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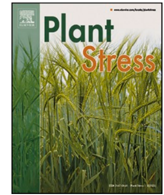

Salicylic acid attenuates salinity-induced growth inhibition in *in vitro* raised ginger (*Zingiber officinale* Roscoe) plantlets by regulating ionic balance and antioxidative system

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ABSTRACT

Ginger (*Zingiber officinalis* Roscoe) is valued as a spice and herbal plant with high economic importance, but its productivity is affected by soil salinity. In the present study, potential of salicylic acid (SA) to reduce salt stress was tested in ginger plantlets grown under *in vitro* conditions. NaCl stress at 150 mM concentration caused a significant decline in growth parameters and photosynthetic pigment contents and a rise in Na^+, Cl^-, H_2O_2, superoxide radical contents, activities of chlorophyllase, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymes. A foliar spray of 0.5 mM salicylic acid showed a remarkable improvement in the growth parameters (around 3-fold increase in shoot number and fresh weight) of stressed plantlets. SA treatment enhanced the contents of chlorophyll a (41%), chlorophyll *b* (51%), total chlorophyll (48.56%), carotenoids (36%) and reduced chlorophyllase activity (18%) under salt stress conditions. NaCl - induced activities of antioxidative enzymes were further increased by SA treatment. An increase of 71.22, 50.84 and 50.45 percent was recorded for SOD, CAT and POD, respectively. A reduction in H_2O_2 content (31.24%) and superoxide production rate (43%) in stressed plantlets was noted after SA application. This treatment also reversed the sodium ion toxicity as revealed by a decline in $Na^+$and Cl^- contents (90%) with a concomitant rise in K^+ ion concentration (25.65%) in stressed plantlets. These results indicated that SA-mediated improvement in ionic balance and antioxidative defense system contributed to the acclimatization of plantlets under salinity. In conclusion, foliar application of 0.5 mM salicylic acid could help recover the reduced growth of stressed plantlets. Therefore, SA treatment could be suggested as a feasible approach to produce salt adapted ginger plantlets *in vitro*. Such plants can perform well on saline soils and could serve as a continuous source of raw material for ginger industry.

1. Introduction

Ginger (*Zingiber officinale* Rosc.), a herbaceous perennial, is one of the most important cash crops of the world native to South-Eastern Asia (Gang and Ma, 2008). It is valued for its rhizomes which accumulates active constituents such as gingerols, shogaols, paradols, zingerone and semiterpenes (Mao et al., 2019). The rhizomes are used worldwide as a spice in preparation of several food items, beverages, soft drinks and in herbal medicines (Gang and Ma, 2008; Tapsell et al., 2006) making it commercially important high demand item in the international markets. Poor seed setting and low rate of propagation by rhizome account for its slow natural regeneration (Ravindran and Nirmal Babu, 2005). Exploitation of rhizomes for commercial purpose limits its availability as planting material. Further, its production is also severely challenged by biotic and abiotic stresses. It is susceptible to soil-borne pathogens such as *Pseudomonas solanacearum, Pythium aphanidermatum* and nematodes (Abbas et al., 2011) and sensitive to drought and salinity (Ahmad et al., 2009; Vivek et al., 2017). All these factors together reduce the overall productivity, affecting its supply to the ginger industry.

Potential of plant tissue cultures in screening for tolerance against various environmental stresses has been well established (Kacem et al., 2017; Muchate et al., 2019). *In vitro* culture system provides the advantage of response being studied under controlled conditions independent of other environmental cues existing at the field level. Further,

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it has been demonstrated that conclusions drawn from cellular or tissue level study can be extrapolated to the whole plant level (Lokhande et al., 2011).

