Effect of salicylic acid on cabbage (*Brassica oleracea* var. Capitata) grown under salinity stress

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Abstract

The effects of salicylic acid (SA) on growth and metabolism of *Brassica oleracea* var. Capitata under salt stress was studied in a hydroponic culture. NaCl at 50 mM concentration and SA at 0.5, 1.0, and 1.5mM concentrations were used as treatments. The results showed that salt exhibited inhibitory effects on shoot and root length, fresh and dry weight, and RWC of the seedlings. NaCl at 50 mM concentration significantly decreased the photosynthetic pigments and protein content and nitrate reductase activity. Sugar and proline content was significantly increased under the influence of salinity. The antioxidant enzymes viz. superoxide dismutase, catalase, and peroxidase activities significantly increased under salinity due to oxidative damage. Graded concentrations of SA played protective role against the salt stress. SA significantly ameliorates the oxidative stress caused by NaCl.

*Keywords:*cabbage; oxidative stress; proline; RWC; salicylic acid;salt stress

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Introduction

Salinity is one of the major environmental problems which occurs due to irrigation through canals, rivers etc. Salinity has adverse effects on agricultural production. Soil salinity may affect different physiological processes viz. osmoregulation, specific-ion-toxicity, and nutritional disorder (Lauchi and Epstein., 1990). Plants face characteristic biophysical and biochemical changes during stress condition due to salinity (Munn, 2002; Nemato and Sasakuma, 2002). Sensitivity of crops to salinity varies with different phenological stages (Bernstein and Hayward, 1958). Hanegawa et al. (2000) described two different types of salt stress such as hyperosmotic and hyperionic. Hyperosmotic salt stresses reduce water availability of plants and thus altering the water status of the plants while in case of hyperionic type of salt stress, there is a consequence of ion accumulation and subsequent toxicity (Munn and Termaat, 1986; Yao et al. 1991; Munn 1993). The growth and metabolism of plants were affected due to higher concentration of salt present in the soil solution. Salts interfere absorption of essential nutrients (Zeinolabedin Jouyban, 2012; Tester and Evenport, 2003; Cornillon and Palloix,

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1997; Halperin et al., 2003). Flores et al. (2000) reported that plant enzymes may be inactivated under salinity. Adjustment of osmotic effect at the level of cytosol and vacuoles is disrupted (Apse et al., 1999). Nitrogen metabolism is affected under salt stress (Abd-Elbaki et al., 2000; Flores et al., 2000; Carillo et al., 2005). Salinity inhibited ammonium assimilation (Chandra et al., 2001; Khadri et al., 2011) and causes alternation in amino acids pool (Lacerda et al., 2001; Ashraf and Bashir, 2003).

It is well known that plants under the various stressful conditions such as sub-optimal temperature, high light and salinity, and pathogen attacks may generate more reactive oxygen species (Asada, 1991; Yamamoto et al., 2003; Halliwell, 2006; Rhoads, 2006). Under the stress conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (Foyer et al., 2005). Mittler (2002) reported cell death due to ROS which cause membrane damage, metabolic enzymes deactivation, and nucleic acids damage. Plants have an antioxidative defense system to protect themselves from ROS released during oxidative stress condition. Antioxidative enzyme activities viz. SOD, CAT, and POX was found to be enhanced under stress condition (Romero-Romero et al., 2005).

Hayat and Ahmed (2007) have recognized salicylic acid (SA) as a plant hormone. SA plays a vital physiological role on growth and development of plants (Khan et al., 2003). The other role of SA is to buttress the abiotic stress tolerance in plants (Janda et al., 2007). SA counters many abiotic stresses viz. heavy metal (Choudhary and Panda, 2004), salinity (Yusuf et al., 2008), low temperature (Tasgin et al., 2003), and high temperature (He et al., 2005). SA has important roles in flowers induction, nutrients uptake, ethylene biosynthesis (Hayat and Ahmed, 2007), stomatal movement (Larque-Saavedra, 1979), photosynthesis (Fariduddin et al., 2003), and various physiological processes including plant growth (Khan et al., 2003).

The objective of the present study was to evaluate the interactive effect of SA and NaCl on *Brassica oleracea* var. Capitata L. Emphasis was laid on how SA as plant growth regulator buttressed the plant defense system against the salt stress.

