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Mechanical wounding of leaf midrib and lamina elicits differential biochemical response and mitigates salinity induced damage in tomato

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Abstract

The objective of the study was to evaluate biochemical response to two different types of wounding damage in leaves of tomato (*Lycopersicon esculentum* Mill.) and also to investigate the influence of pre-wounding on subsequent salt stress exposure. Wounding experiment was performed by small punctures either on the midrib or leaf lamina. Results showed that damage by wounding elicited a rapid increase in H_2O_2 levels within the first few hours of wound stress. H_2O_2 levels, total phenolic and flavonoid levels were significantly higher in midrib damage than either the lamina damage or control conditions. Wounding pre-treatment reduced the toxic effects of NaCl stress in plants. Alleviation of salt induced damage was greater in midrib cuts through the stabilization of relative water content and also an increase in antioxidant scavenging activity. These results confirm that wounding pre-treatment induced cross-tolerance to salinity stress in tomato plants. It is suggested that an early and significantly elevated generation of H_2O_2 with local midrib injury could induce a priming response systemically, thereby providing protection to the subsequent salt stress injury.

Key words: Artificial wounding, mechanical stress, flavonoid, phenol, antioxidant, reactive oxygen species, *Lycopersicon esculentum* Mill.

Introduction

Plants are often exposed to a variety of stress factors including a broad range of biotic or abiotic stressors. Abiotic factors include soil salinity, drought, heat, cold and UV while biotic factors include insects, fungi, and bacteria. Among these stress factors, a major challenge faced by plants is Mechanical Induced Stress (MIS), a term used for physical injury to plants caused by various mechanical stimuli like wind, water movement, attack by herbivore insects and even agricultural practices (Zhang *et al.,* 2013). Plants being sessile do not possess the inherent ability to move away from environmental stress sources. Therefore, plants have developed structural barriers (trichomes, waxy cuticles, thorns, spines and lignification) and chemical deterrents (plant secondary metabolites) to deal with extreme environmental insults (War *et al.,* 2012). Plants also often mediate a wide range of intricate defense responses that are both local (the site of injury) as well as systemic in nature (at the neighboring site) to counter damage to the leaf tissues (Leon *et al.,* 2001; War *et al.,* 2012). Si *et al.* (2018) for instance, demonstrated that mechanical wounding trigger defense response not only in local leaves but also in neighbouring systemic young leaves.

In natural conditions, plant growth and yield are influenced by also a combination of biotic and abiotic stress factors. Climate change and global warming could also potentiate the incidence of these biotic and abiotic factors resulting in reduction in the crop yield (Mittler, 2006). Cross tolerance or cross protection is a term applied to a phenomenon, by which a plant's response to one kind of stress, results in resistance to another form of plant stress (Genoud and Metraux, 1999). Induction of wound response requires a complex signaling network comprising of many structurally different molecules (Leon *et al.,* 2001). It has been suggested that Systemic Wound Response (SWR) is one of the foremost mechanisms to tolerate multiple stress (Si *et al.,* 2018).

Soil salinity is a major abiotic stress factor that often exacerbates oxidative damage due to over production of Reactive Oxygen Species (ROS) causing oxidation of proteins, lipids and DNA (Gill and Tuteja, 2010). Plants have complex regulatory mechanisms to control and minimize the damage to combat the threat posed by the toxicity of ROS (Sewelam *et al.,* 2016). Plants tolerate different abiotic stress factors by accumulation of polyphenols, thereby providing protection during unfavorable conditions. Studying the accumulation of polyphenols in plants is hence a good indicator to predict the extent of abiotic stress tolerance (Tattini *et al.,* 2004). The quenching of ROS by flavonoids can be achieved by various ways including suppression of singlet O_2, inhibition of ROS generating enzymes like lipoxygenase and quenching of cascades of free radical reactions (Mierziak *et al.,* 2014). The location of flavonoids within the outer envelope membrane of chloroplast or in proximity to the ROS production site further confirms their protective role in biotic or abiotic stress (Brunetti *et al.,* 2013). In addition, the transport of respective flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and cell wall, make them more effective scavengers (Brunetti *et al.,* 2013). Incidentally, the plant surface, first point of contact with environment is therefore, enriched in secondary metabolites and provides the earliest defense mechanisms against infections/ herbivore attack in plants (Roda *et al.,* 2003).

Artificial injury in plants by using different tools (razor blade, forceps or hemostat) has been widely used to mimic wounding by herbivores in nature. These tools are used to study the damage caused by insects with distinct feeding behaviors those, that damage either the leaf lamina or midrib (Capiati *et al.,* 2006; Estrada and Niinemets, 2018). The specific type of insect and the distinct feeding behavior dictates the blend of defensive compounds synthesized on the leaf tissues (Walling, 2000). An immediate defense response to the pathogen onslaught in plants is the generation of H_2O_2, at the wounding site, termed as the oxidative burst. This limits the spread of pathogens by contributing to the hypersensitive response and consequently cell necrosis (Gill and Tuteja, 2010). Importantly, H_2O_2 is used for cross talk between different stress combinations as it is thought to mediate rapid systemic signalling and may therefore contribute to cross tolerance response in plants (Niu and Liao, 2016). However, the effect of short-term mechanical wounding on H_2O_2 content, total phenolic and flavonoid content and its subsequent effect on salt tolerance in plants has not been evaluated.

