RESEARCH ARTICLE

Growth of Indian mustard (*Brassica juncea* L.) in response to salicylic acid under high-temperature stress

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ABSTRACT

Pots holding 7 day-old seedlings of Indian mustard (*Brassica juncea* L.) were subjected to differential temperature stress by exposing plants to 30 or 40°C for 24 h. Seedlings were sprayed with double distilled water (DDW) or 10^{-5}M salicylic acid (SA) at the 8-day stage and were sampled at 30 DAS. The plants exposed to temperature stress exhibited a significant ($p < 0.05$) decline in growth, and in levels of chlorophyll, nitrate reductase and carbonic anhydrase activities and photosynthetic parameters. A follow-up treatment with SA protected against the stress generated by temperature and significantly improved the above parameters. Antioxidative enzymes and levels of proline significantly increased in response to SA as well as to temperature stress.

Key words: Antioxidative enzymes; Carbonic anhydrase; Nitrate reductase, Photosynthesis; Proline

Abbreviations: CA - carbonic anhydrase; Ci - intercellular CO_2 concentration; DAS - days after sowing; D.M. – dry mass; F.M. - fresh mass; gs - stomatal conductance; LSD - least significant difference; NR – nitrate reductase; P_N - net photosynthetic rate; SE - standard error

INTRODUCTION

Gaseous emissions due to human activities are substantially adding to atmospheric concentrations of greenhouse gases, particularly CO_2, methane, chlorofluorocarbons and nitrous oxides. In the atmosphere these gases trap heat radiated from the earth and thus increase global mean temperature. This rise in temperature may lead to altered geographical distribution and growing season of agricultural crops by altering the threshold temperature for the start of the season and crop maturity (Porter, 2005)

High temperatures negatively affect plant growth and survival and hence crop yield (Boyer, 1982). According to a recent study (Lobell and Asner, 2003) each degree centigrade increase in average growing season temperature may reduce crop yields up to 17%. High temperature stress directly or indirectly affects plant photosynthetic functions by changing the structural organization and physico-chemical properties of thylakoid membranes (Lichtenthaler et al., 2005). The rate of photorespiration increases with increasing temperature which reduces net photosynthesis (Sage and Sharkey, 1987) and probably the seed yield of the crop.

Rapid and effective measures of plant treatment are necessary so that deterioration of crops due to high temperature can be countered successfully. The application of plant growth regulators is known to play an important role in plant response to stress (Chakrabarti and Mukherjee, 2003). Salicylic acid has recently been recognized as a plant hormone (Hayat et al., 2007). Salicylic acid plays diverse physiological roles in plants including thermogenesis, flower induction, nutrient uptake, ethylene biosynthesis, stomatal movements, photosynthesis and enzyme activities (Hayat et al., 2007). Disease resistance and abiotic stress tolerance are additional roles assigned to SA (Janda et al., 2007). Among abiotic stresses, SA has been reported to counter water stress (Singh and Usha, 2003), low

temperature (Tasgin et al., 2003) and salinity stress (El Tayeb, 2005). The studies on *Phaseolus vulgaris* have revealed that application of salicylic acid induces the chalcone synthase and phenylalanine ammonia-lyase activity that cumulatively confer resistance during stressful conditions (Campos et al., 2003).

The present research was designed with the objective of evaluating changes in the antioxidative enzymes of Indian mustard (*Brassica juncea* L.) plants exposed to temperature stress and treated with salicylic acid. A second objective was to establish a relationship between changes in the activity of antioxidative enzymes and the degree of stress tolerance, in terms of improvement in growth and photosynthesis. The hypothesis tested is that salicylic acid will ameliorate the adverse effects of temperature in Indian mustard.

MATERIAL AND METHODS

Plant material and growth conditions: Seeds of Indian mustard (*Brassica juncea* L. Czern and Coss) cv. Kranti were obtained from the National Seed Corporation Ltd., New Delhi. Healthy seeds were surface-sterilized with 5% sodium hypochlorite followed by repeated washing with deionized water. The sterilized seeds were sown in earthen pots (4 inch inner diameter) filled with sandy loam soil and farmyard manure (ratio 6:1) and allowed to germinate for 7 days. Pots with the 7-day old seedlings were subjected to differential temperature stress by exposing plants to either 30 or 40°C for 24 h. One set of pots remained at ambient temperature (25±2°C).