Materials and Methods

Plant material and treatments

The certified seeds of *Brassica oleracea* var. Capitata were purchased from certified seed agency of Allahabad, Uttar Pradesh, India. The seeds were sown in nursery beds (1m x1m) for experimental plants in the Department of Botany, University of Allahabad, Allahabad (24°47' and 50° 47'N latitude; 81° 91' and 82° 21'E longitude; 78 m above sea level). The seed bed was irrigated as and when required. After 15 days the seedlings were uprooted and washed with tap water to clean root and then washed with distilled water. The seedlings were transferred at the rate of 10 seedlings per box in transparent plastic boxes (height 9 cm, width 17 cm, length 23 cm) each containing 2L Hoagland solution. Hoagland solution was prepared following the method of Hoagland and Arnon (1950). NaCl at 50 mM (T) and salicylic acid at 0.5 (T_1), 1.0 (T_2) and 1.5 (T_3) mM concentrations were prepared in distilled water and used for treatment. After one week of establishment of the seedlings in Hoagland solution, the nutrient medium was replaced with Hoagland solution (2L/box) containing salt and SA according to the treatment. The seedlings in Hoagland solution without treatment were taken as control. The boxes were covered with black papers to avoid the algal growth. The experimental boxes were fitted with aerating tubes and mouth of each pore of box was plugged with cotton to hold seedlings in vertical position. The experiment was performed in a glass house. Boxes were continuously aerated. Sampling was done after 3 days of treatment for biophysical and biochemical analyses.

Measurement of root and shoot length and fresh and dry weight

Root and shoot length of the seedlings was measured with a metric scale and expressed in centimeters. Fresh and dry weight of the seedlings was recorded on an electronic balance.

The samples were oven dried at 70°C for 72 h and then weighed independently for dry weight (DW) determination. FW and DW were expressed in g per plant.

Relative water content

The leaf samples were cut into small discs, weighed for fresh weight (FW) and were immediately floated on distilled water at 25°C in the dark. After 24 h the turgid weight (TW) of discs was measured and they were dried in oven at 70°C for 48 h for dry weight. The RWC was calculated following Bars and Weatherly (1962) as:

RWC (%) = (FW - DW)/(TW-DW) x 100

Estimation of pigment and protein contents

Chlorophyll of the experimental plants was extracted with 80% acetone. The amount of photosynthetic pigments was determined as per Lichtenthaler (1987). Fresh leaf (10mg) was homogenized in 10 mL of 80% acetone and centrifuged. Supernatant was taken and optical density was recorded at 663nm, 645nm and 470nm. Protein content was determined as per the method of Lowry et al. (1951). The amount of protein was calculated with reference to standard curve obtained from bovine serum albumin.

Nitrate reductase activity

Nitrate reductase (EC 1.6.6.1) activity was assayed by modified procedure of Jaworski (1971) based on incubation of fresh tissue (0.25 g) in 4.5 mL medium containing 100 mM phosphate buffer (pH 7.5), 3% KNO_3,and 5% propanol. About 0.4 mL aliquot was treated with 0.3 mL 3% sulphanilamide in 3 NHCL and 0.3 mL 0.02% N-1-naphthyl ethylene diamine dihydrochloride (NEDD). The absorbance was measured at 540 nm. NR activity was calculated with a standard curve prepared from $NaNO_2$ and expressed as µ mol NO_2 g^{-1} FW h^{-1}.

Sugar content

Sugar content was estimated following Hedge and Hofreiter (1962). About 0.25 g sample was homogenized in 2.5 mL 95% ethanol. After centrifugation, the sugar content was determined in the supernatant. The supernatant (1mL) was mixed with 4 mL of anthrone reagent and heated on boiling water bath for 8 min. Absorbance was taken at 620 nm after rapid cooling. Sugar was quantified with the standard curve prepared from glucose.

Antioxidant enzymes assay

Enzyme extract was prepared by homogenizing 500 mg leaves in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged at 15000 g at 4° C for 30 min. The supernatant was collected and used for analyses of superoxide dismutase (EC 1.15.11), catalase (EC 1.11.1.6), and peroxidase (EC 1.11.1.7).

Superoxide dismutase (SOD) activities were determined by the nitroblue tetrazolium (NBT) photochemical assay method following Beauchamp and Fridovich (1971). The reaction mixture (4 mL) contained 63 µM NBT, 13 mM methionine, 0.1 mM ethylene diamintetra acetic acid (EDTA), 13 µM riboflavin, 0.5 M sodium carbonate, and 0.5 mL clear supernatant. Test tubes were placed under fluorescent lamps for 30 min and absorbance was recorded at 560 nm. One unit of enzyme was defined as the amount of enzyme which caused 50% inhibition of NBT reduction.

