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Enhancing Antioxidants Defense System of Snap Bean under NaCl Salinity Using Foliar Application of Salicylic Acid, Spermidine and Glycine Betaine

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Abstract: The adjustments in plant growth, water content, membrane permeability, lipid peroxidation and antioxidant system corresponding to the tolerance to salt stress, were investigated in salt-sensitive snap bean plant foliar treated with salicylic acid (SA), spermidine (Spd) and glycine betaine (GB) during 2012 and 2013 seasons in outdoor pot experiment. Salinity was applied as NaCl form at 0 and 2000 ppm. The concentration of foliar applied treatments consisted of SA at 0 & 1 mM, Spd at 0 & 0.5 mM and GB at 0 & 5 mM, in addition to their combinations. Although NaCl salinity at 2000 ppm significantly reduced the dry weight of root, stem and leaf of snap bean plant and consequently absolute vegetative growth rate (AVGR), an increase in specific leaf area (SLA) values were recorded which refer mainly to the decrease in leaf d.w. The highest significant values in plant organs d.w and AVGR under 0 ppm NaCl salinity recorded with the combined treatments of GB 5 + SA1 + Spd 0.5 and GB 5 + Spd 0.5 which in turn reflected on the increase in pods no./plant, average pod f.w and pod area, while under 2000 ppm NaCl salinity the highest values in plant organs d.w and pod parameters were for GB 5 + SA1 + Spd 0.5 treatment. Under 2000 ppm NaCl, most treatments comparing with control, especially for GB 5 + SA1 + Spd 0.5 treatment led to an increase in leaf water content, non-enzymatic total antioxidant capacity (NEAC) in leaves and superoxide dismutase (SOD) in leaves and pods, while led to a decrease in leaf relative membrane permeability (RMP), malondialdehyde (MDA) equivalents in leaves and pods and NEAC in pods. Under salinity conditions, the highest peroxidase activity in leaves recorded with GB at 5 mM, while in pods the highest values recorded with the combined treatments with GB at 5 mM.

Key words: Snap bean · *Phaseolus vulgaris* · Salt stress · Antioxidants · Salicylic acid · Spermidine · Glycine betaine

INTRODUCTION

Salinity affects almost every aspect of the physiology and biochemistry of plants and lead to osmotic stress and ion toxicity which, significantly reduces membrane permeability, plant growth and yield [1]. Salinity reduces the ability of plants to utilize water and causes not only a reduction in growth rate but also changes in plant metabolic processes [2]. Salinity not only reduced photosynthetic pigments, fresh and dry weights of bean plants, but also declined the stomatal conductance which led to a reduction in intercellular CO_2 concentration and reduces $NADP^+$ regeneration by the Calvin Cycle, which in turn explain the reduction in assimilation rate under salinity conditions [3, 4]. These adverse conditions increase the rate of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), single oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) by enhanced leakage of electrons to molecular oxygen.

Most of ROS are produced in plants under optimal growth conditions as byproducts of aerobic metabolism at a very low level in cell organelles which act as signaling molecules to control various processes, whereas the production of ROS is extremely enhanced under environmental stresses such as drought, salinity and heat stress leading to oxidative stress [5]. The induced oxidative damage in sensitive plants, leads to lipid peroxidation of unsaturated fatty acids in membranes and huge damages in the structure of macromolecules such as pigments, proteins and nucleic acids, whereas tolerant plants are surfeited with mechanisms to combat any increase in ROS levels during environmental stresses

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[5, 6]. Deleterious effects in plant tissue caused by oxidative stress is alleviated by a concerted action of both enzymatic (SOD, POD, etc.) and non-enzymatic (β-carotene, ascorbate, phenolic, etc.) antioxidant systems [6].

Many previous reports concluded that snap bean (*Phaseolus vulgaris* L) is classified as a salt sensitive plant and suffers from growth and yield loss between 10 and 50% at salinity level from 1 to 3 dSm^{-1} [7, 8]. There was a 85% growth reduction on a dry weight basis of bean plants subjected to 96 mM NaCl [8]. Followed researchers studied the antioxidant system in bean plants subjected to different abiotic stress and found an increase the level of lipid peroxidation with concomitant decrease in the activity of SOD, catalase (CAT), ascorbate peroxidase (APX) and POD which led to a reduction in the protection mechanism against oxidative damage induced by abiotic stress [9, 10]. Bean as most legume crops due to their ability to fix nitrogen in the root nodules and their capacity to grow on nitrogen-poor soils, they can be efficiently used for improving saline soil fertility and help to reintroduce agriculture to these lands [11], if we can enhanced its tolerant to oxidative stress.

Salt tolerance plants often activates cell signaling pathways including those that lead to synthesis of ABA, osmoprotectants active metabolites (amino acids, sugars, GB and polyamines), specific proteins and certain free radical scavenging enzymes [11]. Furthermore, many previous reports showed that the exogenous application of osmoprotectants such as GB and phyto-hormones such as polyamines and SA mitigated the adverse effects produced by many environmental stresses, in addition to enhancing the antioxidant defense system in plant cells of many legume crops [1, 12-15].

Salicylic acid is an endogenous growth regulator, actively involved in most stages of plant growth and development such as photosynthesis, stomatal conductance and fruit ripening [16]. It has been reported that exogenous application of SA enhanced the protection of photosynthetic pigments in barely and the maintenance of membrane integrity and decreased the level of lipid peroxidation induced by NaCl salinity [17, 14]. Polyamines (putrescine, spermidine and spermine) are positively charged at physiological pH which allow them to interact with negatively charged macromolecules as DNA and phospholipids, which make them involved in the regulation of cell membranes and modulation of enzyme activities [18]. Polyamines show anti-senescence, anti-stress effects, in addition to its ability to stabilize membrane and cell wall as a result of acid neutralizing and antioxidant capability [15]. GB is thought to protect the plant by stabilizing macromolecules and by balancing water potential between the plant cell and the environment. GB is mainly localized in chloroplasts and plays a vital role in chloroplast adjustment and protection of thylakoid membranes, thereby maintaining photosynthetic efficiency and plasma membrane integrity [1].

