



Salicylic Acid Induced Photosynthetic Adaptability of *Raphanus sativus* to Salt Stress is Associated with Antioxidant Capacity

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Abstract

Despite the plethora of published reports on ameliorative effects of exogenously applied salicylic acid (SA) to plants under salt stress, a critical role of SA in redox balance, photosynthetic and electron transport in mediating salt tolerance in plants is still ignored. The present study was aimed to assess the beneficial effects of SA on photosynthetic electron transport in five radish cultivars during salt stress which may translate into protection from salt-induced oxidative damage. Seeds of five radish cultivars (Red Neck Purple, Early Long White, Minnu Radish, Radish 40-day and Gong Swang Radish) were allowed to germinate for five days after which seedlings were sprayed with different concentrations of SA (0, 2 and 5 mM). After 48 h of SA treatment, seedlings were subjected to salt stress (0, 100, and 200 mM NaCl). Salt stress cause reduction in biomass, chlorophyll contents and PSII photochemistry in all five radish cultivars. However, salt stress causes an increase in proline content, lipid peroxidation, ROS content and antioxidant enzymatic activity. Foliar spray of SA downregulated the absorption, trapping and photosynthetic electron transport fluxes while it increased dissipation flux consequently improving photochemistry of most of the radish cultivars under salt stress. This Improved photochemistry and enhanced antioxidant enzymes activity of radish cultivars by SA application resulted in lower ROS generation and membrane damage. Greater accumulation of compatible solutes due to foliar spray of SA might have additional protective effects on photosynthetic machinery by lowering oxidative stress. Moreover, ameliorative effects of SA were cultivar specific. The study suggested that SA could mitigate the detrimental effects of salt stress by regulating physiological and biochemical mechanisms in radish plant.

Keywords Photochemistry · JIP-test · *Raphanus sativus* L. · Salicylic acid · Salt stress · Reactive oxygen species · Photosynthesis

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Introduction

Salinity is one of the most devastating environmental stresses that reduce crop productivity. Salt stress reduces plant growth by osmotic effect and ionic effect that disturb ion homeostasis (Athar and Ashraf 2009; Zafar et al. 2017). A number of studies suggested that growth yield reduction under saline conditions were associated with secondary effects of salt stress such as oxidative stress, reduction in photosynthesis (Athar et al. 2015; Munns and Tester 2008). Salt-induced osmotic stress caused biosynthesis of ABA and subsequently causes stomatal closure (Munns 2002). At high salt stress, salt may accumulate in apoplast or in cytoplasm or in chloroplast and thus directly affects photosynthetic metabolism (Ben-Rouina et al. 2006; Bose et al. 2017). Similarly, salt stress cause imbalance in generation and consumption of reducing equivalents

(NADPH and ATPs) in chloroplast during chloroplastic electron transport and in Calvin Cycle, respectively (Ogbaga et al. 2018). This results in production of reactive oxygen species (ROS) at acceptor end of PSI thereby causing membrane damage (Foyer and Shigeoka 2011; Foyer et al. 2017). In order to protect plant cellular machinery, antioxidative defense system becomes activated to scavenge ROS. Some studies suggested that enhancing non-enzymatic antioxidants or up-regulation of antioxidant enzymes may protect the plants from salt-induced-oxidative damage or improved salt tolerance in different crop species such as in *Triticum aestivum* (Arfan et al. 2007; Athar et al. 2008), *Torreyia grandis* (Li et al. 2014), and *Vigna radiata* (Khan et al. 2014). Exogenous application of different antioxidants compounds can enhance growth of plants under salt stress by increasing antioxidant enzymatic activities, accumulation of osmolytes and improving plant photosynthetic capacity (Ashraf et al. 2010; Plaut et al. 2013).

Salicylic acid is an important plant growth regulator that modulates plant growth and development under normal or abiotic stress conditions by affecting stomatal conductance, activities of antioxidant enzymes, and nutrient uptake (Ashraf et al. 2010; Noreen et al. 2013). In addition, SA is one of the important signaling molecules which trigger variety of biochemical pathways responsible to counteract abiotic stresses including salt stress. In contrast, some reports are also available in which no positive association was found between activities of antioxidant enzymes and degree of salt tolerance (Munns and Tester 2008). For example, mutants of *Arabidopsis thaliana* lacking functional chloroplastic and cytosolic antioxidant enzymes were more salt tolerant than wild type plants which showed greater antioxidant potential (Miller et al. 2007). It is suggested that salt tolerance of a crop species may depends on interactive effect of stomatal conduction for CO₂ fixation and photosynthetic electron transport to avoid photoinhibition which leads to alter in activities of antioxidants. This aspect is generally ignored in most of the studies, particularly when assessing SA-induced salt tolerance in plants.

Radish (*Raphanus sativus* L.), belongs to *Brassicaceae* family, is moderately salt sensitive or moderately salt tolerant root vegetable (Sonneveld 2000). The present study was aimed to assess as to whether SA could efficiently alleviate the detrimental effects of salt stress on *Raphanus sativus* L. and up to what extent exogenously applied SA has a role in redox balance, photosynthetic electron transport, and ion transport in mediating salt tolerance.

Materials and Methods

Plant Material and Growth Conditions

A pot experiment involving five cultivars of *Raphanus sativus* L. [Red Neck Purple (RNP), Early Long White (ELW),

Minnu Radish (MR), 40 Day Radish (40-D), and Gong Swang Radish (GSR)] was conducted at the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, under the following controlled conditions: Day/Night temperature: 22/18 ± 2 °C, photoperiod: 16 h and humidity: 55–60%. Briefly, plastic pots were filled with sand and four to five seeds/pot were sown and watered with Hoagland nutrient solution. Three days after germination, two same sized plants were kept in each pot after thinning. At 5th day, SA 0 mM (mock spray with distilled water only), 2 and 5 mM (SA was dissolved initially in 70% ethanol and the concentrations of 2 and 5 mM were made up with distilled water along with 0.01% Tween-20) was applied exogenously by foliar spray. Salt treatment was started post 48 h of SA spray. Two NaCl treatments 100 and 200 mM were given for 10 days. Treatment was started from 50 mM and increased gradually to reach the desired concentration. After 24 h of last salt treatment, samples were collected for further analysis. Plants were carefully uprooted from pots and were separated into roots and shoots. Fresh weight of roots and leaves were evaluated on digital scale while length of roots was recorded by using measuring scale. For dry weight, samples were oven dried at 65 °C for 15 days.

Photosynthetic Components

PSII Photochemistry

The maximal efficiency of PSII photochemistry was monitored by fast chlorophyll a fluorescence kinetic analysis using Flour-Pen-FP-100 (PSI, CZ). The plants were placed in dark for 30 min at the room temperature and the light intensity of 3000 μmol m⁻² s⁻¹ was implicated on the leaf area of 5 mm. The initial fluorescence was observed at O-step (50 μs) then on intermediate steps as K, J, I (300 μs, 2 ms, 20 ms) and the maximum fluorescence was observed at P-step at 300 ms respectively. Parameters that were calculated: $F_v/F_m = (F_m - F_o)/F_m$, PIABS = (RC/ABS) [φPo/(1 - φPo)] [ψEo/(1 - ψEo)], ABS/RC = Mo (1/VJ) (1/φPo), DIo/RC = (ABS/RC) - (TRo/RC), ETo/RC = Mo (1/VJ) ψo, and TR/RC = Mo (1/VJ; Cessna et al. 2010).

