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EFFECT OF SALT STRESS ON GROWTH ATTRIBUTES AND ENDOGENOUS GROWTH HORMONES OF SOYBEAN CULTIVAR HWANGKEUMKONG

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Abstract

The adverse effects of NaCl induced salt stress on growth attributes and endogenous levels of gibberellins (GA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) soybean cv. Hwangkeumkong was showed. Plant length, biomass, chlorophyll content, number of pods, 100 seed weight and yield significantly decreased in response 70 mM and 140 mM concentrations of NaCl. Under salt stress, the endogenous GA and free SA content decreased, while a significant increase in the endogenous ABA and JA contents were observed. The results showed that salinity stress drastically reduce growth and yield components of soybean by affecting endogenous growth hormones.

Introduction

Salinity is of vital importance to present day agriculture, as rapid population growth especially in the developing world and consequently increased demand for agricultural products have made salinity oriented problems urgent. Salt stress reduces crop growth and yield in different ways. However, NaCl being the dominant salt in nature elicits two primary effects on plants i.e., osmotic potential and ionic toxicity. Under normal condition the osmotic potential in plant cells is higher than that in soil solution. Plant cells use this higher osmotic potential to take up water and essential minerals through root cells from the soil solution. Under salt stress the osmotic potential in the soil solution exceeds the osmotic potential of plant cells due to the presence of higher concentration of salt, which reduces the ability of plants to take up water and other essential nutrients (Munns *et al*., 2006). On the other hand, Na^+ and Cl^- ions can enter into the cells because of their prevalence and have their direct toxic effects on cell membranes, as well as on metabolic activities in the cytosol (Hasegawa *et al*., 2000). These primary effects of salinity stress causes secondary effects like reduced cell expansion, assimilate production and membrane function, as well as decreased cytosolic metabolism and production of reactive oxygen intermediates (ROS). As a result, in extreme cases, the plants may die under salt stress. In soybean, salinity stress inhibits seed germination and seedling growth, reduces nodulation, and decreases biomass accumulation and yield (Essa, 2002). These effects are induced by osmotically mediated interference with water and nutrient uptake (Brady & Weill, 2002). Salinity stress can also cause severe leaf chlorosis, leaf bleaching and necrosis, and ultimately plant death (Parker *et al*., 1987). More acute

salinity stress symptoms are induced by chloride accumulation in the leaf (Yang & Blanchar, 1993), which includes decreased photosynthesis and formation of super-oxide radicals, which cause membrane damage (Marschner, 1995). Chloride is a major anion in salts derived from fertilizer and sea water (Parker *et al*., 1983).

Salt stress also affects phytohormones which are naturally occurring organic substances, influencing physiological processes at low concentrations either in distant tissues to which they are transported or in the tissue where synthesis occurred (Davies, 1995a). Due to their structural simplicity, plant hormones are not specific enough to match the variety of controlled reactions (Canny, 1985). Contrary to this, it has been suggested that hormones only provide "turn on" or "turn off' signals and that the actual informations are provided by the cell. This scenario is similar to that of calcium, which is now thought to be an intermediate in some hormonal responses (Davies, 1995b). The two hormones for which there is consistent evidence for endogenous regulation in response to environmental stress are ABA and ethylene (Gianfagna *et al*., 1992), although gibberellins, auxins and cytokinins are also implicated in stress response (Levitt, 1980). JA is reported to be involved in diverse developmental processes, such as seed germination, root growth, fertility, fruit ripening and senescence (Creelman & Rao, 2002; Wasternack & Hause, 2002). JA activates plant defence mechanisms in response to insect-driven wounding, pathogens and environmental stresses including drought, low temperature and salinity (Wasternack & Parthier, 1997). SA application has resulted in tolerance of plants to many biotic and abiotic stresses including fungi, bacteria, viruses (Delany *et al*., 1994), chilling (Senaratna *et al*., 2003), drought (Senaratna *et al*., 2003) and heat (Dat *et al*., 1998; Senaratna *et al*., 2003).

