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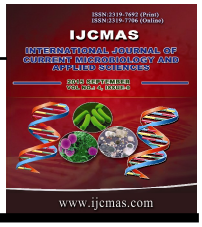

Original Research Article

Salt-Induced Physiological and Biochemical Changes in two varieties of *Linum usitatissimum* L.

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Keywords

Linum usitatisimum, Proline, Protein, Peroxidase, Salt stress

A B S T R A C T

The present investigation was carried out to study the effects of different concentrations of NaCl (0 mM, 25 mM, 50 mM 75 mM and 100 mM) on two varieties of flax (NL- 97 and NL- 260). The extent of influence of NaCl on physiological and biochemical parameters was investigated at seedling stage. Negative effects of NaCl on germination percent, seed vigor index, relative water content, total length and amylase were observed. Germination percent was more affected in variety NL- 97 as compared to variety NL- 260. The protein, proline and peroxidase increased with increasing concentrations of NaCl. Proline levels showed threefold increase at 100 mM NaCl as compared to control in both the varieties. Both proline content and peroxidase activity were more pronounced in NL- 97 as compared to NL- 260. Overall results indicate the contrasting behavior of two flax varieties with respect to salt tolerance capacities. NL- 97 exhibits lesser negative impacts on root length, shoot length and total length compared to NL- 260. Moreover, salt stress resulted in an inhibition of the enzyme peroxidase and lesser accumulation of proline in NL- 260 as compared to NL- 97. Taken together, NL-97 shows comparably higher salt tolerant nature than NL- 260 which might be attributed to higher proline and peroxidase activity under salt stress.

Introduction

One of the major challenges in plant physiological studies is to increase the plant productivity under adverse environmental conditions. Soil salinity is one of the detrimental abiotic stresses and a complex phenotypic and physiological phenomenon in plants that results into reduced crop productivity (Ashraf and Foolad, 2007; Munns and Tester, 2008). The harmful effects of salinity stress on growth and physiology include the reduction in germination rate and seedling growth (Tambhale *et al.*, 2011), and decline in photosynthetic area and biomass production (Mansour and Salama, 2004). In addition, salinity stressed plants show reduced water potential in the root zone causing water deficit, phytotoxicity of ions such as Na^+ and Cl^-, and nutrient imbalance by depression in uptake and/or shoot transport

(Munns and Termaat, 1986). NaCl is the major salt causing salinization and affects morpho-physiological, biochemical and molecular processes, including seed germination, plant growth and water and nutrient uptake in number of crop plants (Kaya *et al.,* 2011).

Flax (*Linum usitatissimum* L.) is economically important food crop grown all over the world. The increasing demand for flax is mainly because it contains high amount of protein, α-linolenic-rich oil, lignans and fiber (Sebei *et al.,* 2007; Sadak and Dawood, 2014). Moreover, consumption of the oil or seed has been reported to have beneficial effects on cardiovascular health and in the treatment of certain cancers, neurological and hormonal disorders and inflammatory diseases (Simopoulos, 2002). It has been reported that flax is a source of at least three classes of commercial products: fibre, seed oil, and nutraceuticals and therefore, increased understanding of the genes affecting the quality and yield of these bio-products will contribute to crop improvement for flax and other oil or fibre producing species (Wang *et al.,* 2012). However, the oilseed crop is often not suitable to grow and improve under conditions of salinity stress and hence, in recent years, there has been increasing interest in recognizing the physiology and molecular biology of salinity stress tolerance in oilseed crops (El-Beltagi *et al.,* 2008).

Increasing salinity levels have been reported to result in reduction in germination, delayed emergence, inhibition of seedling growth, delayed degradation of seed lipids and fluctuating content of linolenic and linoleic acid in different flax varieties (Sebei *et al.,* 2007; Kaya *et al.,* 2011). Despite, there is a little information about the effects of salinity stress on growth physiology and biochemistry of different flax cultivars. Therefore, an attempt was made to evaluate the effect of different NaCl concentrations on physiological parameters and biochemical constituents of flax.

Materials and Methods

Plant material and NaCl stress treatment

Two varieties of flax viz. NL- 97 and NL-260 were procured from Punjabrao Deshmukh Krushi Vidyapeeth, Akola, India. The seeds were soaked in water for 24 hours and allowed to germinate in petriplates (Borosil, India) lined with germination papers. Different concentrations of NaCl (s d fine-chem limited, Mumbai) such as, 0 mM (control), 25 mM, 50 mM, 75 mM and 100 mM were used. The plates were incubated at 25°C for three days and after initiation of shooting, the plates were transformed in light. The experiment was performed in triplicate and each replicate contains 20 seeds.

Growth parameters/physiological parameters

Germination percentage was calculated on fifth day while the observations on root length (cm), shoot length (cm), total length (cm) and relative water content (%) were conducted from 10 seedlings on 12^{th} day after sowing (DAS).