The present study was designed to explore the capability effects of SA, Spd and GB and their combinations as foliar application on motivating the activity of antioxidant defense systems of snap bean plants, exposed to slightly NaCl salinity stress (2000 ppm) and its relation with decreasing the adverse effects of salinity on plant growth and productivity.

MATERIALS AND METHODS

A pot experiment was conducted during the two growing seasons of 2012 and 2013 under outdoor conditions in acid washed sandy soil, at The Experimental Farm, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, in order to investigate the effect of foliar application with salicylic acid, spermidine and glycine betaine and their combinations on improving the status of antioxidant defense systems, which reflected on enhancing vegetative and reproductive growth of snap bean plants under two levels of NaCl salinity.

Experimental Design and Treatments: Ten seeds of snap bean cv. Bronco were sown on 1st of March during 2012 and 2013 seasons in 15-liter plastic pot (30 cm diameter in the top of pot) filled with 14 kg acid washed sandy soil. The seedlings were watered with Hoagland solution [19]. After ten days from germination, seedlings were thinned to three homogeneous seedlings.

The foliar applied treatments consisted of salicylic acid (SA) at 0 & 1 mM, spermidine (Spd) at 0 & 0.5 mM, glycine betaine (GB) at 0 & 5 mM and a mixture of their combinations as shows below. Plants were sprayed four times with 8-day intervals started at the growth stage 14 (unfolding of second trifoliate leaf) of BBCH scale (officially stands for Biologische **B**undesanstalt, **B**undessortenamt und **CH**emische Industrie) which used to identify the phenological development stages of a plant [20]:

- Control
- SA at 1 mM
- Spd at 0.5 mM
- Spd at 0.5 mM + SA at 1 mM
- GB at 5 mM
- GB at 5 mM + SA at 1 mM
- GB at 5 mM + Spd at 0.5 mM
- GB at 5 mM + SA at 1 mM + Spd at 0.5 mM

For salinity levels, pots were divided into two groups, the first was irrigated with full strength Hoagland nutrient solution to serve as control plants (0 ppm NaCl). The second group was the same plus 2000 ppm NaCl in the nutrient solution. The salinity treatment started at the growth stage 15 of BBCH scale (unfolding of third trifoliate leaf). Treatments were arranged in a complete randomized block design with three replicates.

Vegetative Growth Characteristics: Root, stem and leaf dry weights were recorded at full bloom stage (50 days after sowing) in addition to total leaf area per plant (L_A), to calculate specific leaf area (SLA) using equation of Hunt [21]. A simple modification on Hunt [22] equation has been done to calculate absolute vegetative growth rate ($AVGR_{1-2}$) as mg d.w/day, where dry weights of vegetative plant parts (plant without pods) of snap bean plant were recorded at 50 days after sowing (Wv_1) and after 20 days (Wv_2).

$$SLA = L_A / L_W \text{ (total leaf dry weight)}$$
$$AVGR_{1-2} = (Wv_2 - Wv_1)/(t_2 - t_1)$$

Plant Productivity Analysis: The green pods were harvested at the optimum marketable stage of pod growth, where 50% of pods have reached typical length of the BBCH scale [20]. Number of marketable pods per plant, average pod fresh weight, pod area and the ratio between total pods fresh weight and shoot system fresh weight of snap bean plant were calculated. Average pod area were calculated from analyzing images of marketable pods by Image-pro plus software (version 6.2, Media Cybernetics Inc., USA).

Tissue Water Content Measurement (TWC): The leaf water status was calculated on the basis of dry weight (d.w) as:

$$TWC \text{ (ml } g^{-1} \text{ d.w)} = (f.w - d.w) / d.w.$$

Relative Membrane Permeability (RMP): The relative permeability of the cell membrane (%) or the relative electrolyte leakage (%), was determined using a conductivity meter (LYS – DRLANGE) as described by Yang *et al*. [23]. Disks of leaf samples (0.5 g f.w) were placed in test tubes containing 20 ml of distilled water. The test tubes were vortexed for 3 seconds, then the initial electrical conductivity (EC_0) of each sample was measured. After 24 h from storing samples tubes at 4 °C, the electrical conductivity was measured again (EC_1). The same samples were autoclaved at 120 °C for 15 min, cooled to room temperature and conductivity was measured for a third time to determine EC_2. Percent RMP was calculated as

$$RMP (\%) = [(EC_1-EC_0)/(EC_2-EC_0)] X 100$$

Assessing Oxidative Damage and Antioxidants Status: Leaf and pod samples were collected at 60 days after sowing to determine lipid peroxidation, non-enzymatic total antioxidant capacity and the specific activity of superoxide dismutase and peroxidase enzymes.

Lipid Peroxidation: Oxidative damage to membrane lipids produce malondialdehyde (MDA) as a secondary end-product of the oxidation of polyunsaturated fatty acids, which reacts with thiobarbituric acid (TBA) to form a pink solution with maximal absorbance at 532 nm [24]. Lipid peroxidation rates were estimated by measuring the malondialdehyde equivalents according to improving method by Hodges *et al*. [25]. Two groups of the reaction mixture were used with the extraction of plant samples, one of them with TBA and the other without TBA. After heating samples at 95 °C for 30 min. Absorbance at 440, 532 and 600 nm were used for calculation of MDA equivalents with excluding the effect of interfering substances.

