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Vitamins Minimize the Salt-Induced Oxidative Stress Hazards

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Abstract: The interactive effects of some vitamins (folic acid ;vitamin B_9, ascorbic acid ;vitamin c, and coblamin ;vitamin B_{12}) in the presence of NaCl on germination, seedling growth and some relevant metabolic changes of flax (*Linum usitatissimum*) seeds grown at 100, 200 and 300 mM NaCl were studied. In this investigation, flax seeds tolerated NaCl salinity up to 200 mM NaCl; the germination was completely inhibited at 300 mM NaCl. Moreover, salinity induced marked increases in soluble carbohydrates, lipid peroxidation product (MDA) as well as the reduced glutathione and proline contents which were concomitant with sharp decrease in total phenols, ascorbic acid and the total free amino acids contents. Furthermore, NaCl treatment increased the activities of some antioxidant enzymes (superoxide dismutase; SOD, ascorbate peroxidase; APX, ascorbate oxidase; ASO as well as phenol peroxidase; GPX and polyphenol oxidase; POX). On the other hand, the completely non germinated flax seeds (at 300 mM NaCl) showed an amazing capacity for recovery and germination after treatment with either folic acid, ascorbic acid or coblamin. Finally the potentiation of these vitamins was mediated by accumulation of some osmoprotectants such as polyphenols, free amino acids and proline which, associated with increasing of some water soluble antioxidants such as ascorbic acid and glutathione.

Key words: flax; vitamins; antioxidants; osmolytes; salinity.

INTRODUCTION

Linum usitatissimum (flax) plant is considered as one of the most important economic fiber crops in Egypt. It is now second to cotton, and possibly jute, in importance. In addition flax seed oil is one of the richest sources of omega-3 fatty acids.

Salinity is a common environmental challenge in the world and it is one of the major problems that limit agricultural production (Sairam and Tyagi, 2004). Overcoming salt stress is the main issue for increasing plant growth and productivity. For ameliorating salt stress, plants have evolves complex mechanisms that contribute to the adaptation to both osmotic and oxidative stresses caused by salinity. The mechanisms that include osmotic adjustment is usually accomplished by either uptake of organic ions from external solution or by *de novo* synthesis of some compatible solutes (osmoprotectants) such as amino acids and soluble sugars which acting as osmolytes (Bohnert and Shen, 1999; Serrano *et al*., 1999; Shabala *et al*., 2000; Rontein *et al*., 2002; Ashraf and Harris, 2004). Osmoprotectants are neutral molecules that stabilize proteins and membranes against denaturation effect of high concentration of salts (Munns, 2002). Moreover, plant cell must adjust osmotic potential to prevent water losses, maintaining cell turgor under salt stress (Naidoo and Niadoo, 2001). On the other hand, salinity produces oxidative stress in plant tissues (Bartosz, 1997; Rout and Shaw, 2001). The oxidative damage is caused by reactive oxygen species (ROS) which are produced during metabolism (Halliwell and Gutteridg, 1985). ROS can damage essential membrane lipid as well as protein and nucleic acids (Noctor and Foyer, 1998). ROS include superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen (O_2^{-}). The level of oxidative stress is determined by the balance between the rate at which oxidative damage is induced and the rate at which it is alleviated.

To minimize the effect of oxidative stress, plant cell have evolved a complex antioxidant system, which is composed of antioxidant compuonds (glutathione, ascorbate, β- carotene and α- tocopherol) as well as ROS-scavenging enzymes such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), phenol peroxidase (GPX) and glutathione reductase (GR) (Alscher *et al*., 1997; Apel and Hirt, 2004). When ROS production suppresses the antioxidant system capacity, oxidative stress occurs, resulting in protein, DNA damage and lipid peroxidation (Noctor and Foyer, 1998; Shalata and Neumann, 2001).

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Amelioration of the adverse effects of NaCl salinity by vitamin treatments have been reported by many investigators (Shalata and Neumann, 2001; Ali, 2002; El -Bassiouny *et al*., 2005 and El Tohamy and El Gready, 2007; Bassuony *et al*., 2008). In fact, plants don't synthesize vitamins for the benefit of animals but rather because they play the same essential role in plant metabolism. Most vitamins act as cofactors for many enzymes. However, vitamin B_{12} is unique in that, it is found naturally in many plants and animal sources, however, neither plants nor animals can directly synthesize this vitamin. Certain bacterium is capable of producing it and latter adds it to plants (Smith *et al*., 2007).