Seed Vigor

The seed vigor was calculated by the following formula: (Abdul- Baki and Anderson, 1970).

Seed vigor index = (germination percentage × means of total seedling length in cm) / 100

Weight reduction percentage

Fresh weight was calculated from each concentration of NaCl in three replications

from ten seedlings on 12th day and expressed in grams. These ten seedlings of each concentration were kept for drying at 70°C for three days and dry weight was calculated.

Weight reduction percentage was calculated using following formula: (El Goumi *et al.*, 2014).

Fresh weight (FW) percentage reduction: FWPR % = 100 x [1 - (fresh weight salt stress / fresh weight control)].

Dry weight (DW) percentage reduction: DWPR % = 100 x [1 - (dry weight salt stress / dry weight control)].

Relative water content (RWC)

The relative water content was calculated as - RWC % = 100 x [(FW – DW) / FW].

Biochemical parameters

Amylase enzyme extraction and assay

For amylase extraction, 20 seeds were soaked in water for 24 hours and allowed to germinate in petriplates (Borosil) in different concentrations of NaCl. The plates were kept in dark for two days. The extraction of amylase was carried out at the emergence of radicle. Samples of each concentration were homogenized separately in pre-cooled 50 mM phosphate buffer (pH 7.0) maintaining cold conditions.

The homogenate was centrifuged at 12,000 *g* at 5° C for 10 min in a refrigerated high speed centrifuge (Bioera, High speed refrigerated programmable centrifuge, India). The resultant supernatant was used as enzyme source.

The amylase assay was performed as previously described by Miller (1959) wherein the reducing group liberated from starch were measured by reduction of 3,5, dinitrosalicylic acid.

Protein quantification

Proteins were extracted on 12th day from each concentration. The extraction was carried out from 0.5 g and extracted in phosphate buffer pH 7.0 and quantified by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as standard.

Proline determination

The proline was determined from the above seedlings using methods described by Bates *et al.* (1973) and Chinard (1952). The whole seedling (0.5 g) was ground and extracted with 10 mL of 3% sulfosalicylic acid. The extract was filtered through a Whatman filter paper (No. 2). The reaction mixture contains 2 mL filtrate, 2 mL acid ninhydrin and 2 mL glacial acetic acid. The reaction mixture was heated in boiling water bath for 60 min. The reaction was terminated by placing the tubes on ice and the chromophore was extracted using 4 mL of toluene. The absorbance of the chromophore phase was read at 520 nm using a spectrophotometer (Systronics PC based double beam spectrophotometer 2202, India). The amount of proline is expressed as µmoles/g fresh weight.

Peroxidase enzyme extraction and assay

The 12 days old seedlings of each concentration were homogenized separately in 0.1 M Phosphate buffer pH 7.0 by grinding in pre-cooled mortar and pestle, maintaining cold conditions. The homogenate was centrifuged at 17,000 g at 5° C for 20 min in a refrigerated high speed centrifuge (Bioera, High speed refrigerated programmable centrifuge, India). The resultant supernatant was used as enzyme

source. Total peroxidase activity is assayed as described by Putter (1974) and Malik and Singh (1980). The reaction mixture (3 ml) consisted of 0.05 mL guaicol, 0.1 mL enzyme extract and 0.03 mL hydrogen peroxide solution. The reaction was measured spectrophotometrically (Systronics PC based double beam spectrophotometer 2202, India) at 470 nm. Total peroxidase activity was expressed as the units/ L.

Statistical analysis

The data was statistically analyzed by using analysis of variance (ANOVA) SigmaPlot 13.0, Germany. The values were expressed as mean ± standard error (S.E.) of three replications. Differences were considered significant at $p<0.001$, $p<0.01$ and $p<0.05$ level of probability.

Results and Discussion

Effect of NaCl stress on physiological parameters

The effects of different levels of NaCl on physiological parameters are depicted in table 1 and table 2. Significantly reduced germination percent and seed vigor index (Table 1) root length, shoot length, total length and relative water content (RWC) (Table 2) were observed in both the varieties of flax. Increasing NaCl concentration resulted in decrease in germination percentage, seed vigor index and RWC at higher level of NaCl in both NL-97 and NL-260 varieties (Table 1 and Table 2). The successful crop production is mainly determined by seedling establishment as a result of seed germination (Almansouri *et al.,* 2001; Kumar *et al.,* 2007). Therefore, understanding the responses of plants at seedling stages is particularly important for elucidating the mechanisms of salt resistance or sensitivity in plants and their survival (Tambhale *et al.,* 2011). The inhibitory effects on seed germination for the oil seed species at higher NaCl concentration were reported by Debez *et al.* (2004). The decrease in relative water content due to increased NaCl concentration can be due to the solute content of the cells in the salt-treated plants which causes more water to be taken up than in the control plants Munns *et al.* (2006). Seed vigor index was reduced more than 60% at higher concentration of NaCl (75 mM and 100 mM) in both the varieties of flax (Table 1). Decrease in seed vigour index was also reported by El Goumi *et al.* (2014). Sodium chloride inhibited germination via osmotic and toxic effects, which results into altered seed viability in linseed (Sebei *et al.,* 2007).