Salinity stress is a serious threat to crop growth and productivity. Plants have developed several adaptive mechanisms which depend upon activation of molecular processes involved in stress recognition, signal transduction, and expression of stress-related genes and metabolites (Gupta and Huang, 2014). Salicylic acid (SA), a phenolic plant hormone, plays an important role in abiotic stress signaling and has appeared as an inducer of stress responsive pathways (Ma et al., 2017; Moustafa-Farag et al., 2020). Exogenous application of plant hormones including SA has emerged as an effective strategy to alleviate the adverse effects of salinity. Several investigators have used SA to regulate plant adaptation to salt stress under *in vivo* (Jini and Joseph, 2017; Khan et al., 2014, 2010; Miao et al., 2020; Miura and Tada, 2014; Nazar et al., 2011; Souri and Tohidloo, 2019) and *in vitro* (Danaee et al., 2015; Golkar et al., 2019; Govindaraju and Arulselvi, 2018) conditions. However, SA-mediated tolerance depends upon various aspects like the dose and mode of its application, endogenous level in the plant, intensity of salt stress, species type and growth stage (Nazar et al., 2011). Regarding the mechanism of SA-mediated adaptation, studies have revealed its influence on expression of genes involved in osmotic regulation, ion homeostasis, photosynthesis, antioxidative metabolism etc. (Farhangi-Abriz and Ghassemi-Golezani, 2018; Kang et al., 2014; Li et al., 2013). According to Khan et al. (2019) saline stress is managed by various pathways like MAPK1, Programmed Cell Death (PCD) and autophagy. Proteomic studies have revealed that signal transduction-related proteins also respond to SA under abiotic stress conditions (Kang et al., 2012a,b).

Ginger is generally considered as a salt sensitive crop (Ahmad et al., 2009); salinity being a hindrance to achieve optimum growth and rhizome production (Dehghani and Mostajeran, 2010; Vivek et al., 2017). Besides yield reduction, salt stress also reduced the contents of reducing sugars, sucrose, starch, minerals (Yin et al., 2021), total carbohydrates and proteins (Ahmad et al., 2009) in ginger plants. Therefore, improvement of salinity tolerance is necessary to increase rhizome productivity and quality thereby, benefiting the ginger industry. Sustainable strategies involving the use of organic and inorganic amendments can be an effective substitute, but as per the literature survey a very scanty information is available in this regard. One such work dealt with the use of vermicompost and biogas slurry in pot grown plants for improved growth and productivity (Ahmad et al., 2009). Beneficial effect of foliar application of salicylic acid on growth, physiological features and antioxidant system in ginger plants grown under normal greenhouse conditions is known (Ghasemzadeh and Jaafar, 2012, 2013). Therefore, in the present study we examined the role of salicylic acid in alleviating salinity stress. It was aimed to i) assess its effect on *in vitro* growth of ginger plantlets under NaCl stress conditions and ii) conduct biochemical analyses of stressed plantlets to understand the mechanism of action of salicylic acid. As per the available literature, it is the first report demonstrating influence of SA on salt stress in ginger using *in vitro* culture system.

2. Materials and methods

2.1. Plant material and experimentations

The ginger shoot cultures were established by inoculating bud sprouts from rhizome on Murashige and Skoog (MS) medium supplemented with 4.5 mgL^{-1} 6-Benzylaminopurine (BAP) as per the protocol described by Abbas et al. (2011). The cultures were maintained by regular subculture onto the same fresh medium at an interval of 21 days. Individual shoots were inoculated on MS medium containing1-Naphthaleneacetic acid (NAA) (1.0 mg L^{-1}) for root induction. The rooted plantlets were used as the experimental material. All the cultures were incubated at 25± 2° C under 16 h of photoperiod (45 μmol m^{-2} s^{-1}).

To assess the survival and growth attributes under salinity, single *in vitro* raised rooted plantlets were transferred to sterile Soilrite™ supplemented with equal volume (100 ml) of different concentrations of NaCl (0, 50, 100, 150 and 200 mM). Observations were recorded after 14 days which revealed that plantlets did not survive on 200 mM NaCl, whereas 8% and 25% reduction in growth was observed at 100 mM and 150 mM concentrations of NaCl, respectively. Considering significant growth reduction at 150 mM NaCl, this concentration was used for experiments to study the ameliorative effect of SA. Single plantlets of same size and weight were inoculated on Soilrite™ containing 150 mM NaCl and treated with two concentrations of SA (0.5 and 1.0 mM) by two different modes: i) Foliar spray (until shoot was washed-off), ii) Root application by incorporation into the growth medium (Soilrite™). Equal volumes (15 ml) of SA were used for both modes. The plantlets were maintained on the respective treatments for 20 days (with no further SA application). Plantlets inoculated on Soilrite™ containing 0 mM NaCl served as control.