In this study: (1) we analyzed the effect of injury on either the leaf lamina or the midrib using hemostat and forceps respectively. It is hypothesized that the site of injury may dictate distinct biochemical response. In this communication, damage was inflicted by making small punctures rather than large scale disruption of leaf membrane. We reasoned that piercing /sucking insects with piercing mouth parts do not have to cause extensive leaf damage because they can also easily draw plant sap using needle like mouth parts. (2) we also compared the type of wounding pre-treatment with cross tolerance to salt stress injury.

Materials and methods

Experimental Site, Plant Material and Growth conditions: Seeds of tomato (*Lycopersicon esculentum* Mill.) belonging to variety *Arka meghali* was obtained from Indian Institute of Horticultural Research (IIHR, Bengaluru). Germinated seedlings were transplanted to fresh pots (one plant per pot) in green house (12°56‘7” N, 77°35‘3” E) and maintained in moist soil under natural light condition (14 h light / 10 h of dark) at 27-28°C. Relative humidity was maintained around 61-70 % in the green house. All the experiments were conducted between 10.00 am – 2.00 pm to avoid early and late diurnal response.

Application of artificial injury: Wounding experiment was performed to mimic the attack of sap sucking and herbivorous insects that damage either the leaves or stem in the field. Wounding in leaf lamina was performed on 4 weeks-old plants by puncturing the apical compound leaf on the lamina surface using Hemostat (about 6 punctures with 1 cm gap) leaving the midrib untouched (Si *et al.,* 2018). On the other hand, wounding of the midrib was conducted by puncturing with forceps (about 6 punctures with 1 cm gap) along the mid rid of the leaf (Reymond *et al.,* 2000). Leaves samples were collected for biochemical assays at the time interval of 4 h, 24 h and 48 h after wounding stress. For all these stress treatments, plants that were left undamaged, served as untreated controls (referred as controls).

Qualitative detection of H_2O_2

DAB (3, 3’-diaminobenzidine) stain preparation and staining: The qualitative determination of H_2O_2 was performed by DAB staining (Daudi *et al.,* 2012). To prepare the DAB stain, 50 mg of DAB and 45 mL of sterile H_2O was added to a 50 mL falcon tube. The light brownish colour solution was dissolved using a magnetic stirrer and the pH was maintained at 3.0 with 0.2 M HCl. 25 µL of Tween 20 and 2500 µl of 200 mM Na_2HPO4 was then added. The staining solution was stored in an eppendorf tube. The leaves of 4-weeks-old tomato plants were removed from different treatments within 4 h of artificial injury. Thereafter, they were placed in clean centrifuge tubes (2 to 3 leaves per tube). Freshly prepared solution was added to ensure that the leaf remained immersed in the staining solution. The tubes were covered in order to protect them from light exposure. The centrifuge tubes were then placed in a standard laboratory shaker for 4-5 h at 80-100 rpm. After keeping for overnight incubation, removal of chlorophyll was done by immersing in a bleaching solution (acetic acid: glycerol: ethanol = 1:1:3). Then the tubes were carefully placed in a boiling water bath (~90-95°C setting in a water bath) for 15 min. Leaves were visualized for brown precipitates. Photographs of leaves were taken on a plain white background under uniform lighting.

Quantitative determination of H_2O_2: Quantitative determination of H_2O_2 was estimated according to the methodology described by Zhou *et al.* (2006). Leaves samples were collected within 4 h of stress application. Leaf samples were extracted by homogenizing the tissue in 5 % TCA and 0.1 g of activated charcoal. The homogenate was centrifuged at 10000 rpm (4°C) for 10 min and stored for further analysis. The colorimetric reagent solution for H_2O_2 determination was prepared (50 mL) by mixing 10 mg of phenol, 10 mg of 4-aminoantipyrine and 150 U mg^{-1} horseradish peroxidase in 100 mM acetic buffer with pH 5.6. For estimating H_2O_2, 1 mL of sample supernatant was added to 1mL of reagent solution, and absorbance was recorded at 505 nm using UV-Vis spectrophotometer (Shimadzu Inc, Japan). Relative H_2O_2 levels were estimated using a standard graph of concentrations ranging from 50 µmol to 300 µmol H_2O_2.