As the concentration of NaCl increased, significant decrease in root length, shoot length and total length was observed in both NL- 97 and NL- 260 varieties of flax (Table 2). The effects were more pronounced in variety NL- 260 as compared to NL- 97. The results are in agreement with Tambhale *et al.* (2011) and Ben Miled *et al.* (2000). Reduction in growth parameters of flax seedlings due to salt stress were attributed to osmotic effects resulting from salt stress causing disturbance in water balance which further lead to stomata closure, accumulation of toxic ions, damage in cellular organelles and subsequent inhibition of growth (Alves da Costa *et al.,* 2005).

Similarly, percent reduction in fresh weight and dry weight was observed with increasing level of NaCl as compared to control (Table 1). These results revealed inhibitory effects of NaCl at higher concentrations and it appeared to be cultivar dependant. The inhibitory effects were more pronounced in variety NL- 97 as compared to variety NL- 260. Nonetheless, increasing

NaCl levels causes reduction in growth parameters. The present results are in agreement with El Goumi *et al.* (2014) and Tambhale *et al.* (2011), who reported reduction in growth parameters due to enhancing salinity treatments.

Effect of NaCl stress on biochemical parameters

Accumulation of proline as a nontoxic and protective osmolyte under saline conditions can be attributed to osmoregulation (Aziz *et al.,* 1999). Proline functions as hydroxyl radical scavenger (Smirnoff and Cumbes, 1989) and also protects membranes and protein against the adverse effects of high concentration of inorganic ions (Santoro *et al.,* 1992). Data on effects of different concentrations of NaCl on biochemical parameters are presented in table 3. Increasing concentration of NaCl resulted into significantly increased levels of proline in both varieties of flax. At higher concentration of NaCl (100 mM), proline concentration was more than 3 fold as compared to control in both the varieties of *Linum* (Table 3). However, the rate of salt stress-induced proline accumulation was considerably higher in NL- 97 as compared to NL- 260. A cultivar dependent proline accumulation has been reported in rice (Tambhale *et al.,* 2011). The rapid accumulation of proline in response to NaCl has been reported in Safflower (Siddiqi *et al.,* 2011), Chickpea (Singh, 2004) and flax (De- Lacerda *et al.,* 2003). Therefore, increased levels of proline can be used as marker for the identification of salt tolerant genotypes.

Salinity stress produces reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals and metabolic toxicity (Ashraf and Harris, 2004). Scavenging of ROS in plant cells occurs by endogenous protective mechanisms involving antioxidant molecules and enzymes (Türkana and Demiral, 2009) such as ascorbate peroxidase, catalase and peroxidase, together with low-molecular mass scavengers such as ascorbate, glutathione and proline, act as the main defense against ROS produced in various parts of plant cells (Apel and Hirt 2004). Peroxidase activity was enhanced in NL- 97 while there was no specific trend observed in case of NL- 260 (Table 3). Swapna (2003) and Weisany *et al.* (2012) reported increased peroxidase activity in response to increasing levels of NaCl concentrations. Therefore, the increased amount of peroxidase activity may limit the oxidative damage in variety NL-97.

As observed for proline, protein concentration (µm/g tissue) was also found to be increased at each salt level in both the varieties (Table 3). It was ranged from 10.06 (control) to 15.96 (100 mM NaCl) and 10.19 (control) to 31.59 (100 mM NaCl) in variety NL-97 and NL-260 respectively (Table 3). In the present studies, reduction in protein content was observed at higher salinity levels while Agastian *et al.* (2000) reported that soluble protein increases at low salt concentration and decreases at elevated levels. However, a decrease in protein content was noted in rice (Tambhale *et al.,* 2011). The proteins that accumulate under salt stress conditions may provide a storage form of nitrogen that might be re-utilized in post-stress recover (Singh *et al.,* 1987) and contribute towards the better salt stress tolerance of the flax cultivar. Synthesis of enzymes and mobilization of seed reserves are crucial for initiation of germination. However, salt stress either alters it or does not permit the synthesis of specific metabolite required for seed germination (Pattanagul and Thitisaksakul, 2008).

