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DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY IN TWO LYCIUM SPECIES GROWN *EX VITRO* AT HIGH SALINITY IN HYDROPONIC

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Abstract: The effect of salt stress on distribution of alkaline metals in the roots and shoots of *Lycium barbarum* and *Lycium chinense* grown *ex vitro* in hydroponic at three levels of salinity, 0.05 M, 0.1 M and 0.2 M NaCl was investigated. The content of Na^+ and K^+ gradually decreased in the roots and rose in the shoots of *Lycium chinense* with increasing salinity level as compared to control. The content of Na^+ increased sharply in the roots, but increase in the leaves of *Lycium barbarum* was slight. The ratios K^+/Na^+ were higher in the shoots in comparison to roots of both plants. They were reduced more in the roots of *L. barbarum* and in the shoots of *L. chinense* during NaCl treatment. Total antioxidant capacity, measured by FRAP assay and DPPH radical methods changed by the same manner in the leaves of *L. chinense* and were enhanced after treatment with 50 mM NaCl. The results were discussed from the view of mechanisms of action of alkaline metals on the generation of reactive oxygen species (ROS) in different organs and the participation of water soluble (WS-AOC) and lipid soluble (LS-AOC) antioxidants in the defence of plants. WS-AOC was higher and total DPPH and FRAP antioxidant capacity measured was greater in the leaves of *Lycium chinense*.

INTRODUCTION

Plant species growing in the semi-arid regions of the world need to be adapted to environment in which drought strongly affects plant growth. Water availability is the main environmental factor limiting photosynthesis and growth even in plants well adapted to arid conditions. Another source of stress is soil salinity in non-tolerant species (Greenway and Munns, 1980).

Lycium L. (Solanaceae) is a genus of approximately 80 species distributed mainly in Asia, America and Africa (Bernardello, 1986; Miller, 2002; Levin and Miller, 2005). *Lycium* species are long-lived perennial shrubs which produce red or purple, fleshy berries. *Lycium barbarum* and *Lycium chinense* are a unique nutritional and medicinal food. The plants are used in traditional Chinese medicine for the treatment of pneumonia, cough, inflammation and diabetes mellitus. Their physiological characteristics of drought-tolerance and salt-tolerance make them an ideal plants for preventing soil desertification and alleviating the degree of soil salinity, but overexploitation of *Lycium* natural habitats caused its deterioration and decreased the existing populations (Liu et al., 2012). That is why, the development of micropropagation technologies is necessary in the future. *Lycium* species used in the current paper are selected as material to analyze its salt tolerance under different concentrations of NaCl in hydroponic so as to provide fundamental base for vegetation restoration in salinized soils. The investigations showed that treatment of *Lycium barbarum* with 0.1 and 0.2 M NaCl in pot experiments stimulate growth parameters with 20% and 30%, respectively (Wei et al., 2006).

The paper deals with the effect of salt stress on distribution of alkaline metals - K^+ and Na^+ in different organs of *Lycium barbarum* and *Lycium chinense* grown *ex vitro* as hydroponic cultures at three levels of salinity, 0.05 M, 0.1 M and 0.2 M NaCl. The changes in the antioxidant capacity in the leaves of both species is traced too.

MATERIALS AND METHODS

Plant material

Seeds and *in vivo* explants from the species of *L. barbarum* and *L. chinense* were used for developing of *in vitro* multiplication protocol as described by Dimitrova et al. (2016). Seeds originated from China. For induction of shoots multiplication, explants of *L. barbarum* were cultured on Murashige and Skoog (MS) nutrient medium supplemented with sucrose, agar and indolilacetic acid (IAA). For induction of shoots, explants of *L. chinense* were cultured on McCown Woody plant medium supplemented with sucrose, agar, adenine, giberelinic acid, IAA and indol-3-butyric acid. After multiplication, the shoots were transferred to rooting medium based on half strength basal salts MS medium or McCown medium, supplemented with compounds described above. All cultures were

incubated in growth chamber with 16-h photoperiod, light intensity of 100 μmol m^{-2} s^{-1} and 24/18±1ºC day/night temperature. After three weeks of rooting, the shoots were rinsed with Proplant solution. Solution concentrations for shoot and root multiplication were described by Dimitrova et al. (2016).