Determination of Relative Water Content (RWC): RWC was conducted as per the method described in Rampino *et al.* (2006). Whole leaves (0.5 g) were collected within 4 h of midrib and lamina leaf injury or controls, and fresh weight was recorded as soon as the leaves were detached. Leaves were submerged in distilled water for 4 h at room temperature and excess water was removed by quickly keeping the leaves on tissue paper for 30 seconds. The leaf turgid weight was then recorded. Finally total dry weight of the leaves was recorded after drying at 85°C for 24 h. Relative Water Content was then calculated using the formula:

RWC (%) = [(Fresh Weight – Dry Weight) / (Turgid Weight – Dry Weight)] x 100

Total Phenolic compound content (TPC): Total phenolic content in leaves of plants after application of stress were determined by Folin-Ciocalteu method as described by Marinova *et al.* (2005). About 1 gram of fresh leaf from both control and stressed plants was macerated in 25 mL of methanol for 24 h with occasional shaking. 250 µL of the extracted solution was mixed with 750 µL of methanol and 1mL of Folin-Ciocalteu reagent was added. After 5 min, 1mL of Na_2CO_3 (20 %) was mixed to the solution. After 30 min of incubation in dark, absorbance was measured at 765 nm using a UV-Vis Spectrophotometer (Shimadzu Inc, Japan) against a blank sample. Gallic Acid with different concentrations

was used for preparing the standard curve. Total phenol was calculated and was expressed as Gallic Acid Equivalents per gram of fresh weight (GAE g^{-1} of FW).

Total phenol content (w/w) = GAE x V x D x10^{-6} x100/W
GAE= Gallic acid equivalent (mg/ml), V= Total volume of sample (ml), D= Dilution factor, W= Sample weight (g)

Total flavonoid content (TFC): The aluminium chloride method was followed for the determination of the total flavonoid content of the sample based on the methodology described by Dewanto *et al.* (2002). Fresh leaf samples were used for the preparation of leaf extract for flavonoid analysis. About 1g of leaf sample was macerated with 70 % methanol and kept for 24 h with occasional shaking. The extracted sample solution (250 µL) was separately mixed with 75 µL of 5 % $NaNO_2$ followed by incubation for 5 min. After incubation, 10 % $AlCl_3$ (150 µL) was added and then mixed for about 5 min. To this, 500 µL of 1 M Sodium hydroxide was added and by using distilled water, total volume was made up to 2.5 mL. After the incubation period of 30 min, reading for absorbance was taken at 510 nm against a prepared blank using UV-Vis spectrophotometer (Shimadzu Inc, Japan). Quercetin with different concentrations was used for preparing standard curve for quantification of total flavonoids. All the analyses were performed in triplicates for every extract. The total flavonoid was calculated and was expressed as quercetin equivalent (QE g^{-1} of sample).

Total Flavonoid content (w/w) = QE x V x D x10^{-6} x100/W
QE= Quercetin equivalent (mg/ml), V= Total volume of sample (ml), D= Dilution factor, W= sample weight (g)

Determination of antioxidant activity: DPPH leaf disc assay was conducted at 4 h, 24 h and 48 h for those plants which were subjected to midrib and lamina wounding. For the bioassay, fresh leaf discs of every plant material were carried out by using a punch, making a disc with a diameter of 7 mm (Kasote *et al.,* 2018). An aliquot of 350 µL of methanolic solution of DPPH reagent (0.1mM) was then added to leaf discs from both control and treated. The reaction mixture was kept for incubation period of 10-20 min at dark. After the incubation period, the leaf discs were taken away from the wells and subsequently at 515 nm, the reagent solution from the well was being used to take the absorbance in the microplate reader. The DPPH scavenging rate was calculated according to the formula.

Inhibition (%) =Absorbance $_{blank}$ -Absorbance $_{sample}$ /Absorbance $_{blank}$ X100

Salt tolerance analysis: Salt tolerance assay was carried out as per the procedure described by Capiati *et al.* (2006). For this assay, 4 weeks old tomato plants cultivated in green house were subjected to lamina and midrib injury. After 3 h of mechanical injury, salt stress exposure was applied with 200 mM of NaCl (20 mL) to each set of 20 pots comprising of midrib injury, lamina injury and controls (unwounded). After 6 h of salt stress experiment, the leaves were collected for RWC analysis as previously described (Capiati *et al.,* 2006). The salt stress was repeated for another 2 times on alternate days. After 5 days of experiment the leaves were collected for total antioxidant analysis and total chlorophyll estimation.

Estimation of total chlorophyll content: Estimation of total chlorophyll was carried out by the method described by Arnon (1949). For this experiment, 0.2 g of sample was macerated thrice with 80 % acetone until complete discolouration. This extract was centrifuged at 10,000 rpm for 10 min. Then, the supernatant was collected and brought to 25 mL. Finally, 0.5 ml of the supernatant was taken and then diluted with 4.5 mL of acetone. The absorbance was measured at 663 and 645 nm using a UV-VIS spectrophotometer (Shimadzu Inc. Japan) and expressed as mg chlorophyll per gram of fresh weight.

Total chlorophyll= 20.2 (Absorbance 645) + 8.02 (Absorbance 663) x V/1000 x W

Statistical analysis: Statistical analysis for the results obtained was carried out by One-way ANOVA. Means of the significantly different treatments ($P < 0.05$) were separated using Tukey's test. The level of significance was fixed at $\alpha = 0.05$, analyzed using GraphPad Prism (version.8). Every experiment was carried out in triplicates.

