

## Salicylic acid induces differential antioxidant response in spring maize under high temperature stress

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High temperature is one of the important stress factors that affect crops in tropical countries. Plants do evolve or adopt different mechanisms to overcome such stress for survival. It is an interesting subject and has attracted many researchers to work upon. Here, we studied the effect of salicylic acid (SA) on seedling growth and antioxidative defense system in two spring maize (*Zea mays* L.) genotypes viz., CML-32 (relatively heat tolerant) and LM-11 (relatively heat susceptible), under high temperature stress. High temperature induced greater reduction in dry biomass of LM-11 seedlings as compared to those of CML-32. There was a parallel increase in ascorbate peroxidase and glutathione reductase activities in the roots of CML-32 seedlings. However, the activities of catalase and superoxide dismutase decreased and the contents of H<sub>2</sub>O<sub>2</sub>, proline and malonaldehyde (MDA) increased in seedlings of both the genotypes. Application of SA (400 µM) led to increased dry biomass in heat stressed CML-32 seedlings. It improved the efficiency of Halliwell-Asada pathway in roots of CML-32 seedlings as was evidenced by the enhanced ascorbate peroxidase and glutathione reductase activities. The activities of catalase and superoxide dismutase increased in both the tissues of LM-11 seedlings, whereas in CML-32, it was only in shoots, after SA application. Peroxidase activity increased in SA treated seedlings of both the genotypes, though the increase was comparatively higher in CML-32. The contents of H<sub>2</sub>O<sub>2</sub> and MDA decreased and that of proline increased in SA treated seedlings of both the genotypes, under stress conditions. It may be concluded that SA induced differential antioxidant response by upregulating Halliwell-Asada pathway in roots and attaining high POX activity in both the tissues of CML-32 seedlings, under high temperature stress.

**Keywords:** Cereals, Corn, Halliwell-Asada pathway, Heat stress, *Zea mays*

Plants undergo various kinds of stresses, biotic and abiotic, viz., heat, cold, drought, pollution, salinity, pests and diseases, etc., which affect their growth and productivity. Over the time, plants continue to evolve and adopt strategies to overcome such challenges. Considerable research has gone into not only understanding the mechanism behind such acquired strengths but also to look for further scope to protect plants from such stresses<sup>1-6</sup>. In some cases stress factors are applied consciously to benefit crops<sup>7</sup>. In crops, each 1°C increase in temperature above the optimum results in reduced yield up to 17%<sup>8</sup>. Makus and co-workers<sup>9</sup> have reported reduction of ~80% grain yield under high temperature conditions in sweet maize. High temperature inhibits growth and leads to production of reactive oxygen species (ROS)<sup>10</sup>. The generation of ROS can be limited or scavenged by an antioxidant system comprising of low molecular weight antioxidant compounds and a

series of antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX), glutathione reductase (GR) and catalase (CAT)<sup>11</sup>. Antioxidant defense system is thus a component of stress adaptation and its strength is correlated with attainment of thermotolerance<sup>12</sup>. Under severe stress conditions, however, the antioxidant capacity may not be sufficient to minimize the harmful effects of oxidative injury. In this perspective, application of salicylic acid (SA) has been reported to mediate the acclimation of plants to environmental stresses<sup>13</sup>.

Spring maize (*Zea mays* L.) is an important cereal crop of Gramineae family which exhibits an important role in the diversification of the cropping system. Its cultivation in the unconventional spring season provides an opportunity to utilize the fields vacated by other crops e.g. potato and early harvested sugarcane. Besides, it has high yield potential<sup>14</sup> and also used in phytoremediation of heavy metal contaminated fields<sup>15</sup>. Due to these reasons, spring maize is fast attaining an unrivaled place in present

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day agriculture. However, the major abiotic factor which affects cultivation of spring maize is high temperature stress which affects its survival and grain yield<sup>16</sup>.

