Journal of Plant Physiology 277 (2022) 153793

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

The role of ethylene signalling in the regulation of salt stress response in mature tomato fruits: Metabolism of antioxidants and polyamines

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ARTICLE INFO

Keywords:
Antioxidants
Ethylene
Fruit
Never ripe
Polyamines
Tomato

ABSTRACT

Salt stress-induced ethylene (ET) can influence the defence responses of plants that can be dependent on plant organs. In this work, the effects of salt stress evoked by 75 mM NaCl treatment were measured in fruits of wild-type (WT) and ET receptor-mutant *Never ripe* (*Nr*) tomato. Salt stress reduced the weight and size of fruits both in WT and *Nr*, which proved to be more pronounced in mutants. In addition, significantly higher H_2O_2 levels and lipid peroxidation were measured after the salt treatment in *Nr* as compared to the untreated control than in WT. ET regulated the key antioxidant enzymes, especially ascorbate peroxidase (APX), in WT but in the mutant fruits the activity of APX did not change and the superoxide dismutase and catalase activities were downregulated compared to untreated controls after salt treatment contributing to a higher degree of oxidative stress in *Nr* fruits. The dependency of PA metabolism on the active ET signalling was investigated for the first time in fruits of *Nr* mutants under salt stress. 75 mM NaCl enhanced the accumulation of spermine in WT fruits, which was not observed in *Nr*, but levels of putrescine and spermidine were elevated by salt stress in these tissues. Moreover, the catabolism of PAs was much stronger under high salinity in *Nr* fruits contributing to higher oxidative stress, which was only partially alleviated by the increased total and reduced ascorbate and glutathione pool. We can conclude that ET-mediated signalling plays a crucial role in the regulation of salt-induced oxidative stress and PA levels in tomato fruits at the mature stage.

1. Introduction

Salt stress is one of the most significant problems in agriculture worldwide, which negatively affects plant growth and development as well as crop yield and fruit quality (Isayenkov and Maathuis, 2019; Van Zelm et al., 2020). Uptake of sodium by various transporters results in rapid osmotic stress in plants inducing disturbance in water homeostasis, inhibiting growth, and inducing stomatal closure. In the later phase, the ionic stress caused by sodium ion accumulation results in disturbance in the ionic homeostasis, promotes the release of potassium, inhibits various enzyme activities, decreases the efficiency of photosynthesis and generates oxidative stress in plants (Munns and Tester, 2008; Zhao et al., 2020). The successful tolerance to salinity is highly dependent on the sodium exclusion or vacuolar sequestration within the plant cells (Wu, 2018; Zhao et al., 2020). In addition, the synthesis of antioxidants and compatible solutes such as sugars, polyols or proline and the accumulation of chaperones and polyamines (PAs) are also crucial parts of the defence reactions of plants under salt stress (Yu et al., 2018; Chen et al., 2019; Arif et al., 2020; El Moukhtari et al., 2020). At the same time, these processes are highly dependent on various internal and external factors and show time- and tissue-dependent differences, thus many aspects of plant responses to salt stress have remained unanswered (Yang and Guo, 2018; El Moukhtari et al., 2020).

Activation of plant defence responses under salt stress is coordinated by several phytohormones among others by ethylene (ET) (Khan et al., 2017; Arif et al., 2020). The gaseous ET is synthesized from S-adenosylmethionine (SAM) in plants by the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), then the oxidation of ACC to ET is catalyzed by ACC oxidase (ACO) (Sun et al., 2017).

The tomato ripening mutant *Never ripe* (*Nr*) has a mutation in the ET-binding domain of the ethylene receptor NR. Thus the mutant receptor cannot bind ET and as a consequence, the ET signalling, which is dependent upon receptor inactivation by ligand binding, is blocked in the mutant plants (Zhong et al., 2008). *Nr* mutants show insensitivity to

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https://doi.org/10.1016/j.jplph.2022.153793
Received 12 May 2022; Received in revised form 28 July 2022; Accepted 10 August 2022
Available online 14 August 2022

ET in fruit ripening and in some other ET-controlled physiological processes such as triple response, senescence of petals or flower abscission. The *Nr* fruits are also impaired in colour change and softening (Lanahan et al., 1994). In ontrast to fruits, *Nr* mutants showed a growth enhancement in vegetative tissues and exhibited a higher number of leaves as compared to wild-type plants (Nascimento et al., 2021).

ET by concentration- and time-dependent manner can regulate salt stress tolerance or it can trigger programmed cell death in salt-stressed plants (Poór et al., 2013; Riyazuddin et al., 2020). The outcome of the physiological changes upon salt stress is highly dependent on the ET-modulated oxidative stress (Borbély et al., 2019, 2020). ET can enhance oxidative stress depending on the NaCl concentration (Poór et al., 2015), but also can modulate enzymatic and non-enzymatic antioxidants under salt stress (Khan et al., 2014).

Antioxidants regulate the detoxification of reactive oxygen species (ROS, e.g. hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$)) (Foyer and Noctor, 2009). In the Foyer-Halliwell-Asada cycle, the superoxide dismutase (SOD) enzyme catalyzes the conversion of $O_2^{\cdot-}$ to O_2 and H_2O_2, which can be further degraded by catalase (CAT) localised in the peroxisomes, chloroplasts or mitochondria or by the cytosolic or chloroplastic ascorbate peroxidase (APX), as well as by several other enzymes (e.g. guaiacol peroxidases, POD) (Mhamdi et al., 2010; Czarnocka and Karpiński, 2018). There are also non-enzymatic antioxidants, such as the ascorbate (AsA)/dehydroascorbate (DHA) and glutathione (GSH)/glutathione disulfide (GSSG) systems contributing to the moderation of high ROS levels (Foyer and Noctor, 2009). Elevated antioxidant levels are also biochemical indicators for salt stress tolerance in crops (Ashraf and Harris, 2004). However, the activation of enzymatic and non-enzymatic antioxidants shows various patterns under salt stress depending on the NaCl concentration, duration of salt stress as well as on plant species, genotypes, or organs (Abogadallah, 2010). The fine-tuning role of ET in this process is essential (Zhang et al., 2016). ET regulates antioxidant enzymes such as SOD and POD contributing to salt stress tolerance (Peng et al., 2014; Zhang et al., 2016), but many aspects of the action of ET in these processes have not been investigated yet in plants, especially in fruits.