So, the aim of the present study was to elucidate the mechanisms by which flax plant may cope with salinity, this was evaluated by studying the physiological changes induced by salinity stress and to try to enhance salt tolerance in flax seeds exposed to high levels of NaCl salinity by using some vitamins.

MATERIALS AND METHODS

Pure strain of flax seeds (*Linum usitatissimum* L). Cultivar, sakha 2 was obtained from the Agriculture Research Center. Fiber Crops Research Section, Giza, Egypt. The seeds were surface sterilized by dipping in 1% sodium hypochlorite solution for 5 minutes, then rinsed thoroughly with distilled water and germinated in Petri dishes on filter paper (Whatman No.1) saturated with water, vitamins and/or salt solutions. The solutions were replaced every 2-3 days.

Preliminary experiments were done to test the salt sensitivity of flax seeds as well as to choose the proper concentrations of folic acid, ascorbic acid and coblamin. Three different concentrations of sodium chloride were chosen (100, 200, and 300 mM NaCl in ¼ strength Hogland's solution). The proper concentrations of folic acid, ascorbic acid and coblamin were 20μM, 0.5mM, and 2μM respectively. Seedlings were exposed to normal day length with natural temperature (about 22/13 ±2°C and 11 h photoperiod). The percentage of germination was recorded after 2 days from sowing. Radicle protrusion was taken as the criterion for germination. Seedlings were collected after 12 days at the end of the experiment for measuring growth parameters in terms of shoot and radicle lengths of flax seedlings. Moreover, some seedlings were collected for measuring different metabolites, proline and total phenols as well as enzymatic and non enzymatic antioxidants.

Soluble sugars:

were extracted following the method adopted by Homme *et al*., (1992). Sugar free residues were extracted with 1.5N H_2SO_4 following the method adopted by Naguib (1963). Soluble sugars and those resulting after polysaccharides hydrolysis were estimated by anthrone reagent (Fairbairn, 1953).

Soluble proteins:

were extracted according the method described by Hassanein (1977). Water insoluble residues remaining after extraction of soluble proteins were extracted with 1 N NaOH. Soluble proteins and those resulting after insoluble residue hydrolysis were measured by using BIO-RAD protein assay dye reagent according to the method adopted by Bradford (1976).

Free amino acids:

were extracted according to the method described by Vartanian *et al*. (1992) and estimated using ninhydrin reagent (Yemm and Cocking, 1955).

Proline:

were assayed according to the methods described by Bates *et al*., (1973).

Lipid peroxidation:

was determined in fresh tissues by measuring the amount of malondialdehyde (MAD) as a product of lipid peroxidation, by the thiobarbituric acid reaction (Heath and Packer, 1968).

The water soluble antioxidants:

such as glutathione and ascorbic acid were determined by the methods of Schupp and Rennenperg, (1988) and Kampfenkel *et al*., (1995) respectively.

Total phenols:

were extracted and estimated following the method described by Malik and Singh (1980).

Antioxidant enzymes:

were extracted from frozen flax seedlings by using a known volume of phosphate buffer (pH 7).The crude extracts were used for enzyme assay. Superoxide dismutase activity (Cu-Zn SOD EC 1.15.1.1) was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a modification method of Marklund and Marklund, (1974). Catalase (CAT EC 1.11.1.6) activity was assayed following the method of Xu *et al*., (1997). Phenol peroxidase (GPX EC 1.11.1.7) activity was assayed as described by Bergmeyer *et al*., (1974), while polyphenol oxidase (POX EC 1.10.3.1) activity was determined according to the method described by Kar and Mishra, (1976). Ascorbate peroxidase (APX EC 1.11.1.1) and oxidase (ASO EC 1.10.3.3) activities were assayed by the method reported by Cao *et al*., (2004) and Maxwell and Batman (1967) respectively.

Statistical Analysis:

Analysis of variance was conducted using ANOVA one way variance test using Microsoft Excel 2000. Statistical probability values were calculated to quantity levels of significance for each treatment type. The values of analysis of seedlings grown under 100, 200 and 300 mM NaCl were used as a reference controls for vitamin treated ones, as well as they compared also, with the untreated control. Each treatment is an average of three different measurements.