Salicylic acid or ortho-hydroxy benzoic acid has been recognized as a signal hormone for activation of plant defense responses against a number of abiotic stresses. It induced thermotolerance in mustard seedlings, increased chilling tolerance in maize, and played an important role in plant response to salt and osmotic stresses<sup>17-19</sup>. SA has been observed to play an essential role in preventing oxidative damage to plants by detoxifying ROS<sup>20</sup>. It alleviates stress injuries by increasing the activities of antioxidant enzymes<sup>21</sup>. Though SA is known to alleviate the adverse effects of abiotic stresses in plants, relatively little is known about their effect on high temperature tolerance in spring maize. In this study, we explored the potential of SA against high temperature stress by determining variations in (i) seedling growth; and (ii) antioxidative defense system of two spring maize (*Zea mays* L.) genotypes.

## Materials and Methods

### Plant material and experimental design

Seeds of *Zea mays* L. were surface sterilized by adding 2-3 drops of teepol, followed by 4-5 washings with distilled water. Disposable plastic cups (250 cm<sup>3</sup>) were filled with 220 cm<sup>3</sup> of its volume with soil having pH 8.0, electrical conductivity of 0.12 m mho cm<sup>-1</sup> and organic carbon content of 0.51%. Six seeds were sown in each cup and the cups were placed at 25 ± 1°C in the dark for 4 days. On 5<sup>th</sup> day of seedling growth (DSG), 1/3 of the cups were exposed to high temperature stress by transferring them to 40 ± 1°C. Seedlings in half of the remaining cups were sprayed with different concentrations of salicylic acid (10-800 µM) and were kept at normal temperature (25 ± 1°C) for 2 h and then transferred to 40 ± 1°C.

### Growth analysis

Growth was measured in terms of length, fresh and dry biomass and moisture content of shoots and roots on 8<sup>th</sup> DSG.

### Determination of antioxidant enzymes and non-enzymatic antioxidants

Activities of antioxidant enzymes and non-enzymatic antioxidants were determined in the roots and shoots on 6<sup>th</sup> and 8<sup>th</sup> DSG stages. Catalase (CAT, EC 1.11.1.6)<sup>22</sup>, ascorbate peroxidase (APX, EC

1.11.1.1)<sup>23</sup>, glutathione reductase (GR, EC 1.6.4.2)<sup>24</sup>, peroxidase (POX, EC 1.11.1.7)<sup>25</sup> and superoxide dismutase (SOD, EC 1.15.1.1)<sup>26</sup> were extracted and analyzed. Contents of ascorbate<sup>27</sup> and proline<sup>28</sup> were also determined.

### Determination of hydrogen peroxide and malondialdehyde content

H<sub>2</sub>O<sub>2</sub> and MDA contents<sup>29</sup> were determined in the roots and shoots of seedlings of both the genotypes at 6 and 8 DSG stages.

### Statistical analysis

Growth parameters have been presented as mean ± SD of 3 replicates. It was statistically analyzed by applying ANOVA followed by post hoc analysis, the Fisher's LSD (Least significance difference). Data for other biochemical parameters were analyzed by applying two-way ANOVA with interaction followed by LSD.

## Results and Discussion

### Effect of SA on seedling growth

High temperature stress led to a significant decrease in seedling growth of the genotypes (Table 1, Figs. 1 and 2). Root and shoot lengths as well as % moisture content of both the genotypes decreased significantly as compared to their respective controls (Table 1, Fig.1). High temperature caused a more depreciating effect on the growth of LM-11 seedlings compared to that of CML-32 as evident by the % reduction in shoot and root dry biomass of the genotypes (Table 1). Shoot dry wt. of CML-32 showed 6% reduction while it was 23% in LM-11 seedlings. Similarly, in root dry wt., CML-32

Table 1—Effect of salicylic acid (SA) on % increase/decrease in growth parameters of seedlings under high temperature stress

Growth parameters	Heat stress*		SA (400 µM) + heat stress**	
	CML-32	LM-11	CML-32	LM-11
Shoot length	-44.1	-49.5	-29.2 <sup>a</sup>	-33.7 <sup>b</sup>
Shoot fresh wt.	-36.9	-39.0	-5.3 <sup>a</sup>	-7.5 <sup>b</sup>
Shoot dry wt.	-6.4	-22.6	+12.7 <sup>a</sup>	+1.8 <sup>b</sup>
Root length	-32.8	-29.5	-22.8 <sup>a</sup>	-18.2 <sup>b</sup>
Root fresh wt.	-56.2	-68.2	-2.4 <sup>a</sup>	-0.2 <sup>b</sup>
Root dry wt.	-27.7	-51.3	+28.5 <sup>a</sup>	+36.7 <sup>b</sup>

\* and \*\* represent the data in comparison with salicylic acid unsprayed, non-stressed controls.