In addition, ET can influence osmolyte (Sharma et al., 2019), proline (Iqbal et al., 2015; Wang et al., 2020) and PA levels contributing to salt stress tolerance (Quinet et al., 2010). At the same time, the knowledge about the relationship between ET and PAs is significant but the currently available data is contradictory (Pal and Janda, 2017; Takács et al., 2021). Moreover, the interaction between ET and PAs can be dependent on plant organs where the basic level of ET can be significantly different e.g. in leaf as compared to fruit.

PAs are essential polycationic molecules that can regulate growth and development and stress responses of plants as membrane stabilizers or free radical scavengers (Gupta et al., 2013; Alcázar et al., 2020). PAs can be synthesized from L-arginine or L-ornithine amino acids by arginine decarboxylase (ADC) or by ornithine decarboxylase (ODC), respectively, producing the diamine putrescine (Put), which can be a substrate for the biosynthesis of triamine spermidine (Spd) and tetramine spermine (Spm). Spd and Spm are produced by the addition of aminopropyl groups, transferred from decarboxylated SAM (dcSAM), which is synthesized from SAM by SAM decarboxylase (SAMDC) and thus connects PAs formation to the biosynthesis of ET. Spd and Spm synthesis is catalyzed by Spd synthase (SPDS) and Spm synthase (SPMS), respectively, but their levels are also highly dependent on the catabolic processes. The FAD-containing polyamine oxidases (PAOs) are able to convert Spm into Spd or Put (back conversion). In addition, the copper-dependent diamine oxidases (DAOs) show a high affinity for Put producing reactive aldehydes and hydrogen peroxide (H_2O_2) (Moschou et al., 2008; Yu et al., 2019). Based on the first investigations, salt stress increased ET emission but reduced Put level, while increased Spd and Spm contents contributing to salinity tolerance in lettuce (Zapata et al., 2003). Similar changes were found in ET and PA levels in rice leaves (Quinet et al., 2010) and in leaves of the salt tolerant *Solanum chilense* (Gharbi et al., 2016). In addition, changes in PA levels were accompanied by the increased activity of DAO and PAO in the leaves of salt-stressed rice (Quinet et al., 2010). Moreover, it was observed that ET is involved in salt stress acclimation through the regulation of H_2O_2 signalling, which was dependent on PA catabolism in maize (Freitas et al., 2018). However, the effects of ET on PA metabolism can be different in fruits. Salt stress reduced the fresh weight of tomato fruits and increased ET production influencing fruit firmness as well as it elevated both Put and Spd contents in fruits of salt-stressed plants (Botella et al., 2000). The investigation of the effects of active or inhibited ET signalling on PA metabolism in parallel with the antioxidant defence system in mature fruits could improve our understanding of salt stress resistance in plants and the possible effect of saline irrigation water in plant cultivation.

In this work, the effects of NaCl treatment on plant defence reactions mediated by antioxidants and PAs were investigated in the presence or absence of active ET signalling in the agriculturally important tomato fruits. Furthermore, ET-dependent PA catabolism after NaCl treatment was investigated in mature fruits for the first time using wild-type and ET receptor-mutant *Never ripe* tomato plants.

2. Materials and methods

2.1. Plant growth conditions

Wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated at 26 °C for 3 days under darkness (Seeds of homozygous *Nr* plants were a kind gift from Prof. Dr. G. Seymour, University of Nottingham) and healthy seedlings were transferred into perlit for additional 2 weeks. Later, tomato plants were grown according to Poór et al. (2011) in nutrient solution containing 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 0.5 mM KCl, 0.02 mM Fe(III)-EDTA, and micronutrients (1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 10 μM H_3BO_4), which was changed three times a week. Constant environmental conditions were provided to the plants during the whole experiments with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} (PPFD; F36W/GRO lamps, OSRAM SYLVANIA, Danvers, MA, USA), 12/12-h light/dark period, 24/22 °C of day/night temperatures and 55%–60% of relative humidity.

2.2. Treatments and basic measurements

Treatment with 75 mM NaCl was started at the flowering stage of tomato plants and it finished when fruits were in the red ripe stage. The concentration of NaCl was elevated gradually in the nutrient solution, in the first three days after the development of the first flowers and the NaCl concentration was increased from 25 mM to 75 mM preventing the plants from the loss of flower. Nutrient solution containing 75 mM NaCl was changed also 3 times per week during the experiments. Uniform and healthy tomato fruits at the mature stage were selected from at least six different plants. Firstly, fresh weight, the diameter of fruits and the thickness of the pericarp were determined using an analytical scale (Mettler-Toledo; Greifensee, Switzerland) and ruler (Botella et al., 2000), respectively. The experiments were repeated three times and every treatment was performed in six biological replicates. All chemicals used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Determination of H_2O_2 levels in tomato fruits at the mature stage

Levels of H_2O_2 were measured spectrophotometrically according to Takács et al. (2018). After homogenising 0.2 g of the pericarp tissue with 1 mL of trichloroacetic acid (TCA; 0.1%), samples were centrifuged (11, 500 g at 4 °C for 20 min). The supernatant was used in 0.25 mL volume together with 0.25 mL of 10 mM potassium phosphate buffer (pH 7.0)

and 0.5 mL of 1 M potassium iodide (KI) for the determination of H_2O_2. The absorbance of samples was determined spectrophotometrically at 390 nm (KONTRON, Milano, Italy) after 10-min-long incubation in the dark. Levels of H_2O_2 were calculated using a standard curve prepared from H_2O_2 stock solution.

2.4. Determination of lipid peroxidation in tomato fruits at the mature stage

100 mg of fruit pericarps were homogenised in 1 mL of 0.1% TCA and 0.1 mL of 4% butylated hydroxytoluene (BHT). Samples were centrifuged (11,500 g for 20 min at 4 °C) and the supernatant in 0.5 mL volume was added to 2 mL of 0.5% thiobarbituric acid (TBA) dissolved in 20% TCA. These mixtures were incubated at 98 °C for 30 min, then cooled on ice. The absorbance of samples was measured using a spectrophotometer (KONTRON, Milano, Italy) at 532 nm and at 600 nm. Lipid peroxidation was calculated based on the changes in the contents of malondialdehyde (MDA). MDA was quantified using the extinction coefficient of 155 mM^{-1} cm^{-1} based on Ederli et al. (1997).

