Sodium chloride stress induced differential growth, biomass yield, and phytochemical composition responses in the halophytic grass *Aeluropus lagopoides* (L.)

D. A. Animasaun[1*], S. Oyedeji[1], G. G. Joseph[1,2], P. A. Adedibu[1] and R. Krishnamurthy[3]

[1] *Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Nigeria*

[2] *Department of Biological Sciences, Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria*

[3] *C. G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Bardoli, Gujarat, India*

*Corresponding Author: *animasaun.ad@unilorin.edu.ng*

Abstract

This study evaluates the growth and biochemical changes in *Aeluropus lagopoidesi* (L.) Thawaites. induced by different sodium chloride (NaCl) concentrations to understand the effects and tolerance of the plant to salinity regimes. *A. lagopoides* stolons were grown on Hoagland media in three replicates. At four weeks after planting, 0, 100, 300, and 500 mM of NaCl were added to the media as treatments, and this was repeated at 2-week intervals. Data were collected and analyzed on growth and biological yield of the plants at 3, 6, and 9 days after the final salt stress. Also, sodium, potassium, and calcium ions present in the root and shoot of the treated plants were determined, while the number of salt crystals extruded from the leaf was counted. The amounts of proline, amino acids, and total soluble sugars in response to salt concentrations were evaluated. There was a significant variation in the growth of *A. lagopoides* in response to the NaCl concentrations. Generally, a concentration of 500 mM adversely affected plant growth and biomass yield. The concentration of sodium ions in the tissue of treated plants increased with NaCl concentration, while the concentration of potassium and calcium ions decreased. Total amino acid and proline in the plant tissue increased with salinity, while soluble sugar increased from 3-6 days but declined remarkably on the 9th day after NaCl applications. The study demonstrated salt stress tolerance in *A. lagopoides* and suggested its potential as a biotechnological model plant for salt tolerance improvement of economically important crops in high-salinity zones.

Introduction

Soil salinity is a major environmental constraint that adversely affects crop yields in many parts of the world. Salinity affects about one billion hectares of land worldwide due to flooding and ocean surge; it was estimated to increase annually by 10% (FAO, 1988). High sodium ion (Na+) concentration in the soil (sodicity) is toxic to plant cells and metabolic activities. It imparts both ionic and osmotic stresses that adversely affect plant growth, disrupts the ionic equilibrium and elicits salt toxicity in the cells (Flowers and Colmer, 2008). Seawater contains about 40 g L^{-1} of dissolve salts of which sodium chloride usually occurs in the highest proportion (Gleen et al., 1999), and this, of course, cannot be tolerated by most plants. For instance, rice can tolerate less than 3 g L^{-1} of sodium chloride while barley and date palm can tolerate up to 5 g L-1. However, dwarf glasswort (*Salicornia bigelovii*) can tolerate as much as 70 g L^{-1} of sodium salts (Katschnig et al., 2013).

Halophytes are the plants that grow and survive in environments with high concentrations of salts, in particular, sodium chloride (Flowers and Colmer, 2008). Their adaptive mechanism to such environments may involve salt tolerance or/and avoidance (Munns and Tester, 2008). Plants that develop a mechanism to reduce uptake of salts though growing in a saline environment are "facultative halophytes", while those that take up salts but show minimal effects are 'true' or

obligate halophytes. Different plant species employ different mechanisms to mitigate the effects of hyper-concentration of sodium ions in the soil. Some halophytes accumulate proline for osmotic adjustment in response to salinity and abiotic stress, while others show adaption to high salt concentration by regulating a wide array of genes or by evolving unique functional and structural mechanisms such as salt gland, thick suberin layer etc. to withstand salt stress (Kreps et al., 2002; Sobhanian et al., 2010). For instance, to avoid high salt concentration intake, plant species may complete its reproductive life cycle when soil salt concentration is low (such as wet season) to avoid excessive salt uptake. In some cases, excessive salt may be excreted through the leaves, or by the accumulation of the salts in the leaves that later senesce and drop off (Munns and Tester, 2008). Yadav et al. (2012) demonstrated that salt responsive genes isolated from halophytes could be introduced into other plants to achieve salt tolerance.