<sup>a</sup>Differences significant in comparison with high temperature stressed, unsprayed CML-32; and <sup>b</sup>LM-11 seedlings at  $P < 0.01$  (Student's t-test).

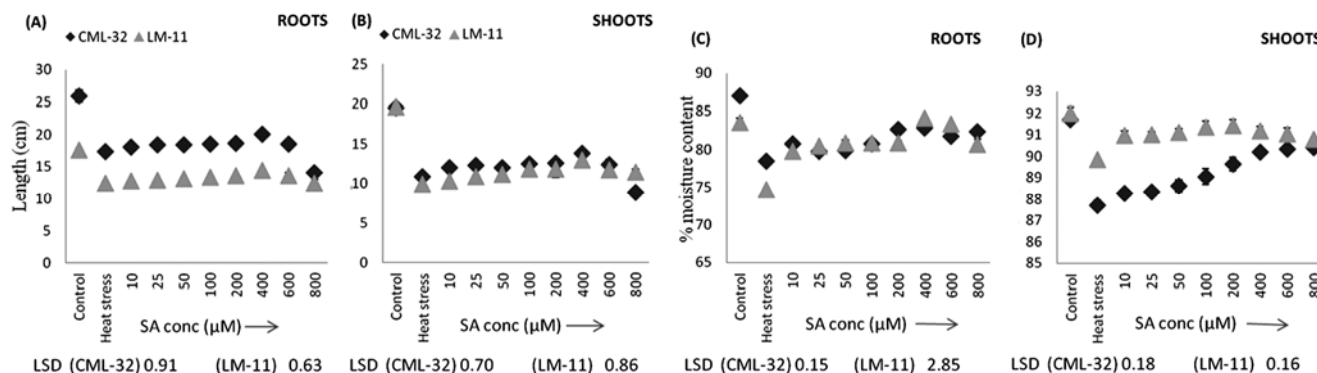


Fig. 1—Effect of different concentrations of salicylic acid on lengths (A and B); and moisture contents (C and D) of roots and shoots of CML-32 and LM-11 seedlings under high temperature stress. [LSD, least significant difference at 5% probability level].

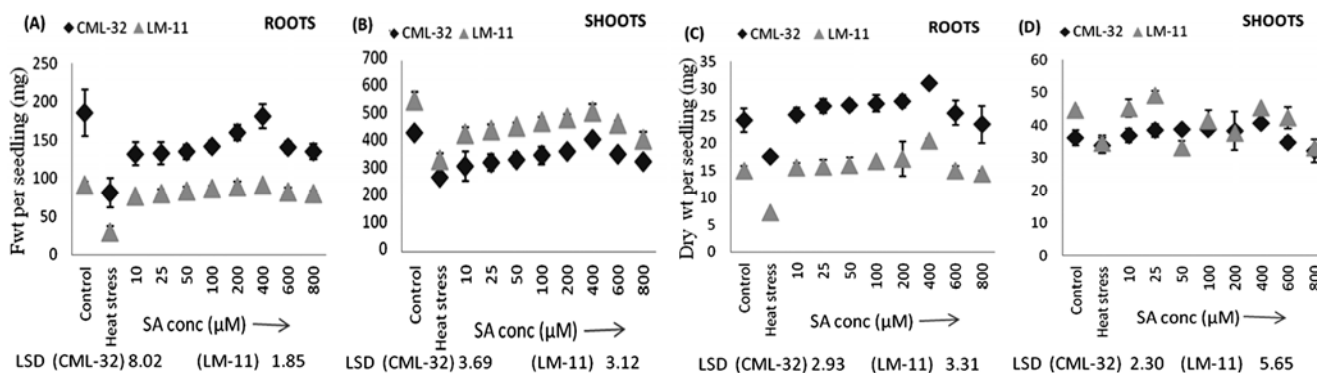


Fig. 2—Effect of different concentrations of salicylic acid on fresh (A and B); and dry biomass (C and D) of roots and shoots of CML-32 and LM-11 seedlings under high temperature stress. [LSD, least significant difference at 5% probability level]