Determination of the activities of antioxidant enzymes in tomato fruits at the mature stage 250 mg of pericarps were homogenised with 1 mL of ice-cold extraction buffer [100 mM phosphate buffer (pH 7.0) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w:v) polyvinyl-polypirrolidone (PVPP)]. Then samples were centrifuged (12,000 g for 20 min at 4 °C) and the supernatant was used for superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD) enzyme activity assays. The extraction for ascorbate peroxidase activity (APX) was performed in the presence of 1 mM ascorbate (AsA). The absorbance of samples was detected by spectrophotometer (KONTRON, Milano, Italy). SOD activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in the presence of riboflavin under light. One unit (U) means the amount of SOD causing 50% inhibition of NBT reduction under light. CAT activity was measured by detecting the consumption of H_2O_2 at 240 nm for 3 min at 25 °C ($\varepsilon_{240} = 39.4$ mM^{-1} cm^{-1}). One unit of CAT activity means the amount of enzyme needed to decompose 1 μmol H_2O_2 per min. POD activity was determined as the increase in absorbance because of the oxidation of guiacol. One unit corresponds to the amount of enzyme producing 1 μmol min^{-1} of oxidised guaiacol. APX activity was measured by the decrease in AsA amount at 290 nm for 3 min at 25 °C ($\varepsilon_{290} = 2.8$ mM^{-1} cm^{-1}). One unit of APX activity means the amount of enzyme needed to oxidize 1 μmol min^{-1} AsA (Tari et al., 2015). All enzyme activities were expressed as U mg^{-1} protein. Soluble protein concentration in samples was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.5. Determination of ascorbate and glutathione levels in tomato fruits at the mature stage

250 mg of pericarps were homogenised with 1 mL of 5% (w/v) TCA. Then samples were centrifuged (12,000 g for 20 min at 4 °C). To assay total AsA, 10 mM dithiothreitol (DTT) was added to the mixture and the excess DTT was eliminated by 0.5% (w/v) N-ethylmaleimide (NEM). AsA concentrations were determined in the mixture of 10% (w/v) TCA, 43% (w/v) H_3PO_4, 4% bipyridyl, 3% (w/v) $FeCl_3$ using spectrophotometer at 525 nm (KONTRON, Milano, Italy).

Glutathione levels were measured using an enzymatic assay containing 100 mM phosphate buffer (pH 7.5), 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 1 mM NADPH, 1 U of glutathione reductase and 20 μL of the supernatant in 1 mL volume. Glutathione was determined at 412 nm using spectrophotometer (KONTRON, Milano, Italy) (Tari et al., 2015).

2.6. Determination of free polyamine levels in tomato fruits at the mature stage

200 mg of fruit pericarps were homogenised in cold 5% (v/v) perchloric acid and then were kept on ice for 20 min. After the incubation, samples were centrifuged (10.000 g for 20 min at 4 °C) and 1 mL of the supernatant was added to 0.4 mL of 2 M NaOH. Then this mixture was vortexed and after the addition of 10 μL benzoyl chloride stored at 25 °C for 30 min. After the incubation, the benzoylated PA derivates were removed from the aqueous phase using 1.2 mL diethyl ether. The organic solvent phase was evaporated to dryness and 200 μL of acetonitrile was added to the residue. Samples were injected into JASCO high-performance liquid chromatography (HPLC) system coupled to an UV detector (JASCO HPLC system, Japan) and equipped with a reverse-phase column (4.6 mm × 250 mm, 5 μm, Apex octadecyl). The mobile phase consisted of a water/acetonitrile, 55/45 (v/v) mixture applied at a flow rate of 1.0 mL min^{-1} (Takács et al., 2016). For the determination of free PAs, standards of Put, Spd, and Spm (Sigma-Aldrich, St. Louis MO, USA) were applied in 1 mM concentration.

2.7. Determination of diamine oxidase and polyamine oxidase activities in tomato fruits at the mature stage

200 mg of fruit pericarps were ground under liquid nitrogen then 0.6 mL of extraction buffer [100 mM K phosphate buffer (pH 6.6) containing 0.2 M TRIS(hydroxymethyl)aminomethane (pH 8.0); 10% glycerol; 0.25% Triton X-100; 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01 mM leupeptin] was added to the fine powder of plant samples (Takács et al., 2016). This homogenate was incubated on ice for 20 min. Samples were centrifuged (7.000 g for 10 min at 4 °C) after the incubation and 0.15 mL of the supernatant was added to 0.6 mL of 100 mM potassium phosphate buffer (pH 6.6), 50 U of catalase in 50 μL volume, 50 μL of 2-aminobenzaldehyde (0.1%) and 150 μL of 20 mM Put for DAO or 150 μL of 20 mM Spd for PAO determination. Samples were carefully mixed and then incubated at 37 °C for 1.5 h. The reaction was stopped by adding 50 μL of 20% (w/v) trichloroacetic acid (TCA). After centrifugation (5000 g, 10 min), the formation of Δ^1-pyrroline was measured at 430 nm using spectrophotometer (KONTRON, Milano, Italy). The enzyme activity was expressed in nmol Δ^1-pyrroline min^{-1} g^{-1} FW ($\varepsilon_{430} = 1.86 \times 10^3$ mol^{-1} cm^{-1}).

2.8. Statistical analysis

Statistical analysis was performed using Sigma Plot 11 software (Systat Software Inc. Erkrath, Germany) where results were analysed by one-way ANOVA, with Duncan's multiple comparison test and differences were considered significant if $P \leq 0.05$. Reported data are means ± SE.

3. Results

3.1. The ET-dependent long-term effects of salt stress in fruit parameters of tomato

To detect the ET-dependent long-term effects of salt stress in fruits of tomato, WT and *Nr* plants were treated with 75 mM NaCl starting from the flowering to the red ripe stage of fruits. Salt stress significantly reduced the weight and diameter, as well as the thickness of the pericarp of mature fruits of WT tomato plants (Fig. 1). Fruits of *Nr* tomatoes were smaller based on the fruits weight and diameter compared to WT at the mature stage but 75 mM NaCl also reduced these parameters of *Nr* fruits (Fig. 1).