Chlorophyll and Carotenoid Contents Estimation

Chlorophyll and carotenoids contents were measured using the protocol of Arnon (1949). Briefly, 0.2 g fresh leaves were grinded in 10 ml of 80% pure acetone. The homogenate was filtered and centrifuged for 15 min at 14,000 rpm, 4 °C. The optical densities of Chlorophyll a, b, and carotenoids were measured using spectrophotometer at 645 nm, 663 nm, and 480 nm respectively.

$$\text{Chl a mg/g F.W} = [12.7(\text{OD}_{663}) - 2.69(\text{OD}_{645})] \times V/1000 \times W$$

$$\text{Chl b mg/g F.W} = [22.9(\text{OD}_{645}) - 4.68(\text{OD}_{663})] \times V/1000 \times W$$

$$\text{Carotenoids mg/g F.W} = [(1000 \times \text{OD}_{470}) - (1.91 \times \text{Chl a}) - (95.15 \times \text{Chl b})]/198$$

W (weight of fresh leaves in g), V (volume of the extract).

Anthocyanin Content Determination

Anthocyanin contents were determined following the protocol of Wagner (1979). Briefly, 0.2 g of fresh leaves were homogenized in pestle mortar with 3 ml solution of 1% methanol:HCl (99:1 v/v). The homogenate was covered with aluminum foil and was placed in dark at 4 °C for 24 h. It was then centrifuged at 3000 rpm for 6 min. The concentration of anthocyanin in the supernatant was quantified by optical density at 550 nm. Anthocyanin concentration was evaluated using molar extinction coefficient $33,000 \text{ cm}^{-2} \text{ mol}^{-1}$.

Proline Estimation

The accumulation of compatible osmolyte proline was determined by using protocol described by Bates et al. (1973). 0.1 g fresh leaf samples were homogenized in 3% sulfosalicylic acid (5 ml) and were filtered using filter paper (Whatman's No. 2). Equal volume of glacial acetic acid and acid ninhydrin were added in 2 ml of filtrate. The reaction mixture was placed in water at 100 °C for 1 h and then was placed in an ice box to cool down. An addition of toluene (2 ml) was made in the mixture and stirred for 15–20 s. The chromophore layer which contains toluene was isolated and the absorbance was measured at 520 nm taking toluene as blank. The concentration of proline was assessed using proline standards (10 mg/100 ml). The following formula was used for estimation of proline:

$$\mu\text{moles per g tissue} = [(\mu\text{g proline/ml} \times \text{ml toluene})/115.5] \times 5/\text{g sample.}$$

Lipid Peroxidation Estimation

Salinity induced oxidative damage to membrane, also known as lipid peroxidation, was assessed by Cakmak and Horst (1991) protocol with slight modifications. 0.2 g fresh leaves were homogenized in pestle mortar with 0.1% (w/v) trichloroacetic acid (TCA) solution (1 ml) and then centrifuged for 15 min at $2000 \times g$. 0.5% thiobarbituric acid (TBA) was prepared in 20% TCA solution. 1 ml of TBA was mixed in 0.5 ml of supernatant. This reaction mixture was heated in shaking water bath at 95 °C for 50 min and then stopped by cooling the tubes in ice box and was centrifuged for 10 min at $12,000 \times g$. Optical density of supernatant was measured at 532 and 600 nm. Lipid peroxidation was evaluated using

$156 \text{ mmol}^{-1} \text{ cm}^{-1}$ (molar extinction coefficient) with the help of following formula:

$$\text{MDA (nmol)} = \Delta(A_{532 \text{ nm}} - A_{600 \text{ nm}})/1.56 \times 10^5$$

ROS Estimation

Reactive oxygen species (ROS) in plant tissues are produced under normal oxygen metabolism. However, dramatic increase in ROS level is observed under stress conditions which can be measured by assessing the amount of hydrogen peroxide (H_2O_2) using protocol of Velikova et al. (2000). 0.2 g fresh leaves were grinded in 0.1% (w/v) TCA solution (2 ml) using pre-chilled pestle mortar and this mixture was centrifuged for 15 min at 9000 rpm. Then, potassium iodide (KI; 0.2 ml) and potassium phosphate buffer of pH 7.0 (0.1 ml) were added in 0.1 ml of supernatant. This mixture was vortexed for 1 min and its absorbance was taken at 390 nm. The concentration of H_2O_2 was interpreted using a standard curve by known concentration of H_2O_2 .

Enzymatic Antioxidants Estimation

Enzymatic antioxidants were done by using protocol of Ananieva et al. (2004). 0.2 g fresh leaves were grinded in pestle mortar using 50 mM pre-chilled potassium phosphate buffer of pH 7.8 (2 ml). The homogenate was centrifuged at 4 °C and $15,000 \times g$ for 20 min. The resulting supernatant was further used for assays. The activity of SOD was assessed using the protocol of Giannopolitis and Ries (1977). Reaction mixture contains: 4 ml distilled water, 2.5 ml of 50 mM potassium phosphate buffer pH 7.8, 1 ml of 13 mM L-methionine, 1 ml of 0.1% (v/v) triton-X-100, 0.5 ml of 1.3 mM riboflavin, 0.5 ml of 50 mM NBT, and 0.5 ml of enzyme extract. The mixture was placed under florescent light of 30 W for 15 min and reaction was stopped by turning off the lights for 10 min. The optical density of this irradiated mixture was determined at 560 nm taking reaction mixture without enzyme extract as a blank. The activity of SOD was assessed by using the standard curve made by known concentrations of NBT. The activity of catalase enzyme was evaluated using the protocol of Chance and Maehly (1955) with few modifications. Reaction mixture of CAT contained: 450 μl of 5.9 mM H_2O_2 , 1 ml of 50 mM potassium phosphate buffer (pH 7.0), and 50 μl of enzyme extract (added at the time of measuring OD). H_2O_2 decomposition changes the OD value of mixture and measured at 240 nm after every 20 s up to 1 min. The catalase activity was assessed by using molar extinction coefficient of H_2O_2 which is $36 \text{ M}^{-1} \text{ cm}^{-1}$ expressed in $\mu\text{mol min}^{-1}$. 1 unit activity of catalase is defined as the change in the absorbance of 0.01 units min^{-1} . The activity of peroxidase enzyme was assessed by oxidation of guaiacol defined as the

change in absorbance of 0.01 units per min using protocol of Chance and Maehly (1955). Reaction mixture contained: 200 μl of 20 mM guaiacol, 250 μl of 40 mM H_2O_2 , 1 ml of 50 mM potassium phosphate, and 50 μl of enzyme extract. The absorbance of reaction mixture was taken at 470 nm after every 20 s up to 2 min. The activity of POD was evaluated by using $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (molar extinction coefficient) expressed in $\mu\text{mol}/\text{min}$.

Statistical Analysis

All the data were statistically analyzed to determine significant variation between different factors using Statistical Software, COSTATv.6.3 (Cohort Software, Berkley, California). Means comparison was done by least significant difference (LSD) test. The statistical analysis was performed on raw measurement values, not on the fold change values.

