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ORIGINAL ARTICLE

Synergistic effect of kinetin and spermine on some physiological aspects of seawater stressed *Vigna sinensis* plants

S.S. Alsokari *

Biology Department, Faculty of Science, Albaha University, Albaha, Saudi Arabia

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Yield

Abstract The effect of kinetin, spermine and their combination on growth vigor, photosynthetic pigments, some metabolites, some enzymes, polyamines and productivity of salt-stressed *Vigna sinensis* plants was investigated. Salt stress reduced all evaluated growth criteria and yield components of used plants. Chlorophyll (CHL (a, b, carotenoids, carbohydrates, protein, spermidine and spermine level as well as and amylase activity were also decreased in response to salinity. On the other hand, proline, K^+, Na^+ and putrescine concentration, and peroxidase activity were increased in the salt-stressed plants. Exogenous application of kinetin and spermine mitigated the deleterious effects of salinity stress on growth and yield of the used plants. Conversely, the combined treatment of kinetin and spermine induced additional reduction in growth and yield of the stressed plants, and the effect appeared to be constitutive. The protective effect of kinetin and spermine on *V. sinensis* plants appeared mainly due to the enhancement effect of these growth regulators on chlorophylls and protein content and polyamines titer.

1. Introduction

Salinity has a considerable effect on world agriculture with as much as half of irrigated areas of land are affected by high salinity, accounting about 7% of total world's land surface (Munns et al., 2002). High concentrations of salts cause ionic, osmotic and oxidative stresses to plants (Simon-Sarkadi et al., 2002; Harinasut et al., 2003; Carlos et al., 2009). Plant responses to these stresses are complex but can be grouped into three general categories: homeostasis, detoxification of free radicals and growth control (Zhu, 2000). Growth control refers to the coordination of stress adaptation and the rate of cell division and expansion. Cytokinins and polyamines are involved in the growth control of the cells subjected to salt stress (Naqvi, 1994; Ali and Abbas, 2003; Tester and Davenport, 2003).

Salinity stress, in many plants, reduces cytokinin export from the root to shoot (Naqvi, 1994; Ali and Abbas, 2003).

* Tel.: +966 056670714; fax: +966 077243286.
E-mail address: alsokari@hotmail.com

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An adequate cytokinin supply is essential for normal plant development and stimulation of a great number of physiological processes which delay leaf senescence, and this can explain why exogenous applications of cytokinin can overcome the effect of salt stress on the growth of many plants (Ambler et al., 1992; Leite et al., 2003). Kinetin is a synthetic cytokinin and there have been studies on the use of kinetin to mitigate the adverse effects of salt stress on plant growth (Aldesuquy and Gaber, 1993; Gadallah, 1999). Aldesuquy and Gaber (1993) found that seeds pre-soaked kinetin (100 mM) nullified the deleterious effects imposed by irrigation with 25% seawater on leaf area, photosynthetic pigments and $^{14}CO_2$ fixation of *Vicia faba* plants at different stages of plant development.

In plants, the diamine putrescine, triamine spermidine and tetramine spermine affect a range of developmental processes such as flowering and senescence (Pandey et al., 2000; Kasinathan and Wingler, 2004). These polyamines have been suggested to afford protection against a large variety of environmental stresses including salinity and potassium deficiency (Chattopadhayay et al., 2002; Simon-Sarkadi et al., 2002). The protective function of polyamines is mainly due to their cationic nature at cellular pH. Polyamines can stabilize cellular structures such as thylakoid membranes by binding to proteins and lipids (Tiburcio et al., 1994). Polyamines have also been proposed to act as radical scavengers and as regulators of K^+ channels in stomata (Kramer and Wang, 1989; Liu et al., 2000; Duan et al., 2008). In this respect, Tipirdamaz et al. (1995) found that treatments of barley seeds with spermine, spermidine and putrescine increase both amylase activity and germination percentage (from 23% to 50% and 45%, respectively) in salt-treated seedlings. Furthermore, they concluded that the adverse effect of salt stress on germination can be partially rectified by polyamines.

