RESEARCH ARTICLE

Changes in growth, pigment content and antioxidants in the root and leaf tissues of wheat plants under the influence of exogenous salicylic acid

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ABSTRACT

The effect of various concentrations of salicylic acid (SA) on the growth, pigment content and the activity of antioxidants was investigated in the laboratory grown wheat plants. The root and shoot growth was affected at higher concentration of SA in early days of growth. The activities of catalase (CAT), ascorbate peroxidase (APX) and guaicol-specific peroxidase (POX) declined with the application of SA (50, 500 and 1000 μM), the decrease being more pronounced with the increase in SA concentrations both in the root and leaf tissues. On the other hand superoxide dismutase (SOD) activity increased with the application of SA. At low concentrations, SA has no effect on the activities of these enzymes in vitro. Salicylic acid at higher concentrations (5- and 10 mM) though inhibited CAT activity, the activities of APX and POX remain unchanged. High concentration of SA increased the level of H_2O_2 and malondialdehyde both in root and leaf tissues. Thus, SA though has been reported to be a signal molecule for inducing various physiological and morphological attributes in plants, this study indicated the negative effect of the compound on growth and the activity of major enzymatic antioxidants.

Key words: antioxidants, oxidative stress, salicylic acid, *Triticum aestivum L.*

INTRODUCTION

Salicylic acid (SA) has been identified as one of the components of the signaling pathway, participating in the regulation of wide range physiological of processes in plants. It has been proposed that the compound acts as a signal molecule in the sequence of metabolic events leading to the expression of systemic resistance to plant pathogens (Yalpani et al., 1993). Exogenously applied SA induces pathogenesis-related proteins and other compounds, which impart an increased resistance to virus and fungal attack (Ward et al., 1993). The role of SA in thermogenesis and other physiological processes is well known (Raskin et al., 1987). In recent years this compound has drawn the attention of researchers because of its role in induction of tolerance to various abiotic stresses (Horva´th et al., 2007).

The effect of SA on the status of antioxidant enzyme activities is a renewed area of research. The compound has been found to enhance H_2O_2 formation but with a negative effect on the activities of H_2O_2 degrading enzymes like catalase (CAT) and ascorbate peroxidase (APX) (Rao et al., 1997). An

increase in guaicol peroxidase (POX) activity with response to SA has been noticed in cowpea (Chandra et al., 2007). It has been suggested that the inhibition of CAT by SA plays a role in mediating stress responses (Horva´th et al., 2002).

The role of SA in the mitigation of stresses of various kinds such as cold, salinity, metal and temperature (Rao and Davis 1999; Popova et al., 2003; Zawoznik et al., 2007) has been studied in different plant species. Exogenous application of SA can regulate the activities of intracellular antioxidant enzymes such as SOD, POX and increase plant tolerance to environmental stresses (Senaratna et al., 2000; Sakhabutdinova et al., 2004). For these studies various concentrations of SA ranging from 0.05 mM to 1 mM have been applied to the plant species. Earlier we have shown that SA at lower concentration has a stimulatory effect on the photosynthetic electron transport activity and phosphate uptake in wheat plants and at higher concentration, the inhibition of these process have been observed (Sahu et al., 2002; 2010). However, to our knowledge, data with regard to the effect of SA at these concentrations on the morphology and biochemical changes including antioxidants in unstressed plants is least known. Also, most of the works related to the influence of SA on the status of antioxidants in unstressed conditions have been carried out in *in vitro* (Conrath et al., 1995), cell suspension cultures and excised leaves (Rao et al., 1997).

The present study demonstrates the dose dependent effects of exogenous SA on root and shoot growth, concentration of chlorophyll and carotenoids (CAR) in wheat plants grown hydroponically. The level of various antioxidants such as CAT, APX, POX, SOD and ascorbate (ASC) under the influence various concentrations of SA in the root and shoot tissues of wheat plants has also been studied. Further the malondialdehyde (MDA) and H_2O_2 content have been measured to find SA generated oxidative stress in wheat plants.