Non-enzymatic Antioxidant Capacity (NEAC): The non-enzymatic total antioxidant capacity in extracts of snap bean leaves and pods were estimated by ammonium molybdate reduction method described by Prieto *et al*. [26]. The antioxidant capacity was expressed as equivalents of ascorbic acid (mg g^{-1} f. w).

Antioxidant Enzymes Assays: Enzyme extract for superoxide dismutase (SOD) and peroxidase (POD) was prepared by first freezing the weighed amount of samples

(1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 5 ml of cold extraction buffer (0.1 M phosphate buffer, pH 7, containing 0.5 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP)) and centrifuged for 20 min at 10,000 x *g* to use the supernatant as enzyme extract.

SOD (EC: 1.15.1.1) activity was determined by nitro-blue tetrazolium (NBT) photochemical assay at 560 nm following the method described by Beauchamp and Fridovich [27]. One unit of enzyme activity was taken as that amount of enzyme which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

POD (EC: 1.11.1.7) activity was determined using *o*-dianisidine as a chromogenic indicator in the presence of H_2O_2 and enzyme extract at 460 nm as described by Shannon *et al*. [28]. The absorbance was read every 30 second interval up to 3 minutes. The reaction mixture consisted of 0.2 ml 5 mM *o*-dianisidine, 0.2 ml 10 mM H_2O_2, 0.1 M potassium phosphate-citrate buffer, pH 5.0 and 0.1 ml enzyme extract in a total volume of 3.0 ml. The peroxidase activity was calculated using extinction co-efficient of *o*-dianisidine and the enzyme activity was expressed as unit mg^{-1} protein.

Statistical Analysis: Data of the two seasons were arranged and statistically analyzed using CoStat software (version 6.4, CoHort Software, USA) according to the method described by Gomez and Gomez [29]. Two-way analysis of variance (ANOVA) was used to test for significant differences among foliar application substances, salinity and their interactions at $P < 0.05$, followed by Tukey's HSD test. One-way ANOVA was used to reveal significant differences across foliar application substances treatments within individual salinity level while a post hoc Tukey's HSD test was used to test for significant differences between individual treatments means.

RESULTS

Vegetative Growth Characteristics: Root, stem and leaf dry weights were recorded which followed by measuring SLA and AVGR of vegetative plant parts (plant without pods) of snap bean plants during critical period of flowering and pod filling (Table 1). All studied vegetative parameters except for SLA were decreased under the effect of treatment with NaCl at 2000 ppm comparing with 0 ppm NaCl. Since, SLA calculated from the ratio between total leaf area and its dry weight, so the increasing in SLA values under 2000 ppm NaCl salinity could refer mainly to the remarkable decrease in leaf d.w under 2000 ppm NaCl where leaf area index was already decreased under salinity stress [30]. This decrease in values of leaf d.w under NaCl salinity lead to an increase in calculated values of SLA. Although, all foliar treatments led to a significant increase in the values of SLA under all salinity levels comparing with control, the highest values under these conditions recorded with GB 5 mM individual application. The superiority of GB application in SLA parameter refer to its effect on increasing leaf area (non-published data), since its effect on the values of leaf d.w were insignificant with control under both salinity levels (Table 1). Predominantly, the highest significant values in root, stem and leaf d.w under both salinity levels recorded with GB 5 + SA1 + Spd 0.5 application. The AVGR values didn't take the same trend as plant organs d.w done for GB 5 + SA1 + Spd 0.5 treatment under both level of salinity, where the higher values in AVGR under 0 ppm NaCl recorded with GB 5 + Spd 0.5 and GB 5 + SA1 + Spd 0.5 treatments respectively. In contrast, under 2000 ppm NaCl salinity, the highest value in AVGR recorded with individual application of SA at 1 mM. Whereas treatment of GB at 5 mM as individual or with all its combinations recorded the lowest levels in AVGR above the control treatment. This decrease in values of AVGR comparing with SA treatment under 2000 ppm NaCl refer mainly to the effect of GB on directed most growth in pod filling stage to developing pods, whereas SA directed the assimilates to stimulate the growth in both vegetative parts of snap bean plant (Table 1) and developed pods (Table 2).

Plant Productivity Characteristics: The most important traits effect on plant productivity are number of marketable pods and average pod weight, where both of them values declined significantly under 2000 ppm NaCl (Table 2). Under this level of salinity, the most effective treatments led to boosting the values of pods no. and pods f.w were the combined treatment of GB 5 + SA1 + Spd 0.5 (5.8 pod no./plant & 3.49 g/pod in 1st season) in addition to individual treatment of SA at 1 mM which recorded 5.4 for pods no./plant and 3.79 g/pod in 1st season comparing with 3.8 & 2.59 for pods no. and pod f.w of control plant respectively (Table 2). These increases in the values of both parameters could refer mainly to the stimulation effect of SA on AVGR

Table 1: Effect of foliar application of salicylic acid (SA), spermidine (Spd), glycine betaine (GB) and their combinations under different levels of NaCl salinity (0 and 2000 ppm) on average dry weight of leaf, stem and root, specific leaf area (SLA: mm^2/mg d.w) and absolute vegetative growth rate (AVGR: mg d.w/day) of snap bean plants in both seasons (2012 and 2013)