2.2. Growth traits

Fresh weight (FW), shoot and root length, and number of shoots were recorded for plantlets from control and each treatment. Biochemical analyses were carried out for plantlets from control, 150 mM NaCl and 150 mM NaCl+ 0.5 mM SA (foliar spray) as this treatment showed maximum foliage growth.

2.3. Photosynthetic pigment contents

Arnon's (1994) spectrophotometric method was used to determine the chlorophyll contents. 100 mg FW of shoots were ground in 8 ml 80% acetone (v/v). The concentration of chlorophyll a, chlorophyll *b* and total chlorophyll was calculated using the following equation:

- *Total Chlorophyll* : $20.2(A645) + 8.02(A663)$
- *Chlorophyll a* : $12.7(A663) - 2.69(A645)$
- *Chlorophyll b* : $22.9(A645) - 4.68(A663)$

The results were expressed as μg g^{-1} FW of the tissues.

Carotenoid content was measured according to the method proposed by Maclachlan and Zalik (1963). The procedure of carotenoid was similar to chlorophyll except that the absorbance was recorded at 480 nm. The concentration of carotenoids was calculated using the following equation:

$$\text{Carotenoid} : \frac{4(A480)\ (Total\ volume)}{Fresh\ weight}$$

The results were expressed as mg g^{-1} FW of the tissues.

2.4. Chlorophyllase activity determination

2.4.1. Enzyme extraction

Shoot samples (200 mg) were powdered in liquid nitrogen and homogenised with 4 ml of pre-chilled acetone (–20 °C). The mixture was centrifuged at 5500 × g at 4 °C for 15 min. and the precipitates were collected. It was re-suspended in the extraction buffer (5 mM potassium phosphate buffer containing 50 mM KCl and 0.25% Triton X-100, pH 7.0) and centrifuged at 12,000 × g for 10 min. The supernatant obtained was utilized as the crude enzyme extract for further biochemical studies.

2.4.2. Preparation of enzyme substrate (chlorophyll)

Chlorophyll extract was prepared from spinach leaves according to the protocol described by Janave (1997). Shoots were ground in liquid nitrogen and homogenised in cold acetone (–20 °C). The extract was centrifuged at 5500 × g at 4 °C for 15 min. Distilled water was added dropwise to the supernatant until the precipitation of chlorophyll. The precipitates were centrifuged at 10,000 × g at 4 °C for 15 min and

re-suspended in acetone after keeping in the dark for 20 min. This chlorophyll extract was stored at –20 °C, followed by separation of pigments using sugar column chromatography (Perkin and Roberts, 1962). The pigment showing maximum absorbance at 663 nm was assumed to be chlorophyll a. Resuspended pigment in 100% acetone was used as substrate for chlorophyllase activity assay.

2.4.3. Chlorophyllase activity assay and determination of chl a concentration

Chlorophyllase (Chlase) activity was determined according to the modified method of Yang et al. (2004). The reaction mixture contained 0.35 mL extract, 0.15 mL of substrate (Chl a) and 1.0 mL of potassium phosphate buffer (5 mM, pH 7.0) and was incubated in dark at 30ºC for 30 min. The reaction was stopped by addition of 2.0 mL cold acetone and 2.0 mL n-hexane. The mixture was centrifuged for 5 min at 12,000 × g and the content of residual substrate in the acetone phase was calculated using the following equation (Ihl et al., 1998):

$$Chla(nmol.cm^{-3}) = 11.30 \times (A_{662} - A_{750}) - 1.11 \times (A_{644} - A_{750})$$

Absorbance was recorded using Hewlett-Packard UV–Vis spectrophotometer (model 8453). The specific activity of chlorophyllase was defined as nano mole of substrate (Chl a) hydrolysed/minute/mg of protein (Arriagada-Strodthoff et al., 2007). The concentration of protein was measured using the method of Lowry et al. (1951) with the calibration curve of bovine serum albumin (BSA).

2.5. Ionic content

Shoot tissues (1000 mg) were finely ground and digested with 5.0 mL mixture of HNO_3 and HCl (1:1). 1.0 mL of acid digested sample solution was diluted with 9.0 mL of deionized water and the ionic content of Na^+, K^+ was determined by atomic absorption spectrophotometry as per the method described by Allan (1996) and Cl^- was determined by precipitation titration with Ag_2NO_3 (Adachi and Kobayashi, 2008). The results were expressed as mg g^{-1} FW of the tissues.