MATERIALS AND METHODS

Plant growth and treatment condition: Seeds of wheat (*Triticum aestivum* L. Var. Sonalika) were collected from Seeds Corporation of India, Bhubaneswar, and were used as the source material for raising the seedlings. The seeds were sterilized with 3% (w/v) freshly prepared filtered solution of commercial clorex, washed thoroughly under tap water and then with distilled water and germinated on moist cotton pads in Petri dishes in dark at 25 ºC for 12 h. The germinated seeds were raised in to seedlings with one-fourth strength Hoagland nutrient medium (Hoagland and Arnon, 1950) containing different concentrations of SA (50, 500 and 1000 μM). A set of plants growing in the medium without SA was taken as control. The seedlings were grown in the plant growth chamber under an 8-h photoperiod at 27 $\pm$ 2 ºC. The light intensity was approximately 200 μmol m^{-2} s^{-1}. Primary leaves and roots from seven day old seedlings were used for analysis.

Plant growth: The length of primary leaves of seven day old plants was taken as plant height (shoot length) and was measured for five different sets of plants.

Pigment estimation: The chlorophyll content was determined following the method as described by Porra et al., (1989). Method of Wellburn (1994) was followed for determination of carotenoid.

Estimation of ascorbate: The ASC content was estimated using the method based on the stochiometric reduction of phosphomolybdenum by ascorbic acid (Mitsui and Ohta, 1961). Fresh leaf tissues were homogenized with 5% metaphosphoric acid and the extraction was centrifuged at 14,000xg for 10 min. The reaction mixture composed of 2 mL ice chilled 2% sodium molybdate, 2 mL 0.15 N H_2SO_4, 1 mL 1.5 x 10^{-3} M Na_2HPO_4 and 1mL supernatant of the extracted sample. The reaction was started by placing the ice-chilled mixture in a thermostat regulated water bath at 60 ºC for 40 min. The reaction tubes were then rapidly cooled in running water and centrifuged. The absorbency of the supernatant was recorded at 660 nm. A standard curve in the range of 0-10 μmole of L-ascorbate was used to calculate the concentration of ASC in the samples.

Lipid peroxidation: Measurement of MDA, a peroxidation product of fatty acids from membrane lipids is the most widely employed method for determination of lipid peroxidation in biological samples (Heath and Packer, 1968). About 0.2 g fresh leaf tissues were homogenized in 5% (w/v) trichloroacetic acid (TCA) and the homogenate was used for estimation of MDA. 5 mL of the reaction mixture consisted of 4 mL of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and 1 mL of tissue homogenate. For blank, tissue

homogenate was replaced by 1 mL of 5% TCA. 5 mL of the mixture with 1 mL of homogenate and 4 mL 20% TCA was used for correction blank. The assay mixture were heated at 95 °C in the water bath for 30 min, cooled immediately and centrifuged at 12,000xg for 10 min. The absorbance of the cooled supernatant was measured at 532 nm. The value of non-specific absorption at 600 nm was subtracted. MDA concentration was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$ and was expressed as nmol g^{-1} fresh weight.

Measurement of H_2O_2: H_2O_2 content was measured according to (Loreto and Velikova, 2001). Approximately 0.2 g of fresh leaf and root tissues were cut into pieces and homogenized in cold with 5 mL of 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 x g for 15 min at 4 °C. 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbency of the mixture was read at 390 nm. The content of H_2O_2 was determined from a standard curve prepared with known concentrations of H_2O_2 and expressed as mmol g^{-1} fresh weight.

Protein extraction: The enzyme extraction process was carried out at 4 °C. For extraction of CAT primary leaves/roots (0.5 g) were cut in to small pieces and were homogenized under ice-cold condition in a pre-chilled mortar and pastel. The buffer used for extraction is 100 mM potassium phosphate buffer, pH 6.5. APX was extracted in the same manner except that the buffer contained 5 mM ASC. 50 mM potassium phosphate buffer, pH 7.4 was used to extract SOD. The homogenates were centrifuged at 12,000xg for 15 minutes and the supernatant so obtained was used as crude enzyme source. Three POX fractions: soluble, ionic and covalent were extracted according to the method of (Gkinis and Fennema, 1978).

The protein content was determined by the modified method of Bradford taking the ratio of absorbance at 590 nm to 450 nm with bovine serum albumin as a standard (Zor and Selinger, 1996).

Antioxidant enzyme activities: The activity of CAT (EC 1.11.1.6) was assayed as described by (Aebi, 1983). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), enzyme extract equivalent to 20 μg protein and 10 mM H_2O_2. The decrease in absorbance at 240 nm due to the consumption of H_2O_2 (extinction co-efficient 40 mM^{-1} cm-1) was recorded for 2 min.