	Leaf d.w. (g)	Stem d.w. (g)	Root d.w. (g)	AVGR	SLA	Leaf d.w. (g)	Stem d.w. (g)	Root d.w. (g)	Absolute □	SLA
Foliar treatments (mM)	---------- 1st season ----------					---------- 2nd season ----------				
	---------- NaCl at 0 ppm ----------									
Control	0.24 bc	0.74 b	0.34 c	52 bc	11.1 d	0.24 b	0.78 b	0.35 c	53 d	10.5 e
SA 1	0.29 ac	0.96 ab	0.49 ac	146 a	13.9 cd	0.28 ab	1.00 ab	0.56 ac	139 b	12.9 de
Spd 0.5	0.35 ab	0.95 ab	0.39 bc	71 bc	19.1 b	0.38 a	1.07 ab	0.48 ac	74 c	18.4 bc
Spd 0.5 + SA1	0.31 ac	0.94 ab	0.42 ac	84 b	17 bc	0.31 ab	1.03 ab	0.48 ac	80 c	16.7 bd
GB 5	0.26 ac	0.91 ab	0.37 bc	33 c	24.5 a	0.27 ab	0.98 ab	0.40 bc	35 e	23.2 a
GB 5 + SA 1	0.23 c	0.78 b	0.49 ac	64 bc	20.3 b	0.23 b	0.86 ab	0.52 ac	69 cd	19.3 ab
GB 5 + Spd 0.5	0.37 a	1.09 ab	0.8 ab	191 a	16.9 bc	0.39 a	1.14 ab	0.90 ab	171 a	16.3 bd
GB 5 + SA1 + Spd0.5	0.38 a	1.21 a	0.84 a	164 a	15.5 c	0.38 a	1.29 a	0.93 a	168 a	14.5 ce
Mean	0.30 A	0.95 A	0.52 A	101 A	17.3 B	0.31 A	1.02 A	0.57 A	99 A	16.4 B
	---------- NaCl at 2000 ppm ----------									
Control	0.17 c	0.49 b	0.16 c	5 e	12.1 b	0.18 c	0.53 a	0.17 b	6 e	11.4 b
SA1	0.26 a	0.95 a	0.36 ac	82 a	18.1 a	0.29 a	1.05 a	0.40 ab	85 a	17.1 a
Spd0.5	0.19 bc	0.57 ab	0.21 bc	38 b	19.9 a	0.2 bc	0.61 a	0.23 ab	40 b	18.9 a
Spd0.5 +SA1	0.25 ab	0.76 ab	0.39 ab	36 b	18.7 a	0.26 ab	0.81 a	0.43 ab	38 b	18.0 a
GB5	0.23 ac	0.54 ab	0.23 bc	19 cd	20.2 a	0.24 ac	0.58 a	0.25 ab	21 c	18.9 a
GB5 +SA1	0.27 a	0.66 ab	0.40 ab	12 ce	19.0 a	0.27 ab	0.71 a	0.46 a	14 d	18.4 a
GB5 + Spd0.5	0.20 ac	0.55 ab	0.31 ac	11 de	17.5 a	0.21 ac	0.56 a	0.32 ab	14 d	16.5 a
GB 5 + SA1 + Spd0.5	0.27 a	0.95 a	0.45 a	19 c	20.0 a	0.27 ab	1.00 a	0.48 a	22 c	19.0 a
Mean	0.23 B	0.69 B	0.31 B	28 B	18.2 A	0.24 B	0.73 B	0.34 B	30 B	17.2 A

Means followed by different letters are significantly different at P< 0.05 level; Tukey's HSD test. Where d.w. = dry weight.
Capital letters for mean of NaCl salinity level, whereas lowercase letters for interaction between NaCl level and foliar treatment.

Table 2: Effect of foliar application of salicylic acid (SA), spermidine (Spd), glycine betaine (GB) and their combinations under different levels of NaCl salinity (0 and 2000 ppm) on pods/shoot f.w ratio, marketable pods no./plant, average pod f.w and pod area of snap bean plants in both seasons (2012 and 2013)

	Pods/shoot ratio (f.w/f.w)	Marketable pods no./plant	Average pod f.w (g)	Pod area (cm^2)	Pods/shoot ratio (f.w/f.w)	Marketable pods no./plant	Average pod f.w (g)	Pod area (cm^2)
Foliar treatments (mM)	---------- 1st season ----------				---------- 2nd season ----------			
	---------- NaCl at 0 ppm ----------							
Control	1.14 ab	4.0 b	3.76 ac	7.4 ab	1.24 a	4.0 b	3.83 ab	7.8 c
SA 1	1.05 b	5.4 ab	3.28 bc	7.0 ab	1.19 a	5.3 ab	3.31 b	7.3 c
Spd 0.5	1.48 ab	5.4 ab	4.47 ab	6.7 b	1.72 a	5.8 ab	4.90 a	7.1 c
Spd 0.5 + SA1	1.16 ab	4.2 b	3.74 ac	7.0 ab	1.27 a	4.5 ab	4.12 ab	7.2 c
GB 5	1.07 b	5.4 ab	3.01 c	7.6 ab	1.26 a	5.8 ab	3.20 b	7.8 c
GB 5 + SA 1	1.55 a	5.2 ab	3.24 bc	7.7 ab	1.75 a	5.8 ab	3.28 b	8.1 bc
GB 5 + Spd 0.5	1.35 ab	6.2 ab	4.61 a	9.0 a	1.56 a	6.3 ab	4.58 ab	9.4 ab
GB 5 + SA1 + Spd0.5	1.37 ab	7.4 a	4.6 a	9.0 a	1.39 a	7.5 a	4.63 ab	9.5 a
Mean	1.27 A	5.4 A	3.84 A	7.7 A	1.42 A	5.6 A	3.98 A	8.0 A
	---------- NaCl at 2000 ppm ----------							
Control	1.36 a	3.8 a	2.59 b	7.2 a	1.46 a	4.0 a	2.74 a	7.6 a
SA1	1.33 a	5.4 a	3.79 ab	8.3 a	1.36 a	5.5 a	3.97 a	8.9 a
Spd0.5	1.34 a	3.8 a	2.83 ab	8.0 a	1.39 a	4.3 a	2.91 a	8.6 a
Spd0.5 +SA1	1.46 a	5.0 a	2.98 ab	8.3 a	1.56 a	5.5 a	3.12 a	8.9 a
GB5	1.31 a	4.0 a	3.95 a	7.7 a	1.35 a	4.3 a	3.98 a	8.2 a
GB5 +SA1	1.19 a	4.8 a	2.82 ab	7.1 a	1.32 a	5.5 a	3.27 a	7.8 a
GB5 + Spd0.5	1.08 a	3.8 a	2.97 ab	7.5 a	1.23 a	4.3 a	2.96 a	7.9 a
GB 5 + SA1 + Spd0.5	1.35 a	5.8 a	3.49 ab	8.6 a	1.44 a	6.3 a	3.70 a	9.1 a
Mean	1.30 A	4.6 B	3.18 B	7.9 A	1.39 A	4.9 A	3.33 B	8.4 A