RESULTS AND DISCUSSION

Seed germination:

It is clearly shown from table (1) that NaCl brought about a marked inhibitory effect on seed germination. Lower concentration (100mM) reduced the percentage of germination to 47% as compared with the control. The germination was completely inhibited in response to the higher concentration of NaCl (300 mM). However, application of folic acid, ascorbic acid or coblamine partially overcame the inhibitory effect of salinity on germination. Flax seeds that fail to germinate at 300 mM NaCl, responded successfully to these vitamin treatments which caused 67%, 34% and 33% respectively increase in germination percentage.

Seedling growth:

s Seedling growth parameters in terms of radicle length and shoot length of treated and untreated flax seedlings are presented in table (1). A pronounced sensitivity in radicle and shoot lengths was displayed in the presence of NaCl, which was found to strongly inhibit their growth at low level of NaCl (100 mM). The shoot and radicle lengths decreased below the control values (52.2 % and 60.5% respectively). It is interesting to note here that any of the applied vitamins had generally an enhancement effect on growth of salinized flax seedlings. This stimulatory effect was more pronounced in folate treated salinized flax seeds.

Metabolic changes:

Salinized flax seedlings accumulated soluble carbohydrate fractions up-to 300 mM NaCl. The maximum soluble carbohydrate accumulation occurred at 200 mM NaCl where the percentage of increase was 63.1% more than the control value. It is interesting to note here that, vitamin treatments had generally favorable effect on the accumulation of total carbohydrate at the expense of the soluble one (Table 2). Furthermore, the data given in table (2) indicated that, salt stress stimulated the accumulation of total soluble protein in salinized flax seedlings. The greatest total soluble protein level was recorded at 300 mM NaCl and the percentage of accumulation exceeded the control value by 186.4%.

It is clearly indicated that all NaCl levels induced an increase in proline content. NaCl at a level of 300 mM promoted proline accumulation up to 170.9% comparing with that of control value. In addition, it was noticeable that coblamine treatment resulted generally in a pronounced increase in proline content of salinized flax seedlings at different concentrations of NaCl. However, the significant gain was about 126.7% (Table 2) as being compared with the corresponding control (300 mM NaCl).

Total free amino acids of salinized flax seedlings was reduced, the percentage of decrease was about 11.1%, 22.2% and 55.5% below the control value, respectively, while any of the vitamins used in the present study induced signific ant stimulatory effect on the accumulation of free amino acids. At 300 mM NaCl, about 55.5% increase in free amino acids was determined in salinized flax seedlings treated with coblamin (Table 2).

Sharp progressive decrease in the total phenol contents was observed in salinized flax seedlings. The reduction in phenol levels was about 23.6%, 61.1% and 69.4% at 100, 200 and 300 mM NaCl respectively below those of the control value (Table 2). The interactive effect of salinity and each of the three vitamins used in the present study provoke pronounced stimulation in total phenol production.

MDA production of salinized flax seedlings were about four times greater than that amounted in control seedlings. The data clearly showed that vitamin treatments not only alleviated the inhibitory effect of salinity stress, but also reduced the lipid peroxidation compared to that estimated in the control seedlings (Table 3).

Changes in antioxidants:

The data given in table (3) clearly indicate that salinity is capable of inducing significant decrease in ascorbic acid level. The reduction was about 36.4% below the control value at 300 mM NaCl. However, each of three vitamins used in the present investigation induced significant stimulatory effect on ascorbic acid production, which was more pronounced in either folate and coblamin treated stressed seedlings. Data presented in table (3) show that, different levels of NaCl induced an increase in the levels of GSH in salinized flax seedlings while a pronounced increase was observed in all vitamins treated salinized flax seedlings. However, GSH detected in salinized flax seedlings (Table 3) treated with either ascorbic acid or coblamin was much more elevated at sever salt stress (300 mM NaCl) than at low salt stress (100 mM NaCl). GSH increased significantly up to about 2 fold after ascorbic acid application.

Salt treatment induced increases in the activity of SOD, ASO, APX, POX, and GPX in flax seedlings. In contrast salt treatment had no significant impact effect on catalase activity. However, moderate level of NaCl (200 mM) reduced catalase activity in flax seedlings to 66.6% below that of the control .This reduction was sharply increased with the increase of the applied NaCl. No significant change was observed in catalase activity at low salinity level. Salinized flax seedlings exhibited an increase in SOD activity with increasing salinity level. Maximum response was observed at 200 mM NaCl. SOD activity increased significantly up to about 3-fold at this level. The percentage of increase in SOD activity was 75% at low salinity level (100 mM) in comparison with that of the control seedlings. At 300 mM NaCl only 41.6% increase in the enzyme activity was observed compared to that of control value (Table 4).