Soybean is a member of family Fabaceae and the world foremost provider of protein and oil. It is often called the miracle crop as it contains high protein content (38–45%) as well as high oil content (20%). Soybean is generally considered to be salt-sensitive (Lauchli, 1984) and soybean plants grown in saline conditions exhibit symptoms of leaf chlorosis, stunting and biomass reduction as a result of chloride induced toxicity (Abel & MacKenzie, 1964). We investigated the effect of NaCl induced stress on hormonal attributes of soybean cultivar Hwangkeumkong when applied at pre-flowering and post-flowering growth stages.

Materials and Methods

General procedure: The experiment was in complete randomized block design (CRBD), with 4 replications per treatment and each replication comprising of 9 plants. Seeds of soybean cultivar Hwangkeumkong were surface sterilized with 5% NaClO for 15 min., and then washed thoroughly with double distilled water. Initially 5 seeds were sown in pots (pot size: 5.5 L) and were later thinned to 3 seedlings per pot at trifoliate stage. Horticulture soil used as growth medium, contained peat moss (13-18%), perlite (7-11%), coco-peat (63-68%) and zeolite (6-8%), while the macro-nutrients were present as follows: NH_4^+~90 mg/L; NO_3^-~205 mg/L; P_2O_5~350 mg/L and K_2O~100 mg/L (Seminis, Korea). The plants were harvested 80 days after sowing (DAS).

Strength of salt stress solution: The NaCl application levels included; control (distilled water), moderate salt stress (70 mM) and high salt stress (140 mM). Salt stress was applied at two growth stages i.e., pre-flowering (27 DAS) and post-flowering (40 DAS). Each pot received a single dose of 500 ml of salt stress solution.

Analysis of soybean growth and yield: The growth parameters i.e. shoot length, shoot and root fresh and dry weights were measured for harvested soybean plants while chlorophyll content of fully expanded leaves was analyzed with the help of chlorophyll meter (Minolta Co., Ltd, Japan). Dry weights were measured after drying the plants at 70ºC for 48 h in an oven (Bohm, 1979).

Analysis of phytohormones: Plant samples were harvested 24 hr after NaCl application and immediately frozen in liquid nitrogen and stored at minus 70ºC. The shoots were lyophilized in freeze drier (Virtis, SP Industries Inc.). The lyophilized plant samples were later crushed to powder for the analysis of plant hormones.

Extraction and quantification of bioactive GA_1 and GA_4: The extraction was based on the already established procedure of Lee *et al*., (1998). Gas chromatograph-mass spectrometer (GC-MS) with selected ion monitoring (SIM) mode was used for the quantification of gibberellins. One µl of the extracted sample was injected in a 30 m × 0.25 mm (i.d.), 0.25 µm film thickness DB-1 capillary column (J & W Co., Folson, USA). The GC oven temperature was programmed for a 1 min hold at 60°C, then to rise at 15°C min^{-1} to 200°C followed by 5°C min^{-1} to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280°C, an ionizing voltage of 70 eV and a dwell time of 100 ms. Retention time was determined by using the hydrocarbon standards to calculate the KRI (Kovats retention indices) value. Three replicates per treatment were used for determination of endogenous bioactive GA_1 and GA_4.

Extraction and quantification of ABA: The endogenous ABA contents were extracted following the method of Qi *et al*., (1998) and Kamboj *et al*., (1999). The extracts were dried and methylated by adding diazomethane for GC-MS SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) analysis. For quantification, the Lab-Base (ThermoQuset, Manchester, UK) data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]-ABA.

Extraction and quantification of JA: The endogenous JA level was extracted according to the protocol of McCloud & Baldwin (1997). The extracts were analyzed with GC-MS SIM (6890N network GC system and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA). To enhance the sensitivity of the method, spectra were recorded in the selected ion mode i.e., in case of JA determination, monitored the fragment ion at m/z= 83 amu corresponding to the base peaks of JA and [9, 10-$^2H_2$]-9, 10-dihydro-JA (Koch *et al*., 1999). The amount of endogenous JA was calculated from the peak areas of endogenous JA in comparison with the corresponding standards. Three replicates per treatment were used for determination of JA.