Means followed by different letters are significantly different at P< 0.05 level; Tukey's HSD test. Capital letters for mean of NaCl salinity level, whereas lowercase letters for interaction between NaCl level and foliar treatment.

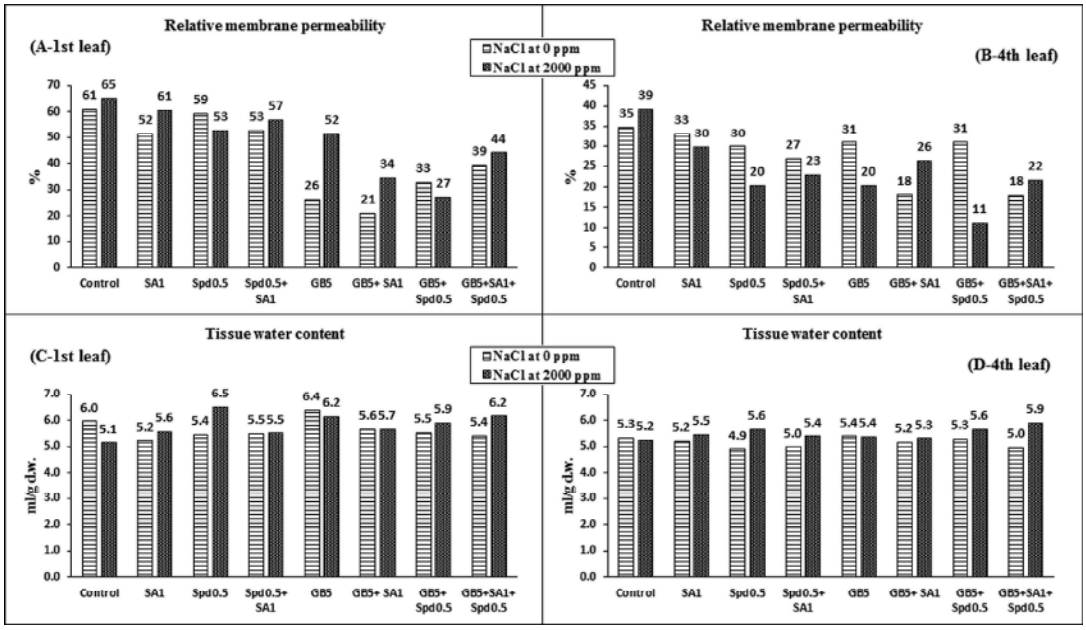

Fig. 1: Effect of foliar application of salicylic acid (SA), spermidine (Spd), glycine betaine (GB) and their combinations on relative membrane permeability % (A and B) and tissue water content (C and D) in snap bean 1st leaf (A & C) and 4th leaf (B & D) under different levels of NaCl salinity (0 and 2000 ppm) in 2012 and 2013 seasons (main of two seasons).

(Table 1) during pod filling stage which reflected on pods productivity. Individual application of GB at 5 mM recorded the highest significant value in average pod f.w (3.95 g), which was not followed by an increase in pods no. where recorded 4 pod/plant. When SA at 1 mM mixed with GB at 5 mM promoted the pods no./plant to reach 4.8 pod/plant in 1st season, which in turn could explain the increase in values of both pods no./plant and pod f.w under the treatment of GB 5 + SA1 + Spd 0.5 (Table 2). All foliar treatments led to an increase in average pod f.w over control under 2000 ppm NaCl, whereas under 0 ppm NaCl, values of pod f.w decreased under individual application of SA at 1 mM and GB at 5 mM comparing with its control. The maximum significant values in pods no./plant, average pod f.w and average pod area under 0 ppm NaCl recorded with combined application of GB 5 + SA1 + Spd 0.5 and GB 5 + Spd 0.5 respectively which could be refer to the boosting effect of individual treatments of SA at 1 mM and GB at 5 mM on pods no./plant, while application of Spd at 0.5 mM led to an increase in pods no./plant and pod f.w (Table 2). Although, values of pods/shoot f.w ratio slightly decreased under individual application of SA at 1 mM and GB at 5 mM than control under 0 ppm NaCl (1.05 & 1.07 comparing with 1.14 in 1st season), treating plants with their mixture boosted the value of pods/shoot ratio to record the highest value (1.55 in 1st season), which mainly refer to the increase in pods no./plant (Table 2) than slight increase in plant f.w [30].