Aeluropus lagopoides, a member of the family Poaceae is a perennial C_4 halophytic grass found in the coastal areas. This makes the plant to be incessantly exposed to high salinity from sea waters. The roots are adventitious, with small leaves that contain epicuticle wax that confers some adaptive characteristics on the plant. *A. lagopoides* could be utilized as fodder for cattle. Members of its genus, *A. littoralis* and *A. lagopoides* are known to survive high salinity with low salt accumulation in the tissue (Bodla et al., 1995). Furthermore, the physiological and biochemical effects of drought and water stresses have been studied in the two species (Vaziri et al., 2011). Also, their molecular and physiological adaptations to salinity have been reported (Mohsenzadeh et al., 2006). Salt tolerance mechanism in *A. lagopoides* as revealed by Express Sequence Tag (EST) indicated the ability of the plant to excrete excess salt (Mehta et al., 2005) and the excreted salts were mainly sodium salts (Barhoumi, 2006).

Chemical nutrients such as proline, soluble sugars, potassium levels, total amino acid, protein and chlorophyll contents have been used to monitor stress in higher plants (Ashraf and Harris, 2004; Mohsenzadeh et al., 2006). It was noted that plants could accumulate a relatively low number of inorganic ions to achieve a water potential gradient between soil and plant (Gulzar et al., 2003). Meanwhile, genes are reportedly involved in signaling, regulation and expression of factors that control osmotic adjustment processes in an abiotic stressed plant (Mehta et al., 2005, Munns and Tester, 2008). Although studies have been conducted on the mechanisms of salt tolerance in halophytic plants, there is a need for further investigation on the physiological and biochemical response of plants to salinity. The present study, therefore, evaluates the physiological and biochemical changes of halophytic plant *Aeluropus lagopoides* in response to sodium chloride (NaCl) of varying concentrations towards improving the current understanding of salt tolerance concerning physiological activities and biochemical constituents of the plant. The findings from the study will provide further information on the utility of biochemicals as precursors to abiotic stress in plants.

Materials and Methods

Plant material and Glasswares

Stolons of *Aeluropus lagopoides* consisting of

3-5 nodes were used for the study. Glasswares used for media preparation and biochemical analysis were of high quality (Pyrex, NY, USA). Glasswares were washed in warm distilled water, rinsed and oven-dried at 120 ^{o}C before use. Chemicals of high analytical grade purchased from Sigma-Aldrich (Germany) and Hi-Media (Mumbai India) were used for the study.

Media preparation and plant establishment

The Hoagland media (HM) used for the study was composed as described by Kane et al. (2006). *Aeluropus lagopoides* (3 nodes) were planted in pots (250 ml capacity) containing vermiculite, irrigated every three days with 150 ml of HM media (HM adjusted to pH 5.8) in a growth chamber at 25±2 °C, 16 hr light (350 µmol m^{-2} s^{-1}) and 8 hr dark cycle. Four weeks after planting, 100 ml each of different NaCl concentrations (0, 100, 300 and 500 mM) were added to the HM as treatments in three replicates. The 0 mM treatment was the experimental control for the study. The salt treatments were renewed every two weeks until the sixth week when plants were harvested. Plants were harvested on the 3rd, 6th, and 9th day after NaCl stress for growth, physiological and biochemical analyses.

Growth Parameters

Plants harvested on the 3rd, 6th and 9th day after salt treatments were evaluated for shoot length, root length, fresh and dry weight. The dry weight was determined by drying at 70°C for 72 hours in the oven and the weight was determined using WENSAR high precision balance (Model 9001).

Salt extrusion and ion estimation

Salt extrusion through the leaf was observed and the number of salt crystals on the upper fully expanded leaf was counted for both the abaxial and adaxial surfaces with the aid of Leica Stereomicroscope (Leica m8, Leica, Germany). The ion contents in the root and shoot tissues were determined according to the methods of Shukla et al. (2012) using Inductively Coupled Plasma Optical Emission Spectrometer (Avio 200, PerkinElmer, UK).

Determination of Na^{+}, K^{+}, Ca^{2+}

The Na^{+}, K^{+} and Ca^{2+} contents of the leaf samples were determined by HPLC using the method described by Boscaiu et al. (2007). Briefly, about 0.2 g (fresh weight) leaf samples were well ground in a mortar. The ions were extracted from the ground samples with 100 mM perchloric acid heated at 95 ºC for 10 min, then centrifuged at 8000 rpm to remove the debris. The supernatants were analysis for Na^{+}, K^{+} and Ca^{2+} by cation exchange chromatography in a HPLC system with a conductivity detector. Elution was carried out in an isocratic flux, using 3 mM HNO_3, containing 0.1 mM EDTA, as per the equipment manufacturer instruction.