Catalase (CAT) activities were assayed as per the method of Cakmak and Marschner (1992). The reaction mixture (2 mL) contained 25 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.2 mL enzyme extract. The activity was determined by measuring the rate of disappearance of H_2O_2 for 1 min at 240 nm and calculated using extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed as enzyme unit g^{-1} fresh weight. One unit of CAT was defined as the amount of enzyme required to oxidize 1 µM H_2O_2 min^{-1}.

Peroxidase (POX) activities were assayed following Mc Cune and Galston (1959). Reaction mixture contained 2 mL enzyme extract, 2 mL sodium phosphate buffer, 1 mL 0.1 N pyrogallol, and 0.2 ml 0.02% H_2O_2 and determined spectrophotometrically at 430 nm. One unit of

Table 1
Effects of salicylic acid on shoot length, root length, fresh weight, and dry weight of cabbage seedlings under salinity

Treatments	SL (cm)	RL (cm)	FW (g/plant)	DW (g/plant)	RWC (%)
C	20.15±0.028	18.9±0.923	3.78±0.145	0.381±0.001	67.78±2.919
T	10.95±0.028[a]	10.9±0.866[a]	2.60±0.002[a]	0.128±0.003[a]	46.78±1.721[a]
T_1	13.65±0.086[a]	14.05±0.028[a]	3.02±0.003[a]	0.166±0.002[a]	52.66±0.025[a]
T_2	14.55±0.144[a]	15.6±0.057[a]	3.20±0.009[a]	0.189±0.002[a]	58.30±0.323[a]
T_3	17.45±0.144[a]	17.85±0.202	3.48±0.155[c]	0.216±0.012[a]	64.68±1.717

Data are mean of three replicates ± SEM. [a]p<0.001, [c]p<0.001 versus C. C: control, T: 50 mM; T_1: 0.5mM, T_2: 1.0 mM, and T_3: 1.5mM concentrations of NaCl and salicylic acid, respectively.

Table 2
Effects of salicylic acid on the pigment contents of cabbage seedlings under salinity

Treatments	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl a+b (mg/g FW)	Carotenoids (mg/g FW)
C	1.410±0.017	1.021±0.078	2.43±0.060	1.150±0.001
T	0.875±0.045[a]	0.531±0.062[a]	1.40±0.017[a]	0.714±0.047[a]
T_1	0.995±0.010[a]	0.699±0.023[a]	1.69±0.033[a]	0.780±0.006[a]
T_2	1.128±0.015[a]	0.986±0.066	2.11±0.050[a]	1.055±0.037[c]
T_3	1.098±0.018[a]	0.742±0.036[b]	1.84±0.054[a]	0.923±0.019[a]

Data are mean of three replicates ± SEM. [a]p<0.001, [c]p<0.001 versus C. C: control, T: 50 mM; T_1: 0.5mM, T_2: 1.0 mM, and T_3: 1.5mM concentrations of NaCl and salicylic acid, respectively.

enzyme activity was defined as the amount which produced an increase of 0.1 OD per minute.

Statistical analysis

Standard errors of means were calculated in triplicates. In addition, analysis of variance was carried out for all the data generated from this experiment, employing one way ANOVA test using GPIS software 3.0 (GRAPHPAD California USA).

Results

Growth of the seedlings under salinity was adversely affected (Table 1). Plant growth was measured in terms of root and shoot length and fresh and dry weight of treated and untreated seedlings. The seedling growth significantly decreased under salt stress as compared with that in combined treatment of NaCl and SA and seedlings of control group. Decrease in the RL, SL, FW, DW, and RWC of seedlings was 42, 45, 31, and 30%, respectively under salinity as compared with control. Maximum growth was recorded in control group. Plants treated with NaCl alone exhibited maximum reduction in growth while others treated with combined treatment of NaCl+SA showed moderate effect on growth. Exogenously applied SA mitigated the effect of salt stress in dose dependent manner. Growth was improved from lower concentration (0.5mM) to higher concentration (1.5 mM) of SA in combination with NaCl. RWC in the seedlings treated with NaCl (50mM) significantly ($p<0.001$) decreased. Treatment T possed maximum value of leaf water potential as compared to other treatments (T_1, T_2, T_3) and control. NaCl in combination with different concentrations of SA showed improved value of leaf water potential in dose dependent manner. The reduction in RWC diminished sharply with the supply of SA from 0.5 to 1.5mM.

The amount of photosynthetic pigments content significantly decreased under NaCl stress (Table 2). The maximum decrease in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids was 37, 47, 42, and 37%, respectively under salt stress as compared with control. Moderateenhancement of 25, 20, 24, and 23% in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, respectively was recorded under highest concentration 1.5mM of SA.