Activities of POX and APX were determined as described by (Rao et al., 1996). For the activity of POX (EC 1.11.1.7), 3 mL of the reaction mixture contained 100 mM potassium phosphate buffer, pH 6.5, 16 mM guaiacol ,10 μl of 10% H_2O_2 and enzyme extract equivalent to 10 μg protein from leaf and 1.5 μg protein from root tissue. The increase in absorbance at 470 nm (extinction coefficient 26.6 mM^{-1} cm^{-1}) was measured for 5 min. For the measurement of APX (EC 1.11.1.11), the reaction mixture contained 100 mM potassium phosphate buffer, pH 7.5, 0.5 mM ASC, 0.2 mM H_2O_2 and the enzyme extract equivalent to 150 μg protein from leaf and 20 μg protein from root tissues. The decrease in absorbance at 290 nm was recorded (extinction co-efficient of 2.8 mM^{-1} cm-1) for 3 min.

The activity of CAT, POX and APX was expressed as nkatal mg^{-1} protein. One katal is defined as 1 mol of substrate transformed by the enzyme in 1 s.

Total SOD (EC 1.15.1.1) activity was determined through the combination of riboflavin photo reduction and nitrite formation as described by (Das et al., 2000). A reaction mixture of 1.4 mL containing 1.11 mL of 50 mM phosphate buffer, pH 7.4, 0.075 mL of 20 mM L-methionine, 0.04 mL of 1% triton X-100, 0.075 mL of 10 mM hydroxylamine hydrochloride, 0.1 mL of 100 μM EDTA was taken in test tubes. The enzyme extracts equivalent to 10 μg of protein was added to the tubes followed by a brief pre-incubation at 37 °C for 5 min. Then 80 μl of 100 μM riboflavin was added to the tubes. The tubes were exposed for 10 min to two 20 W fluorescent lamps fitted parallel to each other in an aluminum coated wooden box. The control tubes contained equal amount of buffer instead of the sample. After exposure to fluorescent lamp, 1 mL of Greiss reagent prepared freshly by mixing equal amount of 0.1% N-(1-Napthyl) ethylenediamine and 1% sulphanilamide in 5% ortho-phosphoric acid was added to each tube. The absorbance of the colour so formed was measured at 543 nm. Corrections were made for the background absorbance at 543 nm without sample and riboflavin.

The activity of SOD was expressed as unit SOD mg^{-1} protein. One unit of enzyme activity is the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

Table 1. Effect of varied concentration of SA on growth and pigment content of seven day old wheat plants. The values presented are the mean of three independent measurements ± SD.

SA (μM)	Root length (cm)	Shoot length (cm)	Chl content (mg g^{-1}FW)	CAR content (mg g^{-1}FW)
0	11.23 ± 0.911	14.73 ± 0.430	1826.92 ± 41.25	271.15 ± 18.92
50	12.35 ± 0.802	14.47 ± 0.858	1778.84 ± 38.26	265.38 ± 11.05
500	9.52 ± 0.628	14.21 ± 0.692	1730.76 ± 23.51	253.84 ± 21.45
1000	6.24 ± 0.548	12.89 ± 0.923	1634.61 ± 21.01	196.15 ± 18.56

In vitro treatments: Soluble proteins were extracted from the seven day old primary leaves of control plants. An equal amount of protein extract (1 mg mL^{-1}) was treated with various concentrations of SA (1, 2, 5 and 10 mM) in test tubes and kept in ice temperature. Activities of CAT, POX and APX were determined after an incubation period of 6 h, 12 h and 24 h following the methods as described above.

Leaching of peroxidase: Five healthy plants were taken and the roots were kept submerged in 10 mL distilled water. The POX activity that occurred due to root leaching was measured after 1 h following the method as already described.

Presentation of data: Each of the experiments described above were performed at least three times and the mean value is presented along with standard deviations (SD) in tables and figures.

RESULTS

Plant growth and pigment content: Although the retarding effect of SA on the rate of plant growth could be observed during early days of growth the same however, was not remarkably discernible at the later stage of plant growth. A marginal difference in shoot height was noticed between control and 1000 μM SA grown plants. A slight decrease in chlorophyll concentration with increased concentrations of SA was observed in wheat plants. The level of CAR however, remained unchanged with 50 and 500 μM SA. Further increase in SA concentration to 1000 μM showed about 20% decline in CAR concentration (Table 1).