Extraction and quantification of free SA: The free SA was extracted as described by Enyedi *et al*., (1992) and Seskar *et al*., (1998). SA was quantified with C18 reverse-phase HPLC (Waters Corp., Milford, MA, USA). The HPLC condition was maintained at fluorescence detector (Shimdzu RF-10AXL, with excitation 305 nm, and emission 365 nm).

Statistical analysis: The data was subjected to analysis of variance (ANOVA SAS release 9.1; SAS, NC, USA) and Duncan's multiple range test (DMRT).

Results

Salinity and soybean growth attributes: Salt stress adversely affected growth attributes of cv. Hwangkeumkong. The shoot fresh and dry weights significantly decreased with elevated NaCl level at both pre-flowering and post flowering stage. Similar results were obtained for root fresh and dry weight parameters. The shoot length decreased insignificantly with the application of salt stress. The chlorophyll contents also reduced significantly with elevated NaCl application. However, the decrease in growth was more pronounced in case of pre-flowering stress application. Pre-flowering NaCl application give least chlorophyll contents as compared to other treatments (Table 1).

The yield parameter i.e., number of pods, pod dry-weight, 100 seed weight and yield were significantly reduced by elevated NaCl levels at both growth stages. NaCl applied at pre-flowering time showed adverse effects on yield components as compared to post-flowering growth stage. All yield components were highest in control, while lowest in plants treated with 140 mM NaCl at pre-flowering growth stage (Table 2).

Salinity and phytohormones: Present results showed that endogenous GA_1 and GA_4 contents significantly reduced with the application of elevated NaCl. We observed that the contents of bioactive GA_1 and GA_4 were higher at pre-flowering growth stage than post-flowering. The GA_4 contents were higher than GA_1 contents (Fig. 1). The abscisic acid was found in much higher amounts in soybean as compared to other endogenous plant hormones. It was observed that the amount of ABA keep pace with growth of plant and maximum contents of ABA were found at later stages of soybean growth and development. The ABA contents in leaves significantly increased with the exposure of soybean plants to elevated NaCl stress (Fig. 2).

Table 1. Effect of salt stress on growth components of cv. Hwangkeumkong.

Treatment	Quantity (mM)	Shoot length (cm)	Shoot weight (g plant^{-1})		Root weight (g plant^{-1})		Chl. content
			FW	DW	FW	DW	
Control	0	98.6^{a}	34.8^{a}	9.04^{a}	13.5^{a}	3.81^{a}	28.3^{a}
NaCl (27 DAS)	70	96.3^{a}	16.5^{b}	3.31^{b}	7.16^{bc}	1.41^{b}	22.9^{ab}
	140	93.3^{a}	12.4^{b}	2.2^{b}	2.12^{d}	0.64^{b}	20.6^{b}
NaCl (40 DAS)	70	95.6^{a}	18.5^{b}	4.98^{b}	8.87^{b}	2.81^{a}	27.7^{a}
	140	87.6^{a}	10.5^{b}	2.34^{b}	3.95^{cd}	1.21^{b}	25.8^{ab}

*In a column, treatment means having a common letter(s) are not significantly different at the 5% by Duncan's Multiple Range Test (DMRT).

Table 2. Effect of salt stress on yield components of cv. Hwangkeumkong.

Treatment	Quantity (mM)	Pods (plant^{-1})	Pod DW (g plant^{-1})	100 seed wt. (g)	Yield (g plant^{-1})
Control	0	11.83^{a}	3.96^{a}	12.6^{a}	3.72^{a}
NaCl (27 DAS)	70	6.3^{bc}	1.19^{b}	9.13^{b}	1.07^{b}
	140	4.0^{c}	0.61^{b}	5.1^{c}	0.54^{b}
NaCl (40 DAS)	70	9.3^{ab}	1.87^{b}	11.4^{ab}	1.61^{b}
	140	7.1^{bc}	0.88^{b}	5.7^{c}	0.76^{b}

*In a column, treatment means having a common letter(s) are not significantly different at the 5% level by DMRT.

