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Ethylene-dependent effects of fusaric acid on the photosynthetic activity of tomato plants

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Abstract

Fusaric acid (FA) is one of the potential toxins produced by pathogenic *Fusarium* species which exerts oxidative stress and cell death in plants. In this work, the effects of different concentrations of FA were investigated on the photosynthetic activity in leaves of wild type and *Never ripe* (*Nr*) tomato plants to reveal the potential role of ethylene under mycotoxin exposure. FA induced a significant ethylene emission from leaves in a concentration- and time-dependent manner. FA (1 mM) decreased the maximal and effective quantum yields of PSII and PSI in both tomato genotypes but photoprotective processes, such as the nonphotochemical quenching and the cyclic electron flow, were activated more effectively in *Nr* plants. However, the lipid peroxidation was higher in leaves of *Nr*. Our result confirmed that *Nr* plants were more sensitive to FA phytotoxicity suggesting the key role of ethylene in the activation of defense responses.

Keywords: assimilation; electrolyte leakage; lipid peroxidation; mycotoxin; photosystem; stomatal conductance.

Introduction

The ascomycete *Fusarium oxysporum* (*F. oxysporum*) is one of the most significant fungal plant pathogens, which results in Fusarium wilt and causes serious economic losses in various plant species worldwide, especially in tomato plants (Marzano *et al.* 2013). The symptoms of *F. oxysporum* infection are well-characterized in tomato, such as loss of turgidity and changes in photosynthetic pigment contents of leaves that eventually cause death (Singh *et al.* 2017). Moreover, xylem vessels are blocked with the mycelium biomass, polysaccharide production, and spore formation by *Fusarium* species; these are considered as the leading causes of water imbalance and

Highlights

- Fusaric acid (FA) induced significant ethylene emission in tomato plants
- FA decreased F_v/F_m and effective quantum yields of PSII and PSI
- NPQ and CEF were activated more effectively upon FA in the lack of ethylene

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Abbreviations: ACC – 1-aminocyclopropane-1-carboxylic acid; Car – carotenoids; CEF – cyclic electron flow; Chl – chlorophyll; EL – electrolyte leakage; ET – ethylene; ETR1 – ethylene receptor 1; F_0 – minimal fluorescence yield in the dark-adapted state; FA – fusaric acid; FB1 – Fumonisin B1; FM – fresh mass; F_v/F_m – maximum quantum yield of PSII; HR – hypersensitive response; MDA – malondialdehyde; NPQ – nonphotochemical quenching; *Nr* – *Never ripe*; PCD – programmed cell death; q_L – estimates the fraction of open PSII centres; q_P – photochemical quenching; RC – reaction centres; ROS – reactive oxygen species; SA – salicylic acid; WT – wild type, $Y_{(I)}$ – effective quantum yield of PSI; $Y_{(II)}$ – effective quantum yield of PSII; $Y_{(CEF)}$ – yield of cyclic electron flow around PSI; $Y_{(NA)}$ – quantum yield of nonphotochemical energy dissipation in PSI due to acceptor side limitation; $Y_{(ND)}$ – quantum yield of nonphotochemical energy dissipation in PSI due to donor side limitation.

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Fusarium wilt in the host plants (Dong *et al.* 2012, Srinivas *et al.* 2019). Due to clogged xylem vessels, the disturbance of water homeostasis and nutrition uptake can affect the synthesis of photosynthetic pigments resulting in decreased photosynthetic activity (Srinivas *et al.* 2019). At the same time, the pathogenicity of *Fusarium* species is mediated by various mycotoxins produced by the fungi, such as lycomarasmin, dehydrofusaric acid, fumonisin B1 (FB1), and fusaric acid (FA), one of the most prevalent mycotoxins (Lievens *et al.* 2008, Rani *et al.* 2009, Singh *et al.* 2017). These toxins can disturb and inhibit the normal plant life in several ways but results of the accurate investigations of these toxins in the host plants are confined and needed to be explored in more details, especially in the case of FA. FA plays a crucial role in disease progression in plants because of its involvement in the Fusarium wilt with several symptoms (Kuźniak 2001, Singh *et al.* 2017). Furthermore, FA is a host nonspecific mycotoxin and its huge production is linked with the severity of disease in plants depending upon the pathogenic *Fusarium* species. FA is accumulated in cereal grains during infection and causes potential toxicity in animals and humans through food (Singh and Upadhyay 2014, Singh *et al.* 2017). However, most of the subcellular changes induced by FA are left unexplored and require much more attention for a comprehensive understanding of hidden mechanisms under stress conditions.

FA is responsible for significant changes in tomato plants, such as necrosis in leaves, shrinkage, and dryness of leaves, and wilting of petioles and stem. Similarly, the root and root hair growth is also reduced due to FA and results in the induction of transitory membrane hyperpolarization (Bouizgarne *et al.* 2006). FA also perturbs various biochemical processes associated with membrane permeability, hindrance of respiration, and disruption of mitochondrial activity in tomato (Singh *et al.* 2017). It is also known that FA increases the electrolytic leakage perturbing the depolarization potential of the plasma membrane that reduces the ATP synthesis and hinders other enzymatic activities responsible for the respiratory disorder and finally, induces cell death in tomato (Singh and Upadhyay 2014). Concurrently, low FA concentrations might evoke several protective responses in plants instead of potential phytotoxic effects and play a signaling role in host–pathogen interactions (Bouizgarne *et al.* 2006). Nevertheless, a scientific study revealed the phytotoxic effects of FA in banana seedlings infected with *F. oxysporum*. The results showed the reduced rates of both transpiration and stomatal conductance as well as damaged membrane system were due to FA (Dong *et al.* 2012). The homeostasis of cells and the status of cell membranes upon toxins are highly dependent on the production and metabolism of reactive oxygen species (ROS) (Gill and Tuteja 2010). ROS can be generated by apoplastic peroxidases, the plasma-membrane-bound NADPH-oxidase or by mitochondria and chloroplasts (Chen *et al.* 2010). The concentration-dependent ROS accumulation can lead to changes in the expression of defense-related genes and/or trigger programmed cell death (PCD) in plants (Sunil *et al.* 2013, Ivanov *et al.* 2018, Khan *et al.* 2018, Farooq *et al.* 2019). In parallel, a defensive mechanism is activated in plants to regulate ROS metabolism (Czarnocka and Karpiński 2018, Noctor *et al.* 2018, Wu *et al.* 2020). Plants have various antioxidants (*e.g.*, ascorbate, glutathione, tocopherol) and antioxidant enzymes (*e.g.*, superoxide dismutase, catalase, ascorbate peroxidases, glutaredoxins, and peroxiredoxins) to limit excess ROS contents (Xia *et al.* 2015). In the case of FA, a decrease in the catalase and ascorbate peroxidase activities and the high ROS production were measured in tobacco cell suspensions after a treatment with the fungal toxin. FA also reduced the ATP content and mitochondrial membrane potential, thus the significant changes in mitochondria can contribute to excess ROS (Jiao *et al.* 2014). In addition, FA evoked oxidative stress (H_2O_2 and $O_2^{\bullet-}$) and perturbed antioxidant enzymatic activities (catalase and ascorbate peroxidase) in leaves and tomato cell cultures (Singh and Upadhyay 2014). At the same time, the effects of FA-induced ROS, especially, the role of chloroplasts and the changes in the photosynthetic activity upon FA have not been investigated yet.