Influence of SA on enzymatic antioxidants: Activity of CAT, the major H_2O_2 scavenging enzyme was found to decline in seven day old plants grown with SA. The decline as compared to control plants was progressive with increase in SA concentration (Figure 1) and was nearly identical both in leaf and root tissues.

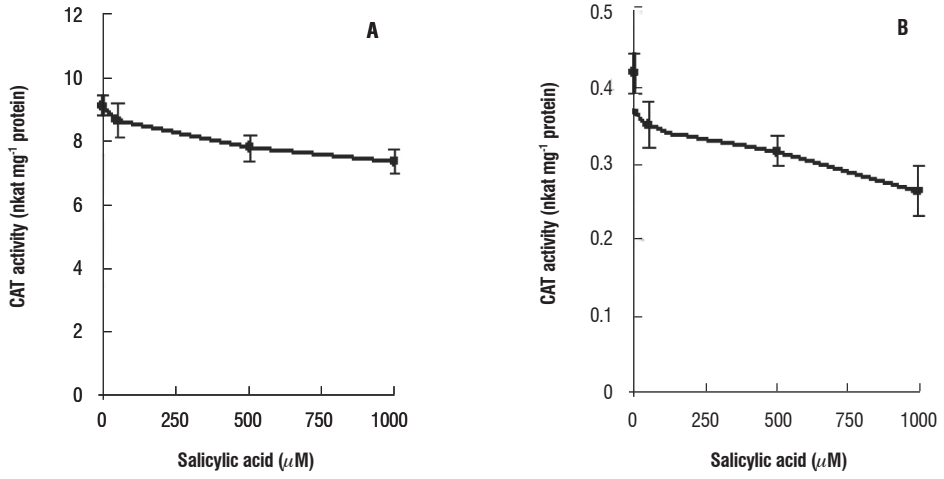

Figure 1. Effect of varied concentration of SA on the activity of CAT in leaf (A) and root (B) tissues of seven day old wheat plant. The enzyme assay was performed in the linear value of enzyme activity with equal amount of protein (10 μg) as described in materials and methods. The mean values were obtained from three independent experiments. The vertical bars in this and subsequent figures represent ± SD.

The enzymatic activity of POX declined significantly both in leaf and root tissues of SA treated plants (Figure 2). The highest decrease was observed in the plants grown with 1000 μM SA. The root tissues though possessed higher POX activity as compared to that of leaf, the relative decrease in activity was almost identical. In root tissues of 1000 μM SA treated plants the activity of the ionically and covalently bound form of POX declined by 45% and 59% respectively in (Figure 3). Both the bound forms of POX remain unaltered in the leaf tissues.

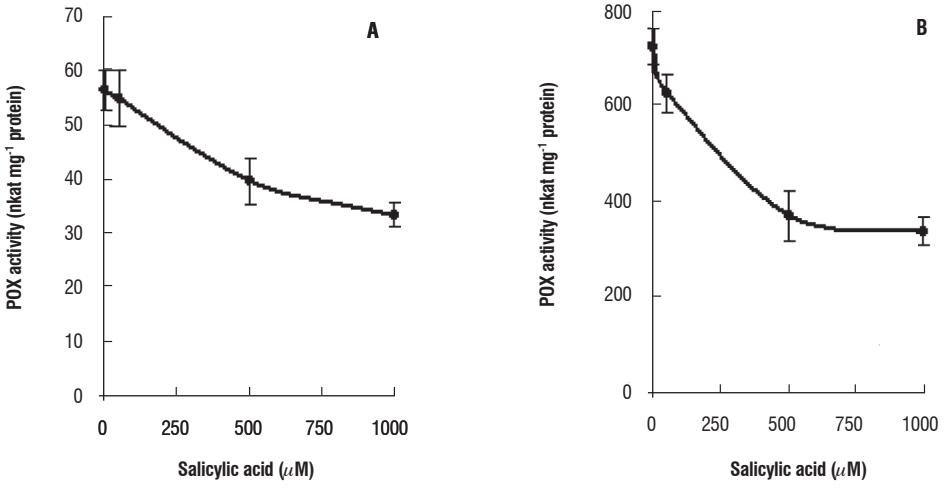

Figure 2. The activity of POX in the leaf (A) and root (B) tissues of seven day old wheat plants grown with different concentrations of SA. The enzyme activity was measured with 10-and 1.5 μg of protein for leaf and root tissues respectively.