recorded 28% reduction whereas LM-11 seedlings showed 51% decrease (Table 1, Fig. 2). Foliar application of different concentrations of salicylic acid (SA) viz., 10, 25, 50, 100, 200, 400 and 600 μM improved seedling growth under high temperature stress in both the genotypes as compared to the untreated high temperature exposed seedlings (Table 1, Figs. 1 and 2). However, 800 μM SA was shown to have inhibitory effect on seedling growth of both the genotypes (Figs. 1 and 2). Lower concentrations of SA had been observed to exhibit stimulatory effects<sup>30</sup> while inhibitory effects were reported with higher concentrations<sup>31</sup>. SA (400 μM) was found to be optimum in alleviating the harmful effects of high temperature by recuperating seedling growth, under stress conditions (Table 1, Figs. 1 and 2). It increased root/shoot lengths, fresh and dry wt. and % moisture content of seedlings of both the genotypes in comparison with untreated high temperature stressed as well as high temperature stressed + other concentrations of SA (Table 1, Figs. 1 and 2). The dry biomass of shoots of SA treated heat stressed

CML-32 and LM-11 seedlings increased by 12.7 and 1.8% while that of roots enhanced by 28.5 and 36.7%, respectively. The increased moisture content of SA treated high temperature stressed seedlings over those of untreated ones (Table 1, Fig. 1) suggests increased water uptake by the roots of these seedlings. Accumulation of inorganic or organic osmolytes is known to increase water uptake in SA treated plants<sup>32</sup>. The increased biomass of SA treated maize seedlings under high temperature stress (Table 1, Fig. 2) appeared to be related with the induction of antioxidant response and protective role of membranes<sup>33</sup>.

#### Effect of SA on antioxidative defense system

Under high temperature stress, APX and GR activities were significantly higher in the roots of CML-32 seedlings as compared to their controls (Fig. 3 A and C). However, the roots of LM-11 seedlings showed decreased APX activity (at 8 DSG stage) but unaffected GR activity, under stress conditions (Fig. 3 B and D). The parallel increase in APX and GR activities in the roots of CML-32

seedlings (Fig. 3A and C) showed that the ascorbate-glutathione cycle was more active in the relatively tolerant genotype as compared to the sensitive one, LM-11, under high temperature stress. The higher ascorbic acid content at 8 DSG stage in the roots of CML-32 seedlings, under stress conditions (Fig. 4A) also supported this inference. Foliar application of SA further improved the efficiency of Halliwell-Asada pathway by increasing the activities of APX and GR in the roots of CML-32 seedlings (Fig. 3), which led to unaffected ascorbate level in their roots as compared to those of untreated high temperature stressed seedlings (Fig. 4A). Although APX activity increased in the roots of LM-11 seedlings, GR activity remained unaffected (Fig. 3D) which indirectly led to reduced pools of ascorbate in their roots (Fig. 4B). APX activity was observed to decrease in the shoots of CML-32 seedlings, under

high temperature, at both the stages of seedling growth (Fig. 3A) which showed that detoxification of H<sub>2</sub>O<sub>2</sub> does not occur through ascorbate-glutathione pathway. Application of SA, in fact, further decreased the activity of APX, though GR activity remained unaffected (Fig. 3) which resulted in reduced oxidation of ascorbic acid leading to its increased content in the shoots at 6 DSG stage (Fig. 4A). In the shoots of high temperature exposed LM-11 seedlings, APX activity increased significantly at 8 DSG (Fig. 3B) while GR showed a significant reduction at 6 DSG stage (Fig. 3D). Foliar application of SA led to a significant increase in APX activity while GR remained unaffected in the shoots of LM-11 seedlings as compared to high temperature stressed untreated seedlings (Fig. 3D). Ascorbate content was observed to remain unaffected under control and high temperature stress in the shoots of LM-11 seedlings