It is known that some significant mycotoxins play a detrimental role in chloroplast structure and function as well as an induction of ROS accumulation in chloroplasts. The surplus ROS results in the degradation of D1 protein, lipid peroxidation of the thylakoid membrane, and photoinhibition of PSI and PSII (Choudhury and Behera 2001, Edelman and Mattoo 2008, Partelli *et al.* 2011, Chen *et al.* 2012, Pospíšil 2012, Xiang *et al.* 2013, Fagundes-Nacarath *et al.* 2018, Eagles *et al.* 2019, Wang *et al.* 2020). For instance, *Eupatorium adenophorum* was treated with tenuazonic acid, which caused necrosis on the leaves and induced direct ROS burst in chloroplasts. This mycotoxin damaged chloroplasts and inhibited the PSII electron transport beyond Q_A as well as caused the reduction of end acceptors on the PSI acceptor side and chloroplast ATPase activity. The excessive chloroplastic ROS production affected the lipids, pigments, proteins, and DNA followed by lipid peroxidation, disruption of the cell membrane, electrolytic leakage, chlorophyll (Chl) breakdown, nuclear damage, and eventually, led to the cell death of plants (Chen *et al.* 2010, 2014). Another significant mycotoxin, FB1, can also induce the cell death due to ROS production (Asai *et al.* 2000, Radić *et al.* 2019). It was found that ROS accumulation resulted in the dysfunction of chloroplasts and provoked the cell death in leaves of *Arabidopsis* (Xing *et al.* 2013). Similarly, FB1 triggered hypersensitive response (HR) by ROS in *Arabidopsis* and increased contents of long-chain bases and long-chain bases phosphates. The phosphorylated compounds of long-chain bases are synthesized by sphingosine kinases (SPHKs) which play an important role in modulating ROS production. It was demonstrated that the overexpression of *Arabidopsis* SPHK1 enhanced ROS accumulation while SPHK1 suppression resulted in reduced ROS contents (Qin *et al.* 2017). Likewise, the effect of lycomarasmin and FA produced by *F. oxysporum* resulted in the cell death of tomato leaf protoplasts (Sutherland and Pegg 1992, Stępień *et al.* 2013). Similarly, the phytotoxic effect of 9,10-dehydrofusaric acid isolated

from *F. nygamai* was detected in pea leaves. In addition, 9,10-dehydrofusaric acid caused tomato leaf chlorosis and hindered root elongation (Brown *et al.* 2012, Bani *et al.* 2014). Thus, the mycotoxin-induced ROS production, Chl breakdown, membrane injury, and the cell death can be results of significant changes in chloroplast structure and photosynthetic activity which can contribute to the pathogenicity but the exact role of various mycotoxins in these processes remaines mostly unclear, especially in the case of FA.

The defense mechanisms of plants are regulated by various phytohormones, such as ethylene (ET) which biosynthesis and effects can be dependent on ROS (Chang 2016). It is known that ROS evoke several ET-triggered responses in various plant species. In addition, molecular studies have revealed that ET and NADPH oxidase function together to control ROS production under various abiotic stress effects (Jiang *et al.* 2013). ET caused stomatal closure in *Arabidopsis* through H_2O_2 production by respiratory burst oxidase homolog protein F (RbohF) (Jiao *et al.* 2013). Moreover, the ethylene receptor 1 (ETR1) and the ethylene insensitive 2 (EIN2)-induced signaling was essential for ROS accumulation (He *et al.* 2011) which contributes to stomatal closure and immunizes plants against pathogens attacks (Mersmann *et al.* 2010). ET regulated the ROS production and hindered the constitutive triple response (CTR1) by activating the mitogen-activated protein kinase 3/6 (MAPK 3/6) signaling cascade (Xia *et al.* 2015). Furthermore, ethylene response factor 6 (ERF6) was also phosphorylated by MPK6 and ERF6 inducing the transcriptional control of ROS-responsive genes. Moreover, both ET and ROS together in a self-amplifying forward loop can induce cell death in plants in response to various environmental stimuli (Wang *et al.* 2013a). It was shown that many mycotoxins induced cell death by rapidly generating gaseous ET. The key effect of ET in DNA fragmentation and PCD induction was reported after the treatment with mycotoxin zearalenone and its derivatives in maize (Repka *et al.* 2017). Moreover, it was found that FB1-induced PCD is also dependent on the ET based on the application of ET receptor mutant *etr1-1 Arabidopsis* plants (Asai *et al.* 2000). Later, it was demonstrated that degradation of Chl and promotion of cell death were more rapid and more extensive in the ET receptor mutant *etr1-1*, but these tendencies were different in other ET receptor mutants (Plett *et al.* 2009). Despite, the role of ET on the regulation of photosynthetic pigment contents and photosynthetic activity has been investigated (Chen and Gallie 2015, Borbély *et al.* 2019), currently acquired knowledge about ET in signaling of mycotoxin-induced physiological changes, especially in photosynthesis, remained mostly incomplete and needs further research. Using ET sensing- or signaling mutants can provide new results to understand the role of ET in FA-induced changes in photosynthetic activity, respectively. There are seven ET receptors (SlETR1-7) in tomato but only five of them can bind ET with high affinity (Kamiyoshihara *et al.* 2012, Shakeel *et al.* 2013, Liu *et al.* 2015). Among these, SlETR3 is known as *Never ripe* (*Nr*) which is the ortholog of the *Arabidopsis* ETR1 (Yen *et al.* 1995, Klee and Tieman 2002). *Nr* plants exhibit insensitivity to ET in all tissues but are able to produce ET, *e.g.*, upon pathogen attack, indicating that mutants are not impaired in ET biosynthesis (Lanahan *et al.* 1994). Using this mutant provides a more precise analysis of the physiological and biochemical functions of ET (Nascimento *et al.* 2020).