Seedlings were sprayed with double distilled water (DDW) or 10^{-5}M of SA at the 8-day stage. Concentration of SA was based on results from an earlier study (Fariduddin et al., 2003). Each seedling was sprayed three times; the sprayer nozzle was adjusted to release 1 cm^3 per spray. Therefore, each plant received 3 cm^3 of DDW or SA solution. Samples were collected at 30 DAS to assess the parameters discussed below.

***Plant growth analysis*:** Plants were removed from pots along with the soil and were immersed in a container filled with tap water. The plants were gently agitated to remove adhering soil particles, and root and shoot lengths were measured using a meter scale. The plants were then placed in an oven set at 80°C for 24 h. The dried plants were weighed to record plant dry matter.

***Chlorophyll and photosynthesis measurements*:** Leaf chlorophyll content was measured using an SPAD chlorophyll meter (Minolta, 502, Japan). The stomatal conductance (gs), internal carbon dioxide (C_i), water use efficiency (WUE), transpiration (E) and net photosynthetic rate (P_N) in intact leaves were measured using a LI-6400 portable photosynthesis system (LI-COR Lincoln, NE, USA), between 11:00 and 12:00h.

***Determination of leaf water potential*:** Leaf water potential was measured using a Psypro water potential system (Wescor Inc., Logan, Utah, USA).

***Determination of proline contents*:** The proline content in fresh leaf samples was determined by adopting the method of Bates et al., (1973). Samples were extracted with sulphosalicylic acid. In the extract equal volumes of glacial acetic acid and ninhydrine solutions were added. The samples were heated to 100°C to which 5 mL toluene was added. The absorbance of the toluene layer was read at 528 nm on a spectrophotometer (Milton & Roy, USA).

Enzyme assays: The activity of carbonic anhydrase (CA) was determined following the procedure described by Dwivedi and Randhawa (1974). Leaf samples were cut into small pieces and suspended in cysteine dihydrochloride solution. The samples were incubated at 4°C for 20 min. The leaf pieces were blotted with tissue paper and transferred to test tubes containing phosphate buffer (pH 6.8). Alkaline bicarbonate solution and bromothymol blue indicator were subsequently added to the tubes. The test tubes were incubated at 5°C for 20 min. The reaction mixture was titrated against 0.05N HCl, after addition of 0.2 mL of methyl red indicator.

The activity of nitrate reductase (NR) was determined in fresh leaf samples by the procedure of Jaworski (1971). This method is based on the reduction of nitrate to nitrite, whose quantity was estimated spectrophotometrically at 540 nm.

For the assay of antioxidative enzymes, leaf tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrolidine. The homogenate was centrifuged at 15,000 rpm for 10 min at 4°C and the supernatant was used as the source of catalase, peroxidase and superoxide dismutase.

Peroxidase and catalase were assayed following the procedure described earlier (Chance and Maehly, 1956).

Catalase was estimated by titrating the reaction mixture, consisting of phosphate buffer (pH 6.8), 0.1M H_2O_2, enzyme extract and 2% H_2SO_4, against 0.1N potassium permanganate solution. The reaction mixture for peroxidase consisted of pyrogallol phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract. Change in absorbance due to catalytic conversion of pyrogallol to parpurogalline was noted at an interval of 20 sec for two min, at 420 nm on a spectrophotometer. A control set was prepared by using DDW instead of enzyme extract.

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Beauchamp and Fridovich (1971). The reaction mixture, containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0-50 mL enzyme extract, was placed under a 15W fluorescent lamp. The reaction was started by switching on the light which was allowed to run for 10 min. The reaction was stopped by switching off the light. A measure of 50% inhibition by light was considered as one enzyme unit.

***Determination of leaf nutrient status*:** The content of leaf nitrogen, inorganic phosphate and potassium was estimated by the method of Lindner (1944), Fiske and Subbarow (1925), and by flame photometry, respectively.

***Statistical analysis*:** A ten replicates were taken in to account. The values for the parameters were subjected to statistical analysis following the standard procedures described by Gomez and Gomez (1984). The means were compared by LSD test to study the significance at 5% level of probability. Standard error of the replicates was also calculated.