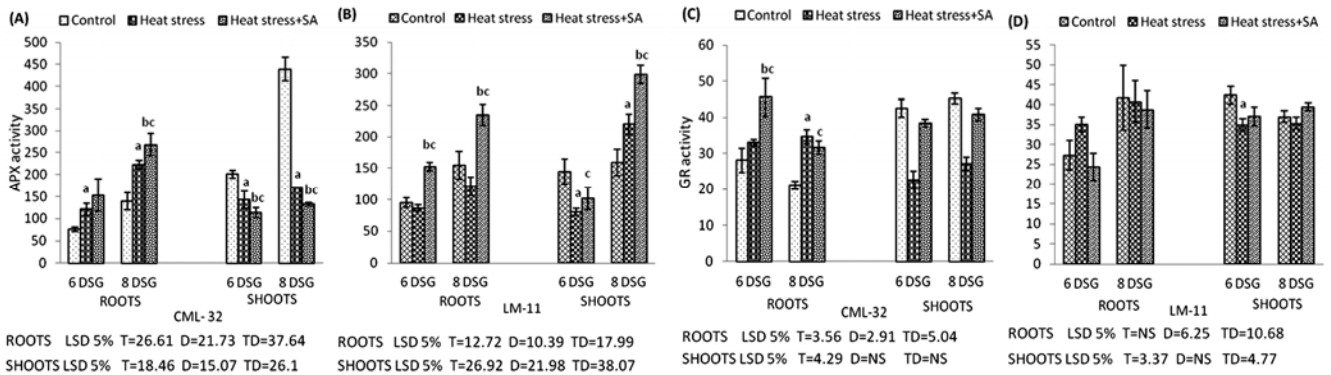


Fig. 3—Effect of 400 μM salicylic acid on ascorbate peroxidase (nmol of monodehydroascorbate formed min<sup>-1</sup> mg<sup>-1</sup> protein) (A and B); and glutathione reductase (nmol of NADP<sup>+</sup> formed min<sup>-1</sup> mg<sup>-1</sup> protein) activity (C and D) in CML-32 and LM-11 seedlings, respectively under high temperature stress. [LSD, least significant difference at 5% probability level; T, treatment; D, days of seedling growth; TD, interaction between treatment and days of seedling growth. ‘a’, ‘b’, and ‘c’, denote level of significance with respect to heat stressed and control; heat stressed and SA+heat stressed; and SA+heat stressed seedlings and control, respectively].

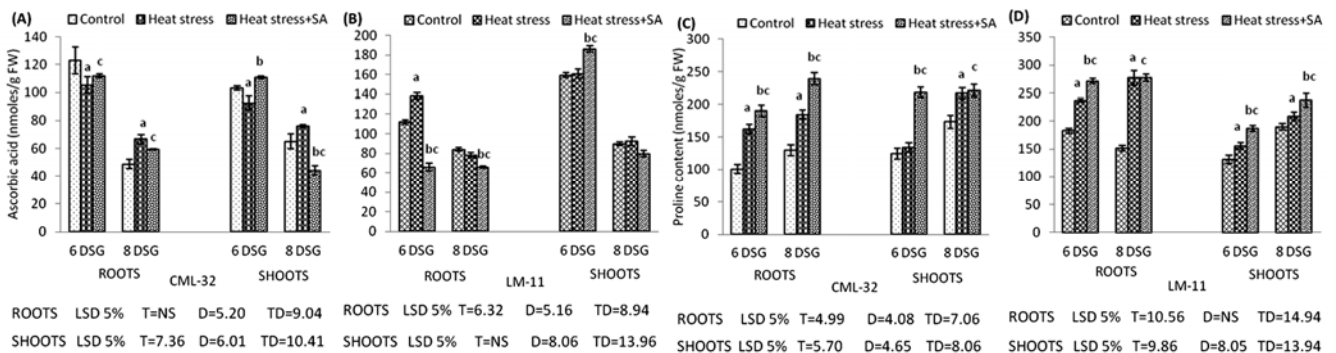


Fig. 4—Effect of 400 μM salicylic acid on the ascorbic acid (nmol g<sup>-1</sup> FW) (A and B); and proline contents (nmol g<sup>-1</sup> FW) (C and D) in CML-32 and LM-11 seedlings, respectively under high temperature stress. [LSD, least significant difference at 5% probability level; T, treatment; D, days of seedling growth; TD, interaction between treatment and days of seedling growth. ‘a’, ‘b’, and ‘c’, denote level of significance with respect to heat stressed and control; heat stressed and SA+heat stressed; and SA+heat stressed seedlings and control, respectively].