In this article, an analysis of the ET-dependent photosynthesis was carried out in leaves of wild-type and ET receptor mutant *Never ripe* (*Nr*) tomato plants exposed to a sublethal (0.1 mM) and a cell-death-inducing concentration (1 mM) of FA to reveal the differences in the main photosynthetic parameters after the mycotoxin treatments. In addition, FA-induced physiological changes were also elucidated in the two tomato genotypes.

Materials and methods

Plant material: Wild type (WT) and ET-receptor mutant *Never ripe* (*Nr*) seeds of tomato (*Solanum lycopersicum* L., cv. Ailsa Craig) were germinated in dark and then plants were grown hydroponically in a greenhouse with 12-h light and 12-h dark period at day/night temperatures of 24/22°C and irradiance of 200 μmol(photon) m^{-2} s^{-1} [PPFD; white LED (5700 K) illumination supplemented with FAR LEDs; *PSI*, Drásov, Czech Republic] and relative humidity of 55–60% for four weeks. The nutrient solution was changed three times a week (Poór *et al.* 2011). The experiments were conducted with 6- to 7-week-old intact plants at the stage of 7–8 developed leaves.

Fusaric acid treatment: Tomato plants were treated in the greenhouse with 100 μM and 1 mM FA concentrations prepared in nutrient solution (Wang *et al.* 2013b) avoiding any artificial injury or wound. Control plants were provided with the nutrient solution only without the addition of FA. The fully expanded leaves on the 3rd or 4th stem from the top were selected for all measurements. The effects of FA on tomato leaves were recorded after 24 and 72 h following the treatment.

Ethylene production: The total ethylene gas produced by tomato leaves was measured after FA treatments with a *Hewlett Packard 5890 Series II* gas chromatograph equipped with a flame ionization detector and a column packed with activated alumina as described by Poór *et al.* (2015). Leaf samples (0.5 g) were incubated with 0.5 mL of deionized water for 1 h in gas-tight tubes under darkness. After the incubation, 2.5 mL of the gas was removed from the flasks with a gas-tight syringe and injected to gas chromatograph. A set of ethylene standards was used to calculate the amount of ethylene generated by the leaves.

Photosynthetic activity: Chl *a* fluorescence and P700 redox state were analysed with *Dual-PAM-100* instrument (*Heinz-Walz*, Effeltrich, Germany) (Klughammer and Schreiber 1994, 2008). Leaves were dark-adapted for 15 min at room temperature before the measurement of the minimal fluorescence yield of the dark-adapted state (F_0,) applying weak measuring light when reaction centres

(RC) are open. The maximal fluorescence in the dark-adapted state (F_m) was determined using a pulse (800 ms) of saturating light [12,000 μmol(photon) m^{-2} s^{-1}]. After illumination with actinic light [220 μmol(photon) m^{-2} s^{-1}], the light-adapted steady-state fluorescence (F_s) was determined and the maximum fluorescence level (F_m') in the light-adapted state was recorded with saturating pulses. Thereafter, the actinic light was turned off and the minimum fluorescence level in the light-adapted state (F_0') was measured by illuminating the leaf with 3-s far-red light [5 μmol(photon) m^{-2} s^{-1}]. The following parameters were determined: the maximum quantum yield of PSII [variable fluorescence (F_v)/maximum fluorescence (F_m)], the minimal fluorescence yield in the dark-adapted state (F_0), the maximal fluorescence yield in the dark-adapted state (F_m), the fraction of open PSII RC (q_L), the quantum yields of PSI ($Y_{(I)}$) and PSII [$Y_{(II)}$], the nonphotochemical quenching (NPQ), the quantum yield of nonphotochemical energy dissipation due to acceptor side limitation [$Y_{(NA)}$], the quantum yield of nonphotochemical energy dissipation due to donor side limitation [$Y_{(ND)}$], and the photochemical quenching coefficient (q_P) (Zhang *et al.* 2014, Poór *et al.* 2019). The ratio of the quantum yield of cyclic electron flow (CEF) to the linear electron flow was calculated as $Y_{(CEF)}/Y_{(II)} = [Y_{(I)} - Y_{(II)}]/Y_{(II)}$ (Lei *et al.* 2014).

Moreover, the stomatal conductance (g_s) and the net photosynthetic rate (P_N) were measured on the fully expanded leaves using a portable photosynthesis system (*LI-6400*, *LI-COR*, *Inc.*, Lincoln, NE), as described by Poór *et al.* (2011). Leaves were illuminated (PPFD of 200 μmol m^{-2} s^{-1}) in the chamber and data were recorded after 10 min. Conditions were constant during the measurements (25°C, 65 ± 10% relative humidity, and controlled CO_2 supply of 400 μmol mol^{-1}).