Phytochemicals determination

The total soluble sugars in the plant tissues were estimated as described by Irigoyen et al. (1992). The resultant mixture was checked at an absorbance of 620 nm on Epoch Micro-Volume Spectrophotometer (Biotech Instruments Inc. USA). A calibration curve was constructed with glucose in the range 20-400 µg/ml (Sigma Aldrich, USA). The total amino acid content was determined following the procedure of Shukla et al. (2012) at an absorbance of 570 nm. Free proline content in the leaves was determined using acid ninhydrin (Bates et al., 1973) at an absorbance of 520 nm on a spectrophotometer (Varian Cary 100/300 UV/VIS Spectrophotometer Agilent,

USA). This was calculated by comparing the absorbance value against a standard curve derived from known concentrations of L-proline with the values expressed as µg mg^{-1} of the fresh weight.

Data analyses

Morphological data collected from three randomly selected plants and biochemical analysis performed in three replicates were subjected to analysis of variance (ANOVA) using SPSS statistical package version 17 for Microsoft Operating System. The means were separated by Duncan's Multiple Range Test (DMRT) at the significance level of $P < 0.05$.

Results

The results obtained from the observations and data analyses showed that responses of *A. lagopoides* varied with the salt concentrations. Normal leaf colouration (green) and shape were observed in 0-300 mM treated plants on the 3rd, 6th and 9th days after salt stress (DSS). But pale green leaves and stunted growth were observed in plants treated with 500 mm NaCl. Some leaves of the plant treated with 500 mM showed chlorosis on the 6th day and a few of them withered on 9-DSS. *A. lagopoides* growth and biological yield in response to NaCl concentrations varied significantly. Shoot developments were similar for 0 and 100 mM treated plants (Table 1) while significantly

TABLE 1

Growth and biological yield parameters of *A. lagopoides* treated with different concentrations of NaCl

Treatment	Shoot length (cm)			Root length (cm)			Fresh weight (mg)			Dry weight (mg)		
	Number of days			Number of days			Number of days			Number of days		
	3	6	9	3	6	9	3	6	9	3	6	9
0 mM	4.73^{ab}	4.93^{c}	5.72^{ab}	2.81^{c}	2.97^{b}	4.32^{a}	196.2^{a}	206.4^{a}	231.2^{a}	89.52^{a}	107.8^{a}	113.4^{a}
100 mM	4.71^{ab}	4.87^{c}	5.11^{bc}	2.71^{c}	2.82^{b}	2.98^{d}	134.2^{c}	139.5^{c}	142.7^{c}	74.09^{c}	77.4^{c}	78.6^{c}
300 mM	5.10^{a}	5.65^{a}	6.14^{a}	3.61^{b}	3.70^{a}	3.76^{b}	178.3^{b}	192.4^{b}	218.3^{b}	81.32^{b}	86.9^{b}	99.7^{b}
500 mM	4.24^{c}	4.19^{c}	4.72^{c}	3.18^{ab}	3.72^{a}	3.25^{c}	138.7^{c}	133.1^{c}	127.9^{d}	58.43^{d}	61.6^{d}	56.8^{d}

Different letter(s) along the columns shows the values are not significant different ($p<0.05$)