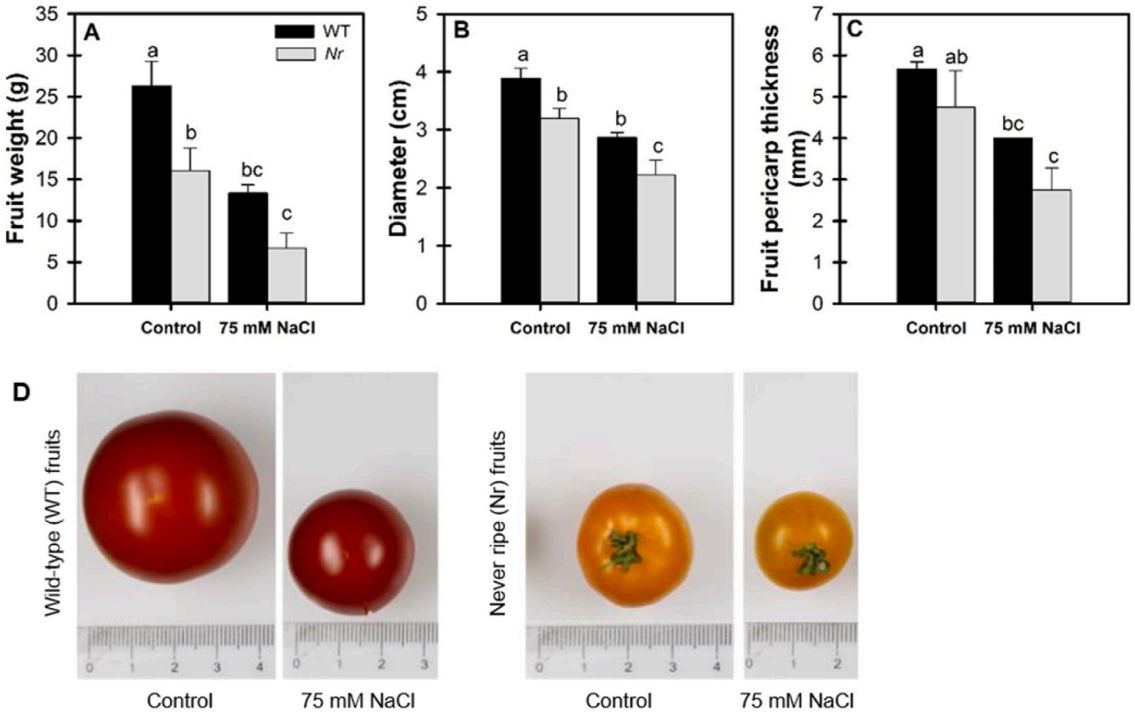

Fig. 1. Changes in the fruit weight (A), fruit diameter (B), and fruit pericarp thickness (C), as well as representative images (D) of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

3.2. Salt stress-induced oxidative stress is dependent on ET in fruits of tomato

Salt stress-induced oxidative stress in fruits was detected based on the measurements of H_2O_2 and MDA content at the mature stage. In contrast to WT, the basically lower H_2O_2 content was significantly elevated by 75 mM NaCl in *Nr*. These changes were not significant in WT fruits (Fig. 2A). The lipid peroxidation (expressed in MDA content) was lower in untreated controls of *Nr* as in WT fruits and it was significantly increased by salt stress in the fruits of both genotypes (Fig. 2B). However, the increase in lipid peroxidation was more pronounced in *Nr* than in WT fruits at the red ripe stage upon salt stress (Fig. 2B).

3.3. ET-mediated changes in the activity of key antioxidant enzymes in fruits of tomato under salt stress

The effect of key antioxidant enzymes on the degree of oxidative stress was also investigated after salt treatment in fruits of tomato at the mature stage. The activity of SOD, which catalyzes the dismutation of superoxide into molecular oxygen and H_2O_2, did not change significantly upon salt stress in the fruits of WT plants (Fig. 3A). In contrast to WT, SOD activity was basically higher in *Nr* fruits and it was decreased by 75 mM NaCl (Fig. 3A). The activity of CAT, which catalyzes the decomposition of H_2O_2 to water and oxygen, changed similarly to SOD, it was reduced by salinity in *Nr* but it did not change in WT fruits at the mature stage (Fig. 3B). The activities of the other two investigated H_2O_2 decomposing enzymes, APX and POD changed in the opposite direction as CAT activity in fruits under salt stress. 75 mM NaCl treatment elevated them in fruits of both tomato genotypes (Fig. 3C and D) but POD activity was significantly higher in *Nr* as compared to WT fruits (Fig. 3D).

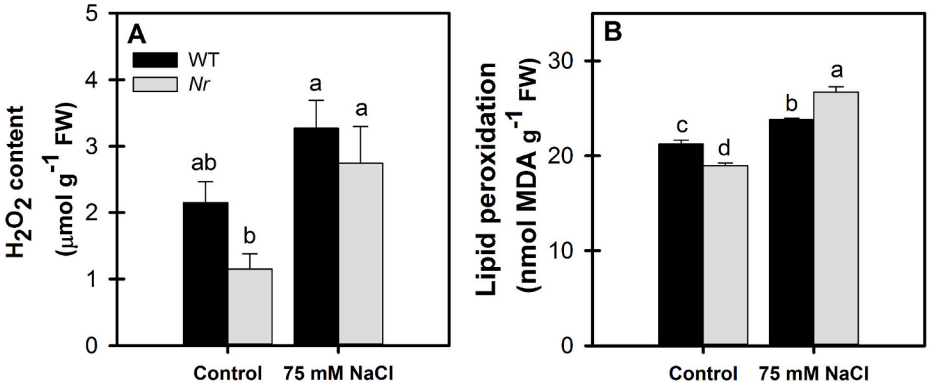

Fig. 2. Changes in the H_2O_2 content (A) and the degree of lipid peroxidation (B) in the fruits of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