Photosynthetic pigment: Chl (*a*+*b*) and carotenoid (Car) contents were determined in accordance with the protocol by Sims and Gamon (2002) with some modifications. Approximately 25 mg of leaves were crushed in 100% acetone and left for 24 h in the dark at 4°C. Samples were centrifuged (12,000 × *g*, 15 min, 4°C) and the pellet was extracted again with 1 ml of cold acetone/Tris buffer solution (80:20, v/v, pH = 7.8) for 24 h in the dark at 4°C. After centrifugation (12,000 × *g*, 15 min, 4°C), the supernatants were collected, and the pigment content was measured by a spectrophotometer (*Kontron*, Milano, Italy) at 470, 537, 647, and 663 nm, respectively.

Lipid peroxidation: Lipid peroxidation was determined based on the malondialdehyde (MDA) content according to Ederli *et al.* (1997). Leaf samples (100 mg) were ground in liquid nitrogen and then, 1 ml of 0.1% trichloroacetic acid (TCA) and 0.4% butyl hydroxytoluene (BHT) were added to avoid further lipid peroxidation. After centrifugation (12,000 × *g*, 20 min, 4°C), 500 μl of supernatant was added to 2 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) and heated at 100°C for 30 min. Samples were cooled down in the next step and their absorbance was determined at 532 and 600 nm by a spectrophotometer (*Kontron*, Milano, Italy). Total MDA content was quantified by using 155 mM^{-1} cm^{-1} molar extinction coefficient and expressed in nmol $g(FM)^{-1}$. All chemicals were purchased from *Sigma-Aldrich* (St. Louis, MO, USA).

Cell viability: Cell viability was determined based on the measurement of the electrolytic leakage according to Czékus *et al.* (2020). Leaf samples (100 mg) were added to 20 ml of ultrapure distilled water and kept under dark conditions at 25°C for 2 h. After that, the conductivity of water (C_1) was determined, then the samples were heated at 100°C for 30 min to completely release the ions from the tissues. Subsequently, samples were cooled to room temperature and the conductivity of the water (C_2) was measured again. The electrolytic leakage (EL) percentage of all the samples was calculated using the following formula: $EL\ [\%] = (C_1/C_2) \times 100$.

Statistical analysis: Each experiment contains at least three biological replicates (at least three plants per treatment) and the entire experiment was conducted three times. Results are expressed as mean ± SE. Statistical analysis was accomplished using *Sigma Plot 11.0* software (*SPSS Science Software*, Erkrath, Germany). Differences between the treatments in the case of each plant species were statistically analyzed by analysis of variance (*ANOVA*) using *Tukey*'s test. The means of each treatment were significant if $p \leq 0.05$.

Results

Ethylene production: Even though phytohormones play a crucial role in defense reactions influenced by photosynthesis, the role of ET in mycotoxin-induced defense responses has remained less-studied. To gain more information about the ET-dependent changes in photosynthesis triggered by FA, the production of ET was measured in the leaves of WT and *Nr* tomato plants after treatments by two concentrations of mycotoxin.

The ET emission was significantly enhanced in the concentration- and time-dependent manner in WT plants, where treatment with 1 mM FA resulted in the highest production of ET (Fig. 1). Similar changes were observed in *Nr* plants treated by FA, but ET production did not show any significant temporal differences in the examined genotypes following the treatments after 24 and 72 h, respectively (Fig. 1).

Photosynthetic activity: The maximal quantum yield (F_v/F_m) of PSII did not show any significant differences after the treatments with 0.1 mM FA during the investigated time period in neither of the genotypes. At the same time, 1 mM FA significantly decreased F_v/F_m after 72 h in both tomato genotypes (Fig. 2*A*). The minimal fluorescence yield of the dark-adapted state (F_0) did not change after 24 h in any FA treatments independently from the active ET signaling but it decreased significantly upon 1 mM FA after 72 h in WT as compared to the control (Fig. 2*B*). The maximal fluorescence in dark-adapted state (F_m) was reduced gradually in accordance with the increasing

FA concentration after 72 h in WT but changes were not pronounced in *Nr* leaves as compared to WT (Fig. 2*C*). The q_L parameter, which is in good correlation with the number of the open PSII RC, did not change during the first 24 h but it significantly declined after 72 h under 1 mM FA treatment in both genotypes. At the same time, no significant differences can be found in all FA treatments as well as between WT and *Nr* (Fig. 2*D*).

The effective quantum yield of PSII [$Y_{(II)}$] significantly decreased upon increasing FA concentration in all treatments. The highest $Y_{(II)}$ reduction was observed in 1 mM FA-treated plants after 24 and 72 h, respectively. Furthermore, $Y_{(II)}$ did not show any significant changes between WT and *Nr* plants after 24 and 72 h (Fig. 3*A*). Similarly, $Y_{(I)}$ showed the same trend as $Y_{(II)}$ after 24 and 72 h in 0.1 mM and 1 mM FA-treated plants, respectively (Fig. 3*B*). However, the photochemical quenching coefficient (q_P) decreased significantly by both FA treatments only after 72 h in both genotypes (Fig. 3*C*). On the contrary, nonphotochemical quenching (NPQ) significantly increased in the concentration-dependent manner by FA within 24 h which was significantly higher in *Nr* leaves. These changes were more pronounced after 72 h in *Nr* plants (Fig. 3*E*). The quantum yield of nonphotochemical energy dissipation in PSI due to donor side limitation [$Y_{(ND)}$] was significantly enhanced in 1 mM FA-treated plants after 24 and 72 h following 0.1 mM concentration as compared to control plants. Nevertheless, no significant changes can be observed between WT and *Nr* plants (Fig. 3*D*). Moreover, a gradual but not significant decline was depicted in the quantum yield of nonphotochemical energy dissipation in PSI due to acceptor side limitation [$Y_{(NA)}$] after 24 h in all FA-treated plants while changes in $Y_{(NA)}$ were not significant after 72 h under FA exposure (Fig. 3*F*).