Table 3
Effects of salicylic acid on sugar and protein content and NR activity of cabbage seedlings under salinity

Treatments	Sugar (mg/g FW)	Protein (mg/g FW)	NR (μmol NO_2 g^{-1} FW h^{-1})	Proline (μmol g^{-1} FW)
C	62.14±0.291	56.05±0.173	70.27±0.072	0.243±0.002
T	102.08±0.29 [a]	45.87±0.447[a]	65.48±0.595[a]	0.538±0.002 [a]
T_1	70.49±0.729 [a]	57.03±0.043	69.33±0.036[c]	0.493±0.006 [a]
T_2	69.47±0.729 [a]	61.33±0.794[a]	71.30±0.126[c]	0.455±0.006 [a]
T_3	64.92±0.729 [c]	59.91±0.115[a]	71.02±0.072	0.417±0.001 [a]

Data are mean of three replicates ± SEM. [a]p<0.001, [c]p<0.001 versus C. C: control, T: 50 mM; T_1: 0.5mM, T_2: 1.0 mM, and T_3: 1.5mM concentrations of NaCl and salicylic acid, respectively.

Table 4
Effects of salicylic acid on antioxidant enzyme activity of cabbage seedlings under salinity

Treatments	SOD(EU g^{-1} FW)	CAT(EU g^{-1} FW)	POX(EU g^{-1} FW)
C	60.55±0.225	27.83±0.038	60.35±0.404
T	66.67±0.537[a]	47.45±0.788[a]	75.62±0.014[a]
T_1	62.31±0.066[a]	43.42±0.038[a]	71.47±0.129[a]
T_2	58.85±0.374[a]	41.39±0.019[a]	68.27±0.447[a]
T_3	57.36±0.121[a]	42.15±0.076[a]	71.27±0.967[a]

Data are mean of three replicates ± SEM. [a]p<0.001, [c]p<0.001 versus C. C: control, T: 50 mM; T_1: 0.5mM, T_2: 1.0 mM, and T_3: 1.5mM concentrations of NaCl and salicylic acid, respectively.

A significant (p<0.001) decrease in protein content was recorded under salinity (Table 3). Maximum 39% reduction in protein was observed in NaCl treatment alone as compared with control. SA in different concentrations viz. from 0.5 to 1.5mM significantly improved the protein content as compared with control. Maximum 6% improvement was recorded in T_3 treatment as compared with treatment with NaCl alone. The significant increase in carbohydrate content was recorded under saline condition. Maximum 39% accumulation in sugar content was recorded in salt treatment alone. SA had moderate effect on sugar content in dose dependent manner.

The nitrate reductase (NR) activity in the leaves of salt treated cabbage seedlings was adversely affected. NR activity significantly decreased under salinity. Maximum inhibition of 6% was recorded in 50mM concentration of salt alone as compared with control. SA in various concentrations improved the NR activity in leaves of the seedlings. NR activity was maximally improved under 1.5mM concentration of SA. Seedlings under control group had maximum NR activity.

In the present study, a significant enhancement in proline content was recorded under salinity. In plants, under saline condition proline accumulation is one of the most frequent modifications induced by stress which was involved in stress resistance mechanisms. Maximum of 55% stimulation was recorded under NaCl alone while SA in different concentrations minimized the accumulation of proline content.

The increased activity of antioxidant enzymes viz. SOD, CAT, and POX were prevalent in the oxidative damage caused by NaCl stress (Table 4). The activity of SOD increased significantly (p<0.001) in response to salt stress alone while in combination with SA activity of SOD was comparatively lower than the salt treatment. The seedlings under combined treatment of salt and different concentrations of SA showed significant alteration in SOD activity to minimize the oxidative damage as compared with salt. CAT activity was stimulated in single NaCl treatment as compared with control. Maximum 2 folds increase in activity of CAT was recorded under salinity while significant changes in CAT activity in combined treatments were observed. SA had moderate effect on CAT activity with maximum improvement of 1fold in T_3 treatment. POX activity had an important role in salt tolerance in plants. The POX activity was

significantly higher under saline condition to tolerate the oxidative damage and survival of the plants. Maximum 1fold POX activity was recorded in treatment T while combined treatment of salt and SA lowered the activity of POX as compared with salt treatment.