(Fig. 4B). Application of SA resulted in increased ascorbate content in their shoots at 6 DSG; however, it decreased, with seedling growth, to comparable levels at 8 DSG stage. The increased ascorbate content in the shoots of SA treated LM-11 seedlings (Fig. 4B) might be related to its increased biosynthesis under oxidative stress<sup>34</sup>, which in turn would have resulted in substrate induced activation of APX activity.

Under high temperature stress, in general, CAT activity was observed to be lower in roots and shoots in comparison with the control values of their respective genotypes (Fig. 5 A and B). The decreased CAT activity in seedlings of both the genotypes could be attributed to their reduced *denovo* synthesis<sup>18</sup>. Reduction in CAT activity under other abiotic stresses has been reported in literature<sup>35</sup>. SA induced CAT activity in both the tissues of LM-11 seedlings as well as the shoots of CML-32 seedlings as observed in this study (Fig. 5 A and B) indicates its role in enhancing the stress tolerance capacity of the genotypes. It shows that Halliwell-Asada pathway remains less operative in the roots and shoots of LM-11 as well as the shoots of CML-32 seedlings even after SA application.

Our results further showed that high temperature stress did not affect POX activity in roots of seedlings of both the genotypes, irrespective of their behavior towards high temperature stress (Fig. 5 C and D). This showed that POX might be the only enzyme responsible for ROS detoxification in LM-11 roots under stress conditions. This could not lead to proper alleviation of ROS species resulting in higher sensitivity of LM-11 towards temperature stress. POX

activity, however, decreased significantly in the shoots of LM-11 but remained unaffected in those of CML-32 seedlings under stress conditions (Fig. 5 C and D). There are contradictory reports in literature which showed increased POX activity in plants under abiotic stresses<sup>36</sup>. Salicylic acid application, in general, led to a significant increase in POX activity in roots and shoots of high temperature stressed seedlings of both the genotypes, though the values were comparatively higher in CML-32 (Fig. 5 C and D). In fact, the roots of CML 32 seedlings showed increased POX activity at 6 DSG and maintained these levels up to 8 DSG stage. The roots of SA treated LM11 seedlings, however, showed increase POX activity at 8 DSG only. (Fig. 5 C and D). The increase in POX activity, after treatment with SA, suggested the effective role of POX activity in protecting the cells against the damaging effects of H<sub>2</sub>O<sub>2</sub>. Besides, SA treated CML-32 seedlings which attained higher activities of POX, endowed them with an upper hand in tolerating high temperature stress. Xu *et al.*<sup>37</sup> have also demonstrated increased POX activity with SA application in Chinese lizard tail (Chameleon plant) under abiotic stress.

The H<sub>2</sub>O<sub>2</sub> content was observed to be significantly higher in the roots and shoots of seedlings of both the genotypes, under high temperature stress, at both the stages of seedling growth (Fig. 6 A and B). Foliar application of SA led to induced antioxidant response in terms of APX, CAT and POX activities which thus resulted in reduced contents of H<sub>2</sub>O<sub>2</sub> (Fig. 6 A and B) The decreased H<sub>2</sub>O<sub>2</sub> content correlated well with reduced or unaffected ascorbic acid content (Fig. 4 A and B) of SA treated seedlings