The ratio of the quantum yield of cyclic electron flow (CEF) to the linear electron flow [$Y_{(CEF)}/Y_{(II)}$] increased significantly only after the 72-h-long 1 mM FA exposure in *Nr* (Fig. 4).

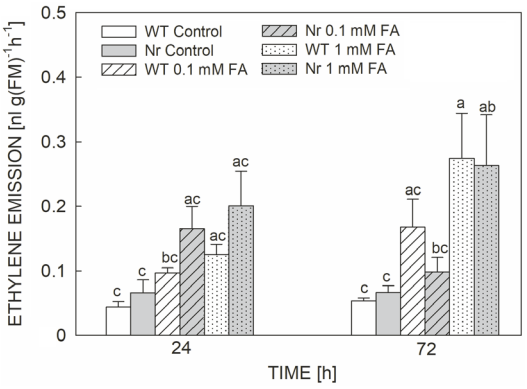

Fig. 1. Changes in the ethylene (ET) production in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

Photosynthetic pigments: Treatment with 1 mM FA induced loss of Chl (*a*+*b*) and Car content after 72 h in leaves of both genotypes as compared to controls (Fig. 5). However, this decrease in Chl (*a*+*b*) was more pronounced in *Nr* as compared to WT leaves (Fig. 5*A*).

Stomatal conductance and net photosynthetic rate: The stomatal conductance was significantly reduced after 24 h under 0.1 mM and 1 mM FA exposure in both plant genotypes and stomata remained closed after 72 h upon all FA treatments, respectively (Fig. 6*A*). Similarly, the net photosynthetic rate rapidly decreased in both WT

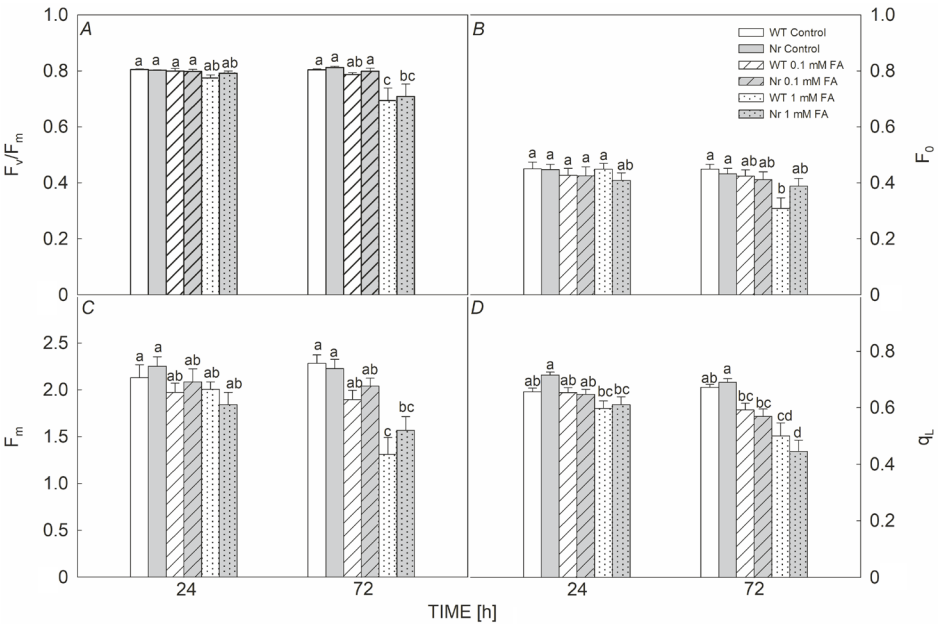

Fig. 2. Changes in the maximum quantum yield of PSII (F_v/F_m) (*A*), the minimal fluorescence yield of the dark-adapted state (F_0) (*B*), the maximal fluorescence in the dark-adapted state (F_m) (*C*), and the fraction of open PSII reaction centres (q_L) (*D*) in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