Hydroponic experiment

Uniform explants were selected and transplanted to polyethylene vessels containing 1.2 l of 1/4 Hoagland solution (Hoagland and Arnon, 1941) (pH 5.9) in growth chamber with a 16-h photoperiod (PAR 100 μmol m^{-2} s^{-1} measured on the upper leaf surface, 25/17±1 ºC day/night temperature, 54/45% relative humidity). The experiments were set as four treatments including control, each treatment with 7 replications. The salt treatment was applied on the 48th day after transplanting of explants when the plants had adapted to the conditions of 1/2 Hoagland nutrient solution and 0 (control), 0.05, 0.1, and 0.2 M NaCl was added as described by Dimitrova et al. (2016).

Determination of ion content

To determine the content of K^+ and Na^+ (mg g^{-1} DW) 0.25 g dry leaf samples were extracted after acidic digestion with Suprapur grade Fluka reagents and analyzed by atomic absorption spectrophotometer (Perkin-Elmer 5000, UK). The K^+/Na^+ ratio was calculated from the content of Na^+ and K^+ (Doumett et al., 2008).

Total antioxidant capacity

Spectrophotometric quantification of water- (WS – AOC) and lipid-soluble (LS – AOC) antioxidant capacity (expressed as equivalents of ascorbate and α-tocopherol, respectively) was performed through the formation of phosphomolybdenum complex (Prieto et al., 1999). The assay was based on the reduction of Mo (VI) to Mo (V) by the sample analysis and the subsequent formation of a green phosphomolybdenum complex at acidic pH. The dry plant samples (0.1 g DW) was ground with pestle and mortar to a fine powder. 3 ml distilled H_2O was added and the suspension was homogenized, transferred to tubes and shaken for 2 h at room temperature in the dark. The suspension was filtered and extraction was repeated with 3 ml distilled H_2O. The pellet was washed again with 2 ml distilled H_2O. For lipid-soluble antioxidant capacity procedure was the same, but the extraction was carried out with hexane as a solvent. The method was optimized and characterized with respect to linearity interval, reproducibility and molar absorption coefficients for the quantitation of water-soluble and lipid-soluble antioxidant capacities expressed as equivalents of ascorbate, and α-tocopherol. Absorption coefficients were: $(3.4 \pm 0.1) \times 103$ M^{-1} cm^{-1} for ascorbic acid, and $(4.0 \pm 0.1) \times 103$ M^{-1} cm^{-1} for α-tocopherol.

DPPH assay

Free radicals scavenging activity was measured from the bleaching of the purple-colored methanol solution of free stable radical (2,2-diphenyl-1-picrylhydrazyl, DPPH$^{\bullet}$) inhibition after Tepe et al. (2006). DPPH$^{\bullet}$ radical is a stable radical with a maximum absorption at 517 nm that can readily undergo

reduction by an antioxidant. The inhibition of free radical DPPH$^{\bullet}$ in percent (I %) was calculated in the following way: I% = (A_{blank}- A_{sampe}/ A_{blank}) ×100,where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, i.e. plant extracts. The reaction mixture consisted of different concentrations from 15 to 180 µg ml^{-1} plant methanol extract, 2.4 ml methanol and 0.1 mM methanol solution of DPPH$^{\bullet}$. Control and tested samples were incubated in the dark for 30 min before spectrophotometrically assayed.

Ferric reducing power (FRAP assay)

The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), ferri 2,4,6-tripiridyl-s-triazine(TPTZ) solution (10 mM TPTZ in 40 mM HCl) and $FeCl_3$-$6H_2O$ (20 mM) in a ratio 10:1:1 (Benzie and Strain, 1996). To perform the assay, 900 µl of FRAP reagent, 90 µl distilled water and 30 µl of plant extract were mixed and incubated at 37 °C for 15 min. The absorbance was measured at 595 nm, using FRAP working solution as a blank. The antioxidant potential of samples was determined from a standard curve plotted using the $FeSO_4.7H_2O$ linear regression. The results were corrected for dilution and expressed as µmol of Fe^{2+} g^{-1} of dried sample.