Plant Water Status and Relative Membrane Permeability: The influence of salt stress (2000 ppm NaCl) on relative membrane permeability (RMP) was more pronounced than its effect on tissue water content (TWC) of snap bean first and fourth leaf from the top of the plant (Figure 1). The decreased values of RMP and TWC in fourth leaf of control plant (without foliar treatments) under both salinity level than first leaf indicated to that the physiological status of different plant leaves not equal, where the responding sensitivity to environmental conditions in youngest leaf is more sensitive than oldest leaf. In 1st leaf, the most effective treatments maximized the decrease in RMP values under both salinity level were GB at 5 mM as individual treatment and with all its combinations (Figure 1:A), whereas in 4th leaf under 2000 ppm NaCl, the lower values recorded with Spd at 0.5 mM, GB at 5 mM and GB 5 + Spd 0.5 respectively (Figure 1:B).

Values of TWC under 2000 ppm NaCl were increased when plants treated with tested foliar treatments for both 1st and 4th leaf of snap bean plant (Figure 1:C & D). The values of TWC in 1st leaf were higher than its values in 4th leaf, which refer to the increase in d.w of 4th leaf than

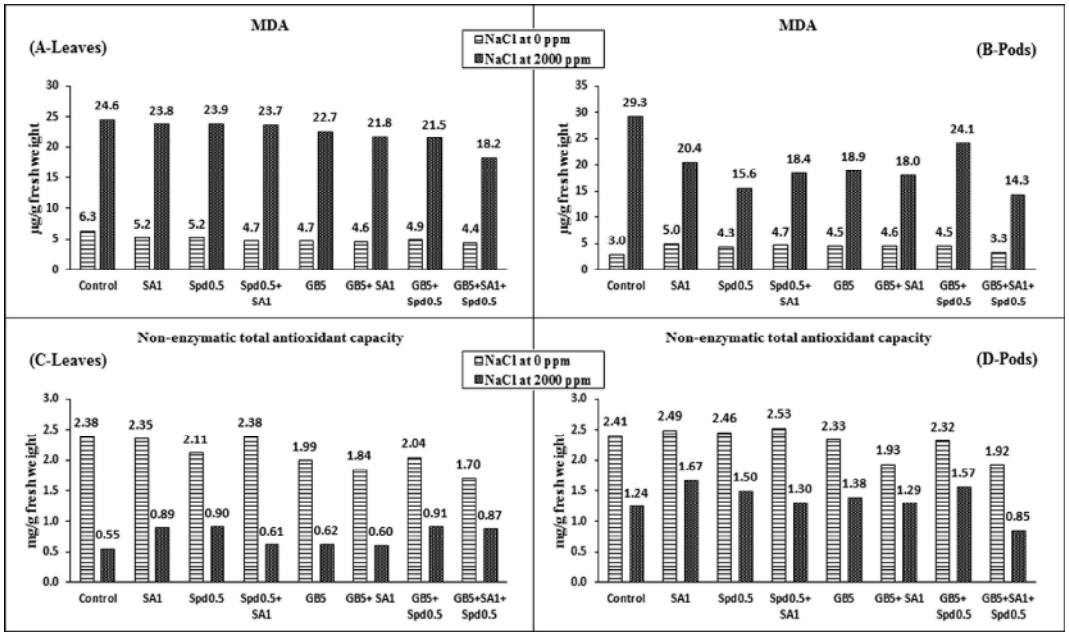

Fig. 2: Effect of foliar application of salicylic acid (SA), spermidine (Spd), glycine betaine (GB) and their combinations on MDA (A and B) and non-enzymatic total antioxidant capacity (C and D) in snap bean leaves (A & C) and pods (B & D) under different levels of NaCl salinity (0 and 2000 ppm) in 2012 and 2013 seasons (main of two seasons).

1st leaf (non-published data), since TWC calculated on the basis of dry weight. The most effective treatments increased the values of TWC under 2000 ppm NaCl were Spd 0.5, GB 5 and GB 5 + SA 1 + Spd 0.5 in 1st leaf (Figure 1:C), whereas for 4th leaf were for GB 5 + SA 1 + Spd 0.5 and GB 5 + Spd 0.5 treatments (Figure 1:D).

Biochemical Changes

Lipid Peroxidation: Malondialdehyde (MDA) is one of the final products of peroxidation to unsaturated fatty acids in membranes. Any increase in ROS causes overproduction of MDA. So, MDA concentration is commonly known as a marker of oxidative stress and the antioxidant status in plant cells [31]. The most obvious observation on MDA concentration was that NaCl salinity at 2000 ppm in control plants increased the levels of MDA to 4-fold in leaves and 10-fold in pods (Figure 2:A & B). Under salt stress, all foliar treatments decreased the levels of MDA in leaves, especially for GB 5 + SA 1 + Spd 0.5 treatment which recorded the lowest values. Although the same previous treatment was the best treatment in reducing MDA level in pods which reached to be a half amount of MDA concentration in control (Figure 2:B), the effect of remain foliar treatments on reducing MDA levels was better in pods than leaves (Figure 1:A & B). Under zero NaCl salinity, all foliar treatments slightly decreased the levels of MDA in leaves, whereas MDA levels in pods slightly increased under same treatments comparing with its control. This observation indicates to that cell membranes in pods are more sensitive than leaves to changes in growth conditions, which in turn indicate to that slightly increase in MDA could refer to slightly increase in ROS which act as a signal leading to increasing the antioxidants level in pods than leaves, especially for non-enzymatic antioxidants (Figure 2:D). This hypothesis also explains why the decrease in levels of MDA in pods under foliar treatments is more pronounced than its values in leaves.