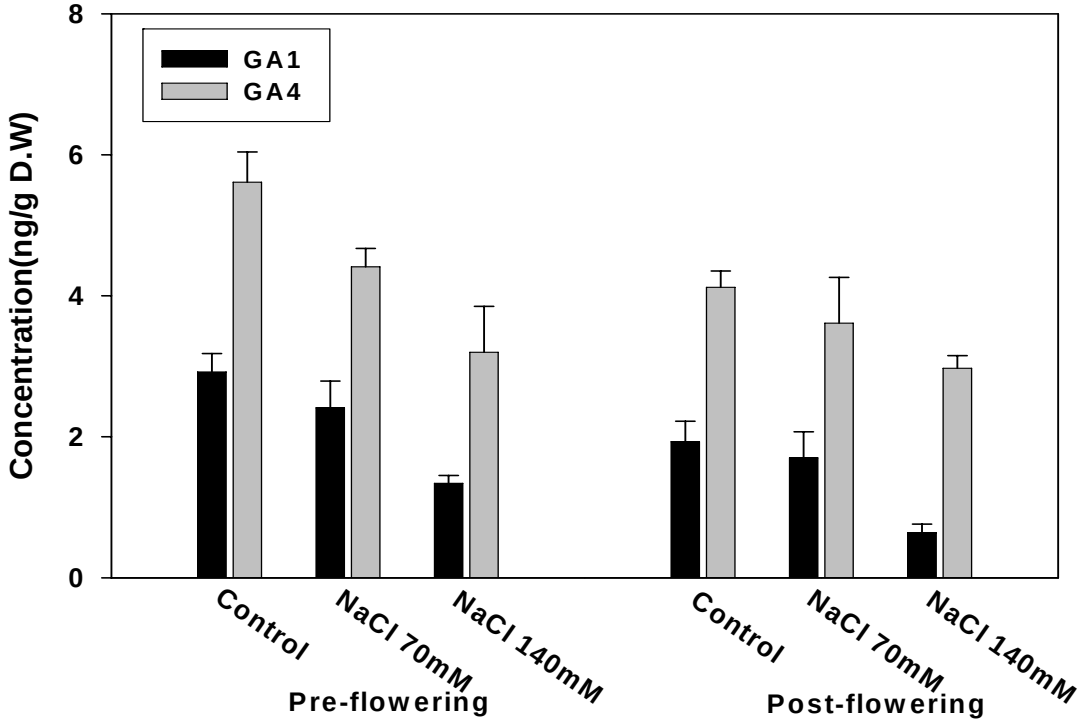

Fig. 1. Bioactive GA_1 and GA_4 content of cv. Hwangkeumkong in response to elevated NaCl.