Statistical analysis

All data reported in this work were mean value of at least five to six independent experiments. The significance of differences between control and each treatment was analyzed by Fisher`s LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

RESULTS AND DISCUSSION

Net accumulation of Na^+ in the plants grown on saline environment is dependent on the balance between its passive influx and active efflux. According to Munns (2002), the ability of plants to regulate the uptake and transport of salts is dependent on the following mechanisms: a/ selectivity of uptake by root cells; b/ preferential loading of K^+ rather than Na^+ into the xylem by the cells of stele; c/ removal of salts from the xylem in the upper parts of roots, the stem and leaf sheaths, based upon exchange of K^+ for Na^+, and d/ loading of the phloem.

Our results showed that both species possessed different mechanisms for regulation of the uptake and transport of salts by roots and shoots (Fig. 1,2). As compared to control, the content of Na^+ is enhanced in the roots of *Lycium barbarum*, while in the roots of *Lycium chinense* it is decreased after treatment with increasing concentrations of NaCl (Fig. 1A). The content of K^+ gradually decreased in the roots of *L. chinense* (Fig. 1B) and in the leaves of *L. barbarum* at the salinity conditions tested (Fig. 2A). The content of Na^+ slightly changed in the leaves of *L. barbarum* after NaCl treatment (in spite of the significance of

differences between control and each treatment), but it gradually increased in the leaves of *L. chinense* (Fig. 2A). The content of K^+ changed in the same manner for *L. barbarum,* but for *L. chinense* it is enhanced as compared to control (Fig. 2B).

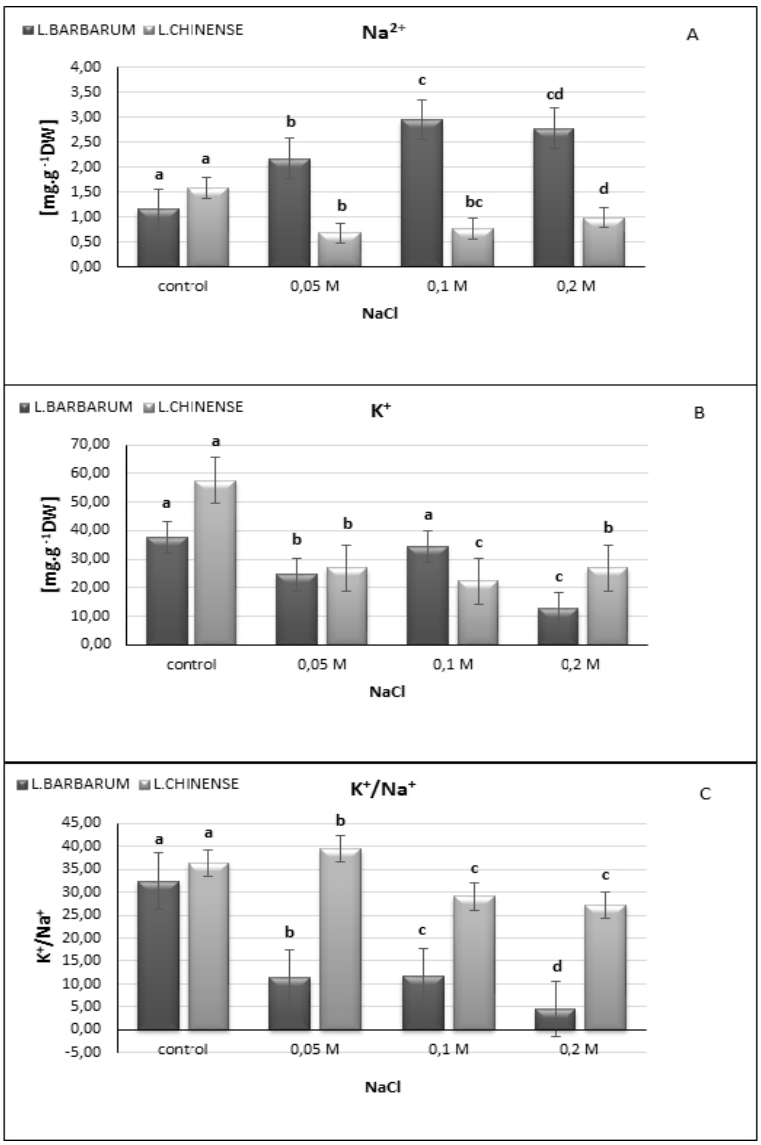

Fig. 1. Accumulation of Na^+ (A), K^+ (B) and K^+/Na^+ ratio (C) in the roots of *Lycium barbarum* and *Lycium chinense* grown in hydroponic at three levels of salinity: 1/ control; 2/ 0.050 M NaCl; 3/ 0.1 M NaCl; 4/ 0.2 M NaCl.