Results

Effect of wounding stress on Relative Water Content (RWC): To study the relative difference in leaf damage caused by two contrasting types of artificial wounding, changes in leaf relative water content (RWC) was determined after 4 h of mechanical wounding. Both midrib and lamina injured plants showed lower RWC, compared with control plants. However, leaf lamina injury resulted in higher RWC (73.3 %) in comparison to midrib injury (62.1 %). As expected, highest RWC was observed in the leaves of control plants (88.3 %). Statistical analysis (ANOVA) resulted in significant difference between control and treated plants at $P < 0.0001$. The results confirmed that local mechanical wounding can induce significant leaf damage resulting in differences in RWC (Fig.1).

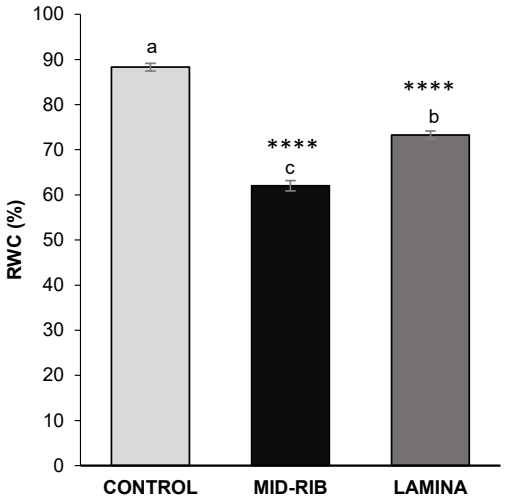

Fig. 1. Comparison of Relative water content (RWC %) observed in control, midrib and lamina injury in *Lycopersicon esculentum*. (Vertical bars indicate mean ± Standard error, where n=3. Mean marked with **** are significantly different from control at $P < 0.0001$). Vertical bars with different letters are significantly different according to Tukey's test ($P < 0.05$)

Effect of wounding stress on H_2O_2 content : To assess the role of mechanical wounding on H_2O_2 content, quantitative determination of H_2O_2 was performed through time intervals. Consistent with the observations on leaf damage with mechanical wounding, H_2O_2 levels were higher in wounded plants in comparison to control H_2O_2 levels at all time intervals. However, H_2O_2 content was highest at 4 h post wounding in midrib and lamina injury. Thereafter, H_2O_2 levels declined in both midrib and lamina wounding (24 h & 48 h) (Fig.2). H_2O_2 levels were higher in midrib injured plants (73.3±0.7 µmol g^{-1}) when compared

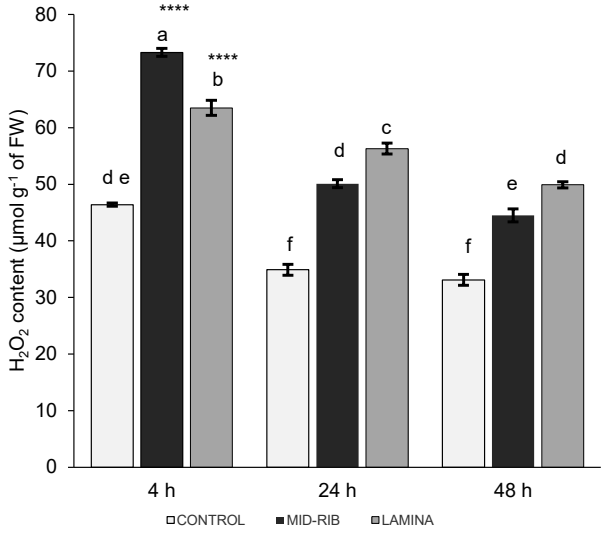

Fig. 2. H_2O_2 levels in µmol g^{-1} of FW observed in control, midrib and lamina injury in *Lycopersicon esculentum*. (Vertical bars indicate mean ± Standard error, where n=3. Mean marked with **** are significantly different from control at P <0.0001). Vertical bars with different letters are significantly different according to Tukey's test (P <0.05)

to lamina injury (63.48±1.3 µmol g^{-1}) at 4 h post wounding (P <0.0001). At 24 h and 48 h intervals, however lamina injury resulted in higher levels in comparison to midrib injury. As evident from Fig. 2, the difference between midrib and lamina H_2O_2 levels decreased through time-intervals. Histochemical detection of H_2O_2 by DAB (3,3'-diaminobenzidine) staining, revealed that localization of H_2O_2 was evident in the areas of wounded parts (site of injury) for both midrib and lamina injuries at 4 h post wounding. The major and secondary veins of the midrib injured leaf as well as the leaf lamina surface in lamina injury showed intense staining in local leaf (Fig.3a). Interestingly, apart from the local leaf, intense amount of H_2O_2 was also observed in the distal systemic leaf of the plants where injury was not imposed (Fig.3b).