Data presented in table (4) show that salt stress induced a large increase in the activity of GOX and POX, under sever salt stress, while a slight increase, was observed concerning the same enzymes at low salinity level. At moderate salinity level, highly significant increase in GPX and POX activities was observed, this increase reached up to about 5-fold. Moreover, the increases in these activities were accompanied by decreases in total phenol content (Table 2). It is clearly indicated that all NaCl levels showed an increase in ASO and APX activities. At 300 mM NaCl enzyme activity was increased up to 144.7% and 132.3%, respectively compared with that of the control value, while the least enzyme activity was estimated at 100 mM NaCl. At this level, only 44% increase in enzyme activity was estimated compared with that of the control.

It is worthy to note that the decreased activity in each of GPX, POX and ASO in salinized flax seedlings treated with any of the applied vitamins (Table 4), was accompanied by large increases in polyphenols and ascorbic acid levels.

Table 1: Effects of folic acid, ascorbic acid, and coblamin on the percentage of germination and growth parameters of *Linum usitatissimum* seedlings grown under salt stress conditions. Each value is a mean of ten replicates ±SE

Parameter Treatment	The percentage of germination	Shoot length (cm)	Radical length (cm)
000 mM NaCl	93%	4.6±0.2	7.6±0.8
100 mM NaCl	47%	2.2±0.1c	3.0±0.2c
200 mM NaCl	13%	0.0	0.0
300 mM NaCl	00%	0.0	0.0
100mM NaCl+ 20µM folic acid	80%	4.9±0.2c	5.1±0.4c
200mM NaCl+ 20µM folic acid	73%	2.4±0.1c	1.3±0.1
300mM NaCl+ 20µM folic acid	67%	0.9±0.2	0.3±0.03
100mM NaCl+ 0.5mM ascorbic acid	73%	3.4±0.3a	2.6±0.2a
200mM NaCl + 0.5 mM ascorbic acid	47%	2.8±0.1	2.1±0.1b
300mM NaCl +0.5 mM ascorbic acid	34%	0.7±0.1	0.3±0.1
100mM NaCl+ 2µM coblamin	80%	4.9±0.2c	5.4±0.4c
200mM NaCl+ 2µM coblamin	60%	1.4±0.1b	0.7±0.04
300mM NaCl+2µM coblamin	33%	0.8±0.2	0.3±0.01

Values with a supercript are significant different from the control. Letter a =* at P > 0.05, b =** at P < 0.01, c =*** at P < 0.001, and absence of letter = non significant.

Table 2: Effects of folic acid, ascorbic acid, and coblamin on total soluble carbohydrates, total soluble proteins, total phenols, free amino acids, and praline contents of *Linum usitatissimum* seedlings grown under salt stress conditions. Each value is a mean of three replicates ±SE

Parameter	Carbohydrate content mg-1 g FW.			Nitrogen constituents mg-1 g.FW.					Total phenols mg-1 g FW.
Treatment	Soluble	Insoluble	Total	Soluble	Insoluble	Total	Proline µg -1g FW.	Free amino acids	
000 mM NaCl	6.5±0.4	105±3.1	±9.85111.	5.9±0.4	5.5±0.2	11±0.4	244±3.2	0.9±0.2	7.2±0.7
100 mM NaCl	7.2±0.1b	98.0±2.4a	105.2±3.5a	7.4±0.3a	5.9±0.6	13±0.3	271±3.6a	0.8±0.1	5.5±0.2b
200mM NaCl	10.6±0.2c	22.3±0.3c	32.9±0.3c	14.5±5.8c	13.4±0.3c	28±0.2c	293±1.4b	0.7±0.3	2.8±0.2c
300 mM NaCl	9.5±1.4a	21.3±2.0c	30.9±0.8c	16.9±0.3c	10.9±0.2c	27±0.3c	419±2.3c	0.4±0.2a	2.2±0.1c