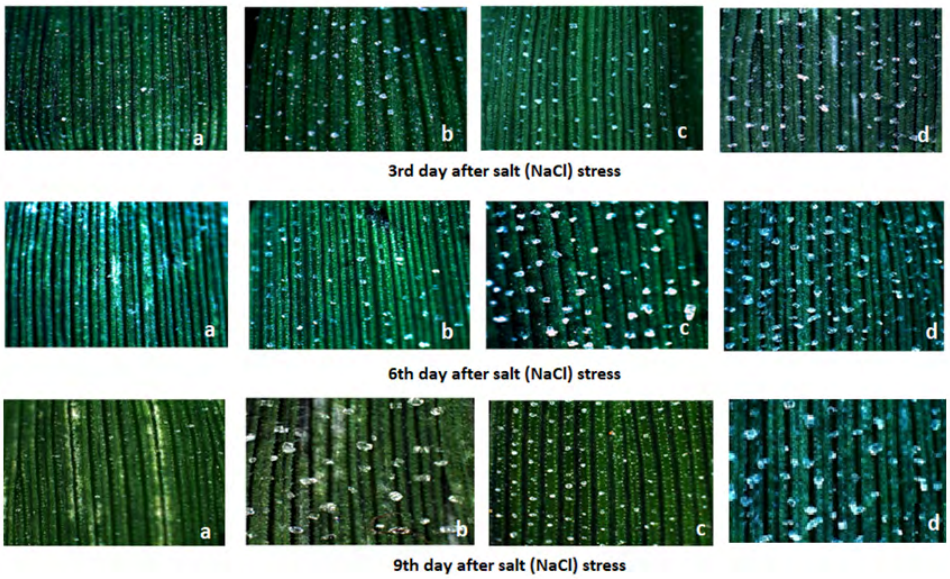

Figure 1: Pattern of salt excretion through the leaves of *A. lagopoides* treated with different concentrations of NaCl on the 3rd, 6th and 9th day after salt stress.
Key: a: 0 mM; b: 100 mM; c: 300 mM; and d: 500 mM

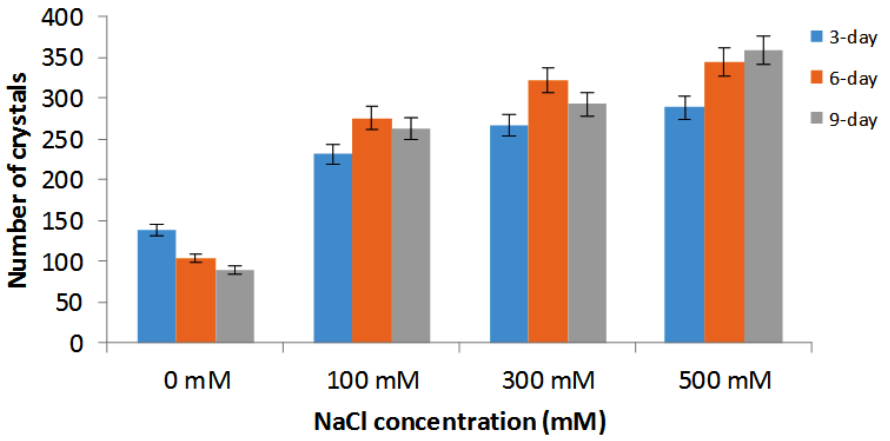

Figure 2: Average number of salt crystal excreted through the leaves of *A. lagopoides* treated in response to different NaCl concentration on 3rd, 6th and 9th day after salt stress

longer roots were recorded on the 9-DSS for 0 mM and reduced for 500 mM. Biological yield (fresh and dry weight) was significantly higher for 0 mM plants, which reduced with increase in salt concentrations. However, 500 mM negatively affected biological yield at all DSS evaluated.

The pattern of salt extrusion with different NaCl concentrations days after salt stress is shown in Fig 1. The number of extruded salt crystals ranged from 89.2-138.0 in 0 mM treated plants with the highest on the 3rd day after salt stress (Fig. 2). The crystal density increased with increase in concentration. All the tested concentrations had the highest number of salt crystals at 6th DSS, except 0 mM that had the highest on the 3rd DSS, which decreased at 6th and 9th DSS. Among the 100-500 mM treated plants, the number of salt crystals increased from 3rd to 6th DSS, and then reduced on the 9th DSS. Meanwhile, a remarkable decrease in average salt crystal number was found in 500 mM treated plant leaves on the 9th DSS compared to the 6th and 3rd DDS.

Sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions present in the treated plant tissues per gram of dry weight are presented in Table 2. The Na^+ content in the root (14.81-192.15 mg/kg DW) and the shoot (8.13-27.54 mg/kg DW) of the salt-stressed plant increased significantly with the increase in concentration, with the highest (192.15 mg/kg DW) in the root of 500 mM treated plants. In contrast,

TABLE 2

Sodium (Na^+), Potassium (K^+) and Calcium (Ca^{2+}) ions accumulated in the root and shoot of *A. lagopoides* treated with different concentrations of NaCl at 9-day after treatment