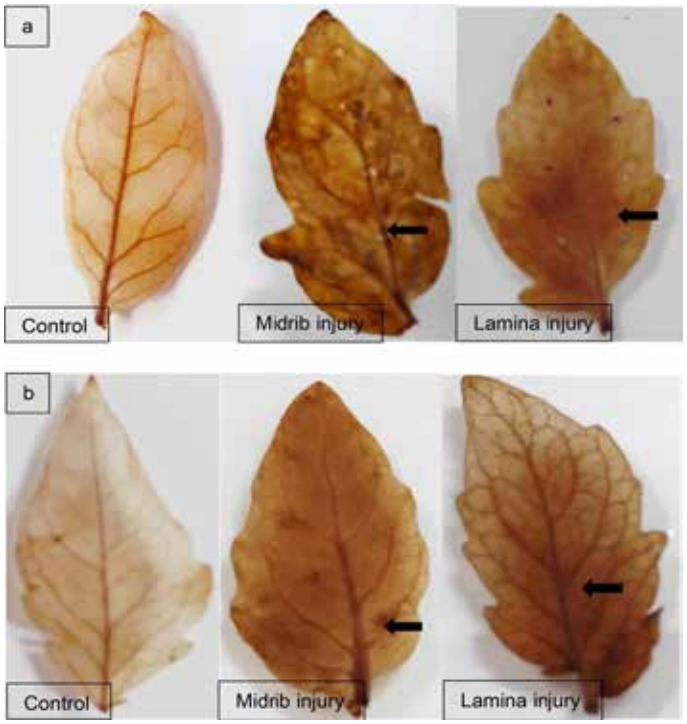

Fig. 3. Enhanced H_2O_2 production visualized as brownish precipitate (4h after stress) in local leaf (a) and systemic leaf (b) after DAB staining (Arrow indicates area of staining)

Influence of wounding injury on total phenol and flavonoid content (TPC, TFC) and antioxidant activity: Total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant activity were significantly altered by wounding stress (Fig.4). Midrib injury increased the total phenolic content at all time intervals tested in comparison with lamina injury. At 48 h, a significant increase of 65 % and 50 % in total phenolic content

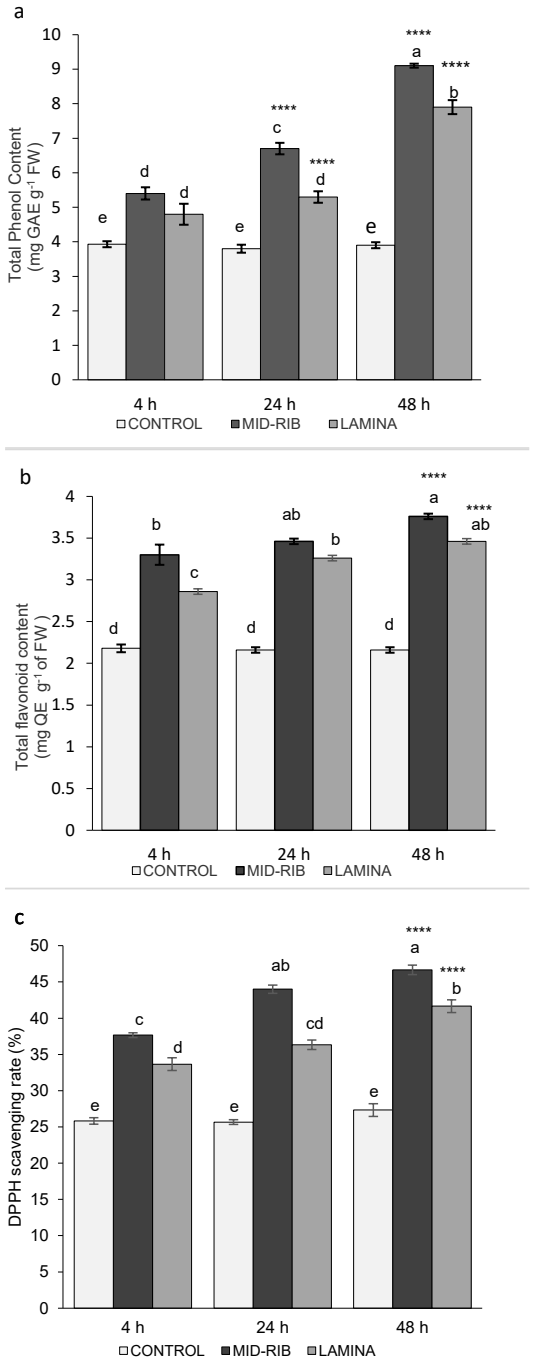

Fig. 4. Total phenol content expressed as mg of Gallic Acid Equivalent per gram of fresh weight (GAEg^{-1}FW) (a); total flavonoid content expressed as mg of Quercetin Equivalent per gram of fresh weight (QEg^{-1}FW) (b); percentage of DPPH scavenging activity at 4, 24 and 48 hours post wounding observed in control, midrib and lamina injury in *Lycopersicon esculentum*. (Vertical bars indicate mean ± Standard error, where n=3. Mean marked with **** are significantly different from control at P <0.0001). Vertical bars with different letters are significantly different according to Tukey's test (P <0.05)

was observed for midrib and lamina injury respectively in comparison to control plants, P <0.0001 (Fig. 4a). Results for leaf total flavonoid content were also consistent with the total phenolic content. At 48 h of treatment, total flavonoid content for midrib injury was 3.76 mg QE/g while the TFC for leaf lamina injury was 3.46 mg QE/g (Fig.4b). However, control total phenolic levels and flavonoid levels did not vary between these time points.

