor of NADPH oxidase), indicating that NADPH oxidase is also responsible to produce H_2O_2 (Jiao et al., 2014). Further, banana leaves and pseudostems showed enhanced ROS accumulation upon *F. oxysporum* infection, which was also inhibited by DPI affirming the significant role of NADPH oxidase for ROS generation under pathogenic attacks, respectively (Liu et al., 2020). Interestingly, another report has documented that both ET and NADPH oxidase operate in conjunction to regulate ROS generation under different environmental stresses (Jiang et al., 2013). Our findings indicated that ROS generation is enhanced upon FA exposure and causes oxidative burst leading to cell death eventually. Similarly, FA treatment resulted in elevated production of ET depicting the involvement of ET in cell death and defence responses of plants under mycotoxin exposure.

Pathogenic attacks or other elicitors induce rapid oxidative stress lasting for several hours. For the detoxification of these toxic ROS, plants have evolved defence mechanisms such as antioxidants promoting plant growth and development without any harm or stress. Various antioxidant enzymes and non-enzymatic antioxidants can be activated by different phytohormones such as ET and suppress or enhance oxidative stress (Ranjbar and Ahmadi, 2015; Poór et al., 2017). Here we studied (the) SOD-, CAT-, APX-, and POD-implemented, ET-dependent ROS scavenging tendencies induced by FA exposure. The activity of SOD which has the capability to dismutase $O_2^{\cdot-}$ to O_2 and H_2O_2, showed higher levels in WT as compared to *Nr* plants under 1 mM FA exposure already followed by 24 h treatment. Interestingly, SOD isoenzymes can be regulated independently at subcellular levels (Wu et al., 2008). We observed no significant changes in the expression pattern of the chloroplastic *SOD-Fe* gene in FA-treated tomato leaves. Concurrently, the gene expression levels of the chloroplastic *SOD-CuZn* and the mitochondrial *SOD-Mn* were significantly increased in both tomato genotypes under both FA concentrations, particularly after the 72 h treatment. Likewise, FA-treated tomato cell cultures and watermelon leaves also depicted a higher SOD activity in a time- and FA concentration-dependent pattern (Kuźniak, 2001; Wu et al., 2008). Similar results were found in another study where SOD levels were reported to be higher in a time-dependent manner upon FA infiltration into the leaves of tomato plants (Singh and Upadhyay, 2014). It is derived from our findings that plants deficient in ET signalling showed lower SOD activity which can contribute to the higher superoxide ($O_2^{\cdot-}$) levels and lipid peroxidation in *Nr* plants. Thus, FA-induced ET can activate SOD serving as a defence reaction of plants upon FA exposure.

The high production of H_2O_2 content was found in FA exposed plants due to possibly partial generation by SOD enzyme, but the balance between H_2O_2 level and its detoxification is based on the activities of POD, CAT, and APX (Li et al., 2021). We observed that CAT activity showed decreasing tendency upon FA treatment in a concentration- and time-dependent pattern in both tomato genotypes, especially in *Nr* leaves. In contrast, the transcript levels of *CAT1* and especially *CAT2* and *CAT3* were increased in 72 h FA treatments and exhibited the maximum expression under 1 mM FA concentration after 72 h in WT and Nr plants. Nevertheless, the expressions of *CATs* were lower in *Nr* leaves in the first 24 h as compared to WT plants. Others also found that FA exposure resulted in reduced CAT activity in FA-treated leaves of tomato plants (Singh and Upadhyay, 2014). Our results coincide with the findings of another study that showed a reduction in CAT activity with time in potato cell suspension cultures under FA exposure (Sapko et al., 2011). These findings indicate that decrease in CAT activity can contribute to higher levels of H_2O_2 which can take part in the initiation of PCD upon the FA exposure. However, our results show that this decline in CAT activity and gene expression is more significant in *Nr* leaves in the first 24 h indicating the defensive role of FA-triggered ET in the rapid regulation of ROS metabolism under FA exposure.

In addition to CAT, APX also plays an essential part in the detoxification of H_2O_2 (Czarnocka and Karpiński, 2018). Treatments with FA increased significantly higher APX activity within 24 h in WT plants but it did not change in *Nr* tomato leaves. In contrast, *Nr* plants exhibited significantly higher APX activity than WT plants exposed to 1 mM FA concentration followed by 72 h, indicating the role of ET in the modulation of this antioxidant enzyme. The expression of *APX1* and *APX2* showed the same trend, they were increased upon 0.1 mM FA exposure after 24 and 72 h in WT plants but did not change in *Nr* leaves. A higher APX activity was also documented in FA-treated tomato cell culture after 48 h (Kuźniak, 2001). Conversely, another scientific study reported the reduced APX activity in FA-treated tomato leaves after 72 h (Singh and Upadhyay, 2014). The potential effect of ET on this enzyme was affirmed by the exogenous treatment of ACC (an ET precursor) which enhanced not only SOD and CAT activities, but also APX activity in bentgrass (Larkindale and Huang, 2004). Intriguingly, treatment of 1-MCP (an ET inhibitor) also induced ROS production and ultimately affected these antioxidant enzymatic activities (Ranjbar and Ahmadi, 2015). Our findings affirmed that ET could induce plant antioxidants enzymes for the detoxification of ROS under mycotoxin exposure. Additionally, our study firstly confirmed the time-dependent regulation of antioxidant mechanisms by ET upon different FA concentrations.