Results

Physiological Parameters

Salt stress significantly decreased the fresh and dry weights of leaves and roots of all five radish cultivars and exogenous treatment of SA significantly improved the biomass of leaves and roots under saline and non-saline conditions (Table 1, Figs. 1a–d, 2, Supplementary Table 1). In addition, application of 2 mM SA was more effective in improving these growth attributes of all radish cultivars under salt stress, particularly at 200 mM NaCl. This is also confirmed from the graphs of fold change for fresh and dry weights of leaves and roots (Fig. 1a–d). The fold change values for 2 or 5 mM SA and 100 or 200 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA})/(\text{Control})]$ and $[(100 \text{ or } 200 \text{ mM NaCl})/(\text{Control})]$ respectively, whereas all the fold change values for 2 or 5 mM SA at 100 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA at } 100 \text{ mM NaCl})/(100 \text{ mM NaCl})]$ and for 2 or 5 mM SA at 200 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA at } 200 \text{ mM NaCl})/(200 \text{ mM NaCl})]$. The fold change values for control (0 mM NaCl, 0 mM SA) is taken as 1.00. The response of each cultivar was significantly different to SA application under salt stress. From Fig. 1a–d, it is apparent that 2 mM SA application particularly at highest salt treatment (200 mM NaCl) had maximum increasing effect on fresh and dry weights of leaves of cv. GSR (2.80-fold) and cv. MR (2.89-fold), respectively. Similarly, the exogenous application of 2 mM SA at 200 mM NaCl increased fresh and dry weights of roots of cv. MR (5.12-fold) and cv. ELW (3.67-fold), respectively. Salt stress also reduced root lengths of all five radish cultivars and SA application particularly 2 mM significantly increased the length of roots (Table 1, Fig. 1e, Supplementary Table 1). However, no

varietal difference was observed among cultivars. Overall, 2 mM SA was found more effective to protect from the damaging effect of higher salt concentration (200 mM) for plant biomass attributes and prominent cultivar differences were observed expect for root length.

Biochemical Parameters

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoids contents were significantly decreased due to salt stress in all cultivars. Foliar application of SA, particularly 2 mM, significantly enhanced these photosynthetic pigments in the leaves of all five radish cultivars under saline and non-saline conditions (Table 1, Fig. 3a–c, e, Supplementary Table 2). From Fig. 3a–c and e, it is apparent that the improving effect of 2 mM SA at 200 mM NaCl on chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents was maximum in cv. ELW (2.26-fold), cv. RNP (2.07-fold), cv. RNP (2.17-fold), and cv. ELW (3.08-fold), respectively. However, effect of SA application in terms of carotenoids/chlorophyll ratio was non-significant under non-saline conditions (Table 1, Fig. 3f, Supplementary Table 2). In contrast, anthocyanin pigments increased with salt stress while SA treatment decreased anthocyanin pigments under non-saline conditions; however, a significant increase was observed in anthocyanin pigments in all radish cultivars, particularly with 5 mM SA application at 100 mM NaCl (Table 1, Fig. 5c, Supplementary Table 2). The response of each cultivar was similar to SA application under salt stress.

Salt-induced reduction in maximum quantum yield of photosystem II (F_v/F_m) and performance index (PI_{ABS}) of different radish cultivars was alleviated by exogenously applied SA (2 & 5 mM SA) under saline and non-saline conditions (Table 1, Fig. 4a, b, Supplementary Table 3). The more pronounced effect was observed by 2 mM SA at highest salt stress (200 mM NaCl). Foliar spray of 2 mM SA at 200 mM NaCl had maximum increasing effect on quantum yield of PSII and PI_{ABS} of cv. ELW (1.14-fold) and cv. GSR (4.57-fold), respectively (Fig. 4a, b). The light absorption flux per reaction center (ABS/RC), trapping energy flux per reaction center (TRo/RC) and energy flux for heat dissipation per reaction center (DIO/RC) increased significantly in all radish cultivars with increasing NaCl concentration, whereas energy flux for electron transport further than Q_A (ETo/RC) decreased with salt stress. However, exogenous foliar spray of SA specifically 2 mM SA significantly decreased the ABS/RC , TRo/RC and DIO/RC , while it increased ETo/RC in salt stress plants of radish cultivars (Table 1, Fig. 4c–f, Supplementary Table 3). The response of radish cultivars to SA treatment under saline condition was significantly different. For example, exogenous application of 2 mM SA at 200 mM NaCl caused a greater decrease in ABS/RC , TRo/RC and DIO/RC of cv. 40-D (0.65-fold),

Table 1 Mean square values from analysis of variance (ANOVA) of data for leaf & root fresh & dry weight, root length, chlorophyll pigments, carotenoid content, proline content, anthocyanin, H₂O₂, MDA, activities of antioxidant enzymes, and different parameters of PSII offive radish (*Raphanus sativus* L.) cultivars treated with varying salicylic acid concentration grown for 21 days under normal and varying saline conditions

Source of variation	df	Leaf F.W	Leaf D.W	Root F.W	Root D.W	Root length	Proline
Salt	2	1.3403***	0.0097***	0.0112***	0.004135***	361.404***	1041.6884***
Varieties	4	0.0911***	0.00049***	0.0018***	0.000324***	36.369***	3046.9792***
SA	2	0.4704***	0.0061***	0.0068***	0.002625***	183.246***	8705.4785***
Salt * Var	8	0.0247***	0.0000378**	0.0003769***	0.0000362***	7.3145***	374.2822***
Salt * SA	4	0.0114***	0.0001015***	0.0001154**	0.0000119*	2.0115*	1929.908***
Var * SA	8	0.0083***	0.000174***	0.0002549***	0.0000431***	1.0015 ns	69.1732***
Salt * Var * SA	16	0.0044***	0.000034***	0.0001.7385***	0.00001.93***	1.3242 ns	55.2546***
Error	90	0.00101	0.00000658	0.000005087	0.00000183	0.7727	9.0941
Source of variation	df	Chl a	Chl b	Chl a/b	Total Chl	Carotenoids	Caroten/Chl
Salt	2	1.21527***	0.25663***	0.14416*	2.54197***	66.7493***	0.99183***
Varieties	4	0.69345***	0.05532***	0.84504***	1.12784***	18.8541***	2.14530***
SA	2	2.05771***	0.34033***	0.42432***	4.09518***	97.0761***	0.25734 ns
Salt * Var	8	0.02814***	0.01088***	0.17544**	0.05266***	2.67181**	0.97846***
Salt * SA	4	0.02269***	0.01593***	0.13517*	0.07219***	5.55971***	1.93860**
Var * SA	8	0.05416***	0.01179***	0.47660***	0.08917***	2.31175**	0.56056***
Salt * Var * SA	16	0.01641***	0.00829***	0.21764***	0.04395***	3.82015***	0.76159**
Error	90	0.00126	0.00029	0.00717	0.00278	0.09936	0.01990
Source of variation	df	Anthocyanin	MDA	ROS	POD	CAT	SOD
Salt	2	6.41210***	10,268,572***	0.107080***	138.119***	0.00239 ns	10.5334 ns
Varieties	4	0.29984***	261,621.57***	0.002354***	252.202***	0.46569***	2510.84***
SA	2	0.04823***	7,178,286.8***	0.124714***	303.446***	3.91749***	7699.26***
Salt * Var	8	0.03912**	324,569.08***	0.001043***	9.57979***	0.01328**	23.9321**
Salt * SA	4	0.36360**	672,020.24***	0.007198**	108.213***	1.15059**	1193.43**
Var * SA	8	0.00215 ns	111,082.52***	0.000468*	3.77459***	0.08638***	66.4431***
Salt * Var * SA	16	0.00332 ns	101,744.48***	0.000339 ns	2.82970***	0.01208**	20.4270**
Error	90	0.00303	4276.634	0.000214	0.35653	0.00289	5.55245
Source of variation	df	Fv/Fm	PI _{ABS}	ABS/RC	DI/RC	ET/RC	TR/RC
Salt	2	0.015167***	8.521946***	6.906631***	0.877742***	1.053783***	2.69903***
Varieties	4	0.001145***	0.119337***	0.189858***	0.102849***	0.067332***	0.08471***
SA	2	0.024110***	11.65833***	6.831498***	1.917576***	1.434105***	1.83063***
Salt * Var	8	0.000682***	0.12407***	0.144295***	0.042574***	0.033087***	0.04809***
Salt * SA	4	0.002954***	0.52446***	0.820822***	0.211912***	0.029482***	0.14344***
Var * SA	8	0.000498***	0.06089***	0.105705***	0.050540***	0.013532***	0.02762***
Salt * Var * SA	16	0.000345***	0.04708***	0.042595***	0.017933***	0.008653***	0.00989**
Error	90	0.0000181	0.00318	0.005415	0.0007204	0.0014432	0.00414