Cytokinins application is known to increase polyamines biosynthesis and increase the level of spermine bound to the chromatin, so it is suggested that polyamines could be involved in cytokinin mode of action (Legocka and Żarnowska, 2002; Sergiev et al., 1995). However, there are no reports in the literature showing the combined effect of kinetin and spermine on salt-stressed plants. Therefore, the present study was undertaken to evaluate the possible role of kinetin, spermine and their combination on growth vigor, some metabolites and productivity of salt-stressed *Vigna sinensis* plants.

2. Materials and methods

2.1. Plant materials and growing conditions

Healthy and equalized seeds of *V. sinensis* L. (var. cult. Cream 7) were surface sterilized with bleach solution (10%) for 10 min and washed thoroughly with distilled water. Then the seeds were divided into four sets according to the soaking solution. The 1st set was soaked in distilled water, whereas the 2nd, 3rd, and the 4th sets were soaked in 0.1 mM kinetin, 0.3 mM spermine and (0.1 mM kinetin + 0.3 mM spermine), respectively, for 24 h. The choice of these concentrations was based on preliminary experiments. After soaking, the thoroughly washed seeds were sown (eight seeds/pot) in plastic pots (30 cm diameter) filled with 3 kg soil (clay: sand; 2/1 v/v). The pots were kept in a greenhouse where the plants were subjected to natural day/night conditions (minimum/maximum air temperature was about 15/25 °C at mid-day during the winter season). After 2 weeks, the plants were thinned to four uniforms per pot and each set was allocated to control and salinity treatments with 12 replicates in each one. Salinity stress was applied by irrigation with 50% seawater from Red sea with a periodical soil washing (each 2 weeks) with tap water. After 2 months of salinity treatments, samples for growth and biochemical analysis were taken. Then all the remaining pots were irrigated with tap water and left for yield analysis. The third upper leaf was employed for different biochemical analyses with three replicates in case of polyamines and eight replicates for other different analyses.

After thinning, the plants received 25 kg N ha^{-1} urea and 25 kg P ha^{-1} potassium dihydrogen phosphate as fertilizers.

2.2. Measurements of leaf area

Leaf area was measured by a Digital Planimeter KP-90 N (PLAKOM).

2.3. Determination of photosynthetic pigments

Photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined using the spectrophotometric method of Lichtenthaler (1987).

2.4. Determination of carbohydrates concentration

Total soluble sugars were extracted and determined by the anthrone method of Riazi et al. (1985). Polysaccharides in a known dry weight were hydrolyzed into simple sugars by acid hydrolysis as described by Sadasivam and Manickam (1996). The sugars content of 0.1 mL of this solution was obtained by the anthrone method Andreani and Gray (1956). The total soluble sugars was subtracted from the total carbohydrates give the value of polysaccharides in each sample.

2.5. Assay of peroxidase activity

A known leaf fresh weight (1 g) was macerated in 3 mL of 0.1 M phosphate buffer pH 7 with a pre-cooled mortar and pestle. The homogenate was centrifuged at 4 °C for 15 min. The supernatant was used as source for peroxidase enzyme within 2–4 h. Peroxidase (EC 1.11.1.7) activity was determined by the guaiacol oxidation method as described by Sadasivam and Manickam (1996).

2.6. Determination of protein concentration

Total soluble protein content of the enzyme extracts was determined with coomassie brilliant blue G250 dye according to Bradford (1976) using bovine serum albumin as standard. Samples were read at 595 nm.

2.7. Assay of α-amylase activity

Extracts for α-amylase activity (EC 3.2.1.1) were obtained with ice cold 0.2 M acetate buffer (pH 4.5), and centrifugation at 20,000 rpm for 30 min at 4 °C. Amylase activity was assayed according to the method adopted by Monroe and Preiss (1990).

2.8. Determination of K^+ and Na^+ concentrations

A known dry weight of the plant leaves was digested in boiling concentrated HNO_3 and made up to known volume with deionized water. K^+ and Na^+ concentrations were measured by flame emission photometry (Gadallah, 1999).

2.9. Determination of proline concentration

Proline was determined using the method of Bates et al. (1973).