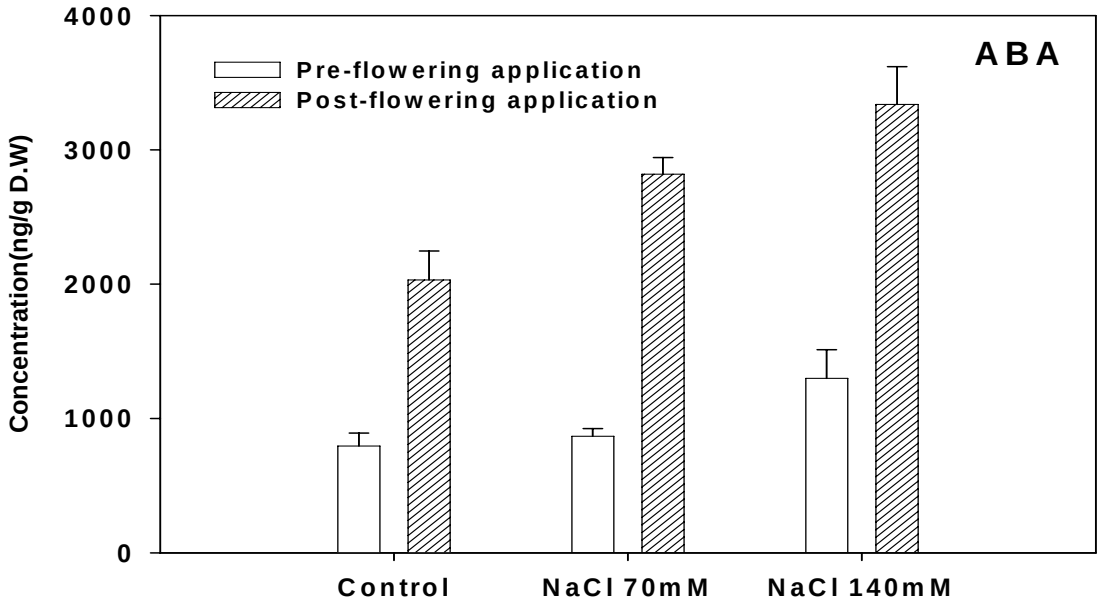

Fig. 2. Endogenous ABA content of cv. Hwangkeumkong in response to elevated NaCl.

Like ABA, the endogenous JA contents of cv. Hwangkeumkong significantly increased at low NaCl (70mM) and high NaCl (140mM), when applied at pre-flowering and post-flowering growth stages. A significant increase in JA contents of the leaves at post-flowering growth stage was observed (Fig. 3). Current study showed that the endogenous SA content of soybean leaves insignificantly decreases, when plants were treated with elevated NaCl levels. Under control condition, SA contents were found higher at post-flowering growth stage. The SA contents were least in the plants treated with NaCl at later stage of growth than pre-flowering stress and control (Fig. 4).

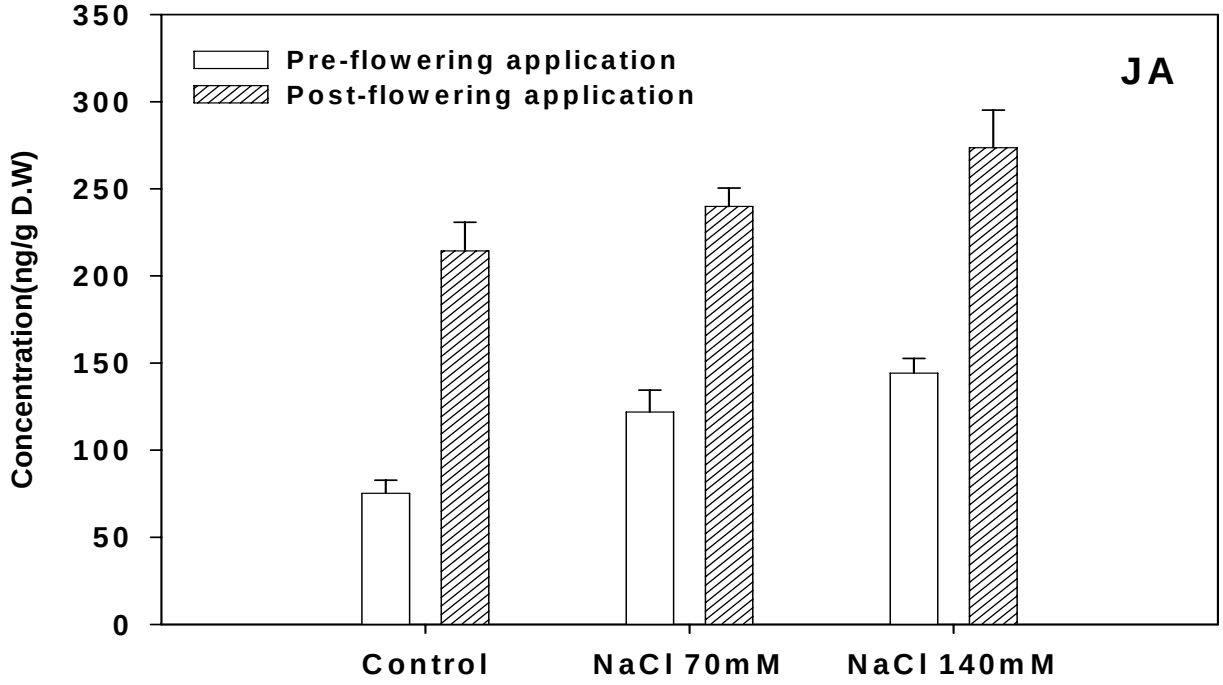

Fig. 3. Endogenous JA content of cv. Hwangkeumkong in response to elevated NaCl.