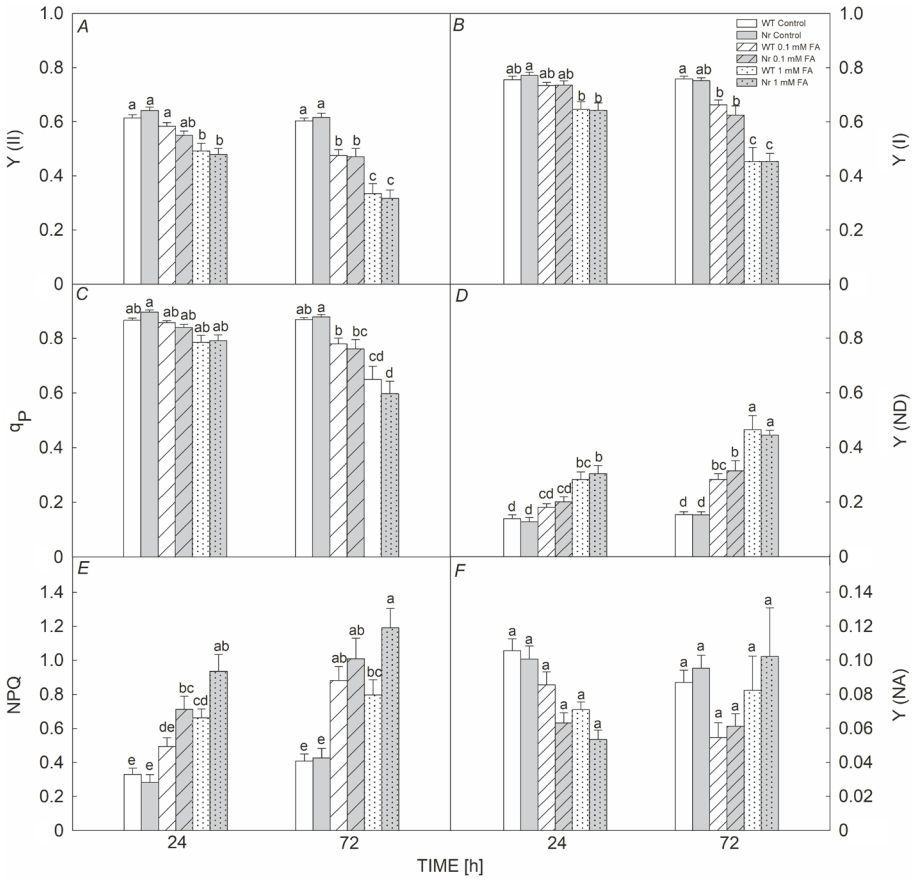

Fig. 3. Changes in the effective quantum yield of PSII [$Y_{(II)}$] (*A*), the quantum yield of PSI [$Y_{(I)}$] (*B*), the photochemical quenching coefficient (q_P) (*C*), the quantum yield of nonphotochemical energy dissipation in PSI due to donor side limitation [$Y_{(ND)}$] (*D*), the non-photochemical quenching (NPQ) (*E*), and the quantum yield of nonphotochemical energy dissipation in PSI due to acceptor side limitation [$Y_{(NA)}$] (*F*) in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, n = 3. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

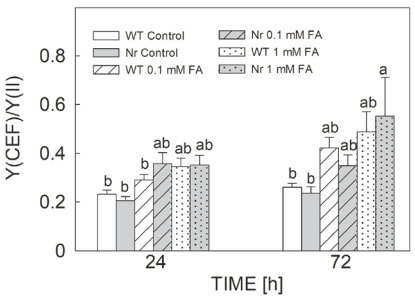

Fig. 4. Changes in the ratio of the quantum yield of cyclic electron flow (CEF) to the linear electron flow [$Y_{(CEF)}/Y_{(II)}$] in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, n = 3. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

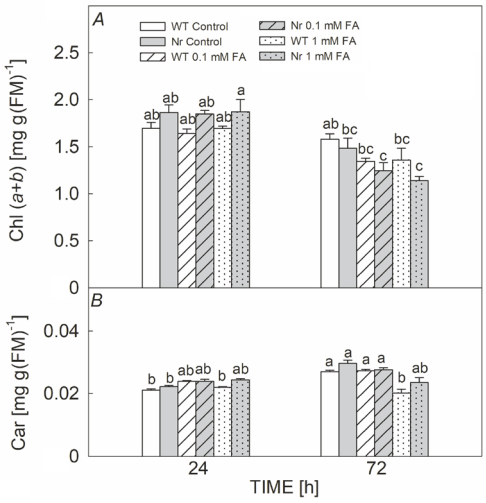

Fig. 5. Changes in chlorophyll (*a*+*b*) [Chl (*a*+*b*)] (*A*) and carotenoids (Car) contents (*B*) in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, n = 3. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

and *Nr* plants upon application of 0.1 mM and 1 mM FA, respectively. Moreover, it was significantly lowered in case of the highest concentration of FA treatment as compared to 0.1 mM (Fig. 6*B*).

Lipid peroxidation and cell viability: Changes in the malondialdehyde (MDA) content represent the product of the final decomposition of lipid peroxidation upon FA application in both WT and *Nr* plants. The MDA content was significantly higher in 1 mM FA-treated plants after 24 and 72 h in *Nr* as compared to 0.1 mM FA treatment and

WT plants. Besides, *Nr* plants showed significantly higher MDA content already after 24 h under 1 mM FA exposure as compared to WT plants (Fig. 7*A*). Similarly, the loss of cell viability based on the electrolyte leakage (EL) from the leaves increased under 1 mM FA exposure after 72 h in both tomato genotypes. However, no significant difference was found between WT and *Nr* plants, respectively (Fig. 7*B*).

Discussion

Several studies have been conducted on the interplay between mycotoxin-induced ROS production and cell death (Singh *et al.* 2017, Farooq *et al.* 2019) but the origin and subcellular effects of ROS remained mostly uninvestigated. The mycotoxin FA produced by *F. oxysporum* f. sp. *lycopersici* plays a key role in the development of Fusarium wilt and results in disease symptoms, such as necrosis mediated by ROS in tomato plants (Stępień *et al.* 2013, Singh and Upadhyay 2014). However, it was found that the degree of cell death is highly dependent on the presence or absence of light (Asai *et al.* 2000, Xing *et al.* 2013), thus, the active photosynthesis and the injury of photosynthetic apparatus can significantly influence the outcome of mycotoxin exposure. In this research work, the FA-induced changes in photosynthetic activity were investigated to reveal the effects of FA on PSII and PSI activities. Defense reactions of plants as well as ROS production and PCD are regulated by various phytohormones under the mycotoxin exposure (Glazebrook 2005, Pare *et al.* 2005, Coll *et al.* 2011, Xing *et al.* 2013, Kurepin *et al.* 2015). Thus, we focused on the role of the gaseous ET in this work using ET receptor mutant *Nr* plants. Moreover, the exposure time and toxin concentration are also determining factors in the onset of disease (Singh and Upadhyay 2014, Singh *et al.* 2017), thus FA was applied in two different concentrations *via* the rooting medium (Wang *et al.* 2013b) and effects of the mycotoxin were recorded at different time-points (24 and 72 h) after the FA exposure.

Our work revealed the enhanced level of ET emission in FA-treated leaves after 24 and 72 h in both tomato genotypes, respectively, depending upon the increasing FA concentration. It is known that ET by a concentration- and time-dependent manner can contribute to both PCD initiation and activation of defense responses of plants (Overmyer *et al.* 2003, Trobacher 2009, Poór *et al.* 2013). Interestingly, it was found that the degradation of Chl and promotion of cell death were more rapid and more extensive in the ET-receptor mutant *etr1-1* after FB1 treatment suggesting the protective role of ET in the case of this kind of *Fusarium* toxin (Plett *et al.* 2009). Moreover, others described that the exogenous treatment with ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) significantly reduced FB1-induced cell death in *Arabidopsis* (Wu *et al.* 2015). Our result based on lipid peroxidation and electrolyte leakage confirmed also that ET plays a crucial role in the activation of defense reaction of tomato plants after FA treatments. ET-dependent changes in photosynthetic activity can also serve as defense reactions of plants under mycotoxin exposure, thus the potential effects of this FA-induced ET production were further investigated on the photosynthetic activity.