Non-enzymatic Total Antioxidant Capacity (NEAC): Although the majority of foliar treatments decreased the levels of NEAC in leaves, the most effective treatments in this decrease were for all foliar treatments containing GB in both leaves and pods of snap bean non-stressed plants comparing with its control (Figure 2:C & D). Whereas, under salt stress conditions all foliar treatments increased the values of NEAC in leaves and pods of snap bean plants over control, except for GB 5 + SA 1 + Spd 0.5 treatment which recorded the lowest value (0.85 mg/g f.w) in pods. This unexpected effect of GB 5 + SA 1 + Spd 0.5 treatment on NEAC in pods could be explained when looking to the value of NEAC for the same treatment in

leaves which recorded near value (0.87 mg/g f.w.) for its value in pods (0.85 mg/g f.w). Such behavior in NEAC (near values in leaves and pods) did not repeated under the effect of all treatments unless for control of non-stressed plants which recorded 2.38 mg/g f.w in leaves and 2.41 mg/g f.w in pods. Exposing plants to NaCl at 2000 ppm decreased the levels of NEAC in leaves and pods under all foliar treatments comparing with non-stressed plants, where decreased in control plants to one quarter of its value in leaves (0.55 comparing with 2.38 mg/g f.w) and half its value in pods (1.24 comparing with 2.41 mg/g f.w). This greater decrease in values of NEAC under salt stress confirmed that snap bean plants is a case sensitive to salt stress. These previous observations (Figure 2:C & D), in addition to the following observation of that, the best treatment led to decrease the values of MDA in both leaves and pods was GB 5 + SA 1 + Spd 0.5 (Figure 2:A & B), leading to hypothesize that the combined treatment of GB 5 + SA 1 + Spd 0.5 balanced the metabolism between leaves and pods (source and sink) under salt stress as was happening under non-stressed conditions.

Antioxidant Enzymes: The same observation recorded for NEAC level in leaves of snap bean where all foliar treatments decrease NEAC values under non-stressed conditions while increasing its value under salt stress (Figure 2:C), repeated for the activity of superoxide dismutase (SOD) in leaves under both salinity levels (Figure 3:A). Whereas in pods of snap bean plant, the activity of SOD was increased under all foliar treatments comparing with its control for both stressed and non-stressed plants (Figure 3:B). Under 2000 ppm NaCl salinity, the most impressive treatments led to boosting the activity level of SOD were all treatments containing GB (Figure 3:A & B), especially in pods (Figure 3:B) which increase the SOD activity to about 12-fold in GB at 5 mM comparing with control (128 unit for GB and 11 unit for control), while in leaves increase SOD activity to 2-fold (106 unit for GB and 56 unit for control).

Peroxidase (POD) activity levels in leaves and pods of snap bean plants under 2000 ppm NaCl salinity recorded the highest levels comparing with 0 ppm NaCl salinity (Figure 3:C & D). These increases in POD activity levels which reached to 5-fold in leaves of control plants under salt stress (696 unit for 2000 ppm NaCl comparing with 132 unit for 0 ppm NaCl) and 2-fold in pods of control plants under same condition (591 unit for 2000 ppm NaCl comparing with 250 unit for 0 ppm NaCl), indicate to that the major defense system in snap bean plants under stress is the deferent isozymes of peroxidase, which in turn indicated to how much snap bean plants are sensitive to any little increase in salinity level, where in tolerant plants, the first defense line coping with oxidative stress is non-enzymatic antioxidants followed by enzymatic antioxidants with SOD in the top of its list, where both of them decreased in leaves and pods of snap bean plants (Figure 2:C & D for NEAC; Figure 3:A & B for SOD). The most effective treatments led to an increase in the levels of POD activity under salt stress were the individual application of GB at 5 mM in leaves, in addition to all treatments containing GB in pods (Figure 3:C & D).

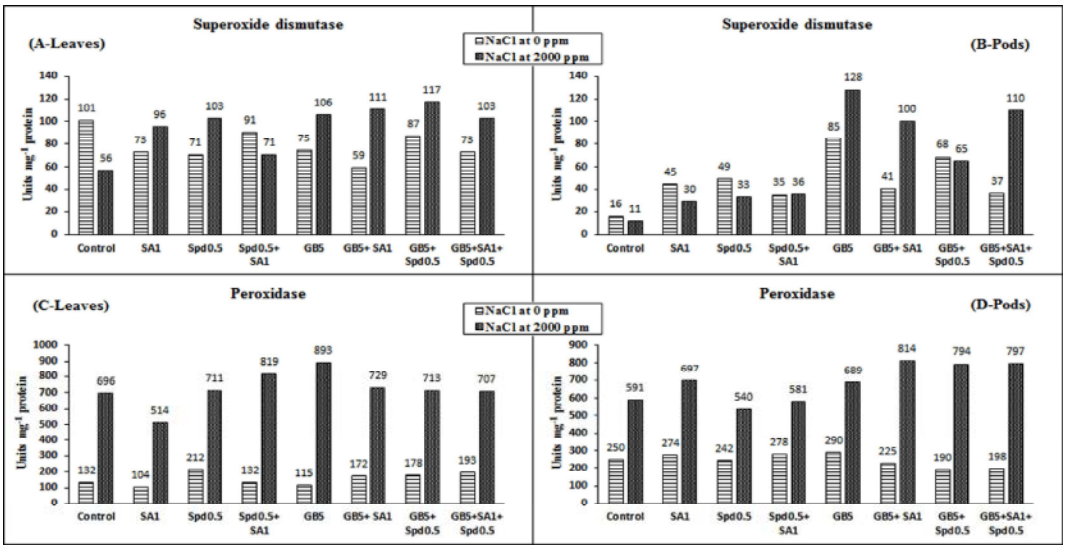

Fig. 3: Effect of foliar application of salicylic acid (SA), spermidine (Spd), glycine betaine (GB) and their combinations on activity of superoxide dismutase (A and B) and peroxidase (C and D) in snap bean leaves (A & C) and pods (B & D) under different levels of NaCl salinity (0 and 2000 ppm) in 2012 and 2013 seasons (main of two seasons).