Var varieties, SA salicylic acid, F.W. fresh weight, D.W. dry weight

*, ** and *** show significance level at 0.05, 0.01 and 0.001, respectively while ns non-significant

cv. 40-D (0.73-fold), and cv. ELW (0.40-fold), respectively and the maximum increase in ET/RC was found in cv. GSR (1.74-fold; Fig. 4c–f).

The results of present work showed that salt stress, SA, and salt stress plus SA treatments caused significant leaf proline accumulation in the leaves of different radish cultivars. However, higher accumulation was observed in

plants treated with 5 mM SA. Exogenous application of 2 & 5 mM SA caused maximum accumulation of proline in salt stressed plants of cv. GSR whereas the reverse was true for cv. MR (Table 1, Fig. 1f, Supplementary Table 4).

Salt stress increased the accumulation of H₂O₂ and MDA contents in leaves of radish cultivars that was decreased by exogenous treatment of SA (2 & 5 mM) in

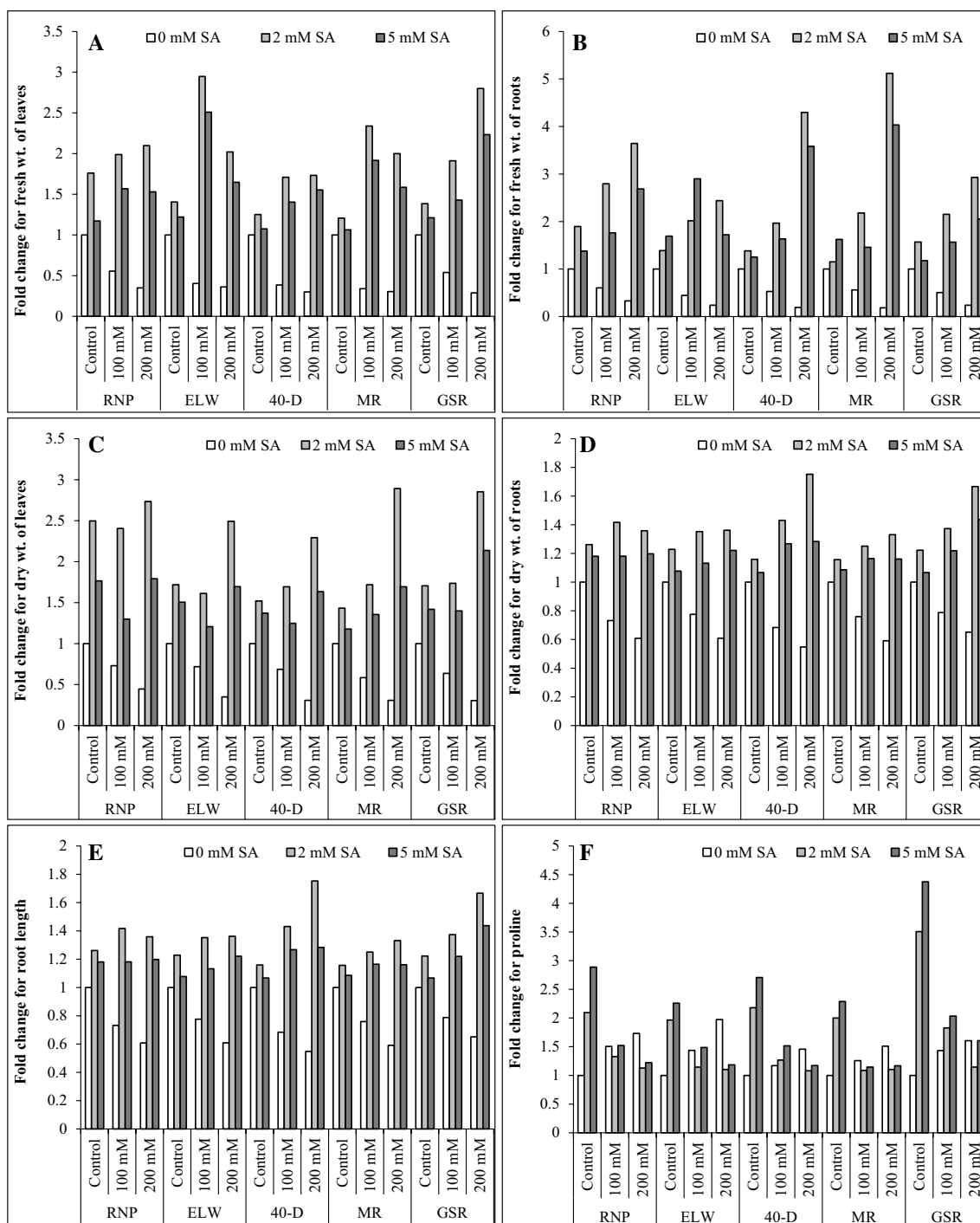


Fig. 1 Effect of Salicylic acid on biomass and proline content of five radish cultivars grown under varying concentrations of salt stress. **a** Fold change for fresh weight of leaves, **b** fold change for fresh weight of roots, **c** fold change for dry weight of leaves, **d** fold change for dry weight of roots, **e** fold change for length of roots, **f** fold change for proline. Fold change (FC) values for 2 or 5 mM SA and 100 or

200 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA})/(\text{control})]$ and $[(100 \text{ or } 200 \text{ mM NaCl})/(\text{control})]$ respectively, whereas FC values for 2 or 5 mM SA at 100 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA at } 100 \text{ mM NaCl})/(100 \text{ mM NaCl})]$ and for 2 or 5 mM SA at 200 mM NaCl are calculated as: $[(2 \text{ or } 5 \text{ mM SA at } 200 \text{ mM NaCl})/(200 \text{ mM NaCl})]$. FC value for control (0 mM NaCl, 0 mM SA) is taken as 1.00

salt stressed and non-salt stressed radish cultivars under investigation (Table 1, Fig. 5a, b, Supplementary Table 4). The foliar spray of 2 mM SA had greater reduction of

leaf MDA contents at both salt concentrations (100 & 200 mM NaCl) in a cultivar dependent manner. For example, 100 mM NaCl, cv. RNP (0.37-fold) and cv. 40-D