RESULTS

The treatments significantly ($p < 0.05$) affected all plant growth characteristics (Figure 1, Figure 2A and 2B). The application of SA (10^{-5}M) alone was highly significant ($p < 0.01$), and increased all growth parameters over the control by 13%, 14.7%, 14.0% and 35.5% for root length, shoot length, fresh mass (F.M.) and dry mass (D.M.) of plants, respectively. However, exposure of plants to either elevated temperature, i.e., 30 or 40°C, inhibited plant growth. The higher temperature (40°C) resulted in greater growth inhibition, decreasing root length, shoot length, F.M. and D.M. of the plants by 53.7%, 45.5%, 68.3% & 56.8%, respectively, below that of the control. However, the follow-up treatment with SA (10^{-5} M) partly overcame the deleterious effects generated by temperature.

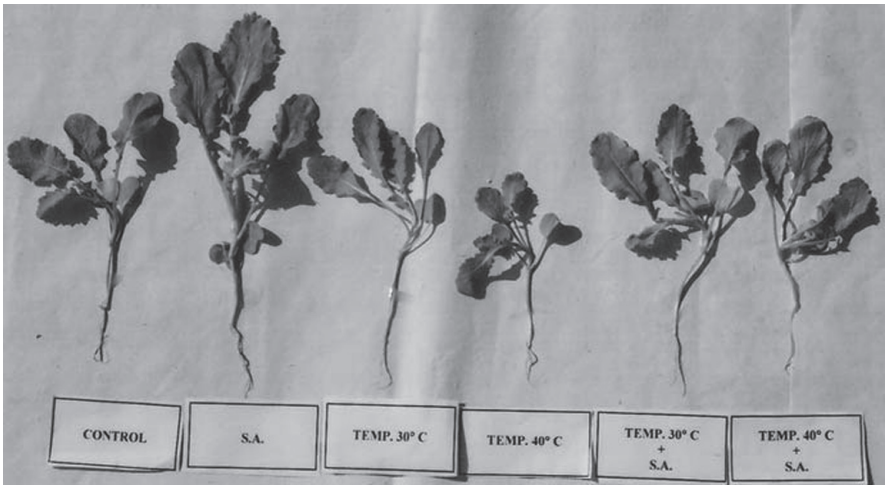


Figure 1. Effect of salicylic acid (10^{-5}M) on the temperature (30°C or 40°C) induced changes on the growth of *Brassica juncea* cv. kranti at 30 day stage (±S.E.).