2.6. Hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$)

Shoot tissues (300 mg) were homogenized in 4.0 ml of solution containing 1.0 mL Trichloroacetic acid (TCA) (0.1% (w/v)), 2.0 mL KI (1 M) and 1.0 mL potassium phosphate buffer (10 mM, pH 8) at 4°C for 10 min in dark. The homogenate was centrifuged at 12,000 × g at 4°C for 15 min and the supernatant was separated and used for H_2O_2 content determination according to the method described by Junglee et al. (2014) with analytical grade H_2O_2 solution prepared in 0.1% TCA as the standard. Extract (1.0 mL) was added to 1.0 mL KI solution (1 M). Following the incubation in dark for 20 min, the absorbance was recorded at 350 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., and Tokyo, Japan). The amount of H_2O_2 was calculated using a calibration curve obtained with H_2O_2 standard solutions prepared in 0.1% TCA and results were expressed as μM g^{-1} FW of the tissues.

The $O_2^{\bullet-}$ production rate was determined spectrophotometrically by measuring the amount of reduced Cyt c produced at 550 nm in the reaction mixture containing 2.0 mM Cytochrome c, 10 μM NADPH, 10 mM potassium phosphate buffer (pH 7.8), O.6 M mannitol, 0.1 mM EDTA and shoot extract in a total volume of 2.0 mL (Doke et al., 1983). The amount of Cyt c was calculated from its reduction spectrum using a molar extinction coefficient of 2.1 × 10^4 M^{-1} cm^{-1}, at 550 nm (Cosi et al., 2007). The $O_2^{\bullet-}$production rate was expressed as reduced Cyt c (μM mg^{-1} FW of the tissues).

2.7. Determination of CAT, POD and SOD activities

Shoot tissues (200 mg) were ground to fine powder in liquid nitrogen which was homogenized in 2.0 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 1.0% (w/v) PVP-40, 2.0 mM Na-EDTA, and 25% Triton X-100. The homogenate was centrifuged at 10,000× g for 15 min and the supernatant was used to determine total soluble proteins (Lowry et al., 1951) and enzyme activities.

CAT (EC 1.11.1.6) activity was measured by subsequent consumption of H_2O_2 at 240 nm for 5 min in 3.0 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7) and 10 μL of 30% H_2O_2 (Rao et al., 1996). One unit of specific activity of CAT was defined as μM of substrate (H_2O_2) hydrolyzed per minute per mg of protein.

SOD (EC 1.15.1.1) activity was assessed by measuring its ability to prevent the photochemical reduction of nitrobluetetrazolium (Giannopotitis and Ries, 1977). The reaction mixture (3.0 mL) consisted of 50 M potassium phosphate buffer (pH 7.8) containing 0.3 μM EDTA, 39.15 μM methionine, 0.225 μM nitrobluetetrazolium, 0.006 μM riboflavin, and 0.05 mL of enzyme extract. One unit of SOD activity was defined as the amount of enzyme that resulted in 50% inhibition of NBT reduction at 560 nm.

POD (EC 1.11.1.7) activity was determined as described by Hammer Schmidt et al. (1982). The reaction mixture consisted of 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, 0.05% guaiacol and 0.05 mL enzyme extract. The POD activity was determined by recording the absorbance changes at 470 nm per min per mg protein. One unit of enzyme (IU) equals to 0.01 ΔOD min^{-1}.

The specific enzyme activities of POD and SOD were expressed as U mg^{-1} protein.

2.8. Statistical analyses

Experiments were conducted in completely randomized design with three replications. Mean values of the treatments and control were subjected to the analysis of variance (ANOVA) and significant difference was shown with Duncan's Multiple Range Test (DMRT) ($P<0.05$) using SPSS (Windows Version 7.5.1, SPSS Inc., Chicago).

3. Results

Compared with control, a significant decline in shoot length (15.85%), root length (19.64%), shoot number (43.13%) and shoot fresh weight (54%) was recorded in NaCl-stressed plantlets. (Table 1).

Treatment with both concentrations of SA applied via both modes improved most of the studied parameters but the effect was most significant with foliar spray of 0.5 mM concentration of SA (Figs 1, 2).