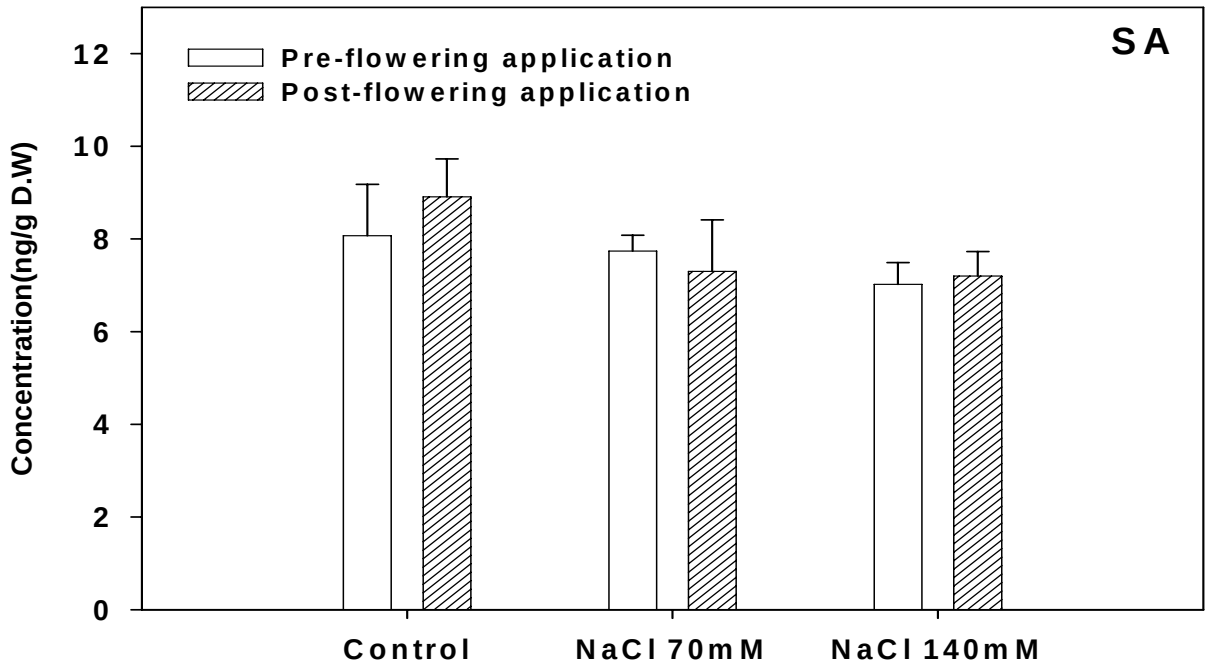

Fig. 4. Endogenous SA contents of cv. Hwangkeumkong in response to elevated NaCl.

Discussion

Salinity is a major problem for agriculture because its adverse effects on plants prevent plants from realizing their full genetic potential. Salt stress afflicts agriculture in many parts of the world, particularly irrigated land. In the present study, NaCl stress significantly decreased growth attributes except shoot length, in which the decrease caused by NaCl stress was insignificant. Similarly the yield attributes also significantly decreased with the application of elevated NaCl stress during pre-flowering and post-

flowering growth stage, although pre-flowering application showed more severe effects on yield components. The reduction may be due to the negative osmotic potential (OP) of the cells, resulting from the higher concentrations of Na^+, which reduced the ability of soybean to take up water and minerals like K^+ and Ca^{2+}.

As a co-factor in cytosol, K^+ activates more than 50 enzymes, which are very susceptible to high cytosolic Na^+ and high Na^+/K^+ ratios (Munns *et al*., 2006). Therefore, apart from low cytosolic Na^+, maintenance of a low cytosolic Na^+/K^+ ratio is also critical for proper functioning of cells (Zhu *et al*., 1998). Under saline conditions, Na^+ competes with K^+ for uptake through common transport systems, since Na^+ and K^+ are physico-chemically similar monovalent cations. Thus, elevated levels of cytosolic Na^+, or in other way high Na^+/K^+ ratios, exert metabolic toxicity by a competition between Na^+ and K^+ for the binding sites of many enzymes (Tester & Davenport, 2003).