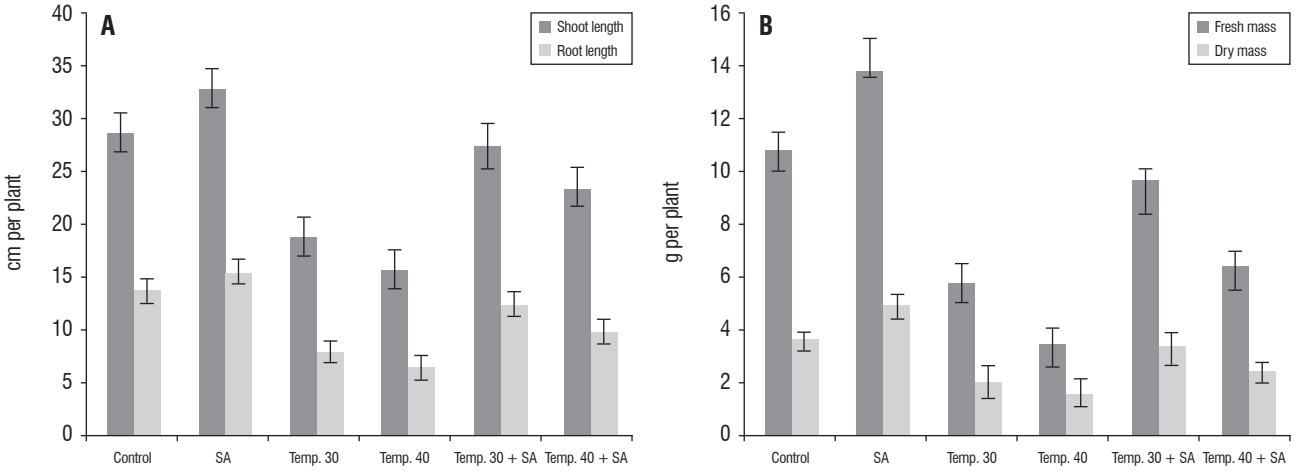
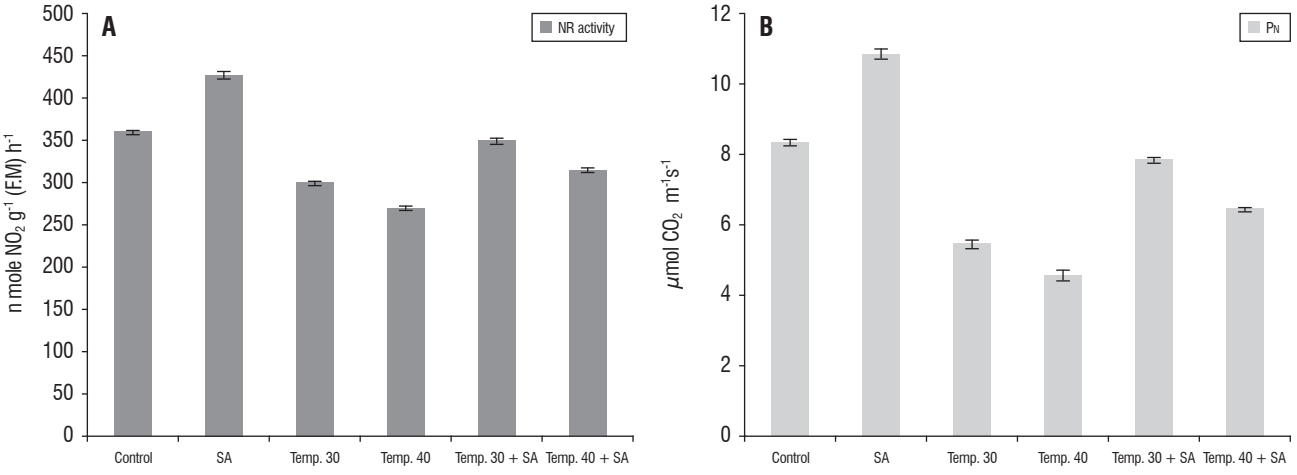


Figure 2. Effect of salicylic acid (10^{-5}M) on the temperature (30ºC or 40ºC) induced changes in (A) shoot and root length (cm) and (B) fresh and dry mass per plant (g) of *Brassica juncea* cv. kranti at 30 day stage (Vertical bar ±S.E.).

The photosynthetic parameters, i.e., gs, Ci, WUE, P_N and E were significantly decreased (Figure 3C-D and Table 1) when exposed to elevated temperature (30ºC or 40ºC). The higher temperature stress (40ºC) proved to be more inhibitory to plant growth compared to the lower temperature stress (30ºC) and decreased root length; shoot length, F.M. and D.M. by 21.5%, 16.1%, 43.2% 45.2% and 32.5%, respectively, as compared to the control. Treatment of the stressed plants with SA (10^{-5}M), however, significantly enhanced all photosynthetic attributes more efficiently in plants exposed to lower temperature (30ºC). Application of SA alone increased all the above parameters significantly.