Treatment	Shoot length (cm)			Root length (cm)		
	Na^+	K^+	Ca^{2+}	Na^+	K^+	Ca^{2+}
0 mM	14.81^{d}	27.97^{b}	11.32^{c}	8.13^{d}	16.50^{a}	5.70^{a}
100 mM	101.71^{c}	32.82^{a}	31.98^{a}	12.42^{c}	10.10^{b}	2.71^{b}
300 mM	112.68^{b}	16.70^{c}	8.76^{d}	18.20^{b}	8.95^{bc}	2.64^{b}
500 mM	192.15^{a}	15.72^{c}	7.25^{b}	27.54^{a}	8.69^{c}	2.62^{b}

Different letters along the columns indicated significant differences ($p<0.05$). The ions were estimated as mg/g of the dry weight

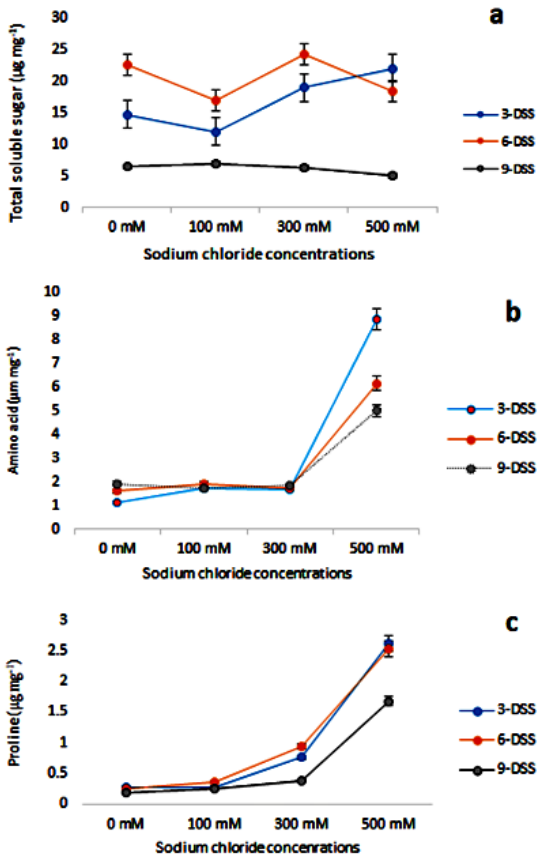

Figure 3: (a) Totaol soluble sugar (b) Total amino acid, and; (c) proline contents of *A. lagopoides* grown in hydroponic culture of different sodium chloride concentrations on 3, 6 and 9 days after salt stress (DSS)

K^+ and Ca^{2+} decreased with increasing NaCl concentration. However, in the root, K^+ was significantly higher in 100 mM (32.82 mg/kg DW) than 0 mM (27.97 mg/kg DW) and decreased as salinity increased from 300 - 500 mM. The trend was also similar for the Ca^{2+}. Generally, the number of ions found in the root was greater than those in the shoot of the salt-treated plants.

The result of total soluble sugar, amino acid and proline in the response of halophytic plant *A. lagopoides* to different NaCl are shown in Figure 3(a-c). The total soluble sugars present in the treated plants' tissue for 0 - 300 mM increased from 3rd to 6th DSS and then decreased sharply on the 9th DSS (Fig. 3a). However, the trend was different for 500 mM plant tissues where total soluble sugar decreased with day of observations after salt treatment i.e. the amount of soluble sugar was higher on the 3rd DSS (22.01 µg mg^{-1}) than the 6th DSS (18.45 µg mg^{-1}) and the least (5.0 µg mg^{-1}) was found on the 9th DSS. In terms of amino acid accumulation, the response of the plant to sodium concentrations of 100-300 mM showed no remarkable difference from the 0 mM, but 500 mM of the salt resulted in high production of amino acid in the plant tissue in order of 3rd DSS > 6th DSS > 9th DSS (Fig. 3b). The proline content increased from 3rd to 6th DSS with an increase in salt concentration (Fig 3c). Proline content ranged from 0.28 - 2.6, 0.24 - 2.53 and 0.19 - 1.68 µg mg^{-1} of the fresh weight on the 3rd, 6th and 9th DSS, respectively. Although there was a slight difference in proline content between 0 mM and 100 mM up to 9 DSS, proline concentration increased by more than twofold

in 300 mM and ten-fold compared with 500 mM on the 3rd and 6th DSS. However, proline content decreased on the 9-DSS in the plant tissue for all the concentrations.