Table 2: Continue

100mM NaCl+ 20µM folic acid	1.8±0.2c	44.9±1.1a	46.7±2.1a	4.9±3.3c	6.2±0.7	11±3.3	214±1.2c	1.1±0.7a	6.3±0.8
200mM NaCl+ 20µM folic acid	4.4±0.6c	30.9±0.9c	35.3±1.4c	4.1±0.2c	5.9±0.5c	9.9±0.2c	507±2.3b	0.9±0.2b	3.2±0.3c
300mM NaCl+ 20µM folic acid	1.5±0.06b	33.7±0.6b	35.2±0.3b	14.0±0.9a	9.2±3.3b	23±0.9b	694±2.2	0.9±0.3c	4.6±0.2b
100mM NaCl+ 0.5mM ascorbic acid	2.7±0.1b	142±4.6	145±4.6	4.8±0.3b	5.2±0.3	10±0.5	163±1.4b	0.9±0.1a	7.8±0.1b
200mM NaCl + 0.5mM ascorbic acid	3.8±0.2c	132±2.9c	136±3.4c	3.8±0.3c	7.9±1.3c	12±3.3c	598±9.4	1.1±0.1a	6.2±0.6c
300mM NaCl + 0.5 mM ascorbic acid	1.3±0.8b	31.5±0.6a	32.8±0.6	16.2±0.1a	8.5±0.6b	25±0.1b	627±2.7b	0.9±0.1c	5.4±0.2c
100mM NaCl+ 2µM coblamin	3.4±0.1a	111±0.6	113±0.3	5.3±0.5a	6.5±0.2a	12±0.4a	157±1.2c	1.2±0.2a	7.2±0.2b
200mM NaCl+ 2µM coblamin	3.2±0.3c	102±2.8c	105±3.2c	8.4±0.4c	7.3±0.3c	16±0.3c	674±4.5c	1.2±0.5c	6.9±0.3c
300mM NaCl+ 2µM coblamin	3.4±0.3a	92.4±2.5	95.8±1.6	13.8±0.5b	8.1±1.0c	22±0.6c	950±1.3c	0.9±0.1b	4.9±0.1b

Values with a supercript are significant different from the control. Letter a =* at P > 0.05, b =** at P < 0.01, c =*** at P < 0.001, and absence of letter = non significant.

Table 3: Effects of folic acid, ascorbic acid, and coblamin on the lipid peroxidation products, and water soluble antioxidants substances of *Linum usitatissimum* seedlings grown under salt stress conditions. Each value is a mean of three different replicates ±SE

Parameter	MDA (lipid peroxidation product) µmol-1 g. FW.	Water soluble antioxidant substances	
Treatment		Ascorbic acid mg-1 g.FW.	Glutathione µmol-1 g.FW.
000 mM NaCl	3.2±0.3	0.22±0.04	19.0±0.8
100 mM NaCl	4.5±0.5	0.19±0.02	20.0±0.3
200mM NaCl	12.7±0.8c	0.15±0.01a	22.0±0.1
300 mM NaCl	14.7±0.03c	0.14±0.003a	26.6±0.4
100mM NaCl+ 20µM folic acid	0.4±0.1b	0.25±0.009	.3±1.320
200mM NaCl+ 20µM folic acid	1.7±0.4c	0.23±0.02a	22.2±0.3
300mM NaCl+ 20µM folic acid	1.6±0.2c	0.26±0.02a	36.4±1.1a
100mM NaCl+ 0.5mM ascorbic acid	1.1±0.1a	0.30±0.01a	20.2±1.6
200mM NaCl + 0.5 mM ascorbic acid	2.8±0.4c	0.34±0.03b	26.0±2.1a
300mM NaCl +0.5 mM ascorbic acid	1.2±0.6c	0.23±0.02b	66.0±2.4c
100mM NaCl+2µM coblamin	1.7±0.3a	0.29±0.02	23.2±0.5
200mM NaCl+2µM coblamin	1.3±0.2c	0.25±0.02a	29.0±1.6a
300mM NaCl+2µM coblamin	0.9±0.1c	0.26±0.006c	62.8±2.6c

Values with a supercripts are significant different from the control. Letter a =* at P > 0.05, b =** at P < 0.01, c =*** at P < 0.001, and absence of letter = non significant.