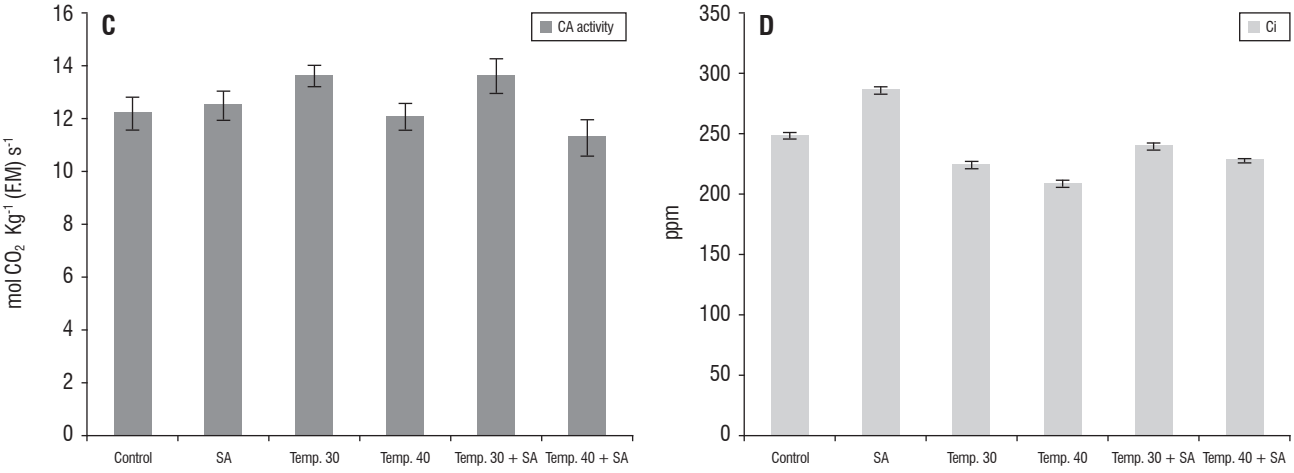


Figure 3. Effect of salicylic acid (10^{-5}M) on the temperature (30ºC or 40ºC) induced changes in (A) leaf NR and CA (C) activities, PN (B) and Ci (D) of *Brassica juncea* cv. kranti at 30 day stage (Vertical bar ±S.E.).