Moreover, at a high concentration, Na^+ can displace Ca^{2+} from the plasma membrane, resulting in a change in plasma membrane permeability. This can be reflected by a leakage of K^+ from the cells (Cramer *et al*., 1989). On the other hand, Na^+ and Cl^- ions can enter into the cells and have their direct toxic effects on cell membranes, as well as on metabolic activities in the cytosol (Hasegawa *et al*., 2000; Cha-Um & Kirdmanee, 2009). These primary effects causes some secondary effects like reduced cell expansion, assimilate production and membrane function, as well as decreased cytosolic metabolism and production of reactive oxygen intermediates (ROS). As a result, in extreme cases, the plants may die under salt stress. Current study confirms previous reports, which suggested that salt stress reduced the biomass of tomato (Kaya *et al*., 2001), pea (Ahmad & Jhon, 2005) and rice (Yeo *et al*., 1999; Masood *et al.*, 2005), although shoot dry weight was more sensitive to salinity than root dry weight (Essa, 2002). In current study, the chlorophyll contents significantly decreased under elevated salt stress, as the chlorophyll contents are sensitive to salt exposure and a reduction in chlorophyll levels due to salt stress has been reported in several plants, such as pea (Ahmad & Jhon, 2005), wheat (Ashraf *et al*., 2002), rice (Anuradha & Rao, 2003) and tomato (Al-Aghabary *et al*., 2004).

Gibberellins regulate all aspects of the life history of plants, from seed germination to vegetative growth and flowering (Ritchie & Gilroy, 1998). In current study, the endogenous bioactive GA_1 and GA_4 contents decreased with NaCl application as compared to control. It suggests that the reduction in growth under salt stress conditions is caused by reduced production of GA. It was also observed that in soybean leaves, GA_4 contents were higher than GA_1, which suggested that non C13-hydroxylation is the major GA biosynthesis pathway in soybean.

ABA is involved in responses to environmental stress such as salinity (Jia *et al*., 2002), and is required by the plant for stress tolerance. As the level of ABA increases during salt and drought induced reduction of water to plants, ABA has been thus postulated to play a central role in signalling for these stress responses (Zeevaart & Creelman, 1988). In current study, we observed that the ABA contents of leaves significantly increased with elevated NaCl stress. This increase in ABA contents hinders soybean growth and development in plants under salt stress and also causes closure of stomata affecting photosynthesis. Presence of higher amounts of endogenous ABA during post-flowering stage, suggests that ABA contents increases along with plant growth and development.

The JA contents also increased with elevated NaCl stress, although the increase was much higher with NaCl applied at post-flowering growth stage. Presence of higher

amounts of endogenous JA during post-flowering stage, suggests that JA contents increases along with plant growth and development. Our current findings confirm the previous reports of Wang *et al*., (2001), who demonstrated that JA generally increase in plants in response to elevated salinity stress. However, our present results do not coincide with Kramell *et al*., (1995), who observed that endogenous jasmonates did not increase when treated with elevated NaCl.

Salicylic acid has enhanced tolerance of plants to many biotic and abiotic stresses including fungi, bacteria, viruses (Delaney *et al*., 1995), chilling, drought and heat (Senaratna *et al*., 2003). It appears that SA has a regulatory role in activating biochemical pathways associated with tolerance mechanisms (Sticher *et al*., 1997). Current investigation confirmed previous report of Wang *et al*., (2001), which demonstrated that JA generally increased and indole-3-acetic acid (IAA) and salicylic acid (SA) declined in response to salinity. The important role of SA in protecting plant is probably played by its ability to induce expression of genes coding not only for PR-proteins but also the extension gene, as found in *Arabidopsis* (Merkouropoulos *et al*., 1999; Narusaka *et al.,* 2003).

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