Table 4: Effects of folic acid, ascorbic acid, and coblamin on the activity of antioxidant enzymes of *Linum usitatissimum* seedlings grown under salt stress conditions. Each value is a mean of three different replicates ±SE

Parameter	GPX	APX	ASO	POX	SOD	CAT
Treatment	Amount of quinon g-1 FW. min-1	mM of ascorbate oxidized g-1 FW. min-1	mM of ascorbate oxidized g-1 FW. min-1	Amount of quinon g-1 FW. min-1	Unit mg-1 protein	µmol of H_2O_2 destroyed g-1 FW. min-1
000mM NaCl	0.18±0.1	22.9±0.4	11.4±1.6	0.24±0.1	1.2±0.6	2.7±0.2
100mM NaCl	0.57±0.02	26.6±0.5	11.9±0.6	0.21±0.1	2.1±0.1	2.2±0.1
200mM NaCl	5.61±0.1c	28.5±1.1b	14.3±0.4	5.87±0.9c	3.1±0.2a	0.9±0.2
300mM NaCl	5.56±1.7c	30.3±0.5b	16.5±1.2	5.78±0.3c	1.7±0.3	0.6±0.3
100mM NaCl+ 20µM folic acid	0.57±0.02	33.8±0.5b	1.6±0.8b	0.23±0.1	2.9±0.2	3.2±0.6
200mM NaCl+20µM folic acid	1.47±0.06c	37.1±0.6b	2.2±0.7b	0.51±0.2c	8.5±0.6a	2.4±0.2
300mM NaCl+ 20µM folic acid	1.73±0.30c	30.7±1.1	1.9±0.1b	1.28±0.5c	2.2±0.5	0.9±0.1
100mM NaCl+0.5mM ascorbic acid	0.51±0.2	28.1±1.2	11.1±0.1	0.31±0.1	4.5±0.5	3.1±0.1
200mM NaCl+0.5mM ascorbic acid	2.26±0.2c	29.4±1.1	13.3±1.2	1.31±0.2c	8.8±0.9	3.2±0.2
300mM NaCl+0.5mM ascorbic acid	1.91±0.2c	31.3±1.1	12.3±0.8	1.52±0.04c	2.4±0.8	0.9±0.3
100mM NaCl+ 2µM coblamin	0.37±0.1	31.3±0.5	13.1±0.4	0.55±0.03	5.4±0.6	3.2±0.8
200mM NaCl+ 2µM coblamin	1.41±0.3c	36.3±0.5b	12.9±0.4	0.68±0.4c	3.4±0.3	0.7±0.1
300mM NaCl+ 2µM coblamin	2.28±0.6c	29.1±1.1	13.2±0.7	2.48±0.03c	2.4±0.1	0.7±0.3

Values with a superscript are significant different from the control. Letter a =* at P > 0.05, b = ** at P < 0.01, c = *** at P < 0.001, and absence of letter = non significant.

Discussion:

The resistance to environmental stress may depend at least partially on the inhibition of ROS production and/or the enhancement of antioxidant level as well as the osmotic adjustment during seed germination and plant development. Indeed, no stage in plant life is more important than germination. The failure of germination may be attributed to the highly negative osmotic potential of the external medium which makes the seeds unable to absorb sufficient water for germination (Cramer *et al*., 1991). The results obtained in the present study show that different levels of salinity induced a marked depressive effect on germination percentage of flax seeds. However, the flax seeds exposed to 300 mM NaCl fail to germinate. Salinity inhibits seed germination through accumulation of toxic ions and/or reduced water uptake which arrested radicle emergence (Hampson and Simpson, 1990; Begum *et al*., 1992). Furthermore excess of Na^+ might cause problems with membranes, enzyme inhibition, disturbance in metabolism which disorganize cell division, elongation and structure as recorded by Ghoulam and Fares, (2001); Nuran and Hüsnü, (2002); Abo-Kassem, (2006).

Completely non germinating seeds of sever salt stress (300 mM NaCl) showed an amazing capacity for recovery and germination after treatment with each of folic acid, ascorbic acid or coblamin (Table 1). The strategy of osmotic adjustment in salinized flax seedlings treated with vitamins might be mediated by accumulation of some compatible solutes (amino acids, sugars or proline) acting as osmolytes. Similar results were obtained by Bohnert and Shen, (1999); Serrano *et al*., (1999) and Shabala *et al*., (2000).