2.10. Determination of polyamines concentration

Extraction and dansylation techniques of Smith and Best (1977) as described by Aldesuquy et al. (2000) were used. Then 10 μL of samples and standards were spotted on thin layer chromatography plates using a Hamilton microsyringe. The plate was developed in a two-solvent system (benzene: triethylamine; 5:1) for 17 cm height. The plate was dried at room temperature and the resulting zones were examined and marked under long ultraviolet wave length (365 nm). The marked areas were determined at 254 nm using a CS-900 Dual Wave Length Scanning Densitometer.

2.11. Statistical analysis

The experiment was a completely random design. The main effect of factors (salinity and growth regulators), and their interaction (salinity × growth regulators) were evaluated by general linear model (two-way ANOVA) using SPSS program. Tests for significant differences between means at $P = 0.05$ were given by LSD test.

3. Results

3.1. Changes in growth criteria

The applied salt stress significantly reduced shoot and root masses, total leaf area in *V. sinensis* plants. Seeds pre-soaking in kinetin or spermine significantly improved these criteria. The interaction (regulators × salinity) was significant, where the combination of kinetin and spermine had a negative effect on growth vigor of the used plants. The effect of spermine, kinetin and their combination appeared to be constitutive (Table 1).

3.2. Changes in photosynthetic pigments

In comparison with the control, chlorophylls a and b, as well as carotenoids level were markedly reduced in *Vigna* plants in response to salt stress. Application of kinetin and spermine partially mitigated the adverse effect of salt stress on these pigments. The interaction (regulators × salinity) was significantly increased, and this reflected the non-significant effect of the dual pre-treatment with kinetin and spermine on chlorophyll a, chlorophyll b and carotenoids concentration of the stressed plants (Fig 1).

3.3. Changes in carbohydrates

Salt stress induced drastic decrease in total soluble sugars concentration of *V. siuensis* plants. Kinetin and spermine alleviated the adverse effect of salt stress on soluble sugars and the effect was more pronounced with kinetin pre-treatment. On the other hand, a non-significant effect was manifested in response to the dual treatment of kinetin and spermine.

Total carbohydrates and polysaccharides concentration was significantly decreased in *Vigna* plants in response to salinity stress. Concerning to total carbohydrates, the effect of growth regulators was insignificant, but the interaction (regulators × salinity) was significant. Kinetin alone enhanced the total carbohydrates concentration of salt-treated plants. The magnitude of increase appeared to be close to the control level. Furthermore, Kinetin mitigated to some extent the adverse effect of salinity on polysaccharides level of used plants. Conversely, spermine either alone or in combination with kinetin induced more reduction in polysaccharides level of salt-treated plants (Fig. 1).

3.4. Changes in Na^+ and K^+ concentrations

Seawater-treated plants had a higher K^+ concentration than control plants. The applied growth regulators induced additional increase in K^+ level of *V. siuensis* plants. Na^+ was greatly increased in salt-stressed plants (> 2-fold) in relation with controls. Spermine either alone or in combination with kinetin increased to some extent Na^+ level of the stressed plants. On the other hand, kinetin appeared to reduce Na^+ content.

Regarding K^+/Na^+ ratio, there was a greater reduction in the values of salt-treated plants as compared with control

Table 1 Effect of seeds pre-soaking in kinetin and spermine on growth criteria *Vigna sinensis* plants grown under seawater stress.

Treatments	Growth criteria				
	Shoot f.wt. (g/plant)	Shoot d.wt (g/plant)	Root f.wt. (g/plant)	Root d.wt (g/plant)	Total leaf area (cm^2)
Control	39.44	6.50	9.24	2.20	780
Kinetin control	45.55	8.07	8.90	1.98	1100
Spermine control	42.30	7.11	10.12	2.01	950
Kinetin + spermine control	34.31	5.53	7.02	1.65	600
Seawater (50%)	13.05	3.55	3.43	0.82	350
Kinetin + 50% seawater	16.92	4.40	4.40	1.07	492
Spermine + 50% seawater	15.75	4.37	4.90	1.15	480
Kinetin + spermine + 50% seawater	10.04	2.95	3.40	0.80	230
LSD ($P < 0.05$)	2.5	0.60	0.65	0.20	61.04