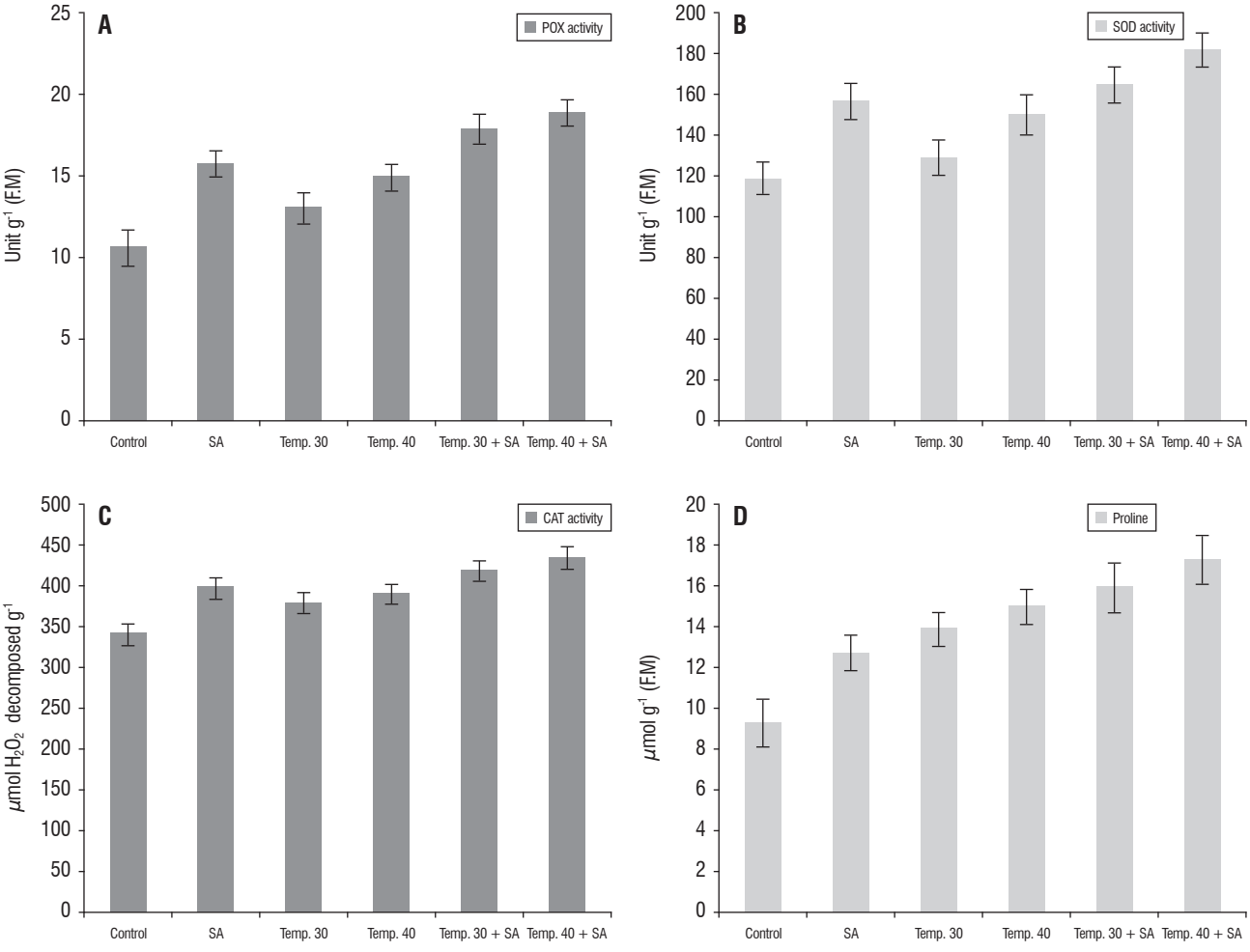


Figure 4. Effect of salicylic acid (10^{-5}M) on the temperature (30ºC or 40ºC) induced changes in (A) peroxidase and catalase activities and (B) SOD and proline content in the leaves of *Brassica juncea* cv. kranti at 30 day stage (±S.E.).

Plants receiving SA alone possessed the maximum value for SPAD chlorophyll and measured 11.9% higher than that of the control (Table 1). However, application of high temperature stress to the plants decreased the value of SPAD by 17.5% below that of the control. The follow-up treatment with SA partly overcame the negative effects generated by higher temperature stress (40ºC) and almost completely by the lower temperature stress (30ºC).

Plants treated with SA alone possessed maximum values for leaf water potential and were about 20.9% higher than that of the control (Table 1). However, application of temperature stress lowered leaf water potential. The follow-up treatment with SA partially overcame the effects generated by temperature stress (30ºC or 40ºC), resulting in increased values of 26.8% and 34.4% respectively, over the control.

Activity of CA was significantly elevated by the SA treatment and was 44.6% higher than that of the control (Figure 4B). However, the high temperature (40ºC) was more deleterious and decreased CA activity by 20.0% below the control, while the lower temperature (30ºC) decreased activity by 15.1% below that of the control. The deleterious effect generated by temperature stress was partly neutralized by the SA (Figure 3B).

Under ambient conditions treatment of the plants with SA enhanced activity of NR (3A). However, application of high temperatures resulted in a significant decrease in enzyme activity. The higher temperature (40ºC) decreased activity by 25% whereas in plants exposed to 30ºC a 16% decline was observed compared with than that of the control. Moreover, the follow-up treatment with SA resulted in significant ($p < 0.05$) improvement in enzyme activity in plants that were subjected to temperature stress.

The activities of antioxidative enzymes (catalase, peroxidase and superoxide dismutase) were significantly enhanced when the plants were exposed to either of temperature stress and SA. Application of SA alone increased activities of catalase, peroxidase and superoxide dismutase by 16.8%, 49.0% and 32.2%, respectively, compared with the control. The maximum activities of these enzymes were recorded from the plants that were exposed to high temperature stress and receiving SA; activities increased by 27.6%, 79.1% and 53.4%, respectively, compared with the control. Control plants had the minimum values for these enzymes (Figs. 4a-c).

The plants incubated under controlled conditions possessed the lowest level of proline (Figure 4D). However, proline level increased, both in response to temperature stress as well as to SA treatment. Interaction of SA with temperature stress significantly ($p < 0.05$) raised proline levels. Plants receiving SA in association with higher temperature stress (40ºC) showed maximum levels of proline.

Treatment of plants with SA significantly increased leaf nitrogen (N), inorganic phosphate (P) and potassium (K) contents by 8.9%, 16.6% & 2.9% respectively, compared with the control (Figure 5). However, leaf N P K content decreased when plants were exposed to either temperature stress. The higher temperature (40ºC) proved to be more detrimental than the lower temperature stress (30ºC). The SA spray partly overcame the deleterious effect generated by high temperature treatment.