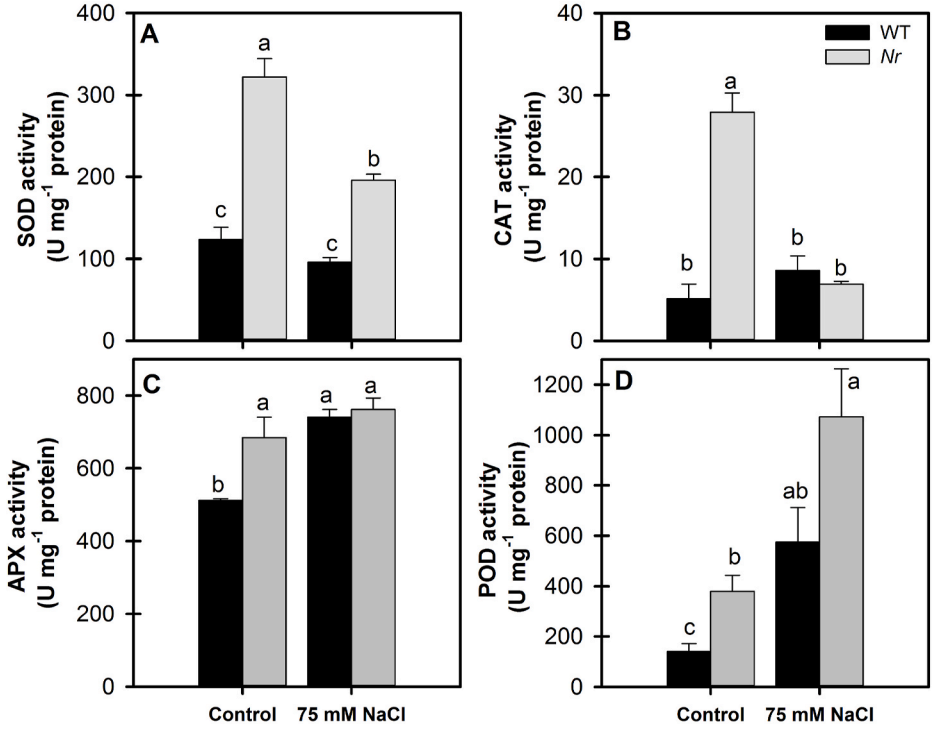

Fig. 3. Changes in the activity of superoxide dismutase (SOD; A), catalase (CAT; B), ascorbate peroxidase (APX; C) and guaiacol peroxidase (POD; D) in the fruits of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

3.4. ET-dependent changes in the non-enzymatic antioxidant levels of fruits under salt stress

The non-enzymatic antioxidants were also investigated in fruits of tomato under salt stress at the mature stage. Contents of total, reduced and oxidised AsA increased only slightly in fruits of WT plants after 75 mM NaCl and did not decrease significantly in *Nr* fruits (Fig. 4A, C, E). However, AsA and glutathione contents were basically higher in *Nr* fruits as compared to WT plants (Fig. 4). Salt stress resulted in a significant increase in total- and reduced-glutathione content of *Nr* fruits, while no significant changes were found in WT (Fig. 4B, C, D).

3.5. ET and PA interaction in fruits of tomato under salt stress

Besides the antioxidant defence, PAs were also investigated in fruits of tomato after salt exposure at the mature stage. The diamine Put content was basically higher in fruits of *Nr* plants as compared to WT, which was further elevated by 75 mM NaCl in *Nr* fruits but did not change in WT (Fig. 5A). The triamine Spd levels did not differ in the control fruits of the two tomato genotypes, however, the salt stress significantly increased it in *Nr* fruits (Fig. 5B). In contrast to Put content, the level of the tetramine Spm was basically higher in WT fruits as compared to *Nr* ones. Spm accumulated upon 75 mM in WT fruits but its concentration did not change in *Nr* and remained at a very low level at the mature stage (Fig. 5C).

3.6. ET-regulated changes in the PA catabolism upon salt stress

Activities of the key catabolic enzymes related to PAs were measured after salt stress in fruits of tomatoes at the mature stage. The activities of DAO and PAO were basically higher in fruits of *Nr* as compared to WT plants (Fig. 6). Salt stress elevated DAO activities in *Nr* fruits but the activity of the PA degrading enzymes did not change significantly in other cases (Fig. 6A and B).

4. Discussion

The role of ET in the defence responses of salt-stressed plants is highly dependent on various external and endogenous factors such as the concentration of salts in the soil, the duration of the stress, the developmental stage of the plants, as well as the organ type that is exposed to salt stress (Poór et al., 2015; Riyazuddin et al., 2020). In contrast to a large amount of available data about salt stress in roots and leaves, the dual role of ET in the developmental processes and defence responses of fruits under salt stress has remained less investigated. Both processes are highly dependent on the activation of antioxidant mechanisms (Dumas et al., 2003; Husain et al., 2020) and PA metabolism (Mattoo and Handa, 2008; Gao et al., 2021). In addition, most of the research works focus on the role of ET in the early, green stage of fruits where the characteristic features of ripened fruits have not been completely formed yet (Faurobert et al., 2007). At the same time, tomato fruits are usually harvested after the mature-green stage when fruits acquire suitable quality for transport and commerce (Wang et al., 2011). Therefore, it is important to evaluate the effect of salt stress on fruit defence responses at the mature, red-ripe stage. In addition, determination of the physiological and biochemical effects of saline irrigation water in mature fruits could serve economical aims, especially in the case of tomato (Incerti et al., 2007). In this work, the effects of moderate salinity (Albacete et al., 2009), 75 mM NaCl were investigated in the presence or absence of active ET signalling in WT and *Nr* tomato fruits.