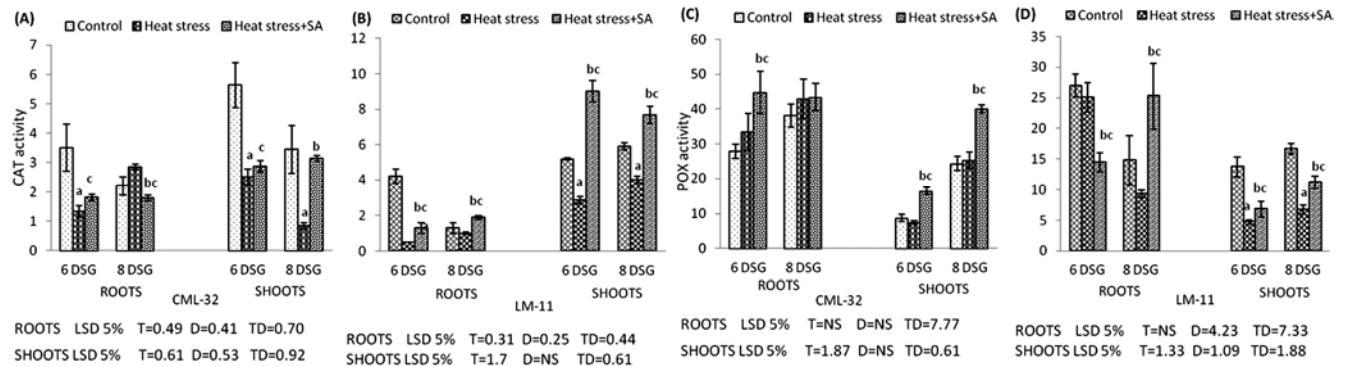


Fig. 5—Effect of 400  $\mu\text{M}$  salicylic acid on catalase ( $\mu\text{ moles of H}_2\text{O}_2\text{ decomposed min}^{-1}\text{ mg}^{-1}\text{ protein}$ ) (A and B); and peroxidase (absorbance  $\text{min}^{-1}\text{ mg}^{-1}\text{ protein}$ ) activity (C and D) in CML-32 and LM-11 seedlings, respectively under high temperature stress. [LSD, least significant difference at 5% probability level; T, treatment; D, days of seedling growth; TD, interaction between treatment and days of seedling growth. 'a', 'b', and 'c', denote level of significance with respect to heat stressed and control; heat stressed and SA+heat stressed; and SA+heat stressed seedlings and control, respectively]

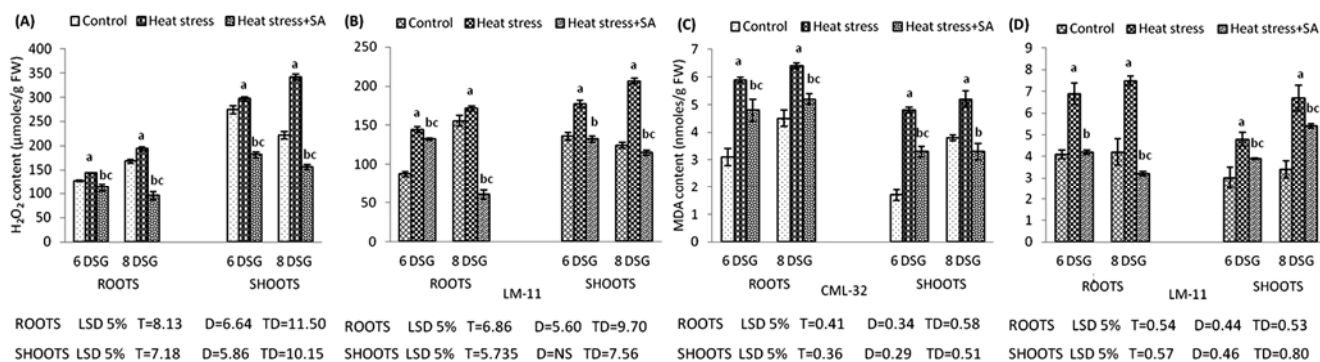


Fig. 6—Effect of 400 µM salicylic acid on the hydrogen peroxide (µ moles g<sup>-1</sup> FW) (A and B); and malondialdehyde contents (n moles g<sup>-1</sup> FW) (C and D) in CML-32 and LM-11 seedlings, respectively under high temperature stress. [LSD, least significant difference at 5% probability level; T, treatment; D, days of seedling growth; TD, interaction between treatment and days of seedling growth. ‘a’, ‘b’, and ‘c’, denote level of significance with respect to heat stressed and control; heat stressed and SA+heat stressed; and SA+heat stressed seedlings and control, respectively]

with increased APX activity (Fig. 3 A and B), under stress conditions. However, shoots of CML-32 seedlings appeared to be an exception with reduced APX activity (Fig. 3A).