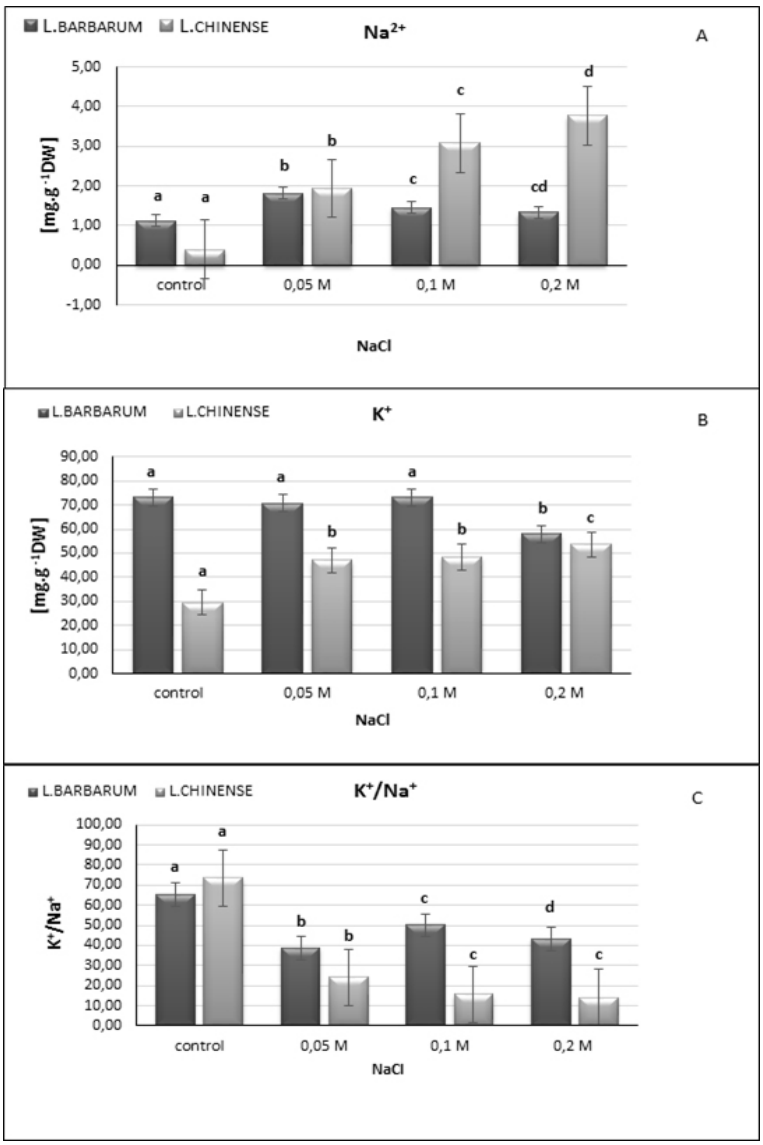

Fig. 2. Accumulation of Na^+ (A), K^+ (B) and K^+/Na^+ ratio (C) in the shoots of *Lycium barbarum* and *Lycium chinense* grown in hydroponic at three levels of salinity: 1/ control; 2/ 0.05 M NaCl; 3/ 0.1 M NaCl; 4/ 0.2 M NaCl.