Discussion

The response of *A. lagopoides* to salt stress varied significantly with different concentrations. The results indicated that plant growth reduced at a high salt concentration which resulted in chlorosis and dead leaves, but at concentrations below 300 mM, the plant was physiologically unaffected. This implies that 300 mM was within tolerable NaCl salinity for *A. lagopoides*. A higher concentration of 500 mM probably exceeded the threshold of salt tolerance in the test plant, consequently resulting in growth reduction and biomass yield (Wu et al., 2015). A similar finding by Sobhanian et al. (2010) demonstrated that biomass yield decreased with a high salinity of NaCl treated plants. At such high concentrations, the gland for salt excretion and other salt regulating process could not mitigate the salt concentration, hence the adverse effects on the plant. Presence of excess sodium chloride will reduce the potassium concentration gradient in plants and affect chlorophyll formation (Wellburn, 1994, Wu et al., 2015), leading to low photosynthetic activities and reduced growth (Atkinson et al., 1967).

The sodium ions accumulated in the excreted fractions were proportional to increasing NaCl concentrations. The salt extrusion through the leaf decreased on the 9th DSS because less salt was available in the media for root absorption. Excess salt absorbed by the roots is subsequently accumulated in tissues within a few days after salt stress, which was vigorously excreted by the leaf (Atkinson et al., 1967). Salt glands are usually distributed on both surfaces of halophytic plants' leaf. The density of such glands will increase with increasing salinity (Munns and Tester, 2008). This is possible because salt excretion (functioning) is an active process (Khan and Gulzer, 2003).

The Na^+ increased due to addition of NaCl which interfered with the influx of other ions, especially K^+ in the treated plants. Though, the presence of Na^+ is beneficial for decreasing osmotic potential, increase in the Na^+ content under salt stress led to Na^+ influx into the plant via roots (Munns and Tester, 2008). This suggests that salt tolerance in *A. lagopoides* was not due to the mechanism of Na^+ uptake restriction, and there may not be a strong Na^+ barrier at the root level in this plant with high salinity. The present results and trend of Na^+, K^+ and Ca^{2+} contents in a salt-stressed plant agreed with Khan and Gulzer (2003), who reported that Na^+ and Cl^- concentrations increased with an increase in salinity, whereas K^+ and Ca^{2+} decreased.

The K^+ transport systems had some affinity for Na^+ as in Na^+/K^+ transporters and Na^+ competed with K^+ for the intracellular influx (Shabala et al., 2006). Therefore, the high external Na^+ concentrations negatively affected intracellular K^+ influx and consequently decrease K^+ content which would affect osmotic adjustment in stomata movement for the survival of the plant (Khan and Gulzer, 2003). However, under drought and water deficit conditions, higher K^+ content had been reported in *A. lagopoides* (Mohsenzadeh et al., 2006). The $Na^{+/}K^+$ ratio was higher in the root than in the shoot and of course, assimilation of salts occurred in the root and the higher the concentration the higher the assimilated

quantity (Sun et al., 2015). Though plants tend to maintain a low ratio of Na^+/K^+ for survival, fluctuations in the ratio were ascribed to Na^+ and K^+ concentration change after addition of NaCl and subsequent recovery process of the plant (Wu et al., 2015).

The relative abundance of proline, amino acid and total soluble sugars are important biochemical precursors of salinity in plants. Increase in total protein content and sugars could be a reason for water loss from the tissues. Accumulation of proline in the plant tissue is an adaptive response to salt stress. It may also serve as carbon and nitrogen storing mechanism when growth is impeded due to stress (Bohnert et al., 1995). Also, soluble sugars, as well as amino acids, could accumulate in plant tissues during stress to mitigate the effect of salinity. This could be achieved through osmoregulation, stabilization of protein structure or mediation as molecular chaperones. The results supported the opinion of Yadav et al. (2012), that increased accumulation of amino acids indicates a higher rate of protein synthesis in plants experiencing salt stress.

Conclusion

The study concludes that *A. lagopoides* has the potential to tolerate high salt concentration by excretion of excess salt except at high concentration of 500 mM, which adversely affected growth. The plant can adjust its osmoregulation mechanism through alteration in proline, total amino acids and soluble sugars production as affected by different concentrations on 3rd, 6th and 9th day after NaCl treatment, an indication of possible adaptation for survival in high salinity environment. The study suggests that the mechanism employed by this plant could be adopted as a model for the proper understanding of the underlying factors in the development of salt and water stress-tolerant varieties of crops.

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