DISCUSSION

The most important yield parameters indicate to the dynamic of vegetative growth and flowering status are pods number and average pod weight [13]. Salinity, due to its osmotic and ionic stress induce multiple responses led to reduce vegetative and reproductive growth constituents in most legume crops. Most legumes are sensitive or moderately sensitive to salinity [32]. It is well-established that plants tolerate environmental stresses including salinity through changing in gene expression leading to increase the synthesis of specific compounds known as osmo-protectants or phyto-protectants such as GB, polyamines, ABA and SA, which in turn increase the osmotic potential of the plant cell in addition to enhancing the antioxidant defense system which allow the metabolic processes to continue under these adverse conditions.

In this study, exposing snap bean plants to NaCl salinity at 2000 ppm reduced the AVGR with all related plant d.w parameters (Table 1), which in turn reduced average pod f.w and pods no./plant (Table 2). As parallel with current results, Cachorro *et al*. [33] reported that increasing salt concentration reduced plant shoot and root growth. As a consequence of the osmotic stress induced by salinity, the turgor of plant cells decreases leading to reduce the size of the growing tissues, the progressive effect of salinity is entering salts to the transpiration stream and eventually injure cells in the transpiring leaves, leading to further reduction in plant growth [2], which confirmed by decreasing the leaf tissue water content under salt stress in current study (Figure 1:C). This reduction in TWC could be refer to that, snap bean plant cells subjected to salt stress has a little ability to maintain cellular membrane integrity, which effect on membranes functions leading to an increase in relative membrane permeability (Figure 1:A & B). These adverse effects of salinity on plant growth, productivity and its water status refer mainly to increasing the levels of ROS in plant cells which resulted from stomatal closure developing a decrease in the concentration of leaf CO_2 and concomitant concentration of $NADP^+$ available to accept electrons from photosystems and thus initiate O_2 reduction with the concomitant generation of ROS [9]. This increase in ROS led to oxidative stress to cells macromolecules which reflected on inactivating enzymes, breaking DNA strands, protein denaturation and increasing the peroxidation of membrane fatty acids when measured as the content of MDA (Figure 2:A & B). Many previous reports showed a gradual increase in lipid peroxidation level with concomitant decrease in activities of the antioxidant enzymes of snap bean plants subjected to different abiotic stress such as drought and salinity [9, 10]. Under the effect of oxidative stress, lipid and membrane integrity are among the key factors in plants survival [31]. Plants divert their energy from growth to maintenance under abiotic stress [11]. Since lipid biosynthesis is energy dependent, which could hardly occur under salinity, the preservation of membrane lipids is the most efficient way to maintain functional membranes [31]. Moreover, under salt stress, the reduction of NEAC (Figure 2:C & D) and SOD (Figure 3:A & B) as a first line of antioxidants defense system could concluded that the key factor in enhancing plant growth is by decreasing the MDA levels and increasing the activity of antioxidants defense system.

The positive effects of SA at 1 mM, Spd at 0.5 mM and GB at 5 mM on plant growth and productivity is more pronounced under salinity conditions. Since, it led to an increase in plant organs d.w, AVGR and SLA (Table 1) and pods parameters (Table 2). Reducing relative membrane permeability using the application of SA, Spd and GB under saline conditions (Figure 1:A & B) refer to the reduction in MDA levels (Figure 2: A & B) and alleviated the amount of TWC (Figure 1:C & D), NEAC (Figure 2: C & D) and SOD (Figure 3:A & B).

The previous results of SA treatment confirmed by Khan *et al*. [34] who reported that treated mung bean plant with exogenous SA, enhanced the photosynthetic pigments and the maintenance of membrane integrity through reducing ROS levels and increasing the activities of antioxidant enzymes which induces protective effects on plants under salinity. Polyamines have a complex functional role in regulating cellular signaling and metabolism during stress and help maintain cellular ROS homeostasis by scavenging free radicals and activating antioxidant enzymes during stress conditions [35]. Spermidine has the ability to modulate ion balance of the cell and interact with anionic molecules, such as DNA, RNA, proteins and membrane lipids as it has polycationic nature at physiological pH leading to an increase in membrane stability index, relative water content, plant growth and pod yield [36, 30]. In the present study, the best increase in antioxidants defense system was under the application of GB at 5 mM. The most suggested role of GB is its involved in scavenging ROS and in protecting antioxidant enzymes [37]. Increasing SOD activity under GB treatment suggested that GB may protected membrane stability from salt-induced oxidative damage. In addition to SOD, increasing the protection level against oxidative damage should require fast removal of H_2O_2 by other scavenging systems, such as POD which also considerable increased under GB treatment. These results agreed well with those of Hu *et al*. [38], who observed that enhanced salt tolerance under GB treatment in

perennial ryegrass was mainly related to the elevated SOD, CAT and APX activity and alleviation of cell membrane damage by reducing lipid peroxidation and improving the ion homeostasis under salt stress.

CONCLUSION

Based on the decreasing values obtained from plant biomass, TWC, relative membrane permeability, NEAC and lipid peroxidation, it is clear that snap bean plant cv. Bronco is sensitive to salt stress. Increasing the capacity and activity of antioxidants defense system in salt-sensitive snap bean plant achieved by using foliar application of SA at 1 mM, Spd at 0.5 mM and GB at 5 mM. These treatments have the ability to maintain higher constitutive activity of SOD, POD and NEAC resulting in lower the peroxidation of membrane lipids, which enhancing the tolerance of snap bean plant to salt stress. It could be suggest using the application of combined GB at 5 mM + SA at 1 mM + Spd at 0.5 mM for multiply the green pods yield through enhancing the antioxidant defense system.

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