Uptake and distribution of Na^+ within the roots connected to the effects of K^+ and is related to the K^+/Na^+ root selectivity (Jeschke, 1972). To confer salt stress tolerance, in many plant species, the achievement of a high K^+/Na^+ ratio is more important than simply maintaining low concentrations of Na^+ in the tissues (Maathuis and Amtmann, 1999). Our results showed that K^+/Na^+ ratio is higher in the roots of control plants of *L. chinense* than of *L. barbarum* and it is reduced at great extent in *L. barbarum* after NaCl treatment (Fig. 1C). The values of K^+/Na^+ ratio measured in the shoots of both control plants are approximately twice higher than that in its roots. Nevertheless, this ratio is more reduced in the leaves of *L. chinense* than *L. barbarum* after treatment with increasing NaCl concentrations (Fig. 2C). The investigations showed that in wheat, salt tolerance is related to the enhanced K^+/Na^+ selectivity (Gorham, 1990). In contrast to dicotyledonous plants, in monocots the maintenance of a lower K^+/Na^+ ratio in shoots is of greater significance, because of their lower capacity for Na^+ storage and higher requirement for K^+ and compatible organic solutes (Glenn et al., 1999). Our preliminary results suggested that *L. chinense* is more tolerant to salt stress than *L. barbarum* because the ratios of fresh weight/ dry weight are more enhanced with increasing salinity level for its shoots. The leaf content of proline is higher in control plants of *L. chinense* as compared to *L. barbarum* and changed significantly during stress (Dimitrova et al., 2016). That is why, irrespective of decreased K^+/Na^+ ratio in the leaves of *L. chinense* than *L. barbarum* after treatment with increasing NaCl concentrations, free proline level is decisive in protection and adaptation of shoot`s growth under saline conditions.

The leaves of both species differed in the means of measured total antioxidant capacity (Fig. 3, 4). Control of *L. chinense* possessed higher WS-AOC and LS – AOC than control of *L. barbarum*. The values of the first parameter decreased in both plants with increasing salinity level (Fig. 3A), while for the second one the highest value is established at 0.1 M NaCl in *L. barbarum* (Fig. 3B). Total antioxidant capacity measured by DPPH and FRAP methods changed by the same manner in the leaves of *L. chinense* and is highest at 0.05 M NaCl. DPPH and FRAP assays for the leaves of *L. chinense* showed highest values after the same treatment (Fig. 4A, B), while FRAP assay did not showed significant difference between control and addition of 0.05 M NaCl in the leaves of *L. barbarum* (Fig. 4A). The researchers which studied community dynamics and predominant characteristics of encroachers in semi-arid grassland ecosystems in North America established that one of the encroaching species (*Larrea tridentata*) has higher carotenoid contents and greater DPPH-radical scavenging capacity than the non-encroachers (*Lycium fremontii*) (Liu and Guan, 2012). Higher carotenoid content and greater DPPH-radical scavenging capacity characterize more tolerant to environmental conditions species. Our preliminary investigations showed that chlorophyll a/chlorophyll b ratio is enhanced and chlorophyll a+b/ carotenoids ratio slightly decreased during treatment with increasing concentrations of NaCl from 0.05 M to 0.2 M in more tolerant *L. chinense* than in *L. barbarum* (Dimitrova et al., 2016). WS-AOC is higher in the leaves of *L. chinense* than in *L. barbarum*.

Flavonoids prevailed in this fraction (unpublished data). It is known that these compounds are more effective antioxidants than ascorbate and α-tocopherols in scavenging ROS because of the possession of phenolic - OH groups (Sakihama et al., 2002).