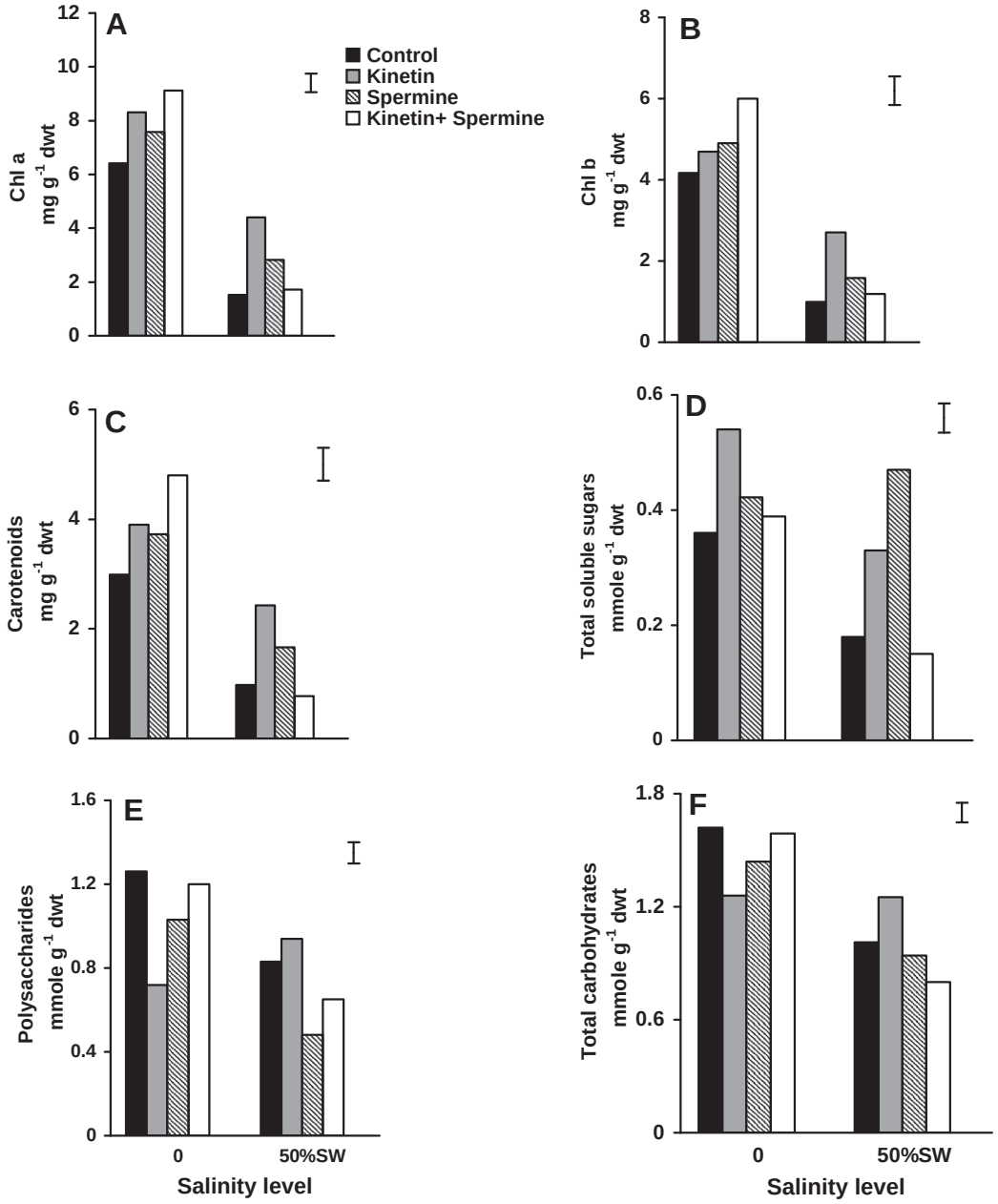

Figure 1 Effect of seeds pre-soaking inkinetin and spermine on photosynthetic pigments and carbohydrates concentration of *Vigna sinensis* plants grown under seawater stress. The vertical bars represent LSD values at $P < 0.05$.

plants. Kinetin and spermine pre-treatments significantly improved this ratio in the stressed plants. However, the dual treatment of kinetin and spermine attenuated this ratio (Fig. 2).