DPPH scavenging rate is a useful measure to study antioxidant activity of plant extracts. Plants with midrib and lamina injury showed significant increase in DPPH scavenging rate in comparison with the control plants. Leaves with midrib injury produced significantly higher level of DPPH scavenging rate (46.6 %) at 48 h compared with leaf lamina injury (P <0.0001) (Fig.4c).

Effect of wounding pre-treatment on salt tolerance: To analyze the effect of pre-treatment by wounding on cross-tolerance towards saline stress, plants which were initially stressed by artificial wounding (either midrib or lamina) were further subjected to NaCl stress treatment. Pre-treated plants showed tolerance to NaCl stress after 5 days of salt stress treatment in comparison to control plants (no pre-treatment imposed) (Fig.5). Plants that were wounded in midrib or lamina (wounding alone experiment) showed lower RWC compared to unwounded plants (refer to –NaCl; Fig.6). However, RWC was significantly higher in plants pre-treated by both types of wounding followed by the salt stress treatment. Between the two different types of wounding, plants treated with midrib injury followed by salt stress showed higher RWC (71.6 %) in comparison to plants pre-treated with lamina injury (65.6 %). A significant difference between midrib and lamina injury, post salt treatment was observed (refer to +NaCl; Fig.6).

Fig. 5. Photograph of tomato plants (*Lycopersicon esculentum* Mill.) after 5 days of salt stress treatment post wounding stress

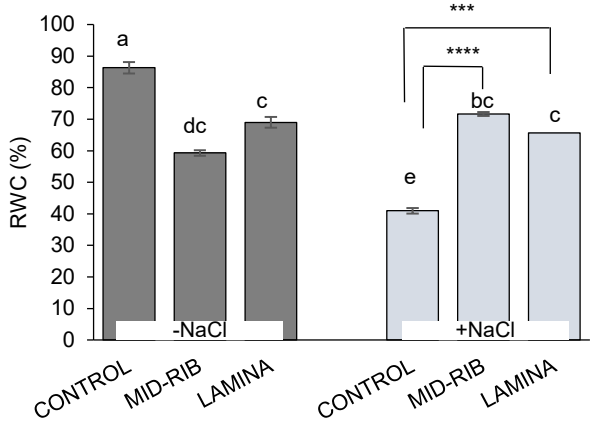

Fig. 6. RWC in control, midrib and lamina injury for both wounding alone (referred as –NaCl) and wounding + NaCl (referred as +NaCl) (b). For the wounding alone experiment, plants that were unwounded served as control. For the combined stress treatments, plants that were unwounded but treated with NaCl (200mM) served as controls. (Vertical bars indicate mean ± Standard error, where n=3. Mean marked with *** and **** are significantly different from control at P=0.0001 and P <0.0001 respectively).. Vertical bars with different letters are significantly different according to Tukey's test (P <0.05)

Analysis of antioxidant activity and chlorophyll content in pre-treated plants: Remarkable increase in DPPH scavenging rate was observed in plants with midrib injury (57 %) and lamina injury (49 %) in comparison to control plants (32.5 %), significant at P <0.0001 (Fig.7a). Also, plants that were pre-treated with wounding followed by salt stress exposure, also showed greater accumulation of total chlorophyll contents. Midrib injury resulted in higher total chlorophyll content (1.13mg) in comparison to control plants (0.9 mg) per gram of fresh weight, significant at P < 0.0001. However, differences in total chlorophyll content between midrib and lamina injury remained non-significant (Fig.7b).