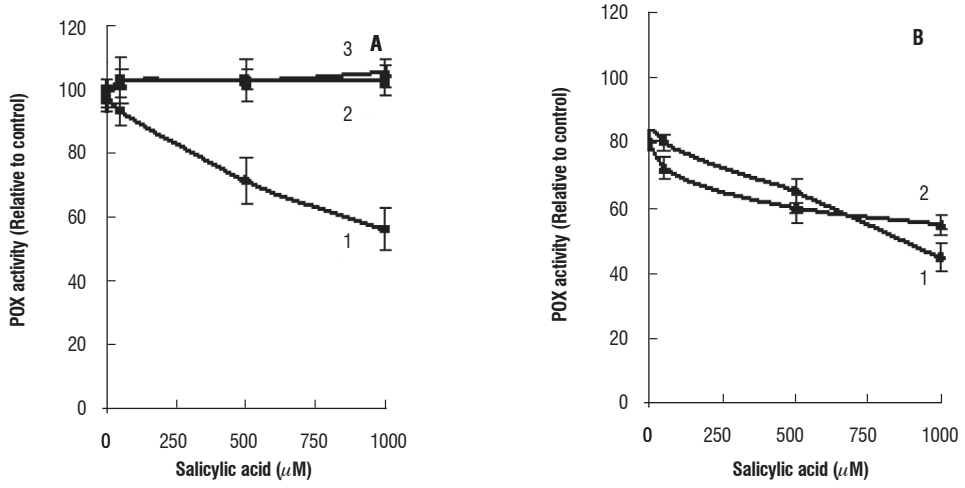

Figure 3. Relative activity of soluble (1), covalently bound (2) and ionically bound (3) POX in leaf tissues of seven day old wheat plants grown with and without SA (A). (B) represents the relative activity of covalently bound (1) and ionically bound POX (2) in root tissues of SA-grown wheat plants.

The activity of APX in plant tissues grown with 50 μM SA, did not show any change. However, the activity declined about 20% in the leaves of wheat plants grown with 500 and 1000 μM SA in comparison to control plants. The decline in APX activity in root tissues was also dependent on SA and a maximum of 35% inhibition (from 165 nkat mg^{-1} protein to 108 nkat mg^{-1} protein) as compared to control was noticed with 1000 μM SA (Figure 4).

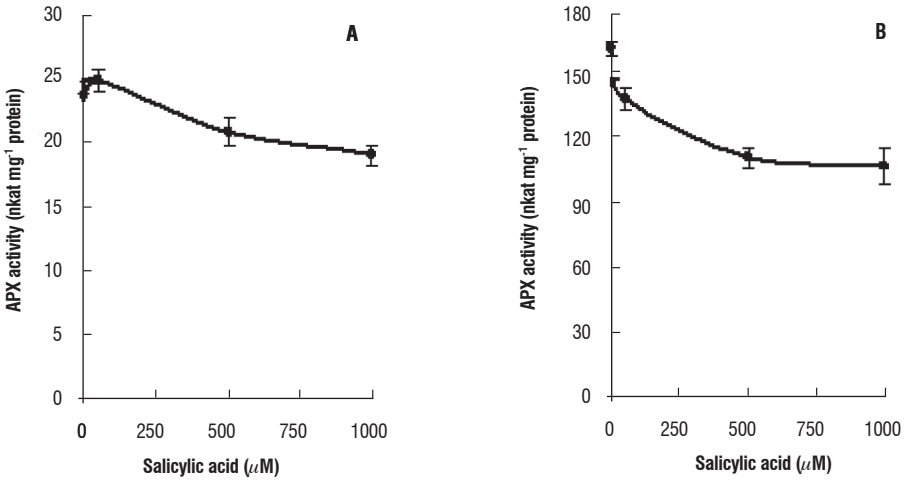

Figure 4. The activity of APX in the leaf (A) and root (B) tissues of seven day old wheat plants grown with different concentrations of SA. The enzyme activity was measured with 150-and 20 μg of protein for leaf and root tissues respectively.