3.5. Changes in proline and protein concentrations

Proline was markedly increased in salt-stressed plants. Used growth regulators did not affect the proline level of these plants. The interaction (regulators × salinity) was significant. Kinetin alone or in combination with spermine, in control conditions, significantly increased proline concentration in *Vigna* plants as compared with corresponding untreated plants.

Protein level was significantly reduced in the salt-treated plants. Seeds pre-soaking in kinetin and spermine enhanced the protein content of these plants. The interaction (regulators × salinity) was significant. The dual pre-treatment with kinetin and spermine lowered the protein level of the used plants (Fig. 2).

3.6. Changes in α-amylase and peroxidase activities

Salinity stress induced massive decrease in α-amylase activity of *Vigna* plants. The used growth regulators enhanced the amylase activity of these plants and the effect was more pronounced with kinetin pre-treatment.

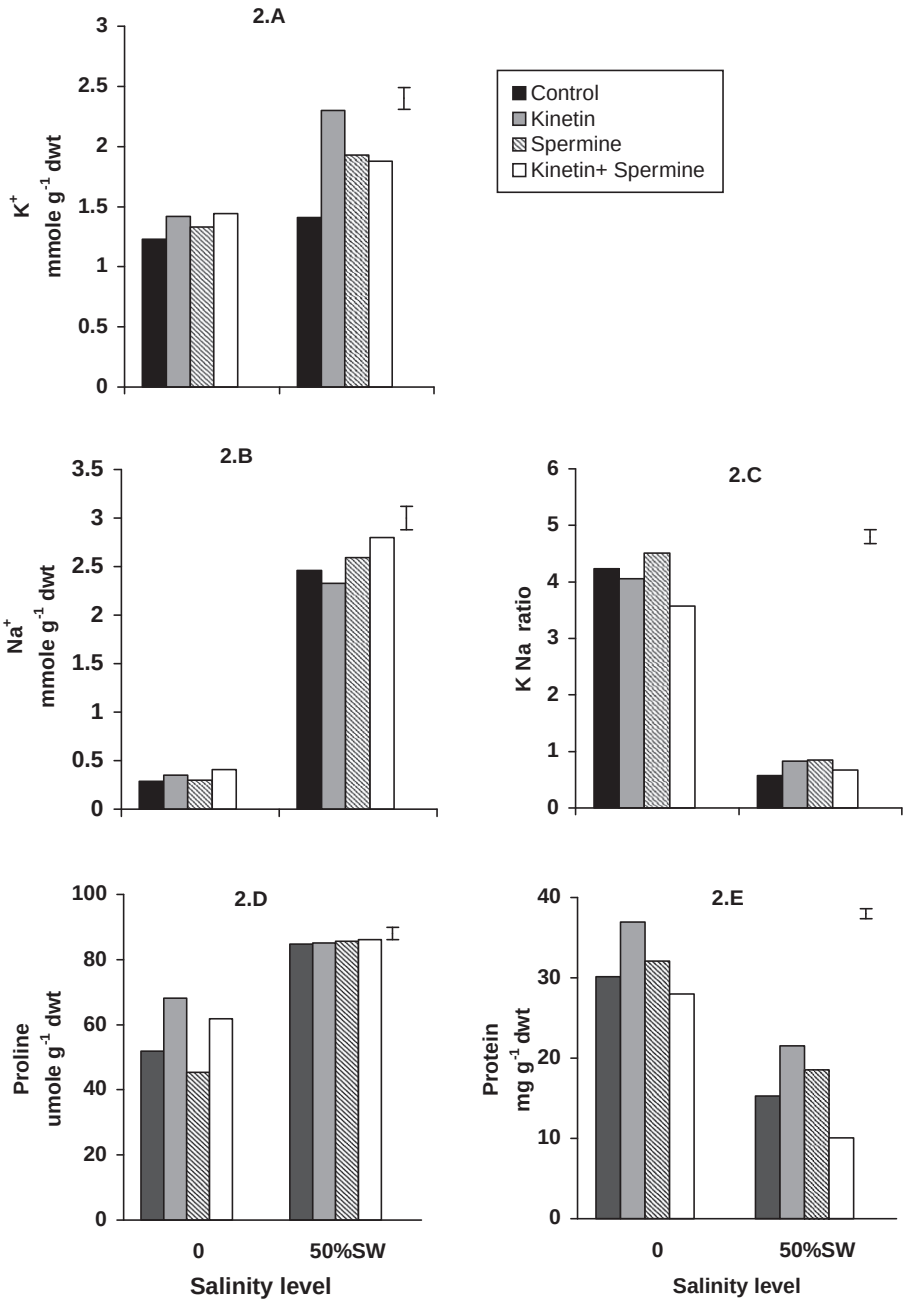

Figure 2 Effect of seeds pre-soaking in kinetin and spermine on K^+, Na^+, proline and protein concentrations of *Vigna sinensis* plants grown under seawater stress. The vertical bars represent LSD values at $P < 0.05$.

Concerning the peroxidase activity, the salt-stressed plants had a higher enzyme activity (> 6-fold) than the corresponding control plants. Used growth regulators reduced this increase and the effect was more elicited with spermine pre-treatment (Table 2).

3.7. Changes in polyamines level

Salt-stressed plants had a higher putrescine and lower spermidine and spermine concentration as compared with corresponding controls. The results clearly showed that total polyamines were increased in the salt-stressed plants. Pre-treatment with kinetin and spermine induced additional increase in putrescine level of salt-stressed plants. Furthermore, these growth regulators enhanced the production of spermidine and spermine of the stressed plants. The interaction (regulators × salinity) was significant. This reflected the different trend of changes in case of the dual pre-treatment with kinetin and spermine. This treatment greatly reduced putrescine and increased spermine concentration of *V. siuensis* plants as compared with non-treated plants (Table 2).