Discussion

The plants exposed to salinity exhibited drastic change in biophysical and biochemical parameters. Inhibition of plant growth is a common feature under saline condition (Munns, 2002; Ruiz et al., 2005; Abbas et al., 2010; Akram et al., 2012). Our results showed significant overall reduction in all parameters under salinity. Salinity inhibited root and shoot growth in *Brassica* (Jamil et al., 2006; Neumann, 1995). Reduction in biomass under saline condition is also observed by Jeannette et al., (2002) and Keshavarzi (2011). Amiri et al. (2010) recorded decreased dry weight in *Cynara scoolymus* and *Echinacea purpurea* under saline condition. Exogenously applied SA increased dry weight and plant growth under salinity (Gunes, 2007; Wang et al., 2007). The decreased photosynthetic pigments under saline stress may be due to inhibited biosynthesis of the pigments. The increased activity of chlorophyllase enzyme under salinity reduced the photosynthetic pigment content (Yasar et al., 2008; Noreen and Ashraf, 2009; Kusvuran, 2010; Nazarbeygi et al., 2011). Plant growth regulator diminished the adverse effect of NaCl and enhanced the pigment content (Khodary, 2004; Shi et al., 2006; Alsokari, 2009; Zeid, 2011). Our results also showed enhancement in pigment content by application of SA.

The amount of carbohydrate significantly increased when plants were exposed to salinity (Munns, 1993). The results are in agreement with Sirigam et al. (2011) who reported increased sugar content in *Oryza sativa* under saline condition. Under abiotic stress condition sugar serves as a signaling molecule to avoid the oxidative damage (Hoekstra et al., 2001). Niazietal (2005) and Jat and Sharma (2006) reported improved value of sugar under salinity due to application of plant growth regulators. Our results showed the significant decrease in protein content under salinity which is in conformity with that of *Dioscorea rotundata* (Jalal et al., 2008), rice (Amirjani, 2010) and tomato (Doganlar et al., 2010). Tammam et al. (2008) reported decreased protein content in *Triticum avesticum* under salinity. SA has positive effect when used in combination with NaCl. SA improved the protein content under salinity. In *Hordeum vulgare*, increased protein content was reported due to application of plant growth regulators (Sarwat and El-Sherif, 2007). NR activity was determined in leaves of the seedlings to demonstrate the effect of SA on nitrate assimilation under salinity. Results showed that NR activity significantly decreased under NaCl stress. Decreased NR activity under salinity is documented in cashew (Viegas et al., 1999) and tomato (Debouba et al., 2007). Whereas, in combined treatment with SA, the activity of NR slightly enhanced. Similar result was reported by Singh et al. (1997).

Proline is one of the important components of the defense system of plants to mitigate stress. In our results, proline increased under salt stress. Mahajan and Tuteja (2005) suggested that accumulation of proline is beneficial under stress condition. Increased proline under NaCl stress is also reported in alfalfa (Mezni et al., 2010), eggplant (Abbas et al., 2010), *Capsicum annum* (Chookhampaeng, 2011), sunflower (Akram et al., 2012), okra (Saleem et al., 2012), and pea (Noreen and Ashraf, 2009). Whereas, the exogenous supply of SA significantly decreased accumulation of proline under NaCl (Sakhabutdinova et al., 2003; Gautam and Singh, 2009). The researchers have also reported similar results in combined treatment of NaCl+SA.

Antioxidant defense system contains a variety of non-enzymatic and enzymatic antioxidants. SOD, CAT and POX are the key enzymes of defense system which play an important role in oxidative defense mechanism (Ashraf, 2009; Sabir et al., 2011; Akram et al., 2012). The increased antioxidant enzyme activities indicate a protective mechanism against oxidative damage under salinity. SOD activity enhanced under NaCl stress to avoid the oxidative damage caused by ROS (Sabir et al., 2011; Abbaspour 2012). Increased activity of CAT has been found under NaCl stress. Similar results

were also reported in soybean (Comba et al., 1998), tobacco (Bueno et al., 1998), cucumber (Lechno et al., 1997), mulberry (Sudhaker et al., 2001), and rice (Swapna, 2003). In our study we observed that CAT activity improved significant level under combined treatment of salt + SA. POX activity has played an important role in salt tolerance mechanism in plants. Increased activity of POX was observed and it has been reported in wild beet (Bor et al., 2003) and in tomato (Mittova et al., 2000).

Conclusions

The present study gives sufficient evidence to conclude that the plant growth is significantly suppressed under salinity. Results clearly evince the positive interaction between SA and salt which significantly influenced the plant growth and metabolism. Salicylic acid, as plant hormone, plays an important role to protect the plant from oxidative damage prevalent in salt stress. SA has potential to help stressed *Brassica oleracea* var. Capitata tolerate the adverse effect of salt. Thus SA has buttressed the defense system of the cabbage seedlings subjected to salt stress.

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