This treatment increased the shoot and root length (58% and 44%, respectively), number of shoots (3-fold) and plantlet fresh weight (3-fold) compared to untreated stressed control (Table 1). Root application effect was much lower than that seen under foliar application, representing 29.4% and 20% increase for shoot and root length, respectively. SA alone showed only slight increase in the measured parameters over control.

NaCl reduced the contents of photosynthetic pigments with maximum reduction observed in chl b (51.20%) and minimum in carotenoid (36%). Foliar application of 0.5 mM SA significantly increased chl a, chl b, total chlorophyll and carotenoid contents by 69%, 200%, 100% and 78%, respectively (Table 2). NaCl stress also increased the activity of chlorophyllase enzyme by 2-fold which suggested its role in reducing chlorophyll contents. SA treatment reduced chlorophyllase enzyme activity by 50% compared to the stressed plants.

Stressed plantlets showed elevated Na^+ and Cl^- ion contents (100%) and reduced K^+ ion contents (30%). SA treatment decreased $Na^+$and Cl^- ion accumulation by approximately 90% and increased K^+ion (25.65%) contents resulting in improved K^+/Na^+ ion ratio (Table 3).

Compared to control, NaCl stress registered a rise in superoxide radical generation (82%) and H_2O_2 content (46.65%). SA application impeded the salt-induced ROS production as indicated by 30% reduction in concentrations of superoxide and H_2O_2 (Table 4). Activities of SOD, POD and CAT were increased by 24%, 24% and 50%, respectively in

Table 1
Effect of foliar application of salicylic acid on various morphological parameters of *Z. officinale* plantlet under *in vitro* salt stress.

Treatment	Shoot length (cm)	Root length (cm)	Number of shoots	Total Fresh Weight (g)
0 mM NaCl (Control)	4.16 ± 0.26bc	2.8 ± 0.19b	4.66 ± 0.87b	0.74 ± 0.09b
150 mM NaCl	3.5 ± 0.35d	2.25 ± 0.18d	2.65 ± 0.32e	0.34 ± 0.06e
0.5 mM SA	4.13 ± 0.16bc	2.8 ± 0.22b	4.7 ± 0.32b	0.75 ± 0.19b
1.0 mM SA	4.14 ± 0.20bc	2.8 ± 0.16b	4.68 ± 0.47b	0.73 ± 0.34b
150 mM NaCl +0.5 mM SA (root)	4.23 ± 0.28b	2.7 ± 0.37bc	3.33 ± 0.66c	0.56 ± 0.20cd[a]
150 mM NaCl +1.0 mM SA (root)	3.66 ± 0.61d	2.20 ± 0.21d	3.0 ± 0.9de	0.40 ± 0.13d
150 mM NaCl +0.5 mM SA (Foliar)	5.56 ± 0.26a	3.2 ± 0.24a	6.66 ± 0.71a	1.09 ± 0.09a
150 mM NaCl +0.5 mM SA (Foliar)	5.56 ± 0.26a	3.2 ± 0.24a	6.66 ± 0.71a	1.09 ± 0.09a
150 mMNaCl +1.0 mM SA (Foliar)	4.0 ± 0.02c	2.6 ± 0.15c	3.5 ± 0.12d	0.59 ± 0.13c

[a] Values are the mean ± SE (n=3). Means with different letters designate a significant difference at $P < 0.05$ using DMRT

stressed plants compared to control. SA treatment further increased these activities by 100 % (CAT), 70% (SOD) and 56 % (POD) (Table 4).

4. Discussion

The present investigation was undertaken to improve salinity tolerance of ginger plantlets grown *in vitro*. Several studies have suggested that exogenous application of salicylic acid increase its endogenous levels and attenuate the adverse effects of salinity (Jini and Joseph, 2017). SA-mediated protection has been associated with its role as a signal molecule in activating plant's defense system that includes osmoregulation, ROS scavenging, ion homeostasis etc. (Kang et al., 2014; Ma et al., 2017).

Salt stress significantly reduced the growth of ginger at seedling (Yin et al., 2021) and vegetative (Ahmad et al., 2009) stage. In the present study also, significant decline in growth parameters of NaCl-stressed ginger plantlets was recorded. The results showed that roots were more sensitive than shoots. Application of SA alleviated the growth inhibition, the effect being most significant at low (0.5 mM) concentration. Maximum improvement was seen in shoot number and plantlet fresh weight.