3.8. Changes in yield components

The applied salt stress reduced all yield components of *Vigna* plants, i.e., shoot dry mass, straw mass, economic yield, fresh

Table 2 Effect of seeds pre-soaking in kinetin and spermine on α-amylase and peroxidase activities, and polyamines titer of *Vigna sinensis* plants grown under seawater stress.

Treatments	α-Amylase activity (μmol/min)	Peroxidase activity (U g^{-1} f.wt.)	Polyamines (nmol g^{-1} f.wt.)		
			Putrescine	Spermidine	Spermine
Control	1.01	2.61	36.76	13.24	6.62
Kinetin control	0.82	1.71	50.35	16.80	8.38
Spermine control	1.03	3.56	37.55	13.60	11.74
Kinetin + spermine control	0.64	1.30	26.14	2.40	16.50
Seawater (50%)	0.30	20.0	162.0	0.68	0.70
Kinetin + 50% seawater	0.88	11.54	230.0	4.66	0.98
Spermine + 50% seawater	0.72	6.50	200.0	0.86	1.46
Kinetin + spermine + 50% seawater	0.79	7.13	104.03	0.76	3.20
LSD ($P < 0.05$)	0.04	1.00	9.00	0.40	0.50

Table 3 Effect of seeds pre-soaking in kinetin and spermine on yield and yield components of *Vigna sinensis* plants grown under seawater stress.

Treatments	Yield and yield components					
	Shoot d.wt. (g/plant)	Straw weight (g/plant)	Total seeds f.wt. (g/plant)	Total seeds number/plant	Ten seeds d.wt. (g)	Harvest index
Control	10.20	6.6	5.57	7.0	8.25	48.59
Kinetin control	12.28	6.62	6.64	8.0	8.92	45.56
Spermine control	13.95	6.45	8.04	8.0	9.70	50.52
Kinetin + spermine control	9.50	4.87	5.06	6.0	7.80	45.52
Seawater (50%)	2.70	2.57	0.16	1.33	1.07	6.21
Kinetin + 50% seawater	3.80	3.50	0.52	1.67	2.40	12.50
Spermine + 50% seawater	3.56	3.03	0.42	1.67	2.00	10.79
Kinetin + spermine + 50% seawater	1.70	1.75	0.00	0.00	0.00	0.00
LSD ($P < 0.05$)	0.50	0.34	0.20	1.2	0.52	4.78

and dry seed masses, seeds number and harvest index. The straw weight appeared to be the lowest affected one. Seeds pre-soaking in kinetin and spermine markedly enhanced the yield of salt-stressed plants. On many occasions, the interaction (regulators × salinity) was significant. This reflected the adverse effect of the dual treatment of kinetin and spermine on yield of salt-stressed plants (Table 3).