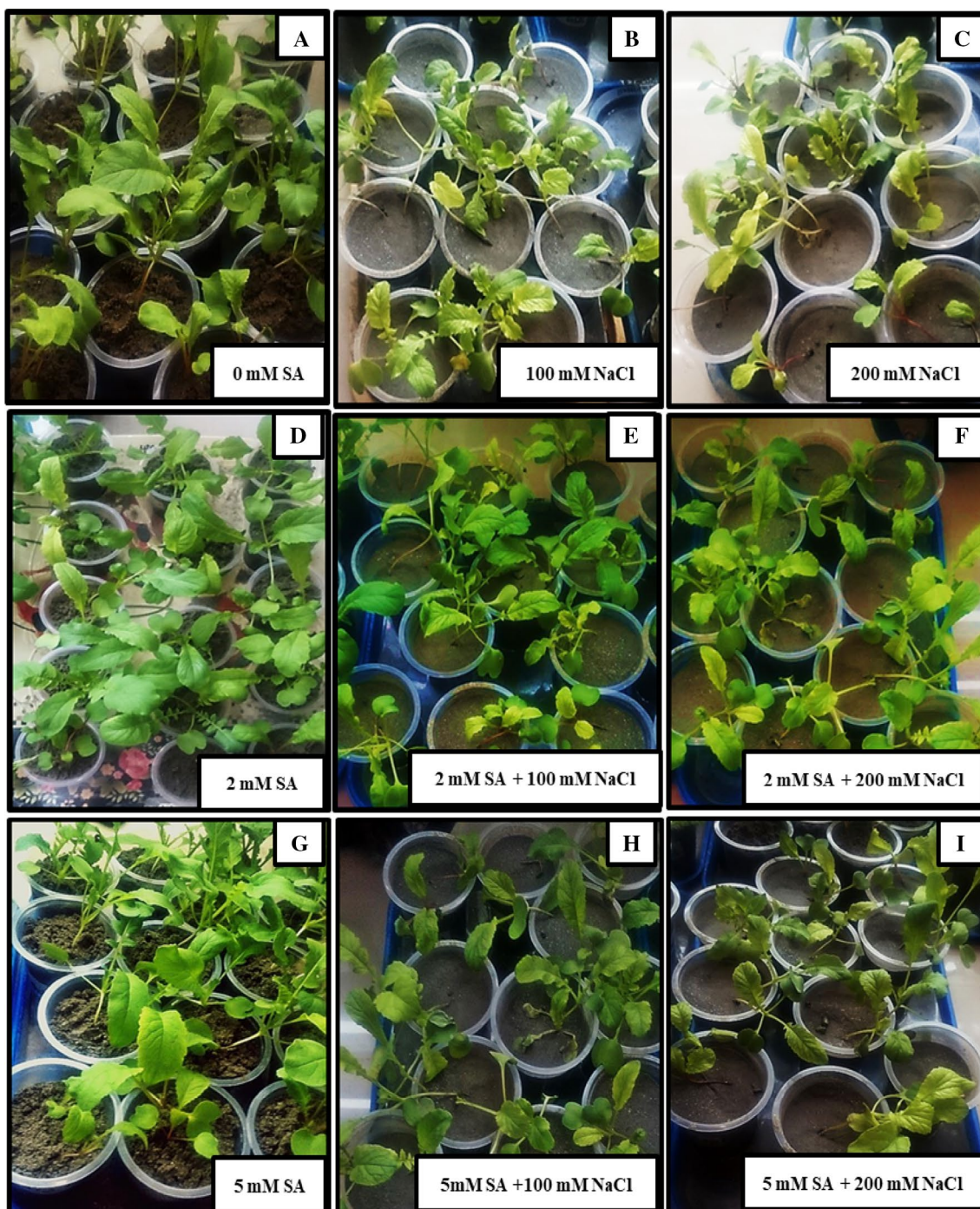


Fig. 2 Representative plants of different radish cultivars grown under varying concentrations of salt stress and salicylic acid. **a** 0 mM SA, **b** 100 mM NaCl, **c** 200 mM NaCl, **d** 2 mM SA, **e** 2 mM SA plus 100 mM NaCl, **f** 2 mM SA plus 200 mM NaCl, **g** 5 mM SA, **h** 5 mM

SA plus 100 mM NaCl, **i** 5 mM SA plus 200 mM NaCl. For each treatment, the representative plants belong to different cultivars and are placed randomly for this figure panel

(0.37-fold), had maximum reduction while at 200 mM NaCl cv. MR (0.35-fold) had maximum decrease in MDA content as compared to other cultivars. For leaf H_2O_2 , the response of each cultivar was significantly different to SA

treatment under non-saline condition; however, in saline conditions the interactive term (Salt * Var * SA) was not significant.

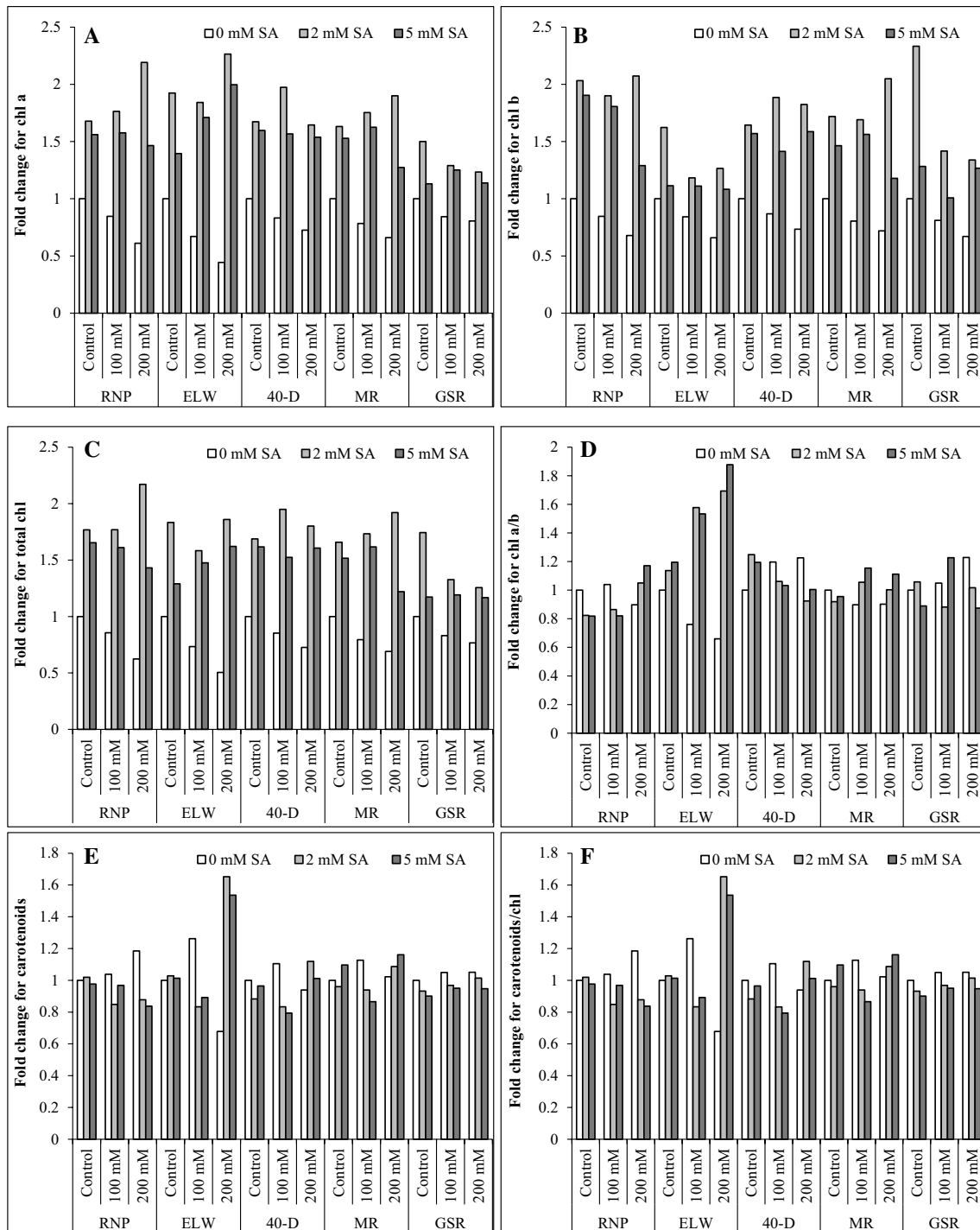


Fig. 3 Effect of Salicylic acid on photosynthetic pigments of five radish cultivars grown under varying concentrations of salt stress. **a** Fold change for Chl a, **b** fold change for Chl b, **c** fold change for total Chl, **d** Fold change for Chl a/b, **e** fold change for carotenoids, **f** fold change for carotenoids/total chl. Fold change (FC) values for 2 or 5 mM SA and 100 or 200 mM NaCl are calculated as: [(2 or 5 mM SA)/(Control)] and [(100 or 200 mM NaCl)/(Control)] respectively,