Salinity did not affect only germination but also reduced shoot and radicle growth (Table 1). The reduction in growth parameters of salinized flax seedlings might be attributed to the osmotic effect resulting from salt stress which cause disturbances in water balance of the stressed plants, leading to stomatal closure, accumulation of toxic ions, damage in cellular organelles and subsequent inhibition of growth (Alves da costa *et al*., 2005). The exogenous application of folic acid, ascorbic acid, or coblamin alleviated partially or completely the adverse effect of salt stress on growth of flax seedlings. Folic acid has become the most prominent of B-complex vitamins despite of its essential biochemical function of transporting single carbon fragments in amino acid metabolism and nucleic acid synthesis (Andrew *et al*., 2004). Coblamin is necessary for the rapid synthesis of DNA during cell division (Smith *et al*., 2007). Ascorbic acid is one of the most powerful antioxidant (Noctor and Foyer, 1998; De Tullio, 2000; Smirnoff and Wheeler, 2000). The ability to donate electrons makes ascorbic acid the main ROS-detoxifying compound in aqueous phase. Ascorbic acid can directly scavenge superoxide hydroxyl radicals and singlet oxygen and reduce H_2O_2 to H_2O via ascorbate peroxidase reaction (Noctor and Foyer, 1998). Ascorbic acid regenerates tochopherol providing membrane protection (Thomas *et al*., 1992). In addition, ascorbic acid carries out a number of non-oxidant functions in the cell. It has been implicated in the regulation of cell division, cell cycle progression from G_1 to S phase (Liso *et al*., 1988; Smirnoff, 1996) and cell elongation (De Tullio *et al*., 1999).

Metabolic changes due to exposure of flax to salt stress are also observed during seed germination and seedling development. Data in table (1) indicate a general increase in soluble sugars at the expense of the insoluble and total carbohydrates, which manifested a pronounced increase in salinized flax seedlings, similar results with obtained by Khattab (2007). In fact, sugars act as osmoprotectants counteracting the toxic effect of Na^+ and Cl^- in many plants (Everad *et al*., 1994). On the other hand, the link between ROS production and photosynthetic metabolism are particularly important (Rossel *et al*., 2002). Soluble sugars feeding of the oxidative pentose phosphate (OPP) pathway can enhance NADPH production, which is a major cofactor of ROS scavenging pathways such as ascorbate-glutathione cycle (Gaetani *et al*., 1989; May *et al*., 1998).

Moreover, the pronounced increase in proline level in salinized flax seedlings at the expense of total amino acids was observed (Table 2). The reduction in free amino acid content which was observed in response to different salinity levels may attribute to the disturbance in amino acid metabolism which was shifted to the accumulation of proline (Boggess *et al*., 1976).

Under salt stress conditions, proline accumulation in plants increased for the osmoregulation (Gunes *et al*., 1996; Aziz *et al*., 1999). Many plants accumulate proline as a non toxic and protective osmolyte under saline or other some stress conditions (Cayley *et al.,* 1992). Its accumulation is caused by both the activation of its biosynthesis and inactivation of its degradation (Mattioni *et al*., 1997). Proline protects membranes and protein against the adverse effects of high concentration of inorganic ions (Paleg *et al*., 1984 and Santoro *et al*., 1992). Proline also function as hydroxyl radical scavenger (Smirnoff and Cumbes, 1989; Hoque *et al*., 2007).

Increase in polyphenol content in different tissues under salt stress has been reported in many plants (Agastian *et al.,* 2000; Pokorny, 2001). Our data are in disagreement with this idea mainly because salinity induced sharp decrease in polyphenol content in salinized flax seedlings particularly with increasing salinity levels. Similar results were obtained by Ksouri *et al*., (2007). The decrease in polyphenol content was contaminant with a pronounced increase in polyphenol oxidase and peroxidase activities which consume phenols as substrate. Vitamin treatments stimulated the accumulation of phenolic compounds in salinized flax

seedlings and this increase may be one aspect of the role played by vitamins in alleviating the suppressive effects of high salinity levels. Polyphenols possess ideal structural chemistry for free radical scavenging activity Another mechanism underlying the antioxidative properties of phenolic compounds is the ability of flavonoids to decrease membrane fluidity (Rice-Evans *et al.,* 1997; Gaballah *et al*., 2006).

Moreover, salt stress induced membrane injury which may therefore due to changes in the membrane lipids or protein or both (Scandalios, 1993). Lipid peroxidation is the symptom most easily ascribed to oxidative damage (Zhang and Kirkham, 1996). Our results were consistent with this view because we observed a pronounced increase in MDA production in salanized flax seedlings. The growth inhibition at 300 mM NaCl was correlated with 4.5-fold augmentation of MDA concentration. The increase in lipid peroxidation might be due to incapability of endogenous antioxidants to scavenge all ROS resulted from salt stress. Any of the vitamins used in the present study consistently reduced salt-induced accumulation of MDA; and this was attributed to antioxidative properties of these vitamins which scavenge the ROS resulted from salt stress, consequently flax seedlings treated with folic acid, ascorbic acid, or coblamin were much more protected from oxidative damage (Shalata and Neumann, 2001).