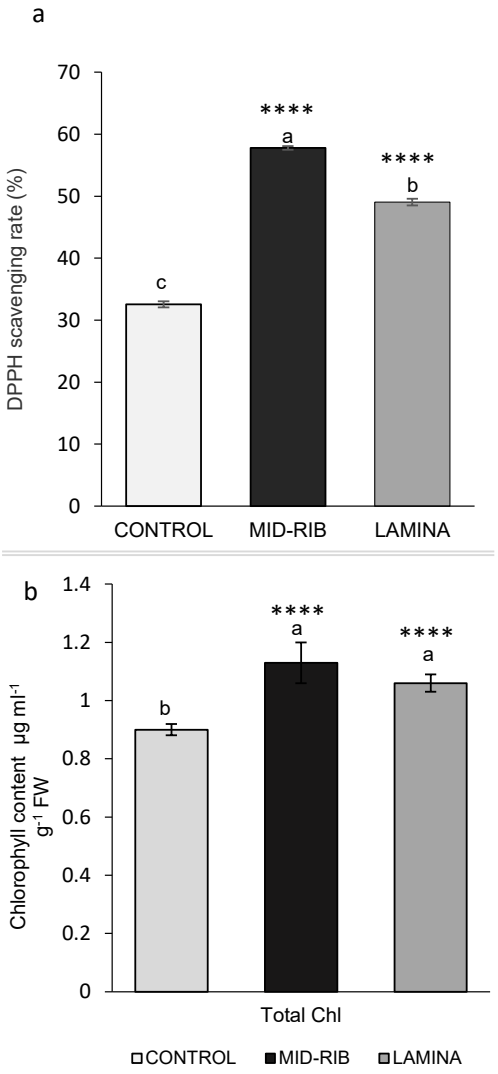

Fig.7 DPPH scavenging activity (a) and chlorophyll content expressed in µg $ml^{-1}g^{-1}$ FW (b) observed in control, midrib and lamina injury post salt stress treatment in *Lycopersicon esculentum*. (Vertical bars indicate mean ± Standard error, where n=3. Mean marked with **** are significantly different from control at P <0.0001). Vertical bars with different letters are significantly different according to Tukey's test (P <0.05)

Discussion

In the present study, it was shown that both the types of wounding resulted in considerable leaf damage in tomato. It has been reported that water deficit stress is a crucial component

of mechanical wounding (Raymond *et al.,* 2000). However, wound inflicted on leaf lamina and midrib showed differential biochemical response. Relative water content (RWC) is a measure of leaf's hydration status relative to the maximum water holding capacity at full turgidity status (Capiati *et al.,* 2006). Relative changes in water content in leaves reveal the extent of stress experienced by the plant as damaged tissues undergo drastic water loss and severe disorder in cellular structures (Leon *et al.,* 2001; Si *et al.,* 2018). Relative water content was found to be lower in both lamina and midrib injury in comparison to control plants, instructive of membrane damage in leaves. It has been hypothesized that injury to midrib could result in considerable decrease in water availability to leaf lamina downstream of the midrib injury. As the major veins are necessary for transport of water to leaves, injury to midrib leads to secondary water desiccation stress apart from wounding stress in leaves (Estrada and Niinemets, 2018). Indeed, results from this study showed that leaves exposed to local midrib injury showed greater damage as revealed by lower RWC in comparison to leaf lamina injury.

Several studies show that under both physiological water deficit conditions and mechanical wounding, levels of reactive oxygen species are elevated (Capiati *et al.,* 2006; Si *et al.,* 2018). In this study, the two different sites of mechanical wounding (midrib and lamina) allowed us to compare H_2O_2 levels between the two treatments. H_2O_2 analysis was quantitatively assessed in addition to the qualitative diaminobenzidine staining methods reported in a previous study. It has clearly been demonstrated that levels of H_2O_2 maximises at 4-6 h in response to wounding after which H_2O_2 levels decline (Orozco-Cardenas and Ryan, 1999). Quantitative analysis of H_2O_2 levels revealed that both types of mechanical wounding induced a quick response, reaching a maximum level at 4 h of stress, followed by a decline in H_2O_2 after 24 h and 48 h respectively. Therefore, our study corroborates the earlier finding that hydrogen peroxide levels progressively increased and then declines in the leaves of tomato with wounding. In addition, H_2O_2 levels in midrib injury were more than in lamina injury at 4 h. Estrada and Niinemets, (2018) observed that midrib injury generated higher biogenic volatile organic compound emissions (BVOCs) than lamina. The higher BVOC emissions observed in midrib might be indicative of elevated ROS formation in leaf veins (Estrada and Niinemets, 2018). Therefore, the observations from the present study imply that midrib injury generates higher levels of H_2O_2 in comparison to lamina due to damage to the vascular bundles.

Histochemical analysis using DAB procedure confirmed that midrib and lamina injury showed higher H_2O_2 levels in comparison to control leaves. Moreover, H_2O_2 accumulation was also shown in systemic leaves, distal from the site of injury. Localization of H_2O_2 in systemic leaves indicates the potential role of H_2O_2 in activating the systemic wound response to alert remote distal tissues of an imminent stress injury (Orozco-Cardenas and Ryan, 1999; Si *et al.,* 2018). Production of H_2O_2 due to oxidative burst upon wounding is considered as a universal stress signal for protection from a potential damage from mechanical injury by herbivory (Orozco-Cardenas and Ryan, 1999; Leon *et al.,* 2001). Taken together, the timing of response from the analysis of H_2O_2 accumulation (shown to be at 4-6 h) and signal transmission to distal tissues may be a crucial adaptive defense response to herbivory attack (Orozco-Cardenas and Ryan, 1999).