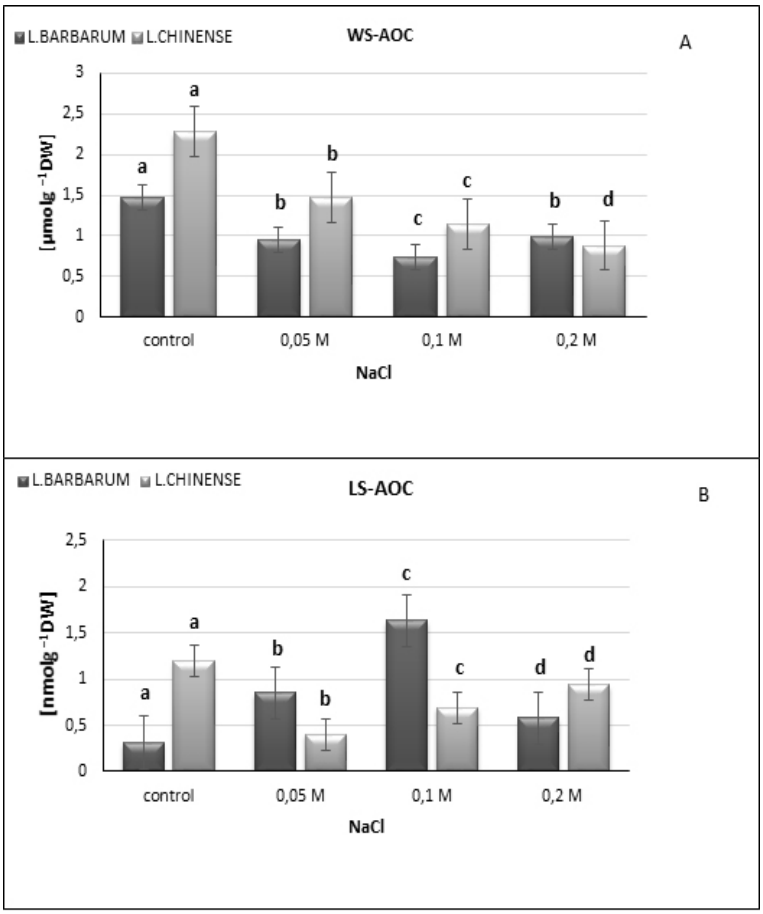

Fig. 3. Changes in leaf water-soluble antioxidant capacity (WS-AOC) – A and lipid-soluble antioxidant capacity (LS-AOC) – B of *Lycium barbarum* and *Lycium chinense* grown in hydroponic at three levels of salinity: 1/ control; 2/ 0.05 M NaCl; 3/ 0.1 M NaCl; 4/ 0.2 M NaCl.

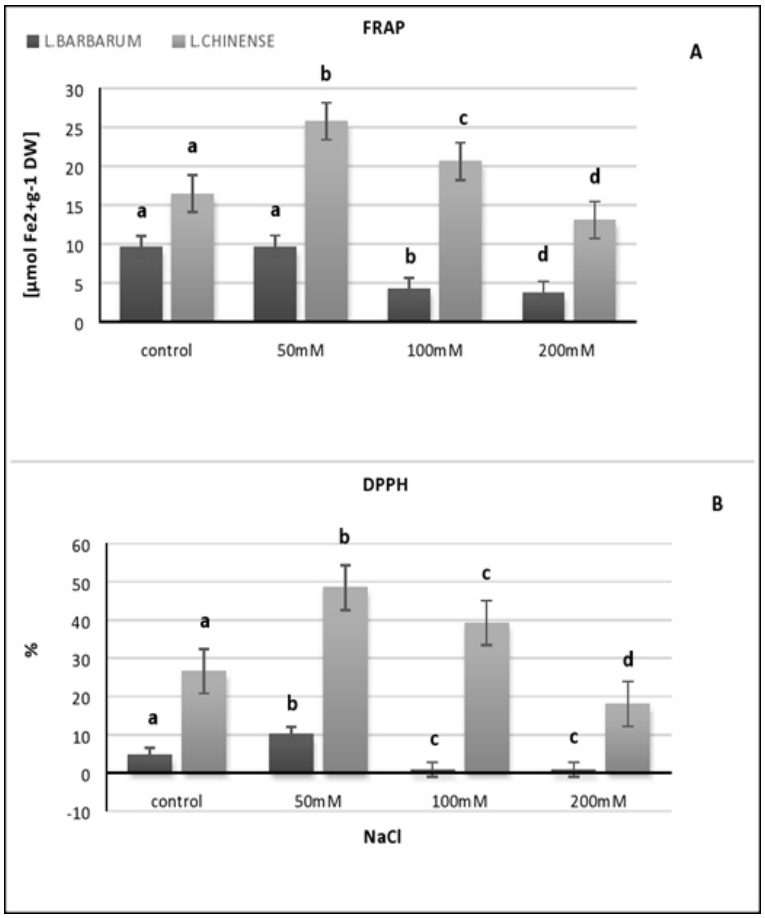

Fig. 4. Changes in leaf antioxidant activity measured by FRAP (A) and DPPH (B) assays in *Lycium barbarum* and *Lycium chinense* grown in hydroponic at three levels of salinity: 1/ control; 2/ 0.05 M NaCl; 3/ 0.1 M NaCl; 4/ 0.2 M NaCl.