whereas FC values for 2 or 5 mM SA at 100 mM NaCl are calculated as: [(2 or 5 mM SA at 100 mM NaCl)/(100 mM NaCl)] and for 2 or 5 mM SA at 200 mM NaCl are calculated as: [(2 or 5 mM SA at 200 mM NaCl)/(200 mM NaCl)]. FC value for control (0 mM NaCl, 0 mM SA) is taken as 1.00

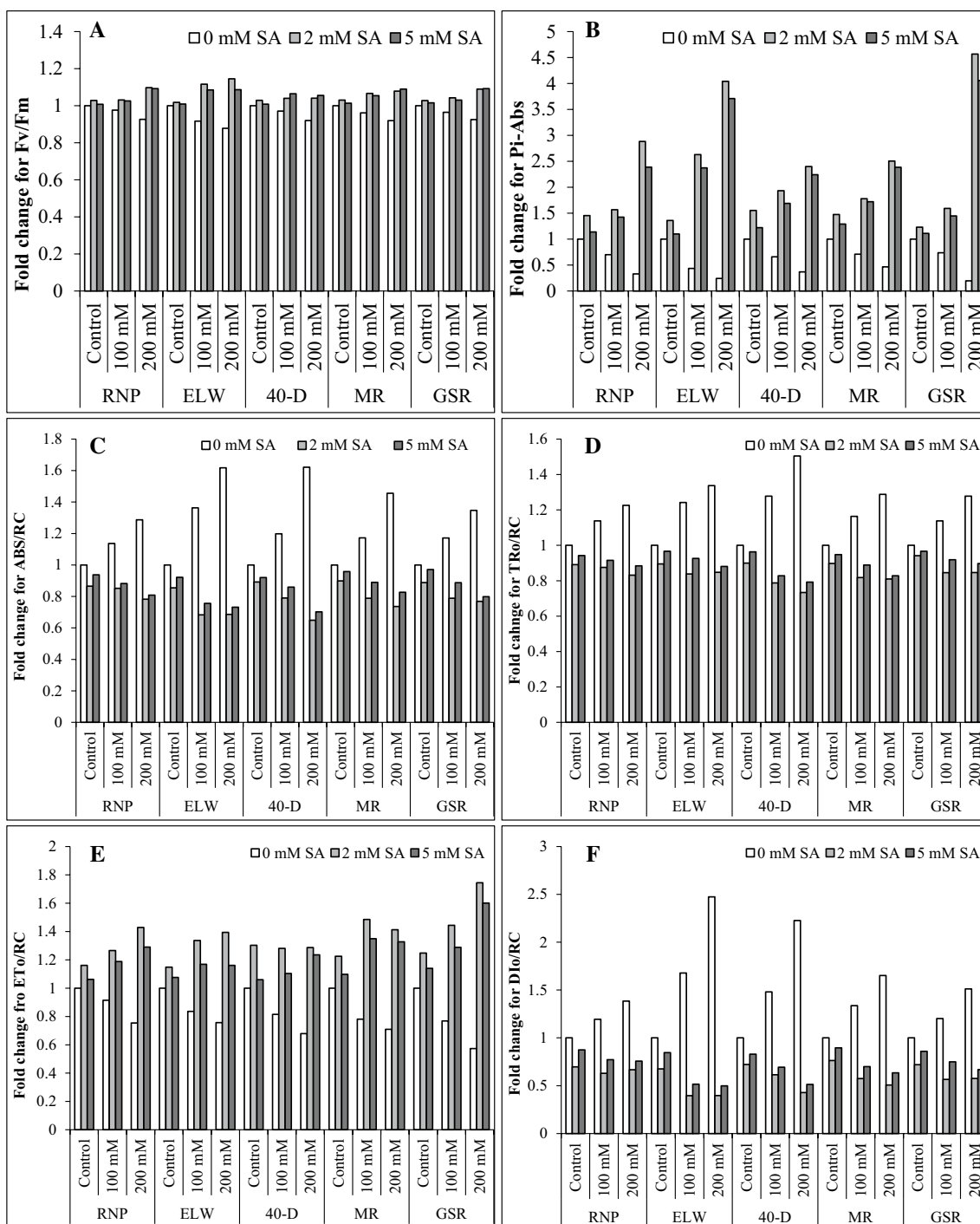


Fig. 4 Effect of Salicylic acid on photochemistry of PSII of five radish cultivars grown under varying concentrations of salt stress. **a** Fold change for Fv/Fm, **b** fold change for Pi_{Abs}, **c** fold change for ABS/RC, **d** fold change for TRo/RC, **e** fold change for ETo/RC, **f** fold change for Dto/RC. Fold change (FC) values for 2 or 5 mM SA and 100 or 200 mM NaCl are calculated as: [(2 or 5 mM SA)/(Con-

trol)] and [(100 or 200 mM NaCl)/(Control)] respectively, whereas FC values for 2 or 5 mM SA at 100 mM NaCl are calculated as: [(2 or 5 mM SA at 100 mM NaCl)/(100 mM NaCl)] and for 2 or 5 mM SA at 200 mM NaCl are calculated as: [(2 or 5 mM SA at 200 mM NaCl)/(200 mM NaCl)]. FC value for control (0 mM NaCl, 0 mM SA) is taken as 1.00