ET plays a pivotal role in ripening and in the post-harvest physiology of tomatoes (Van de Poel et al., 2014). In addition, ET regulates plant defence responses against biotic and abiotic stress such as salt stress, which can influence fruit quality at harvest and during storage (Incerti et al., 2007). Based on our results, the ET insensitive *Nr* fruits showed high sensitivity to salt stress at the end of the fruit development as compared to WT plants, where 75 mM NaCl also reduced the weight and diameter of tomato fruits. It is well known that ET controls fruit development and ripening by interacting with auxins and abscisic acid. It shows a characteristic pattern in the induction of fruit development and

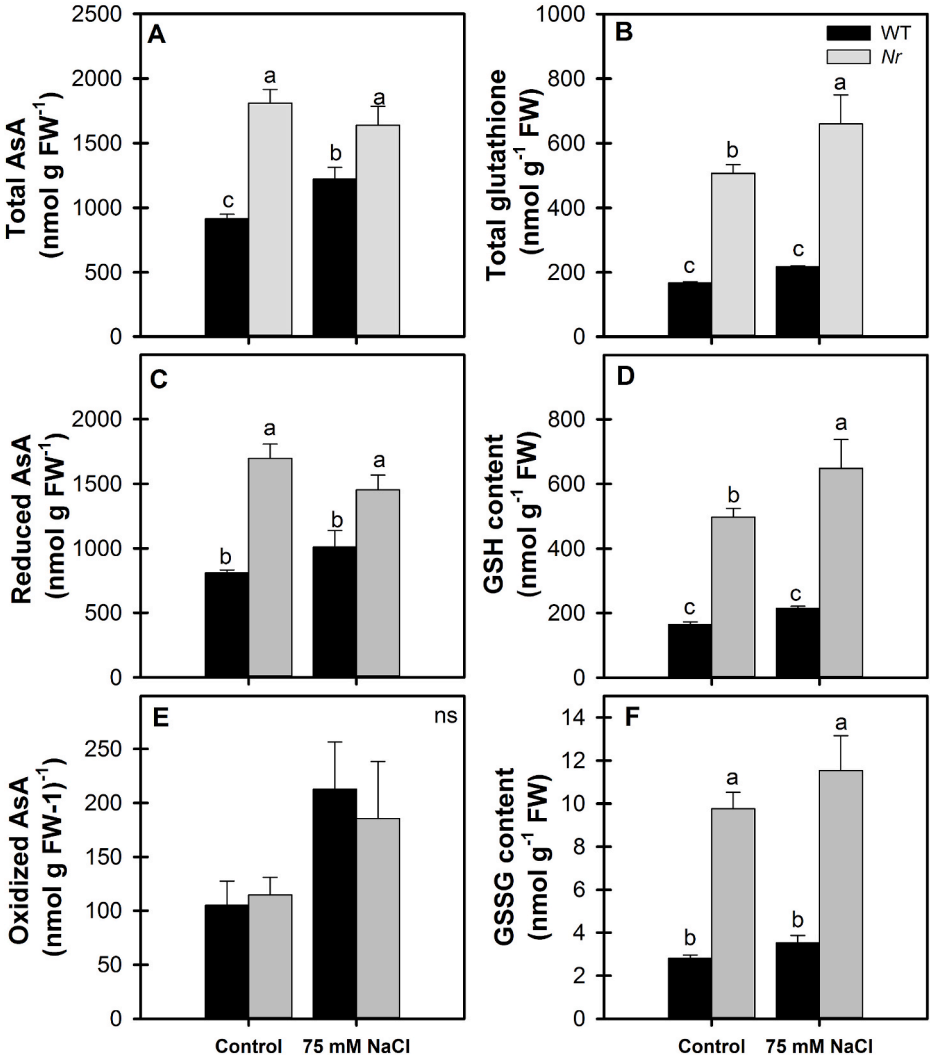

Fig. 4. Changes in the content of the total ascorbate (AsA; A), total glutathione (B), reduced AsA (C), reduced glutathione (GSH; D), oxidised AsA (E), and oxidised glutathione (GSSG; F) in the fruits of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

the late phase of ripening by the accumulation of sugars, change of colour and the cell wall composition, as well as flavour and aroma production in fruits (McAtee et al., 2013; Seymour et al., 2013). These processes were disturbed by salt stress from the starting of flowering. Our results showed that in the absence of active ET signalling, fruits are smaller as compared to WT after 75 mM NaCl treatment and the decrease was more significant in *Nr* as compared to WT (Decrease in fruit weight: 49% in WT; 58% in *Nr*) suggesting the key role of ET in the regulation of fruit development and defence under salt stress.

Plant defence responses to salt stress are dependent on various endogenous metabolites and antioxidants (Munns and Tester, 2008; Zhao et al., 2020), which show significant organ-specificity in tomato (Monteiro et al., 2011). It is well known that the activities and contents of antioxidants in tomato fruits show fluctuation according to the cropping season, cultivar, and environmental stress conditions such as light and temperature (Dumas et al., 2003; Zushi and Matsuzoe, 2009). At the same time, at the end of the ripening process, the scavenging of ROS and high antioxidant capacity is crucial from the aspect of food quality and tolerance of storage (Ali et al., 2019). We found that salt stress resulted in oxidative stress in fruits, which was manifested in H_2O_2 accumulation and enhanced lipid peroxidation in both tomato genotypes. Similar results, higher ROS levels and MDA contents were found earlier by Zushi et al. (2009), Monteiro et al. (2011), and Murshed et al. (2014) in fruits of salt-stressed tomatoes. At the same time, fruits of *Nr* plants were more sensitive to 75 mM NaCl and these fruits showed higher increase in H_2O_2 levels and in lipid peroxidation suggesting the key role of ET in the activation of defence against oxidative stress in fruits under moderate salt stress. Formerly, it was found that ET, depending on the salt concentration can induce both programmed cell death and tolerance to salt stress depending on ROS metabolism in roots of tomato (Poór et al., 2015). Here we found a higher degree of oxidative stress for the lack of active ET signalling under salt stress, which suggests that ET can activate various salt tolerance mechanisms via regulating ROS metabolism at this NaCl concentration in WT tomato fruits.

The degree of salt stress-generated oxidative stress and metabolism of ROS are dependent on the level and activities of enzymatic and non-enzymatic antioxidants, which can be controlled by ET (Riyazuddin et al., 2020). We found that the SOD and CAT activities were not changed significantly under salt stress in WT fruits at the red ripe stage but the activities of APX and POD were significantly increased in the pericarp contributing to scavenging of H_2O_2 and moderating the salt-induced oxidative stress in these fruits. Similar results were found in the case of change in SOD and CAT activities in salt-treated tomato fruits by 50 and 100 mM NaCl at the red ripe stage (Murshed et al., 2014). The activity of all investigated enzymatic antioxidants were basically higher in *Nr* fruits suggesting that the accumulation of ROS was controlled by higher activity of ROS scavenging systems in the absence of active ET signalling in control tissues. At the same time, activities of SOD and CAT were significantly decreased by 75 mM NaCl in *Nr* fruits but the activity of POD was significantly induced by salt treatments in the mutant