The other H_2O_2 scavenging enzyme, POD exhibited concentration- and time-dependent changes after FA treatments in both investigated tomato genotypes. POD activity was increased followed by 24 h in 1 mM FA exposed plants which was more elevated after 72 h. In parallel, POD activity was observed significantly higher in WT as compared to *Nr* leaves indicating the protective and regulatory part of ET to modulate high ROS levels under FA exposure. Others also found that FA-treated tomato cell cultures showed high POD activity after 48 h of treatment suggesting its potential to detoxify ROS production under mycotoxin exposure (Kuźniak, 2001).

Besides enzymatic antioxidants, other key antioxidants for instance AsA and GSH also play a crucial part in scavenging ROS under mycotoxin exposure in plants. AsA content showed a significant decline in FA-treated tomato leaves especially in *Nr* plants than WT leaves followed by 72 h. This also indicates the protective role of ET in the modulation of ROS levels by antioxidants upon mycotoxin exposure. Other results also confirm this, e.g. ET treatment in *Actinidia deliciosa* also enhanced antioxidant activities (Park et al., 2008). In addition, higher levels of total AsA were found in tomato leaves treated with ethephon but no significant difference was reported for GSH content (Chen et al., 2013). At the same time, FA exposure in tomato cell cultures did not change significantly the total AsA levels after 48 h following the treatment (Kuźniak, 2001). Similarly, both AsA and GSH levels were found to be higher in tomato seedlings 36 h followed by the mycotoxin Beauvericin exposure (Loi et al., 2020) confirming the different actions of mycotoxins. In contrast to AsA levels, GSH content was elevated by 72-h-long

1 mM FA exposed WT and *Nr* plants. Concurrently, WT plants showed significantly higher GSH content than *Nr*, also confirming the protective role of ET in the regulation of non-enzymatic antioxidant levels. It is well known that the role of GSH is important to predict plant defence responses or PCD in plants under stress conditions (Czarnocka and Karpiński, 2018). It was found, that the overexpression of a lipid transfer protein AtLTP4.4 enhanced the total GSH level in *Arabidopsis* and improved the survival of plants upon mycotoxin deoxynivalenol exposure (McLaughlin et al., 2015). In addition, GSH alleviated the AAL toxin-evoked plant cell death in *Arabidopsis* leaves through SA- and ET signalling pathways (Sultana et al., 2020). Therefore, ET exhibits its plausible involvement in the induction of antioxidants for defence responses to mitigate ROS accumulation under FA exposure for the survival of plants.

5. Conclusions

We can conclude that FA induced rapid ROS production responsible for the activation of ET signalling. The lethal, 1 mM FA treatments significantly elevated $O_2^{\cdot-}$ and H_2O_2 levels in both WT and *Nr* leaves after 72 h. In parallel, the antioxidative defence mechanisms of plants were also activated. 1 mM FA activated NADPH oxidase and elevated the transcript levels of *RBOH1* in WT plants after 72 h. SOD and POD activities were also increased in 1 mM FA-treated WT leaves as compared to *Nr* leaves in ET-dependent manner after 72 h, however, CAT activity and the expression of its encoding genes showed ET-dependent decrease in WT plants while remained unaffected in *Nr* plants in all treatments. In contrast, the APX activity was significantly enhanced in *Nr* as compared to WT leaves after 72 h. Furthermore, AsA level was decreased under 1 mM FA exposure in *Nr* plants while WT plants showed ET-dependent increase in GSH levels upon 1 mM FA treatment for 72 h. Conclusively, ET serves as a potential candidate to alleviate mycotoxin-induced stress by activating antioxidant mechanisms to detoxify excess ROS accumulation for the proper functioning and normal growth of plants. These findings could assist in future research on mycotoxin-induced alleviation of environmental stresses in economically important crops. Keeping in view the available data, ET could play a crucial role in plant defence responses by activating ET-dependent antioxidant mechanisms and by the upregulation of defence-related genes under pathogenic attacks. There are several studies conducted on the effects of fungal infections and mycotoxins on plants, however, more research could be done focusing on their exact action mechanism and on control strategies to reduce their phytotoxic effects on crops either by priming agents to boost plant defence responses or by other approaches to decrease or inhibit fungal infections and accumulation of mycotoxins in plants.

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Author contributions

Conceptualization, P.P.; investigation, I.N. and Z.C.; writing—original draft preparation, I.N.; writing—review and editing, I.N., Z.C., P.P., and A.Ö.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.02.047.

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