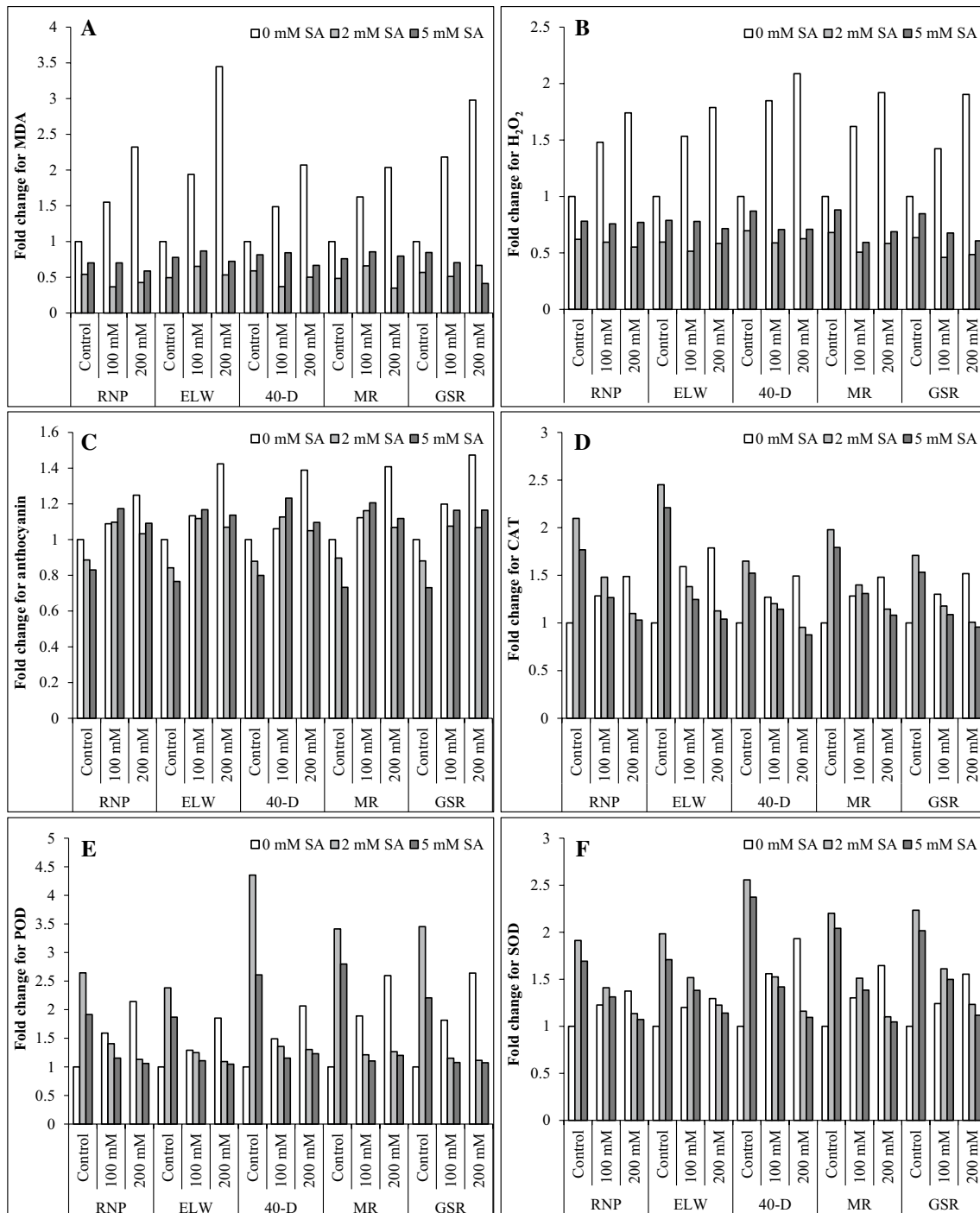


Fig. 5 Effect of Salicylic acid on lipid peroxidation, ROS, anthocyanin and enzymatic antioxidants of five radish cultivars grown under varying concentrations of salt stress. **a** Fold change for MDA, **b** fold change for H₂O₂, **c** fold change for anthocyanin, **d** fold change for CAT, **e** fold change for POD, **f** fold change for SOD. Fold change (FC) values for 2 or 5 mM SA and 100 or 200 mM NaCl are calcu-

lated as: [(2 or 5 mM SA)/(control)] and [(100 or 200 mM NaCl)/(control)] respectively, whereas FC values for 2 or 5 mM SA at 100 mM NaCl are calculated as: [(2 or 5 mM SA at 100 mM NaCl)/(100 mM NaCl)] and for 2 or 5 mM SA at 200 mM NaCl are calculated as: [(2 or 5 mM SA at 200 mM NaCl)/(200 mM NaCl)]. FC value for control (0 mM NaCl, 0 mM SA) is taken as 1.00

Antioxidant Enzymes

The activities of SOD and CAT remained unchanged due to salt stress while the application of SA increased their activities significantly under saline and non-saline conditions (Table 1, Fig. 5d, f, Supplementary Table 4). All the radish cultivars show significantly different behavior due to SA application under salt stress. The treatment of 2 mM SA at 100 mM NaCl showed maximum activities of SOD and CAT in the leaves of cv. GSR (1.61-fold) and cv. RNP (1.48-fold), respectively while at 200 mM NaCl cv. GSR (1.23-fold) and cv. MR (1.14-fold) showed greater SOD and CAT activities, respectively (Fig. 5d, f). In different radish varieties, POD activity significantly increased upon salinity treatment as well as due to exogenous SA application under saline and non-saline conditions (Table 1, Fig. 5e, Supplementary Table 4). The increasing effect of 2 mM SA at 100 & 200 mM NaCl on activities of POD was maximal in cv. RNP (1.40-fold) and cv. MR (1.26-fold), respectively (Fig. 5e).

Discussion

Plant growth regulators and signaling molecules like salicylic acid have the potential to overcome the deleterious effects of abiotic stresses on plants. In the present study, exogenous application of SA had ameliorative effect on all five radish cultivars. However, lower concentration of SA application was more effective as compared to higher concentration of SA (5 mM SA). The effectiveness of low concentration of SA was also reported in *Brassica juncea* (Fariduddin et al. 2003) and *Triticum aestivum* (Hayat et al. 2005). In addition, growth promoting effect of SA depends on type of cultivar and degree of salt stress. For example, at 100 mM NaCl salt stress, SA application caused maximal increase in leaf dry weight in cultivar RNP, whereas the least growth improvement was found in radish cultivar ELW. However, at 200 mM NaCl salt stress, SA application caused growth improvement in cv MR followed by cv. GSR. Similar genotypic differences in growth due to SA application under saline conditions has already been observed in different crop species such as in *Triticum aestivum* (Arfan et al. 2007), *Helianthus annuus* L. (Noreen and Ashraf 2008), *Zea mays* L. (Khodary 2004; Gunes et al. 2007). SA-induced increase in biomass (Table 1, Fig. 1a–e, Supplementary Table 1) of salt affected plants might have been due to the enhanced photosynthetic capacity, and/or reduction oxidative stress (Khan 2003; Fariduddin et al. 2003; Szepesi et al. 2005).

In salt stressed plants of radish cultivars, the chlorophyll (a, b & total chlorophyll; Table 1, Fig. 3a–c, Supplementary Table 2) and carotenoids contents (Table 1, Fig. 3e, Supplementary Table 2) decreased with increasing salinity level.

This decrease in chlorophyll might be due to reduction in the biosynthesis of chlorophyll. For example, while working with *Pennisetum typhoides* Reddy and Vora (1986), showed that salt stress destabilized protein complexes involved in Chl biosynthesis. In addition, salt stress increased the activity of chlorophyllase enzyme. In another study, it has been reported that salt stress caused degradation of the chlorophyll a/b binding proteins in light harvesting complex II (Levitt 1980).