The SOD activity in leaf tissues of wheat plants treated with 50 μM SA remained unchanged. However, the activity of the enzyme increased by 25% and 37% respectively in leaves of plants grown with 500 and 1000 μM SA. The same trend of change in SOD activity was also observed in root tissues of SA grown plants. The increase in SOD activity in root tissues of wheat plants grown with 500 and 1000 μM SA was about 28% and 41% over the control plants (Figure 5).

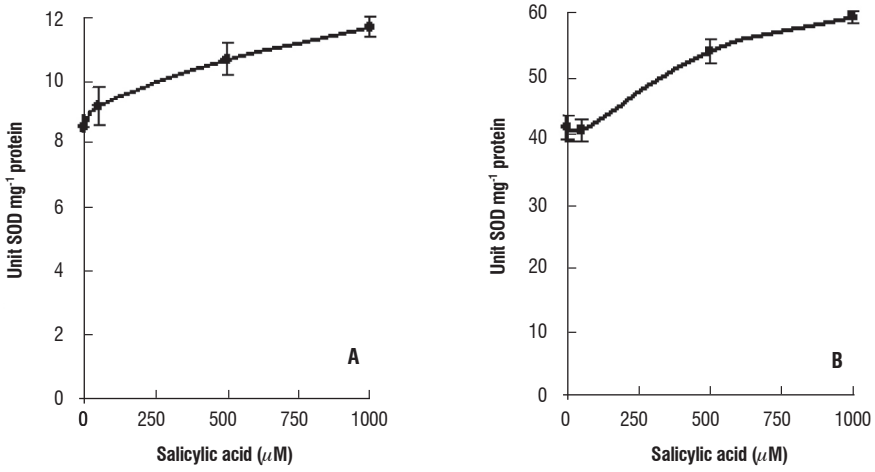

Figure 5. Effect of exogenous SA on SOD activity of leaves (A) and roots (B) of seven day old wheat seedlings. For assay 10 μg of protein was used.

The ASC content of the leaves and roots of control and SA grown-plants remained almost identical (Data not shown).

In vitro influence of SA: When the enzyme extract was incubated with 5 mM SA, the activity of CAT after 12 and 24 h of incubation was observed to be declined by 12% and 35% respectively. With 10 mM SA, the activity of CAT declined by 32% after an incubation period of 6 h. However, the *in vitro* effect of SA on APX and POX was not observed even at 10 mM SA after 24 h incubation (Table 2).

Table 2. *In vitro* effect of high concentration of SA (5 and 10 mM) for long term on the activity of CAT, POX and APX. The values presented are the mean of three independent experiments ± SD. Values in the bracket indicate the relative activity compared to control.

Enzyme	SA (μM)	Incubation period (h)			
		0	6	12	24
		(nkat mg^{-1} protein)			
CAT	0	7.92±0.305 (100)	7.79±0.281 (100)	8.20±0.67 (100)	7.43±1.01 (100)
	5	-	6.25±0.21 (80)	6.95±0.86 (84)	4.89±0.63 (66)
	10	-	5.358±0.71 (68)	6.30±1.11 (77)	4.85±0.47 (65)
POX	0	42.61±2.64 (100)	42.58±3.36 (100)	47.40±0.60 (100)	44.26±2.40 (100)
	5	-	44.39±1.76(104)	47.55±1.07 (100)	46.50±1.81 (105)
	10	-	41.20±1.24 (100)	51.44±1.23 (108)	45.23±2.30 (102)
APX	0	35.43±2.36 (100)	34.42±2.41 (100)	-	34.857±1.5 (100)
	5	-	35.85±1.06 (104)	-	35.00±1.95 (100)
	10	-	36.07±1.03 (104)	-	35.57±1.12 (102)

Leaching of peroxidase: Since a large decline in POX activity was observed in roots and leaves of SA treated wheat seedlings, the leaching of POX from the roots to the external medium was measured. When the activity of the leached POX was compared to control, about 50% decline in activity was observed both in 500 and 1000 μM SA grown wheat plants. The percentage decline in leached POX activity was almost identical to the relative activity of soluble POX both in root and leaf tissues of SA grown wheat plants (Figure 6).