Despite accumulation of soluble carbohydrate fractions as well as proline for osmotic adjustment, salinized flax seeds fail to germinate under sever salinity stress (Table 1), therefore, the strategy of osmotic adjustment conferred by these osmoprotectants is not sufficient to protect salinized flax seeds. On addition to the osmotic adjustment, salinized flax seedlings have evolved a complex antioxidant system which is composed of molecular antioxidant (glutathione) as well as ROS-scavenging enzymes such as SOD, APX, and GPX. In fact, an increase in the activity of antioxidant enzymes under salt stress could be indicative of an increased production of ROS and a build-up of a protective mechanism to reduce the oxidative damage triggered by salt stress (Meneguzzo *et al*., 2000; Chaparzadeh *et al*., 2004). A significant increase in SOD activity was observed in salinized flax seedlings (Table 4). The activity of SOD was slightly enhanced at sever salt stress, to an extent which is far less that induced by moderate salinity level (200 mM NaCl). Even though a high SOD activity protect the plant against the superoxide radical because it converts $O_2^{\cdot-}$ to H_2O_2 which is also a ROS. H_2O_2 should be scavenged by CAT, APX, and GPX (Badawi *et al*., 2004a). Elevated SOD activity, without an accompanying increase in the ability to scavenge H_2O_2 can result in cytotoxicity by the even more destructive hydroxyl radical generated from H_2O_2 (Gosset *et al*., 1994a). In some plant species, the increase in catalase activity may be used as a marker for salt tolerance (Sudhakar *et al*., 2001; Sairam *et al*., 2002 and Bor *et al*., 2003). Our result were inconsistent with this view mainly because salt stress induced a reduction in catalase activity, this results in accumulation of toxic level of H_2O_2 which may be the main reason in inhibition of germination at high salinity level (300 mM NaCl). Moreover, the increase in the activities of APX, ASO, GPX, and POX has been reflected by the reduced levels of polyphenols and ascorbate contents (Tables 2 & 3). These enzymes withdraw polyphenols and ascorbate as substrates, which may be a second reason for inhibition of germination at highest salinity level. The decreases in ascorbic acid and polyphenols in response to high salinity levels were also observed by sairam *et al*., (2005) and Ksouri *et al*., 2007) respectively. Moreover, salinized flax seedlings treated with either folic acid, ascorbic acid or coblamin have much better hydrogen peroxide scavenging mechanism as manifested by continuous increase in CAT and APX activities up to highest salinity level (Table 4), resulting in lower H_2O_2 content. It is possible that these antioxidant enzymes succeed to capture ROS ($O_2^{\cdot-}$ and H_2O_2) at high salinity level. Consequently, flax seeds succeed to germinate under severs salt stress.

Marked increases in ascobate and glutathione were observed in salanized flax seedlings treated with folic acid, ascorbic acid or coblamin (Table 3). In this respect, vitamin treatments provoke a reduction in the activity of ASO which in turn sqenches ROS to a low level via the accumulation of ascorbate (Tables 3 and 4).

Glutathione play an important role in the protection against oxidative stress. It is involved in the ascorbate / glutathione cycle and in the regulation of protein thiol-disulphid redox status of plants in response to biotic and abiotic stresses (Mullineaux and Rausch, 2005).

In conclusion, salinized flax seedlings were thus ill equipped to face salt stress. The sensitivity of flax seeds to salt stress may be due to the decreased activity of catalase, increased activities of oxidases and peroxidases as well as the subsequent decreases in ascorbate and total phenol levels.

Consequently, the mechanism of exogenous application of some vitamins on alleviating salt stress hazards was mediated by accumulation of some osmoprotectants and increasing some antioxidants.

Abbreviations:

AS ascorbic acid- ASO ascorbic acid oxidase- APX ascorbic acid peroxidase- POX polyphenol oxidase- GPX Phenol peroxidase- SOD superoxide dismutase- CAT catalase- MDA malondialdehyde- GR glutathion reductase.

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