Our results demonstrated that the exogenous treatment

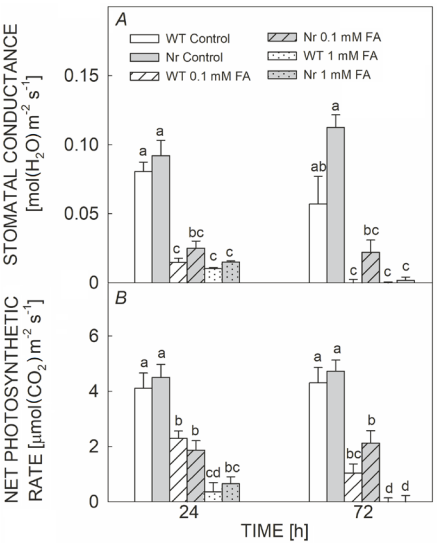

Fig. 6. Changes in the stomatal conductance (*A*) and net photosynthetic rate (*B*) in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

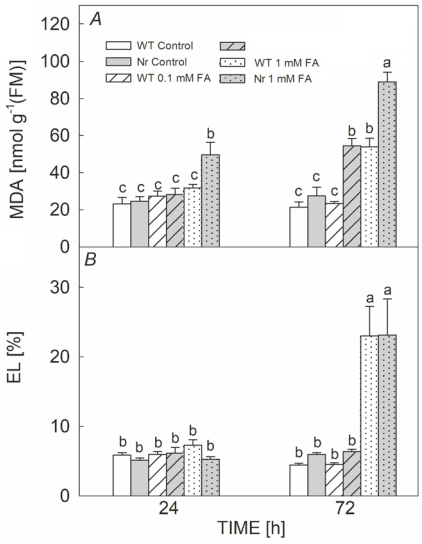

Fig. 7. Changes in the content of malondialdehyde (MDA) (*A*) and electrolyte leakage (EL) (*B*) in fully expanded leaves of wild type (WT; *white columns*) and ethylene-insensitive *Never ripe* (*Nr*; *grey columns*) tomato plants treated with 0.1 mM or 1 mM fusaric acid (FA) for 24 and 72 h. Means ± SE, $n = 3$. Bars denoted by *different letters* are significantly different at $P \leq 0.05$ as determined by *Tukey*'s test.

with 1 mM FA caused a significant decrease in the F_v/F_m after 72 h independently of the presence or absence of active ET signaling. This result confirmed for the first time that FA by a concentration-dependent manner can disturb the efficiency of PSII and damage the photosynthetic apparatus. At the same time, changes in F_0 and F_m were more significant in WT plants but the decrease in q_L was more pronounced in *Nr* leaves. However, the effects of ET/ACC on photosynthesis seem to be contradictory (Ceusters and Van de Poel 2018). Borbély *et al.* (2019) found that neither low nor high concentration of ACC influenced the F_v/F_m parameter in tomato, but it decreased q_L, which displays the fraction of open PSII RC. Thus, changes in this parameter upon FA can be dependent on FA-induced ET.

Time- and concentration-dependent changes in $Y_{(II)}$, q_P, and NPQ were recorded after FA treatments because of photoinhibition in PSII by FA exposure. The photoinhibition of PSII by tenuazonic acid and FB1 has also been reported earlier (Guo *et al.* 2020, Zavafer *et al.* 2020). Thereafter, the excessive photoexcitation pressure is exerted at PSII RC resulting in ROS production in the form of $O_2^{\bullet-}$, $^1O_2$, and H_2O_2. These oxidant species are detrimental to other electron transport components and might damage the protein structure of PSII, respectively (Liu *et al.* 2012, Zhang *et al.* 2014). Plants have evolved several photoprotective mechanisms to lessen the deleterious effects of ROS accumulation by activating defense systems, such as HR at the infection site, antioxidant production, and NPQ operation (Xing *et al.* 2013). NPQ mechanisms are capable to disperse excessive energy captured by LHCII (Liu *et al.* 2012). Moreover, the increase in NPQ is a good indicator of the onset of photoprotective mechanisms, which are mostly related to the xanthophyll cycle and the formation of ΔpH across the thylakoid membranes (Miyake 2010, Zhang *et al.* 2014). Our findings revealed that NPQ increased by FA in the concentration-dependent manner and *Nr* showed a significant elevation of NPQ as compared to WT. Exogenous ACC treatment also decreased $Y_{(II)}$ which was accompanied by increasing NPQ in tomato leaves (Borbély *et al.* 2019). Moreover, others also confirmed that ET significantly affected NPQ in *eto1-1* ET overproducing and *ctr1-3* constitutive ET response of *Arabidopsis* mutants. These plants showed the inhibition in the conversion of violaxanthin to zeaxanthin, thus in the efficiency of the xanthophyll cycle due to their impaired violaxanthin deepoxidase activity. Based on this observation, the excess ET or constitutive ET signaling inhibited the activity of xanthophyll cycle in *eto1-1* or *ctr1-3* (Chen and Gallie 2015). Based on our results, FA-induced ET played a role mainly in the protection mechanism of photosynthesis through the development of NPQ and CEF. Not only $Y_{(II)}$ but also $Y_{(I)}$ was downregulated by FA. These changes occurred in parallel with higher $Y_{(ND)}$ and lower $Y_{(NA)}$. Besides, the reduction of PSI acceptor side and NADPH accumulation led to photoinhibition of PSI. Usually, the overproduction of NADPH results from a reduced level of carbon fixation that can eventually increase the production of hydroxyl radicals (Kalaji *et al.* 2012, Zhang *et al.* 2014). Consequently, Chl triplets are formed due to the overreduced acceptor side of PSI evolving harmful singlet oxygen. Moreover, NADPH accumulation speeds up the Mehler reaction which ultimately produces harmful superoxide radicals. Eventually, ROS accumulation causes damage to PSI by photoinhibition (Huang *et al.* 2011). In contrast, the gradual increase in $Y_{(ND)}$ parameter can be caused by induction of CEF under FA stress, especially after 1 mM FA treatment. Hence, CEF plays a crucial role against photoinhibition by protecting PSI, while conversely, CEF can direct extra electron flow towards O_2 and NADPH to decrease ROS production. In addition, CEF also has the capacity to consume excessive reduced NADPH *via* NADPH dehydrogenase-dependent route (Shikanai 2007, 2014; Huang *et al.* 2011, Zhang *et al.* 2014). Furthermore, CEF plays an important role across the thylakoid membrane by producing a proton gradient *via* shifting electrons from PSI to PQ which is essential for PSII protection by dispersing excessive light energy (Munekage *et al.* 2002, Takahashi *et al.* 2009, Jahns and Holzwarth 2012). Our results showed the ascending trend of the CEF parameter in a FA concentration-dependent manner. Moreover, $Y_{(CEF)}/Y_{(II)}$ like NPQ was significantly higher in *Nr* as compared to WT following 1 mM FA after 72h. Therefore, CEF has also been involved in NPQ mechanisms as well as xanthophyll cycles depending upon the proton gradient (Zhang *et al.* 2014). These results suggest the photoprotective role of ET in FA-induced physiological changes. However, it was found that the ACC-induced NPQ and $Y_{(CEF)}/Y_{(II)}$ failed to protect the photosynthetic apparatus efficiently and facilitated the increasing formation of ROS (Borbély *et al.* 2019). Considering these observations and other results (Chen and Gallie 2015, Borbély *et al*. 2020), ET is suggested as a participant of CEF and NPQ, and therefore it plays a pivotal role in the regulation of photoprotection under the mycotoxin exposure.