In this study, the decline in H_2O_2 generation with time (24 h and 48 h) occurred concomitantly with the enhanced DPPH scavenging rate indicative of enhanced antioxidant capacity to counter ROS levels in midrib and lamina injury. Crucially, both types of wounding resulted in higher TPC and TFC levels at all time points tested. Activation of the antioxidant defence mechanism is crucial in plants for protection against all forms of oxidative damage. Plants are well adapted to control and minimize the damage caused by ROS toxicity in cells (Sewelam *et al.,* 2016). For instance, progressive development in anthocyanin pigmentation has been reported when young leaf lamina of *Pseudowintera colorata* (Raoul) Dandy, were pierced with a fine beading needle (Gould *et al.,* 2002). Increased accumulation of phenol in 'Bianca' grapevine (*Vitis vinifera* L.) and certain phenylpropanoids such as stilbenes were demonstrated upon wounding stress (Chitarrini *et al.,* 2017; Vannozzi *et al.,* 2012). In addition, midrib injury caused higher accumulation of TPC and TFC in comparison to lamina injury at all time points analysed. Leaf midrib is comprised of densely packed cells of collenchyma and lignified xylem cells and membrane injury to these cells results in greater stress in comparison to lamina injury (Estrada and Niinemets, 2018). It can therefore be assumed that the observed elevated production of stress protective phenols and flavonoids could be indicative of increased stress in midrib injury. More importantly, induced defence responses occur within a short span to activate defense related signalling pathways and, serves to cater to the imposed nutritional needs for damage repair by regulating production of plant metabolites (Leon *et al.,* 2001).

We observed that pre-treatment of tomato plants with both the types of wounding stress provided tolerance from the deleterious impact of a subsequent exposure to salinity stress. There was no difference in stress phenotype between pre-treatment by midrib and lamina injury and subsequent NaCl stress exposure. This can be also observed in the analysis of chlorophyll content which did not differ significantly between midrib and lamina injury. NaCl stress has been shown to inhibit the production of photosynthetic pigments (Khan, 2003). Therefore, higher chlorophyll content with both the types of wounding pre-treatment suggests that pre-treatment ameliorates NaCl stress toxicity by leading to an increase in chlorophyll synthesis. However, plants treated to the midrib type of wounding stress prior to the salt stress treatment showed increased tolerance as revealed by an improvement in the stress performance indicators (RWC: indicator of plant water status and antioxidant activity: indicator of radical scavenging capacity). In plants, systemic wound response (SWR) is crucial for cross-tolerance to multiple stresses. It can be inferred from results presented here that an early and significantly elevated generation of H_2O_2 with local midrib injury could induce a priming response systemically. This in turn, stimulates the inherent antioxidant molecules, protecting the plant from subsequent exposure to another stress. Undeniably, many lines of evidence from studies conducted in other plants reveal that pre-treatment with H_2O_2 by exogenous application improve salt tolerance through a mechanism of induced resistance (Niu and Liao, 2016). Seed priming with H_2O_2 at lower levels, plays a significant protective role against oxidative stress during salinity (Ashfaque *et al.,* 2014), temperature (Wang *et al.,* 2014) and heavy metal toxicity (Bai *et al.,* 2011). The exogenous application of H_2O_2 in plants has been shown to mitigate the toxic effects of NaCl by enhancing ROS scavenging capacity and improving

photosynthetic performance (Hossain *et al.,* 2015; Khan *et al.,* 2018). Pre-treatment of seedlings with combined application of H_2O_2 and abiotic stress leads to renewal of redox-homeostasis and also alleviation of oxidative damage to macro molecules (Khan *et al.,* 2018).

It has been demonstrated that biotic and abiotic stresses stimulates cytosolic Ca^{2+} triggering the production of H_2O_2, which diffuses into surrounding cells as a messenger. Regulation of H_2O_2 in plants during stress is mediated by calmodulin (CaM), a ubiquitous calcium-binding protein (Niu and Liao, 2016). Importantly, it was shown that cross-tolerance in plants involves the participation of calmodulin (CaM) in shared responses to wounding and high salinity stress (Capiati *et al.,* 2006). An initial burst of H_2O_2 with wounding reported in the present study may therefore, suggest a crucial link interconnecting the response triggered by wounding and NaCl stress with the mechanism involving calmodulin reported earlier (Orozco-Cardenas and Ryan 1999; Capiati *et al.,* 2006). This is also consistent with a previous study confirming that wounding induced H_2O_2 generation primarily functions as signalling molecule which in turn, enhances the antioxidant system in mitigating oxidative stress during subsequent exposure to freezing stress (Si *et al.,* 2018). Additional molecular evidence is however, required to elucidate the pathway involving H_2O_2 in wounding induced salt stress protection in plants.

In conclusion, the results from the present investigation highlighted the crucial short term response exhibited by tomato plants after exposure to two different types of leaf injury. Furthermore, short-term pre-treatment with wounding stress showed positive effect on salt tolerance, providing strong evidence for systemic wound response possibly through H_2O_2 production by wounding. Thus, additional studies on the role of H_2O_2 in systemic wound response will help in contributing towards crop improvement by priming phenomenon.

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