CONCLUSIONS

On basis of novel results we asserted assumption that *Lycium chinense* is more salt tolerant in comparison to *Lycium barbarum* at the salinity conditions tested. Irrespective of the higher Na^+ accumulation and lower K^+/Na^+ ratios in the leaves of *Lycium chinense* during treatment with increasing NaCl concentrations, WS-AOC prevailed and measured total DPPH and FRAP antioxidant capacity is greater than in *Lycium barbarum*.

REFERENCES

1. Benzie, I.F.F., Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay*, Anal. Biochem*., 239: 70-76.
2. Bernardello, L.M. 1986. Revisión taxonómica de las species sudamericanas de *Lycium* (Solanaceae*), Bol. Acad. Nac. Cienc Córdoba*, 57: 173-356.
3. Dimitrova, V., Georgieva, T., Markovska, Y. 2016. Influence of salt stress on some physiological characteristics of two *Lycium* varieties grown *ex vitro* in hydroponic, *Ann. del`Univ. de Sofia "St. Kliment Ohridski*", *Faculte de Biologie*, 101: 141-148.
4. Doumett, S., Lamperi, L., Checchini, L., Azzarello, E., Mugnai, S., Mancuso., S., 2008. Heavy metal distribution between contaminated soil and *Paulownia tomentosa* in a pilot-scale assisted phytoremediation study: influence of different complexing agents, *Chemosphere*, 72: 1481-1490.
5. Geenway, H., Munns, R.1980. Mechanisms of salt tolerance in non-halophytes, *Ann. Rev. Plant Physiol*., 22: 131-160.
6. Glenn, E.P., Brown, J.J., Blumwald, E. 1999. Salt tolerance and crop potential of halophytes, *Crit. Rev. Plant Sci*., 18: 227-255.
7. Gorham, J. 1990. Salt tolerance in *Triticeae*: K/Na discrimination in synthetic hexaploid wheats, *J. Exp. Bot*., 41: 623-627.
8. Jeschke, W.D. 1972. Wirkung von K^+ auf die fluxeund den transport von Na^+ in gerstenwurzeln K– stimulierter Na^+- efflux in der wurzelringe, *Planta*, 106: 73-90.
9. Levin, R.A., Miller, J.S. 2005. Relationships with intribe Lycieae (Solanaceae): paraphyly of *Lycium* and multiple origins of gender dimorphism, *Am. J. Bot*., 92: 2044-2053.
10. Liu, N., Guan, L. 2012. Linkages between woody plant proliferation and plant physiological traits in southwestern North America, *J. Plant Ecol*., 5: 407-416.
11. Liu, Z., Shu, Q., Wang, L., Yu, M., Hu, Y., Zhang, H., Tao, Y., Shao, Y. 2012.Genetic diversity of the endangered and medically important *Lycium ruthenicum* Murr. revealed by sequence-related amplified polymorphism (SRAP) markers, *Biochem. Systematics Ecol*., 45: 86-97.
12. Maathuis, F., Amtmann, A. 1999. K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios, *Ann. Bot*., 84: 123-133.
13. Miller, J.S. 2002. Phylogenetic relationships and the evolution of gender dimorphism in *Lycium* (Solanaceae), *Syst. Bot*., 27: 416-428.
14. Munns, R. 2002. Comparative physiology of salt and waterstress, *Plant Cell Environ.*, 25: 239– 250.
15. Prieto, P., Pineda, M., Aguilar, M. 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolibdenum complex:Specific application to the determination of Vitamin E, *Anal. Biochem*., 269: 337-341.
16. Sakihama, Y., Cohen, M.F., Grace, S.C., Yamasaki, H. 2002. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants, *Toxicology*, 177: 67-80.
17. Tepe, B., Sokmen, M., Akpulat, H.A., Sokmen, A. 2006.Screening of the antioxidant potentials of six *Salvia* species from Turkey, *Food Chem.,* 95: 200-204.
18. Wei, Y., Xu, X., Tao, X., Wang, P. 2006. Growth performance and physiological response in the halophyte *Lycium barbarum* grown at salt-affected soil, *Ann. Appl. Biology*, 149: 263-169.