Mode of SA application is a significant factor influencing its efficacy. Contrasting results on the effectiveness of foliar and root application have been reported by several investigators (Gharbi et al., 2018; Souri and Tohidloo, 2019; Methenni et al., 2018; Khan et al., 2019). Souri and Tohidloo (2019) reported shoot as well as root growth on foliar application of SA and foliar spray showed better root growth than root application. In the present study, foliar spray (0.5 mM) proved more effective than root application in improving the growth characteristics. Under foliar spray, the recorded effect was higher for shoot growth than root growth. Surprisingly, root application also improved shoot growth more than root growth. These observations suggest that the SA-mediated stress response mechanism operating at shoots and roots was not the same.

One reason could be that there is a difference in the absorption of SA by shoots and roots. Foliar sprays of SA are often carried out to wash-off, with the possibility that could enter via stomata and that soft mechanical damage can activate defense response (Benikhlef et al., 2013) whereas absence of such damage during root application could be the cause of less defense metabolism in roots. Further, differential uptake of SA by shoot and root can also affect their endogenous concentration. Synthesis of SA takes place in leaves (chloroplast) and under stress it spread all over the plant to participate in SAR (Fragniere et al., 2011). Although the mechanism of SA assimilation and transport is still elusive, it can be hypothesized that endogenous SA content is higher in shoots than roots. This hypothesis gets support from the results of Gharbi et al. (2018), who reported higher endogenous SA content in shoots than in roots under all conditions *i.e.* control (unstressed), stressed and SA treated stressed plants. They also noted that exogenous SA increased endogenous SA in shoots but not in roots in unstressed as well as stressed plants, indicating more SA absorption by shoots over roots. Although we did not measure the endogenous SA, based on the above cited literature it can be said that under foliar spray, better absorption of exogenous SA and its higher endogenous levels in shoots could be the reason for better shoot growth than root growth compared to root application.

Another reason for low efficacy of root application to improve root growth could be that exogenous SA may decrease its own synthesis in

Fig. 1. Effect of foliar application of salicylic acid on growth of ginger plantlets under *in vitro* salinity stress: a) control (unstressed), b) 150 mM NaCl, c) 150 mM NaCl+0.5 mM SA, d) 150 mM NaCl+1.0 mM SA.

Fig. 2. Effect of 0.5 mM SA application on growth of stressed (150 mM NaCl) ginger plantlets: a) root application, b) foliar spray.

Table 2
Effect of foliar application of SA on photosynthetic pigments of *Z. officinale* grown under *in vitro* salt stress.[a]

Treatment	Chl a (μg g^{-1} FW)	Chl b (μg g^{-1} FW)	Total Chl(μg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Chlorophyllase (nM min^{-1}mg^{-1})
0 mM NaCl	9.14 ± 0.11a	3.73 ± 0.32b	14.25 ± 0.18b	201.83 ± 0.21b	7.28 ± 0.16b
150 mM NaCl	5.37 ± 0.16b	1.82 ± 0.01c	7.33 ± 0.11c	128.4 ± 0.12c	15 0.28 ± 0.18a
150 mM NaCl +0.5 mM SA	9.01 ± 0.07a	6.09 ± 0.30a	16.31 ± 0.15a	220.26 ± 2.13a	8.79 ± 0.20b

[a] Values are the mean ± SE (*n*=3). Means with different letters designate a significant difference at $P < 0.05$ using DMRT. Chl: chlorophyll, FW: fresh weight of the tissues.

Table 3
Effect of foliar application of SA on ionic content of *Z. officinale* grown under *in vitro* salt stress.[a]

Treatment	Na^+ (mg g^{-1})	K^+ (mg g^{-1})	Cl^- (mg g^{-1})	K^+/Na^+
0 mM NaCl	19.73 ± 0.63c	50.96 ± 0.17b	25.14 ± 1.43a	2.58 ± 0.01a
150 mM NaCl	47.16 ± 0.54a	34.83 ± 0.83c	54.83 ± 0.95c	0.73 ± 0.10c
150 mM NaCl +0.5 mM SA	24.3 ± 0.69b	56.33 ± 0.72a	35.06 ± 0.63b	2.31 ± 0.01b

[a] Values are the mean ± SE (*n*=3). Means with different letters designate a significant difference at $P < 0.05$ using DMRT

roots thereby not maintaining its sufficient concentration to show stress defense response. According to Gondor et al. (2016), SA may affect its own synthesis by modulating the expression and activity of phenylalanine ammonia lyase (PAL) and reported decreased SA synthesis in wheat root tissues treated with exogenous SA.