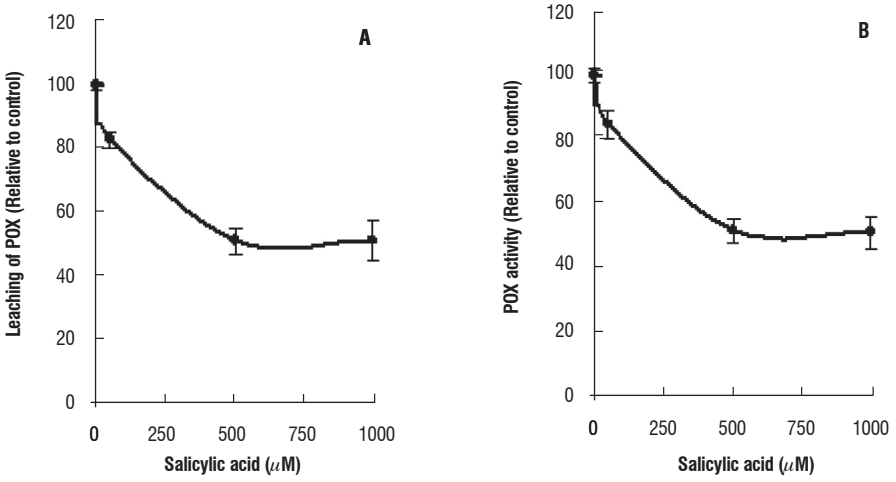

Figure 6. Relative leaching of POX from the roots of seven day old wheat plants grown with varied concentration of SA (A); (B) depicts the relative activity of soluble POX in SA-grown wheat plants.

Influence of SA on lipid peroxidation and H_2O_2 content: No change in MDA content was noticed in leaves and roots of 50 μM SA grown wheat plants. MDA content of the leaves of plants treated with 500 and 1000 μM SA elevated by 26% and 38% respectively over the control plants. However, lipid peroxidation was comparatively more in the roots of these plants than the leaves. The MDA content increased in roots of 500 and 1000 μM SA treated plants by 39% and 61% respectively as compared to control plants (Figure 7).

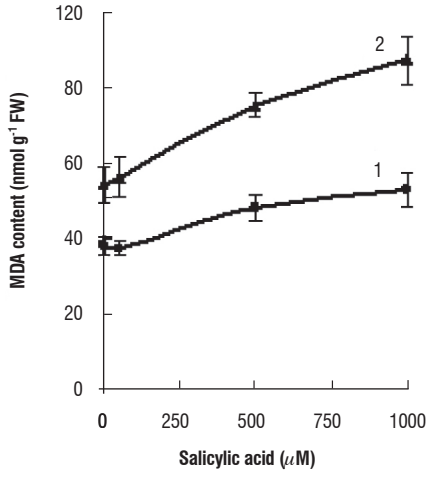

Figure 7. Effect of exogenous SA on MDA content of leaves (1) and roots (2) of seven day wheat seedlings.

The content of H_2O_2 did not show any change both in the leaves and roots of wheat plants grown with 50 μM SA. However, a significant increase in the level of H_2O_2 was noticed in plants treated with higher concentration of SA (Figure 8).

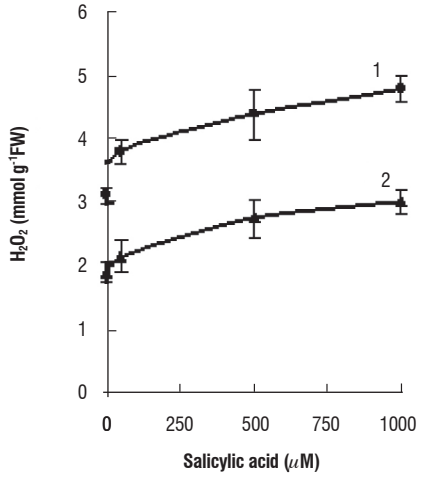

Figure 8. H_2O_2 content measured in the leaf (1) and root tissues (2) of control and SA co-cultivated wheat plants.

DISCUSSION

In the present study, we have shown that treatment of SA at higher concentration affects the growth of wheat plants. The average shoot length of SA treated plants decreased as compared to control plants. Growth of roots of treated plants was strongly affected as compared to shoots. Furthermore, a dose dependent decrease in chlorophyll content was also observed in SA grown plants. The decrease in chlorophyll content and reduced plant growth have been already observed in barley plants treated with SA at the concentration range closely similar to this study (Pancheva et al., 1996).