Besides the effects of FA on the photosynthetic apparatus, the effects of FA on stomatal conductance and CO_2 assimilation were also investigated. Our results exhibited a significant reduction in the stomatal conductance and CO_2 assimilation in both 0.1 mM and 1 mM FA-treated plants as compared to controls after 24 and 72 h, respectively. These findings coincide with the earlier studies with the strong stomatal closure and reduction in photosynthetic activity due to FA exposure (McElrone *et al.* 2003, Wu *et al.* 2008, Poór and Tari 2012, Singh *et al.* 2017). However, changes in the size of stomatal aperture are in a close relation with the water-use efficiency and water uptake (Romero-Aranda *et al.* 2001). Therefore, FA exposure caused stomatal closure after 24 h in both tomato genotypes influencing water uptake by plants. Although, *Nr* mutants showed basically a higher stomatal conductance and assimilation and thus a higher biomass production (Nascimento *et al.* 2020), FA exposure rapidly reduced these parameters. The stomatal closure restricted CO_2 uptake in FA-treated plants and led to the suppression of photosynthetic activity (Sapko *et al.* 2011, Chen *et al.* 2015). In addition, it was also reported that ET induced stomatal closure in several plant species (Desikan *et al.*

2006, Ceusters and Van de Poel 2018). Thus, the ET-induced decrease in stomatal conductance contributed to the ET-induced inhibition of the net CO_2 assimilation rate, which limited CO_2 diffusion to leaf mesophyll. It has also been reported that FA resulted in a decreased Chl content in the seedlings of watermelon (Wu *et al.* 2008), which contributed to the decreased photosynthetic activity. Furthermore, FA induced leaf wilting and necrosis (Wilhelmová *et al.* 2005, Singh *et al.* 2017). Similar tendencies were found in case of our results, 1 mM FA induced loss of Chl (*a*+*b*) and elevated EL from the leaves of tomato after 72 h. However, the decrease in Chl (*a*+*b*) was more pronounced in *Nr* leaves suggesting the role of ET in this process.

The degree of necrosis is dependent on the ROS-induced lipid peroxidation (Czarnocka and Karpiński 2018). Interestingly, the MDA content was significantly higher in *Nr* plants as compared to WT after 24 and 72 h under 1 mM FA treatments. These results showed that the lack of active ET signaling resulted in higher oxidative stress after FA exposure. Others found similar results in the case of abiotic stressors, where ET-receptor *Arabidopsis* mutants (*etr1-3*) deficient in ET signaling were more sensitive to excessive salt stress (Wang *et al.* 2009). At the same time, the electrolyte leakage increased in both WT and *Nr* leaves after the 72-h-long FA exposure as it was measured in the case of other *Fusarium* toxin such as FB1 (Asai *et al.* 2000, Plett *et al.* 2009). Thus, the ET-regulated defense reaction (*e.g.*, NPQ, CEF) could only delay but not inhibit the cell death progression under FA exposure.

In conclusion, several mycotoxins are responsible for the induction of cell death in plants but their effects on photosynthesis are less studied. FA is one of the potential toxins produced by pathogenic *Fusarium* species. In the current study, the ET-dependent effects of FA were investigated in leaves of intact tomato plants. FA induced a significant ET emission from leaves in a concentration- and time-dependent manner suggesting ET's role in the regulation of defense- and/or cell death mechanisms. Based on the measurement of photosynthetic activity, FA significantly reduced F_v/F_m as well as $Y_{(I)}$ and $Y_{(II)}$ which were accompanied with less efficient photoprotection in WT leaves. Although the treatment of tomato plants with both concentrations of FA decreased the effective quantum yield in both photosystems after 72 h, the photoprotective processes, such as NPQ in parallel with CEF were activated more effectively in the leaves of *Nr*. At the same time, the lipid peroxidation and the loss of Chl (*a*+*b*) were higher upon FA treatments in this tomato genotype. Conclusively, *Nr* tomato plants were more sensitive to FA phytotoxicity as compared to WT leaves, where inhibition of photosynthetic activity is an important step, suggesting the key role of ET in the activation of defense responses under the mycotoxin exposure.

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