Although root is the first organ to come in contact with salt ions, its toxic effects are mainly on leaves (Bernstein, 2019). Leaf is the major site of biochemical processes and salt uptake adversely affects these processes (Bernstein, 2019). Therefore, direct application of SA right at the site of damage could have resulted in better recovery compared to root application in the present study. According to Ahmad et al. (2019) SA may impact root metabolism but its transportation to the leaves makes them the main organ to activate the associated changes. Our results are in agreement with those of Golkar et al. (2019) and Danaee et al. (2015) who reported SA- mediated improved *in vitro* growth under salinity. In *in vivo* studies also, SA application significantly improved the decreased rates of germination and growth under salinity in rice (Jini and Joseph, 2017), *Dianthus superbus* (Ma et al., 2017) and tomato (Naeem et al., 2020). All these reports indicate the role of SA in growth regulatory mechanisms and reducing salinity induced damage (Ardebili et al., 2014; Gautam and Singh, 2009).

Several studies have established a close relationship between growth and photosynthesis (Shao et al., 2014). In the present study, salinity reduced the contents of photosynthetic pigments such as chl a, chl b, total chlorophyll and carotenoids, which is in agreement with the results reported in *Torreya grandis* (Li et al., 2014) and *D. superbus* (Ma et al., 2017). Santos (2004) have demonstrated that salt-induced decrease in chlorophyll content was due to reduced accumulation of 5-aminolaevulinic acid (ALA), a precursor of chlorophyll. Another reason for this decline could either be due to down regulation of enzymes involved in chlorophyll synthesis and/or upregulation of chlorophyll degradation enzymes such as chlorophyllase (Sarangthem et al., 2011). This explanation was validated by the results of our study wherein stressed

Table 4
Effect of foliar application of salicylic acid on ROS production and antioxidative enzyme activities of *Z. Officinale* under *in vitro* salt stress.

Treatment	H_2O_2(μmol g^{-1}FW)	O_2^-Production rate(μM min^{-1g} $^{-1}$FW)	CAT activity(μM min^{-1}mg^{-1}protein)	POD activity(U mg^{-1}protein)	SOD activity(U mg^{-1}protein)
0 m MNaCl	478.33 ± 0.78c	0.4 ± 0.20c	2.11 ± 0.15c	4.78 ± 0.60c	7.42 ± 0.36c
150 mM NaCl	701.56 ± 0.63a	0.73 ± 0.29a	3.21 ± 0.69b	5.96 ± 1.16b	9.22 ± 0.71b
150 mM NaCl +0.5 mM SA	490.83 ± 0.37b	0.51 ± 0.52b	6.45 ± 0.64a	9.32 ± 0.43a	15.78 ± 0.76a

Values are the mean ± SE (*n*=3). Means with different letters designate a significant difference at $P < 0.05$ using DMRT, FW-fresh weight of the tissues

plantlets showed a rise in chlorophyllase activity (2-fold) with a consequent decline in chlorophyll contents. Although being a negative outcome of stress, reduction in chlorophyll content has been considered as an adaptive characteristic as this leads to reduced light absorption thereby causing photoprotection (Elsheery and Cao, 2008). During photosynthesis, carotenoids act as collectors of light energy and quenchers of chlorophyll and oxygen. They also function as antioxidants which neutralize ROS generated under stress (Verma and Mishra, 2005). However, in the present study, reduced carotenoid contents under salinity indicated that they were not involved in the protection mechanism.