Exogenous application of SA in heat tolerance, chilling injury, salt and osmotic stress, alleviation of metal toxicity is well known (Dat et al., 1998; Janda et al., 1999; Borsani et al., 2001; Metwally et al., 2003). It has been noticed that exogenous SA can regulate the activities of intracellular antioxidant enzymes such as SOD, POX and increase plant tolerance to environmental stresses (Senaratna et al., 2000; Sakhabutdinova et al., 2004). The effect of SA on the biochemical changes of antioxidants in the *in vivo* condition in the absence of abiotic stress is not studied in detail. The present findings showed that wheat plants grown with different concentrations of SA for seven days recorded a decrease in activity of CAT, POX and APX both in the root and leaf tissues in concentration dependent manner. Inhibition of CAT and APX activity mediated by SA has been observed in the excised leaves of *Arabidopsis* (Rao et al., 1997). Though SA is known to inhibit CAT in many plants, the exact mechanism of inhibition is still not clear. Salicylic acid and other phenolics serve as one electron donor for CAT and POX (Durner and Klessig 1995; 1996) to convert oxidized intermediates in to an inactive and partially reduced state. It has been suggested that SA mediated inhibition of CAT may be due to chelation of heme Fe (Ruffer et al., 1995). Identification of the 240 kDA SA binding protein (SABP) that has CAT activity, binds reversibly with SA and is thus inhibited is assumed to be one of the causes for inhibition of CAT activity (Chen, and Klessig, 1991). Decrement of catalase mRNA level after SA treatment has been reported in barley plants (Zeshuang et al., 1998). The lack of inhibition of CAT by 1 and 2 mM SA tested *in vitro* in the present study indicates that in wheat binding of SA to CAT might not be the exact explanation for inhibition of the activity. In contrast, the reason for the *in vitro* inhibition of CAT at higher concentration of SA (5 and 10 mM) and/or no inhibitory effect on APX and

POX is not known and warrants further study. To explain in another way, SA is known to inhibit heme content of the plant (Molina et al., 1999). Salicylic acid-mediated inhibition of heme containing cytochromes (Cytochrome f_{554}) has been reported in our earlier study (Sahu et al., 2002). These observations together suggest that SA after entering in to the plant probably could be metabolized in to some intermediate compound that is responsible for inhibition of CAT.

The treatment of SA stimulated SOD activity in leaf and root tissues with each of the SA concentration tested. Plant cells regulate H_2O_2 levels by coordinating activities of H_2O_2 generating enzymes such as SOD and H_2O_2 degrading enzymes such as CAT, POX, APX and GR (Creissen et al., 1994). These workers have suggested that CAT inhibition by SA elevates endogenous level of H_2O_2 which result from the oxidative burst associated with the hypersensitive response or from metabolic processes such as photosynthesis, photorespiration and oxidative phosphorylation (Chen et al., 1993). The results in this work have shown that in response to SA-induced inhibition of the H_2O_2 degrading enzymes, there is an increase in H_2O_2 level, the accumulation of which in roots and leaves of wheat plants treated with 500 and 1000 μM SA might be generating oxidative stress as evidenced by the increase in the content of MDA leading to the reduced growth of the shoot and root in these plants. Lipid peroxidation products induced by SA as observed in this study have been noticed in tobacco cell suspension culture (Anderson et al., 1998).

All the three forms of POX (soluble, covalent and ionic bound) declined in root tissues of SA co-cultivated plants. However, the decline in activity of the bound form of POX was not noticed in leaf tissues. The results obtained from the measurement of leaching of POX suggested that probably there is per se decrease in the synthesis level of the enzyme in response to SA. The *in vitro* non-inhibitory effect of SA on POX further substantiated the assumption that SA might not be playing a direct role in inhibiting the activity of these enzymatic antioxidants, the mechanism of which remains to be investigated in detail.

In conclusion, while SA has been reported to be the inducer of various physiological and biochemical effects in plants, it has a negative effect on the growth and the activity of enzymatic antioxidants. The effect of the compound is concentration dependent. It is therefore worth to find out the concentration of SA applied for and the exact role of SA interfering the biosynthesis of these enzymes.

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