Photosynthesis is one of the premier physiological processes that govern the plant growth and productivity. Plant photosynthetic capacity can be measured through CO₂ fixation rate or functional activity of photosystem II (PSII). It has been documented that reduction in photosynthetic capacity due to salt stress is due to structural and functional damages to photosystems and electron carriers. However, structural damages to photosystems occur at high salt stress or for a longer period of stress such as exposure of plants to 150–200 mM NaCl salt stress for more than three weeks or 300–400 mM NaCl for 10–15 days (Kalaji et al. 2016). In the present study, the salt stress significantly decreased the quantum yield of PSII and performance index (PI) in all cultivars radish while the application of SA increased quantum yield of PSII and PI in all radish cultivars under saline conditions (Table 1, Fig. 4a, b, Supplementary Table 3). A positive correlation between PI and active reaction center density, quantum yield of electron transport and primary photochemistry was recorded. Therefore, exogenous application of SA improved the photosynthetic activity of salt stressed plants of radish cultivars by stabilizing PSII and electron transport. Previous studies suggested that SA treatment can protect the function of PSII by increasing Φ_{PSII} under environmental stresses (Shi et al. 2006; Khalid et al. 2015). It is well established that changes in quantum yield of PSII are associated either with inhibition in electron transport from donor side (damage to oxygen evolving complex OEC) or reduction in pool of oxidize Q_A^- (Kalaji et al. 2016). In the present study, salt stress increased the ABS/RC and TRo/RC along with reduction ETo/RC in all radish cultivars which indicated that effective antenna size become increased due to inactivation of reaction centers (Table 1, Fig. 4c–e, Supplementary Table 3). Moreover, this result in over-reduction of Q_A^- and efficiency of electron transfer from Q_A^- to Q_B also reduced due to salt stress. Thus, dissipation of absorbed energy as heat (Dio/RC) might have increased by active reaction centers which results in overall lowering in PI_{ABS} . However, the adverse effect of salt stress on donor end of PSII was greater than that on acceptor end of PSII as has earlier been observed in wheat plants (Mehta et al. 2010). These results can be explained in view of the argument of Zhang and Xing (2008) who reported that salt-induced osmotic stress affected antenna size heterogeneity with no change in acceptor end of PSII, while salt-induced

ionic stress affected both donor and acceptor ends of PSII. Improvement in quantum yield of PSII and PI_{ABS} due to SA application might have been due to increase in density of active reactive centers per antenna chlorophylls thereby lowering in ABS/RC, TR/RC. These results are in parallel with those of Ghassemi-Golezani and Lotfi (2015) who found that exogenous application of SA enhanced the quantum yield of PSII and electron transfer efficiency in mung bean plants under salt stress. From these reports and results from the present study, it can be inferred that salt stress not only reduced the photosynthetic pigments in antenna, but it also disturb the conformation of antenna pigment assemblies that resulted in efficiency of PSII. This is further supported by the fact that salt stress increased the MDA in all radish cultivars and exogenous application of SA lowered the MDA contents along with improved the chlorophyll contents and carotenoids in salt stressed plants of radish cultivars. Exogenous application of SA substantially enhanced the photosynthetic rate in wheat (Arfan et al. 2007) and cotton (Noreen et al. 2013), which may be associated with higher CO_2 assimilation rate, pigment level or activities of antioxidants. In contrast, application of high concentration of SA reduced photosynthetic rate by lowering in photosynthetic pigments, amount and activity of rubisco, and affecting ultrastructure of chloroplast (Pancheva et al. 1996; Uzunova and Popova 2000; Sahu 2013) or reducing quantum efficiency of PSII by inhibiting electron transport in guard cells of tomato (Poór and Tari 2012). Thus changes in SA-induced photosynthetic capacity of radish cultivars might have been linked with osmoprotection and antioxidant capacity of plants.

Salt stress causes the generation of reactive oxygen species in chloroplast, a wide variety of osmoprotectants and antioxidants become accumulated to protect thylakoidal protein complexes. Proline is one of the potential osmoprotectants having role in osmotic adjustment, protecting PSII activity, as antioxidant, and stabilization of cell membrane (Szabados and Savaouré 2010). Results of present study showed that SA treatment and salt stress increased proline accumulation; however, the increase in proline accumulation was more due to SA application (Table 1, Fig. 1f, Supplementary Table 4). Similar increase in proline by SA treatment has earlier been observed in *Panax ginseng* (Ali et al. 2007), *Solanum lycopersicum* (Mimouni et al. 2016) and *Cucumis sativus* (Youssef et al. 2018). A positive association between accumulation of proline and PI_{ABS} has been found which indicated that SA-induced increase in PSII efficiency can be linked to proline accumulation. However, exogenous application of 5 mM SA caused greater accumulation of proline with relative lower PI_{ABS} in salt stressed plants of different radish cultivars than with those of applied with 2 mM SA. These results suggest that contribution of proline in improving PSII efficiency of salt stressed plants radish cultivars is

partial. Other factors may include non-enzymic and enzymic antioxidants (Foyer and Shigeoka 2011; Foyer et al. 2017).

Anthocyanins are group of flavonoid pigments that act as non-enzymic antioxidants (Taiz et al. 2015). In the present study, salt stress increased the anthocyanin pigments in radish cultivars while SA treatment decreased their amount (Table 1, Fig. 5c, Supplementary Table 2). Similar increase in anthocyanins due to salt stress has already observed in Pelargonium (Breš et al. 2016) and spinach (Xu and Mou 2016). However, SA application decreased anthocyanin pigments under non-saline conditions but increase in anthocyanins was observed under SA plus salt stress. Stress induced anthocyanins are thought to function as ROS scavengers (Agati et al. 2012). Szepesi et al. (2008) demonstrated that pre-treatment with SA increased anthocyanin accumulation under saline and non-saline conditions. Based on our results, it can be suggested that stress increased ROS accumulation and in order to detoxification of ROS radicals SA increased production of anthocyanins as non-enzymic antioxidants. Anthocyanin accumulation is mainly localized to upper epidermis where it shields lower mesophyll cells from light intensity or lower oxidative stress. Thus, it is assumed that SA treatment reduced the oxidative stress thereby increasing anthocyanin accumulation.

The malondialdehyde (MDA) content is considered as key indicator to show lipid peroxidation level (Shen et al. 2014). In the present study, the salt stress increased H_2O_2 and MDA content in all radish cultivars, while SA application reduced the concentrations of both H_2O_2 and MDA content, which indicated that the salt stress caused oxidative damage and SA application lowers the oxidative damage (Table 1, Fig. 5a, b, Supplementary Table 4). While working with five cultivars of turnip (*Brassica rapa* L.) Noreen et al. (2010) reported that decrease in photosynthetic capacity of turnip cultivars due to salinity stress is related with higher ROS production. Similarly, seedlings of *Torreya grandis* treated with SA had lower electrolyte leakage and MDA content (Li et al. 2014). Generally, plants activate their antioxidant enzymes (SOD, CAT and POD etc.) for protecting their tissues from lethal effects of ROS accumulated due to salt stress (Foyer et al. 2017).

In the present study, the foliar spray with SA significantly increased POD, CAT and SOD activity which is inversely related with MDA and H_2O_2 , which indicate foliar spray with SA ameliorated the salt-induced oxidative damage to PSII by modulating activities of antioxidant enzymes (Table 1, Fig. 5d–f, Supplementary Table 4). These results can be explained in view of the argument of Bose et al. (2017) that SA activates multiple pathways to scavenge ROS in chloroplast to protect photosynthetic machinery including battery of antioxidant enzymes. Signaling between SA and ROS is closely related, as ROS signaling is inhibited by SA which stimulates the activation of defensive genes (Xu and Brosche 2014).

Conclusion

Salt stress adversely affect the physiological and biochemical properties of radish plant causing growth inhibition, destruction of photosynthetic machinery, ROS accumulation, increased membrane damage, and reduced efficiency of PSII. However, SA treatment reduced the detrimental effect of salt stress by combination of osmolytes, non-enzymic and enzymic antioxidants. However, mode of action of SA in ameliorating the adverse effects of salt stress on radish cultivars depends on concentration of SA application. As observed in previous studies, that higher dose of SA become inhibitory to some photosynthetic processes is another supportive argument in this regard.

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Compliance with Ethical Standards

Conflict of interest The authors declared no conflict of interest.

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