4. Discussion

Seawater salinity markedly reduced shoot and root masses as well as total leaf area of *V. sinensis* plants. The underlying causes are complex and range from physiological drought, decrease in the photosynthetic surface, the cost of osmotic adjustment, and nutrient deficiencies to the accumulation of free radicals (Simon-Sarkadi et al., 2002; Harinasut et al., 2003). Application of kinetin and spermine mitigated adverse effect of salinity on growth vigor of the used plants. This is in agreement with previous findings of many authors (Gadallah, 1999; Chattopadhayay et al., 2002; Ali and Abbas, 2003). Salt stress is known to reduce cytokinins and spermine level in plant shoot, and this can explain the protective effect of exogenous application of these growth regulators on growth of many plants grown under salt stress conditions (Ali and Abbas, 2003; Leite et al., 2003; Duan et al., 2008).

The observed reduction in chlorophylls and carotenoids concentration in response to salt stress appeared to run in close parallelism with the results of Aldesuquy and Gaber (1993) who found that high seawater (25%) level reduced the photosynthetic pigments level of broad bean plants. Seeds pre-soaking in kinetin and spermine partially mitigated the deleterious effect of salt stress on these pigments. This ameliorative effect of kinetin application was also reported by Aldesuquy and Gaber (1993). In connection with these results, Chattopadhayay et al. (2002) found that the exogenous application of spermine enhanced the total chlorophyll level of salt-stressed rice plants. This enhancement effect of kinetin and spermine may be attributed to increased stability of thylakoids membranes (Gadallah, 1999; Pandey et al., 2000; Chattopadhayay et al., 2002) and plastids biogenesis (Aldesuquy and Baka, 1998).

The reduction of chlorophyll content and the inhibition of carbon metabolism and photosynthetic activity (Aldesuquy and Gaber, 1993; Chattopadhayay et al., 2002) led to the observed reduction of total carbohydrates, polysaccharides and total soluble sugars concentration in salt-stressed plants. Kinetin and spermine pre-treatments alleviated the adverse effects of salt stress on total soluble sugars concentration and this is at least partially due to enhanced amylase activity (Table 2) and chlorophyll content. In this respect, Gadallah (1999) found that kinetin application increased soluble sugars accumulation in salt-stressed wheat plants growing under aerobic

and anaerobic conditions. Kinetin pre-treatment greatly enhanced total carbohydrates and polysaccharides concentration of the stressed plants and this is mainly due to enhanced chlorophyll content.

It is accepted that competition exists between Na^+ and K^+ leading to a reduced level of internal K^+ at a high external NaCl level (Gorham and Bristol, 1990; Gadallah, 1999). Data presented here indicated that the internal K^+ level was not reduced but increased and Na^+ accumulated, in salt-stressed plants. In this result, salinity was induced by seawater, which contains K^+ and Ca^{2+} in addition to the major cation Na^+. Sodium–calcium interaction under salinity has been reviewed recently (Munns et al., 2002; Tester and Davenport, 2003; Easton and Kleindorfer, 2009) and it can be concluded that Ca^{2+} is important for the maintenance of K^+ transport in the presence of Na^+ via some regulation of non-selective cation channels. However, K^+/Na^+ ratio reduced in the stressed plants. This low K^+/Na^+ ratio can disturb various enzymatic processes in the cytoplasm and protein synthesis (Tester and Davenport, 2003). Kinetin and spermine pre-treatments enhanced the K^+/Na^+ ratio of salt-stressed plants and this is in accordance with the findings of Gadallah (1999) and Chattopadhayay et al. (2002). These results may indicate that the provided growth regulators exert some effects at the membrane level for increasing K^+ selectivity which is considered to be important in salt tolerance.