High temperature stress downregulated SOD activity in seedlings of both the genotypes with the exception of CML 32 roots at 6 DSG stage (Fig. 7). Reports regarding reduced SOD activity under low temperature stress are also available in literature<sup>38</sup>. SA application did not affect SOD activity in roots but increased its activity, at 6 DSG stage, in the shoots of CML-32 seedlings (Fig. 7A). The relatively sensitive genotype showed increased SOD activity in both the tissues at 8 DSG stages after SA application in high temperature stressed seedlings (Fig. 7B). Thus, SA improved the stress tolerance efficiency of relatively sensitive genotype LM-11 by effectively increasing the activity of SOD in these seedlings. These findings are in alignment with Shi *et al.*<sup>39</sup> who reported similar increased SOD activity in SA treated high temperature stressed *Cucumis sativa* L. seedlings.

When the corn seedlings were exposed to high temperature, proline content increased in seedlings of both the genotypes (Fig. 4 C and D). An increase in proline content in response to high temperature stress has been observed in other plants as well<sup>40</sup>. Proline has been reported as an important osmolyte which maintains redox metabolism by removing excess levels of ROS and re-establishing cellular redox balance<sup>41</sup>. Application of SA increased the content of proline in seedlings of both the genotypes under high temperature stress. In fact, it increased the proline content of LM 11 roots at 6 DSG stage and maintained the same levels by 8 DSG (Fig. 4 D). The increase in proline content with SA treatment was

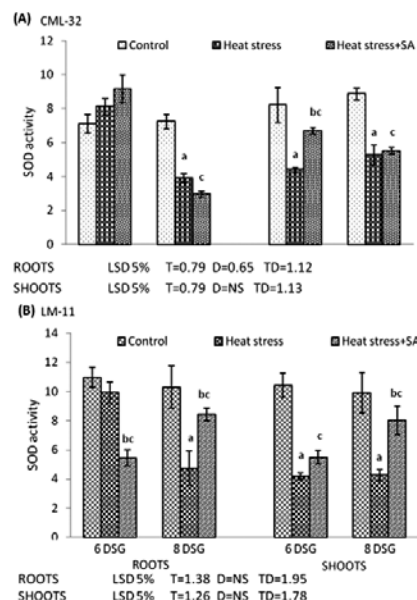


Fig. 7—Effect of 400 µM salicylic acid on superoxide dismutase activity (min<sup>-1</sup> mg<sup>-1</sup> protein) in CML-32 (A); and LM-11 (B) seedlings under high temperature stress. [One unit is equal to the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol. LSD, least significant difference at 5% probability level; T, treatment; D, days of seedling growth; TD, interaction between treatment and days of seedling growth. ‘a’, ‘b’, and ‘c’, denote level of significance with respect to heat stressed and control; heat stressed and SA+heat stressed; and SA+heat stressed seedlings and control, respectively]

high enough to bypass the values of control seedlings (Fig. 4 C and D). It could be attributed to the increased activities of proline biosynthesizing enzymes and decreased activities of proline oxidizing enzymes<sup>42</sup>. It may be inferred that SA induced elevation of proline content helps in improving the antioxidant responses of maize seedlings under high temperature stress.

Malondialdehyde (MDA) has been reported as the most abundant individual aldehydic lipid breakdown product and is a widely used marker of oxidative lipid injury<sup>43</sup>. High temperature stress resulted in an increase in MDA content in roots and shoots of seedlings of both the genotypes at different stages of seedling growth (Fig. 6 C and D) which indicated increased membrane injury. Drastic increase in lipid peroxidation due to high temperature stress has been already reported in pearl millet<sup>2</sup> as well as wheat<sup>44</sup>. When SA was foliar sprayed, there was a reduction in MDA content of seedlings of both the genotypes, under high temperature stress, (Fig. 6 C and D), which could be attributed to their enhanced antioxidant defense system. Our results are in agreement with others who reported that SA treatment prevented lipid peroxidation in *Ctenanthe setosa* plants<sup>45</sup>.

It may be concluded that SA induced differential antioxidant response especially by upregulating Halliwell-Asada pathway in roots and attaining high POX activity in both the tissues of CML-32 seedlings under high temperature stress.

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