Table.1 Effect of NaCl on germination percent, seed vigor index, fresh weight and dry weight at seedling level in two Linum varieties

Salt stress	Germination (%)		Seed Vigor Index (%)		Fresh weight (g)		Dry weight (g)	
	NL-97	NL-260	NL-97	NL-260	NL- 97	NL- 260	NL- 97	NL- 260
0 mM	92.03±0.61	96.00±10.15	11.81±0.56	10.95±0.71	0.42±0.03	0.408±0.016	0.034±0.003	0.037±0.001
25 mM	89.33±2.60	92.00±02.96	06.97±0.36	07.08±0.34	0.33±0.04	0.391±0.019	0.039±0.002	0.045±0.001
50 mM	83.67±3.76	81.67±01.76	05.80±0.45	05.43±0.23	0.28±0.03	0.415±0.017	0.037±0.001	0.045±0.003
75 mM	68.00±2.31	76.67±02.60	03.70±0.22	03.49±0.25	0.29±0.06	0.385±0.006	0.036±0.001	0.046±0.001
100 mM	56.67±2.40	71.67±02.19	03.02±0.27	03.72±0.22	0.21±0.01	0.228±0.017	0.044±0.002	0.041±0.001
Significance	*	*	*	*	***	*	NS	NS

Note: The values are average of three replications. *, **. *** indicate significance at p<0.001, p<0.01 and p<0.05 respectively. NS indicates not significant

Table.2 Effect of NaCl on growth parameters and relative water content at seedling level in two Linum varieties

Salt stress	Root Length (cm)		Shoot Length (cm)		Total Length (cm)		Relative Water Content (%)	
	NL-97	NL-260	NL-97	NL-260	NL-97	NL-260	NL-97	NL-260
0 mM	5.62±0.80	6.11±0.79	7.21±0.32	5.29±0.19	12.84±0.64	11.41±0.74	91.93±0.90	90.77±0.63
25 mM	3.53±0.13	4.05±0.54	3.98±0.05	3.63±0.08	07.51±0.17	06.93±0.63	89.80±0.50	88.22±0.84
50 mM	3.63±0.13	3.09±0.17	4.16±1.27	3.48±0.20	07.79±0.16	08.95±0.40	88.88±1.15	87.09±0.30
75 mM	3.99±0.17	1.73±0.14	4.11±0.34	2.82±0.62	08.10±0.19	07.20±0.47	84.64±2.38	89.98±0.55
100 mM	3.71±0.25	3.24±0.37	4.08±0.10	2.68±0.09	07.80±0.29	07.69±0.41	88.18±2.25	88.51±1.09
Significance	*	*	**	*	*	***	**	*

Note: The values are average of three replications. *, **. *** indicate significance at p<0.001, p<0.01 and p<0.05 respectively. NS indicates not significant

Table.3 Effect of NaCl on biochemical parameters at seedling stage in two Linum varieties

Salt stress	Proline content (µm/g tissue)		Protein content (mg/g)		Peroxidase activity (Units/g)		Amylase activity (Units/g)	
	NL-97	NL-260	NL-97	NL-260	NL-97	NL-260	NL-97	NL-260
0 mM	12.29±1.52	05.30±0.56	10.03±0.12	10.19±1.02	05.33±0.81	05.67±0.46	132.66±04.29	130.75±10.86
25 mM	14.89±2.07	05.97±1.15	13.59±1.33	12.25±0.32	05.80±0.66	06.52±0.25	127.01±08.54	136.79±06.35
50 mM	20.37±2.63	08.44±0.73	13.73±0.57	21.36±2.26	09.07±1.51	04.79±0.69	134.22±11.82	129.93±05.78
75 mM	33.65±1.02	10.45±2.15	15.70±1.37	22.12±1.45	10.69±2.04	04.98±0.51	121.40±05.33	124.71±15.64
100 mM	37.70±2.16	15.12±1.83	15.96±0.87	31.59±1.02	11.17±0.43	07.94±2.17	120.47±13.23	121.36±06.28
Significance	*	**	**	*	***	NS	NS	NS

Note: The values are average of three replications. *, **. *** indicate significance at p<0.001, p<0.01 and p<0.05 respectively. NS indicates not significant.

In the present investigation inhibitory effects of NaCl was observed at higher levels of NaCl levels (Table 2). Similarly decrease in amylase activity at higher levels (75 mM and 100 mM) of NaCl in different crops (Almansouri *et al.,* 2001; Ashraf *et al.,* 2002). This reduction in enzymatic activity may be one of the factors for reduced assimilate transportation and reduction in growth at seedling stage.

In conclusion, from the results obtained in the present investigation, we can conclude that overall NL-97 exhibited lesser negative effects of NaCl on root length, shoot length and total length at seedling stage as compared to NL- 260 variety of flax. Additionally, NL- 97 showed better tolerance to salt stress than NL- 260 which might be attributed to high accumulation of proline accompanied by increased peroxidase activity. NL- 97 showed tolerance for the high capacity to limit oxidative damage by increasing the activity of antioxidant enzyme such as peroxidase. All these parameters might have played an important role in its salt tolerance nature.

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