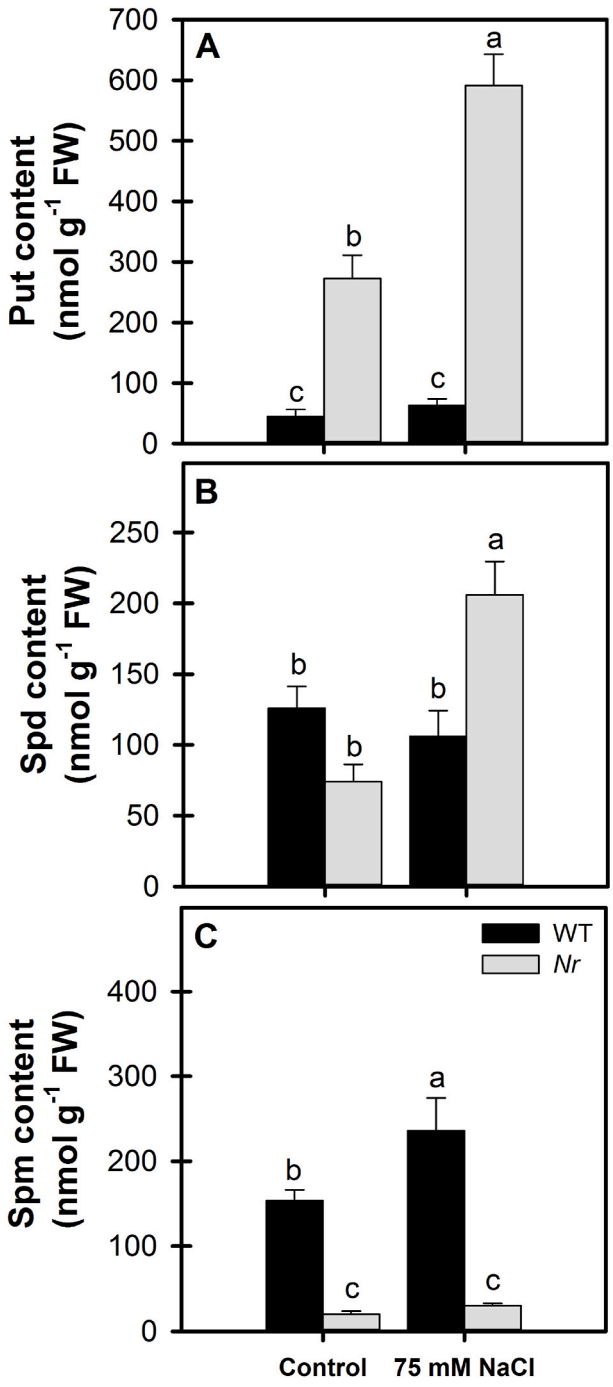

Fig. 5. Changes in the content of free putrescine (Put; A), spermidine (Spd; B), and spermine (Spm; C) in the fruits of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

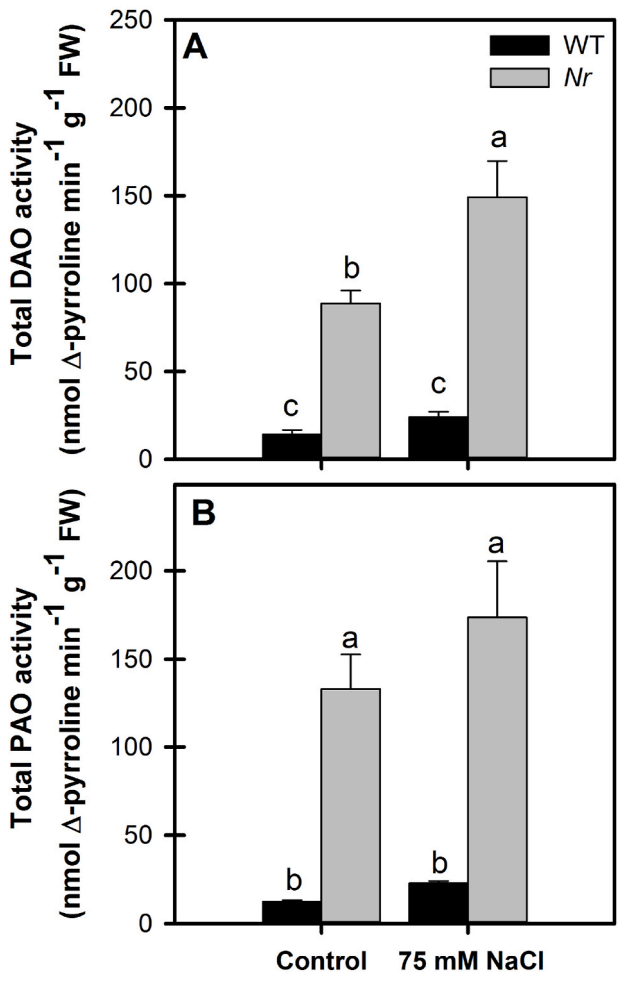

Fig. 6. Changes in the total activity of diamine oxidase (DAO; A) and polyamine oxidase (PAO; B) in the fruits of wild-type (black columns) and ET receptor mutant *Never ripe* (grey columns) tomato plants treated with 75 mM NaCl via the rooting medium after the start of flowering. Means ± SE, n = 6. Means marked with different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple comparison test.

tomatoes at the mature stage. Similar to our results, Monteiro et al. (2011) measured also reduced SOD and higher POD activity in *Nr* tomato fruits after 100 mM NaCl treatment. Although the basic levels of these antioxidant enzyme activities were significantly higher in *Nr* fruits, the salt stress-induced drop in SOD and CAT activities is very significant in the lack of active ET signalling as compared to WT. These results suggested that decreased activities of enzymatic antioxidants contributed to the harmful effects of NaCl-induced oxidative stress in mature fruits of *Nr* based on the changes in fruit weight and diameter.

These and other observations in salt-treated *Arabidopsis* (Peng et al., 2014) suggest that ET can regulate H_2O_2 accumulation by SOD and decomposition by APX and POD in mature fruits of salt-stressed tomato plants limiting the degree of salinity-induced oxidative stress.