The data presented showed that proline concentration was greatly increased in the salt-stressed plants and this is compatible with the fact that many higher plants accumulate free proline in response to salt stress (Roosens et al., 1999). Several possible roles have been attributed to this supra-optimal level of proline: osmoregulation, detoxification of free radicals, conservation of nitrogen and energy for the post-stress period and regulating the stress protective proteins (Lutts et al., 1999; Roosens et al., 1999; Khedr et al., 2003). Conversely, protein concentration was reduced in salt-stressed plants. This reduction could result from impairment protein synthesis and/or increased protein degradation by salinity stress (Duby, 1994). Kinetin and spermine pre-treatments did not affect proline level but enhanced the protein concentration of salt-stressed plants. This is compatible with the general reputation that spermine and kinetin are anti-senescence agents and retard protein and chlorophyll loss in detached leaves (Gadallah, 1999; Huang et al., 1990; Pandey et al., 2000).

It can be seen from this result that α-amylase activity was reduced in salt-stressed plants and the used growth regulators enhanced this activity. These results could explain the observed reduction of total soluble sugars in salt-stressed plants and the beneficial effect of spermine and kinetin pre-treatments. The beneficial effect of spermine on α-amylase activity under salt stress was also observed by Tipirdamaz et al. (1995) in barley. On the other hand, peroxidase activity was markedly increased in broad bean plants by salt treatment. This anti-oxidant enzyme increased the ability of higher plants to scavenge the toxic active oxygen which accumulate under stress conditions (Ali and Abbas, 2003; Harinasut et al., 2003). This result also pointed out clearly that kinetin and spermine pre-treatments reduced the increase in peroxidase activity and this reflected the protective effect of these regulators on the used plants. In this respect Kwon and Kim (1995) found that peroxidase activity increased with leaf senescence, and kinetin and spermine treatments reduced this increase.

The observed increase in putrescine and reduction of spermidine and spermine level of the salt-stressed plants were also reported in maize plants. These changes could result from increased arginine decarboxylase (ADC) activity and impairment of higher polyamines biosynthesis from putrescine (Willadino et al., 1996). Others, observed the decrease (Benavides et al., 1997) or increase (Kasinathan and Wingler, 2004; Duan et al., 2008) of all these biogenic amines in salt-stressed plants. Kinetin and spermine pre-treatments, over all conditions, markedly increased putrescine, spermidine and spermine concentrations of the used plants. This is consistent with the findings of Liu et al. (2000) that enhanced callus growth of soybean hypcotyl by kinetin treatment was accompanied by the accumulation of endogenous putresine, spermidine and spermine. Furthermore, Kinetin and spermine were found to promote ADC and ODC activities which induce polyamines biosynthesis (Huang et al., 1990; Mo and Pua, 2002).

The dual treatment with kinetin and spermine increased spermine concentration of and reduced putrescine and spermidine level of the used plants and this could explain the observed reduction of plant growth. This is possibly because this treatment elevated the internal spermine concentration to a level that inhibits putrescine and spermidine biosynthesis. However, the lack of data about the combined effect of kinetin and spermine on ADC and ODC activities makes the explanation elusive and it needs further works. In this context, Theiss et al. (2002) found that elevated intracellular spermine in response to exogenous spermine application reduced ODC activity and consequently reduced putrescine and spermidine concentrations which are important for the normal growth of *Chalamydomonas reinhardtii* cells.

The adverse effect of salt stress on *V. sinensis* plants growth and metabolism was reflected on plant yield. The enhanced effect of kinetin and spermine on the yield of salt-stressed plants was basically due to enhanced seed biomass which result from enhanced growth vigor, chlorophylls content, K^+/Na^+ ratio and polyamine titer during the vegetative growth stage. This is compatible with the finding of Gadallah (1999) that exogenous application of 10 ppm kinetin improved growth and grain yield of salt-stressed wheat plants. The negative effect of the dual treatment of Kinetin and spermine on growth vigor, protein content and polyamines titer could explain the adverse effect of this pre-treatment on the obtained yield.

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