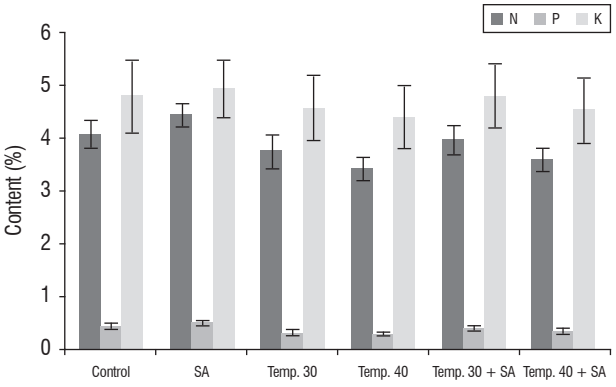


Figure 5. Effect of salicylic acid (10^{-5}M) on the temperature (30ºC or 40ºC) induced changes in nitrogen (N) content (%), phosphorus (P) content (%) and potassium (K) content (%) in the leaves of *Brassica juncea* cv. kranti at 30 day stage (±S.E.).

DISCUSSION

Plant growth and development is complex and determined by several endogenous and exogenous factors. Among the internal factors, hormones play a vital role in regulating growth and development. The role of SA in plant growth regulation was the first physiological process to be reported (De-Kock et al., 1974). Thereafter, both exogenous and endogenous levels of SA have been shown to impart various effects (Raskin, 1992).

In the present study it was observed that temperature stress reduced plant growth, SPAD chlorophyll value, photosynthetic attributes, nitrogen, inorganic phosphate and potassium content and leaf water potential (Figs. 1-3 and 5;

Table 1) and hence may ultimately lead to an overall decrease in crop yield. Brief exposure of plants to heat stress during seed filling can accelerate senescence, diminish seed set and seed weight and reduce yield, as reported earlier (Siddique et al., 1999). This phenomenon is a result of the plant tending to divert resources to cope with the heat stress at the expense of photosynthesis and ultimately plant growth and development. However, exogenous application of SA reverses the effect of heat stress on *Brassica juncea* (Figure 3C). A similar growth promoting effect of salicylic acid in *Brassica juncea* plants exposed to temperature stress was reported by Cong et al., (2008). Salicylic acid activates a novel gene BjDREB1B encoding a DRE (dehydration responsive element) binding protein, leading to elevated level of proline thereby provided tolerance to the plants against harmful effects of temperature stress (Cong et al. 2008). The induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid application which results in the synthesis of certein phenolic compounds that play an important role in conferring resistance against various abiotic stresses including temperature stress (Campos et al. 2003) further supports our results. Moreover, it was also reported earlier that wheat seedlings raised, from grains soaked in 10^{-5}M of SA possessed more leaves and higher F.M. and D.M. compared with those which were water-soaked (Hayat et al., 2005).

With the onset of high temperature regime, *Zygophyllum qatarense* produced polymorphic leaves and tended to reduce transpirational water loss by showing bimodal stomatal behavior (Sayed, 1996), thereby altering gaseous exchange (Hopkins, 1995). Therefore, plants subjected to heat stress had lower Ci as compared to the control (Figure 3D). Decrease in P_N (Figure 3C) is generally assumed to be attributed to reduced CO_2 supply (Hsiao, 1973). At the sub-cellular level major modifications occur in the chloroplast, leading to significant changes in photosynthesis. For example, heat stress reduced photosynthesis by changing the structural organization of thylakoids (Karim et al., 1997). Studies have revealed that specific effects of high temperature on the plant photosynthetic membrane results in the loss of grana stacking or its swelling. In response to heat stress, chloroplasts in the mesophyll cells of grape plants become round in shape, the stroma lamellae become swollen, and the contents of the vacuole form clumps, whilst the cristae were disrupted and mitochondria became empty (Zhang et al., 2005). Such changes result in the formation of antenna-depleted photosystem-II (PS-II) and hence reduced photosynthetic and respiratory activities (Zhang et al., 2005). The cumulative effect of all these changes resulted in poor plant growth (Figs. 2a-b). Moreover, heat stress reduced gs (Table 1) leading to reduced carbon assimilation and consequently low biomass production (Medrano et al., 2002).

Water use efficiency (WUE) of plants also decreased under severe stress conditions (Table 1). PS II is highly thermo-labile and its activity is greatly reduced or even ceases under high temperatures (Camejo et al., 2005), which may be due to properties of thylakoid membranes where PS II is located (Mac Donald, 1997). Stomatal conductance and net photosynthesis are inhibited by moderate heat stress in many plant species due to decrease in activation state of rubisco (Monson et al., 1982). In the present study, however, application of SA enhanced the SPAD chlorophyll value and photosynthetic rate and also partially overcame the deleterious effect of the heat stress, if administered as a follow-up treatment (Table 1 and Figure 3C). This is deduced from the present observation that application of SA was beneficial in enhancing total chlorophyll and P_N value under normal conditions and also under temperature stress. Enhanced P_N and chlorophyll by SA application was also reported earlier (Fariduddin et al., 2003).