The outcome of oxidative stress induced by salinity in fruits can also be dependent on the levels of non-enzymatic antioxidants such as AsA and glutathione, which can be regulated by ET (Khan et al., 2014). We found that the content of the oxidised AsA increased upon salt stress in WT fruits in parallel with the increasing APX activity. Other authors also measured higher AsA content and elevated APX activity under 100 mM NaCl treatment in fruits of tomato at the red ripe stage (Murshed et al., 2014). At the same time, AsA levels and APX activity were basically higher in *Nr* as compared to WT fruits, which did not change significantly upon salinity in the ET mutant at the mature stage. Therefore, the active ET signalling can regulate AsA levels in fruits both under normal conditions and salt stress.

It was observed that the fruit growth in *Nr* is highly reduced, which can be a consequence of several factors, such as the accumulation of other hormones during the cell division and elongation phase in the absence of active ET signalling (Sravankumar et al., 2018). Among the parameters affecting fruit size, the activity of enzymatic and non-enzymatic antioxidants is only one factor. Since enzymatic antioxidant activities as well as total and reduced AsA and GSH pools exceeded

those of WT in the control fruits of *Nr*, the tissues could maintain low H_2O_2 and MDA levels. When the plants were exposed to salt stress, the activity of H_2O_2-generating SOD remained at the level of untreated control, but H_2O_2-degrading enzymes were upregulated in WT leading to small increase in the H_2O_2 level of tissues, which was not significant. However, in *Nr* fruits the activities of both SOD and CAT were reduced and APX activity remained at control level under high salinity. Thus H_2O_2 accumulation and increased lipid peroxidation cannot be prevented by the activation of POD and by the enhancement of total and reduced GSH pool. These ET-regulated non-enzymatic antioxidants together with enzymatic antioxidants can reduce the harmful effects of the oxidative part of salt stress but on the other hand the control of salt-damaged macromolecules can also contribute to the development of salt tolerance in WT fruits. However, the lack of SOD and CAT activation under salt stress was only partially alleviated by the increased total and reduced ascorbate and glutathione pool in *Nr* plants.

Besides the ET-mediated control on the degree of oxidative stress, the mechanisms of salt tolerance in fruits can also be regulated by other components such as PAs, however, the data about the interaction between ET and PAs under stress conditions is often contradictory in vegetative tissues (Pal and Janda, 2017; Takács et al., 2021).

The levels of PAs are dependent on their biosynthesis, conjugation, catabolism, and transport (Tiburcio et al., 2014). These processes are scarcely investigated in fruits and knowledge about the role of ET in these processes is very limited. Earlier it was found that Put content was slightly higher in *Nr* leaves as compared to WT ones (Takács et al., 2021). Similar results were obtained in present work for Put content of fruits both under control and stress conditions. In *Nr* fruits, the accumulation of Put and the higher activity of PA catabolism can contribute to higher ROS accumulation at the mature stage (Moschou et al., 2008), which results in smaller fruits under salt stress. In contrast to ROS accumulation, the exogenous Put exposure could contribute to maintain the water and cation-anion balance in the different rice cultivars under salt stress (Quinet et al., 2010).

It is well-known that PAs can bind different anionic macromolecules (e.g. DNA, RNA, chromatin, and proteins) and protect them under various abiotic stress effects, such as salinity (Alcázar et al., 2020). Spm, which significantly accumulated in the leaves and roots of salt-stressed tomato plants, plays a crucial role in the development of salt tolerance (Szepesi et al., 2009; Takács et al., 2017). Our results proved that salt stress induced the accumulation of Spm in fruits of WT contributing to healthier fruits. Since Spm accumulation was inhibited in *Nr*, its protecting role was not effective in the mutants under high salinity. These results also suggest a tight connection between ET signalling and PA levels in tomato fruits under salt stress. Based on our results, we can conclude that intact ET signalling can contribute to Spm accumulation and Put conversion to higher PAs normally in WT tomato fruits under moderate salt stress serving the defence responses of plants, while in *Nr* fruits the high Put level and DAO activity can contribute to higher oxidative stress resulting in smaller fruits.

5. Conclusion

In conclusion, long-term, moderate salt stress caused by 75 mM NaCl during tomato fruits development has an impact on the defence reaction, on the activation of enzymatic and non-enzymatic antioxidants and PAs metabolism, which showed ET dependency. Salt stress resulted in smaller fruits at the mature stage based on the weight and diameter in *Nr* tomato as compared to WT ones confirming the crucial role of ET in the development and defence responses of tomato fruits. Since exposure to 75 mM NaCl resulted in a significantly higher increase in lipid peroxidation and H_2O_2 content in *Nr* as compared to their enhancement in WT fruits, this suggests that the active ET signalling contributes to the moderation of salt-stress-induced oxidative stress at this ripening stage. In addition, ET regulated the key antioxidant enzymes in fruits of tomato at the mature stage under salt stress. The basically higher SOD and CAT activities significantly decreased whereas APX activity did not change in *Nr* fruits upon salinity contributing to a higher rise of oxidative stress compared to respective untreated controls in the mutants, while APX and POD activities increased in WT plants. Besides the accumulation of AsA and GSH, the increased level of Spm is also an integral part of the effective salt stress tolerance mechanisms of matured tomato fruits. Significant Spm accumulation was observed in WT but it remained at a very low level and did not change in *Nr* fruits while Put and Spd increased after the salt treatment. Additionally, DAO activity, which participates in the Put degradation and H_2O_2 accumulation, was elevated in *Nr* fruits, contributing to oxidative stress and to the development of smaller fruits. These results indicate that the ET-mediated signalling associated with NR receptor plays a crucial role in the regulation of salt-induced oxidative stress and PA metabolism in tomato fruits at the mature stage.

Funding

This work was supported by the grants from the National Research, Development and Innovation Office of Hungary—NKFIH (NKFIH FK 124871 and 138867) and by the UNKP-21-5-SZTE New National Excellence Program of the Ministry of Human Capacities and the University of Szeged Open Access Fund (5826). Péter Poór was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

CRediT authorship contribution statement

Zoltán Takács: Investigation, Writing – original draft, Writing – review & editing. **Zalán Czékus:** Investigation, Writing – review & editing. **Irma Tari:** Writing – review & editing. **Péter Poór:** Conceptualization, Writing – original draft, Writing – review & editing.

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.

Acknowledgments

We are grateful to Bécs Attiláné for her professional assistance.

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