Foliar application of 0.5 mM SA to stressed ginger plantlets increased the contents of all the photosynthetic pigments with a maximum increase of 5-fold seen in chl b. Similar results on enhanced pigment contents were obtained in *T. grandis* (Li et al., 2014) and *D. superbus* (Ma et al., 2017). SA might stimulate chlorophyll *b*iosynthesis or reducing chlorophyll degradation. In our study, rise in chlorophyll could be associated with the reduction (50%) in chlorophyllase activity. This mechanism could further be supported by the findings of Kohli et al. (2018) wherein SA- induced increase in chlorophyll contents was related to the decline in expression of chlorophyllase gene in *Brassica juncea* L. According to Idrees et al. (2010), SA-induced rise in photosynthetic pigments could be attributed to its role in promoting *Rubisco* activity and photosynthesis.

Salinity-induced disturbance in the uptake of ions has been reported (Isayenkov and Maathuis, 2019). In the current study, increased Na^+ and Cl^- ion concentration and reduced K^+ ion concentration were noticed in stressed plantlets. Absorption of Na^+ ions adversely affects the intracellular content of potassium ions and hence the K^+/Na^+ ratio (James et al., 2011). The reduction of K^+concentration in tissue may be due to direct competition between K^+ and Na^+ ions at plasma membrane, Na^+induced inhibition of K^+ transport process in xylem tissues and/or K^+ efflux from the roots (Chen et al., 2007). In the present study, SA treatment reduced $Na^+$and Cl^- ion accumulation and increased K^+ions resulting in improved K^+/Na^+ ratio. Similar effect of SA on ionic levels in NaCl-exposed *Arabidopsis* (Jayakannan et al., 2013), mungbean (Khan et al., 2014) and rice (Jini and Joseph, 2017) has been reported. Jayakannan et al. (2013) provided evidence on the role of SA in restoring membrane potential and reducing K^+ efflux via GORK channels, which could be a mechanism towards attaining ion homeostasis.

It is well documented that salinity causes oxidative stress leading to the generation of ROS (Kang et al., 2014). We also observed a rise in superoxide radical generation and H_2O_2 contents in salt stressed plantlets but application of SA significantly reduced their production. Sakhabutdinova et al. (2004) noticed down regulation of ROS in SA treated roots of young wheat seedlings. To repair the ROS driven damage, plants switch on antioxidative defense system (Gill and Tuteja, 2010; Okatan, 2020) wherein expression and coordinated activities of antioxidant enzymes lead to attain a balance between generation and removal of ROS. SOD is the first enzyme involved in detoxification of superoxide radical into H_2O_2 which in turn is scavenged by POD and CAT to produce water and oxygen. In the present study, activities of SOD, POD and CAT were higher in stressed plants compared to control and the activities further increased upon SA treatment. Similar observations on SA-mediated rise in antioxidant enzyme activity has been reported (Ma et al., 2017; Miura and Tada, 2014; Nazar et al., 2011). These findings indicate the role of SA in controlling the expression of genes encoding antioxidative enzymes (Kang et al., 2014). The possible mechanism could be through ROS regulation (Rizhsky et al., 2004). Low levels of ROS results into activation of enzymes (NADPH oxidases) of ROS signaling pathway resulting into amplified ROS signals to attain cellular redox balance (Miller et al., 2004). This in turn triggers the activation of defense genes including antioxidative enzymes. Agarwal et al. (2005) while studying the effect of SA on antioxidant system in wheat proposed that H_2O_2 produced in response to SA treatment might have induced the synthesis of transcription factors associated with induction of antioxidant enzymes. Our results indicate that enhanced activities of enzymes helped to cope with the ROS driven damage enabling ginger plantlets to survive under saline conditions.

5. Conclusions

Salinity showed significant adverse effects on all the measured parameters of ginger plantlets grown under *in vitro* conditions. Application of SA alleviated the stress and improved the growth characteristics. This action of SA could be attributed to its involvement in pigment biosynthesis, maintaining ionic balance and activating enzymatic antioxidative defense system in plant tissues. The results of the present study suggested that foliar spray of SA (0.5 mM) under *in vitro* conditions could be an efficient strategy for rapid production of salt adapted ginger plantlets. It is required to check whether these results can be extrapolated to the whole plant level so that SA application could be proposed as an approach to relieve the symptoms of salt stress under field conditions.

Authors contribution statement

A. Hundare contributed in conduct of experiments, interpretation of results, V. Joshi contributed in interpretation of results, data analysis. N. Joshi contributed in interpretation of results, data analysis and preparation of manuscript. All the authors contributed to the study conception and design and approved the manuscript.

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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