The activities of CA and NR decreased in plants subjected to heat stress. The activity of NR is known to depend on the concentration of its substrate (NO_3) (Solomonson and Barber, 1990). The decrease in NR activity in heat stressed plants serves as a biochemical adaptation to conserve energy by stopping nitrate assimilation at the initial stage. However, application of SA (10^{-5}M) was found beneficial in enhancing the activities of NR and CA while applied under ambient conditions and also help partially in overcoming the negative effect of heat stress (Figure 3A,B). Induction of carboxylation efficiency and NR activity by SA was reported earlier (Fariduddin et al., 2003).

The activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) and the level of proline exhibited increases in response to SA or heat stress application (Figs. 4a-d). Heat stress induces oxidative stress. For example, generation and reaction of activated oxygen species (AOS), which causes the autocatalytic peroxidation of membrane lipids and pigments subsequently leads to membrane permeability and modification of its functions (Xu et al., 2006). Recent studies show that some signaling molecules may increase

the antioxidant capacity of cells (Gong et al., 1997; Dat et al., 1998). Extreme temperatures led to accumulation of certain organic compounds termed osmolyte, including sugars, polyols, and proline. Proline is known to occur widely in higher plants and normally accumulates in response to environmental stress (Kavi Kishore et al., 2005). The treatment of plants under stressed conditions results in an increase in proline contents over the control, and led to induction of peroxidase activity and ascorbate peroxidase (Chakraborty and Tongden, 2005).

Increased SOD and CAT activities were observed under heat stress after SA application in Kentucky bluegrass (He et al., 2005). Proline is reported to act as a protective osmolyte (Hartzendrof and Rolletschek., 2001), membrane stabilizer (Bandurska, 2001) and ROS scavenger (Matysik et al., 2002). The accumulation of proline is an enzyme-regulated process, which is synthesized from the amino acid glutamate. The enzymes are reported to be elevated under stressed conditions (Sumithra and Reddy, 2004). The accumulation of proline in plants exposed to heat stress (Wang and Zhang, 1993) has also been reported earlier. This could be a possible explanation for SA- or heat stress- (or a combination of both) mediated elevation in the activity of the antioxidative enzymes of plants and also of an increase in proline accumulation (Figure 4A-D).

Table 1. Effect of salicylic acid (10^{-5}M) on the temperature (30ºC or 40ºC) induced changes in leaf SPAD chlorophyll value, leaf water potential (Mpa), gs, WUE and transpiration of *Brassica juncea* cv. kranti at 30 day stage (±S.E.). Data are the mean of three independent replicates (n=3)

Treatments	SPAD value	Leaf water potential	g_s	WUE	Transpiration
Control	28.50 ± 0.753	-0.62 ± 0.012	0.344 ±0.0117	1.99 ±0.105	3.20 ±0.096
SA	31.90 ± 0.634	-0.49 ± 0.015	0.398 ±0.0113	2.41 ± 0.036	3.81 ±0.075
Temp. 30ºC	26.10 ± 0.723	-1.22 ±0.031	0.296 ±0.0119	1.45 ±0.064	2.46 ±0.040
Temp. 40ºC	23.50 ± 0.681	-1.34 ± 0.035	0.270 ±0.0115	1.13 ±0.057	2.16 ±0.021
Temp. 30ºC+ SA	27.90 ± 0.519	-0.80 ± 0.012	0.338 ±0.024	1.95 ± 0.067	2.97 ±0.051
Temp. 40ºC + SA	26.80 ± 0.764	-0.98 ± 0.032	0.320 ±0.010	1.57 ±0.104	2.60 ± 0.062
LSD at 5%	0.42	0.059	0.02	0.13	0.12

CONCLUSIONS

It can be concluded from the present observation that application of salicylic acid enhanced the activity of antioxidative enzymes in Indian mustard plants under elevated temperatures. The elevated activity of antioxidative enzymes counter the direct as well as indirect effects of temperature stress thereby, improving the photosynthetic efficiency, metabolism and growth in mustard plants.

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