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INFLUENCE OF SALT STRESS ON SOME PHYSIOLOGICAL CHARACTERISTICS OF TWO *LYCIUM* VARIETIES GROWN *EX VITRO* IN HYDROPONICS

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Abstract: *Lycium chinense* is one of the two species of boxthorn in the family Solanaceae from which the goji berry is harvested, the other being *Lycium barbarum*. They differ morphologically. The plants are used in traditional Chinese medicine for the treatment of pneumonia, cough, inflammation, and diabetes mellitus.

We studied the effect of salt stress on growth parameters, pigment and proline contents in *Lycium* varieties grown *ex vitro* as hydroponic cultures at three levels of salinity, 50 mM, 100 mM and 200 mM NaCl solution and look for suitable markers for salt tolerance. Content of proline, which is selected as stress marker, increased more in the leaves of *Lycium barbarum*. The ratios of fresh weight/ dry weight were enhanced with increasing salinity level for the roots and shoots of *Lycium chinense*, while this parameter decreased gradually for *Lycium barbarum*. The ratio of chlorophyll a/ chlorophyll b changed in the same manner in the leaves of *Lycium chinense and Lycium barbarum*. The ratios of chlorophyll a+b/ carotenoids are reduced in the leaves of both species because the carotenoid content increased during NaCl treatment. Our results suggest that selected markers, such as ratios fresh weight/ dry weight of roots and shoots, chlorophyll a/ chlorophyll b, chlorophyll a+b/ carotenoids and leaf proline content are sensible, but insufficient for characterization of salt tolerance of *Lycium barbarum* and *Lycium chinense*, which are micropropaged by BioTree Ltd, Bulgaria.

INTRODUCTION

Species from the genus *Lycium* (Solanaceae) are perennial shrubs, inhabiting arid and semiarid regions of Asia, America and Africa, and its ripe fruits are unique from the view of pharmacology and medicine (Li et al., 2007). Their special physiological characteristics of drought-resistance and salt-resistance make them a suitable plant to prevent land desertification and alleviating the degree of soil salinity (alkalinity), which is very important for the ecosystem and agriculture in the remote areas (Zheng et al., 2011). The great international economic interest in the production of *Lycium* is shown. The billion investments for cultivation, conservation, processing, packing and distribution are made. There is business interest in any sort of production on the basis of *Lycium*: drying fruits, packed as a juice, extract, snacks and etc. Because of overexploitation and deterioration of *Lycium* natural habitats, the number and individuals of these species has dropped considerably in recent decades and *in situ* conservation strategies should be adopted to protect and restore all existing populations (Liu et al., 2012). Micropropagation of these endangered species is also recommended, but the breeding of high quality varieties is extremely urgent. In future, analysis of chemical constituents, important for medicine and genetic structure analysis will assist in breeding excellent germplasm of *Lycium* species (Liu et al., 2012). *Lycium* varieties used in the current paper are propagated and rooted according to the technology, prepared by BioTree Ltd., Bulgaria (No RD 12-11/15.05.2015).

In the present study we look for the sensible physiological markers of the salt tolerance in the *Lycium* varieties – *Lycium barbarum* and *Lycium chinense* grown *ex vitro* in hydroponic at three levels of salinity, 50 mM, 100 mM and 200 mM NaCl solution.

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. For induction of shoots, explants of *L. barbarum* were cultured on 4 $g.l^{-1}$ Murashige and Skoog nutrient medium - MS (1962), supplemented with 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar. For shoots multiplication MS medium was supplemented with 1 $ml.l^{-1}$ indoleacetic acid (IAA, Duchefa Biochemie). For induction of shoots, explants of *L. chinense* were cultured on 2.37 $g.l^{-1}$ McCown Woody plant medium (1981), supplemented with 24.3 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar and 0.3 $ml.l^{-1}$ adenine. For shoots multiplication the medium was supplemented with 0.125 $ml.l^{-1}$ gibberellic acid (GA_3, Duchefa Biochemie), 1$ml.l^{-1}$ IAA and 0.5 $ml.l^{-1}$ indol-3-butyric acid (IBA, Duchefa Biochemie). 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. The pH of all media

was adjusted to 6.0 using 0.1 N HCl and 0.1 N NaOH before autoclaving. 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 1.5 $ml.l^{-1}$Proplant solution (Smith and McCown, 1983).

Hydroponic experiment. The experiments were set as four treatments including control, each treatment with 7 replications. The 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 a growth chamber with a 16-h photoperiod (PAR 100 μmol m^{-2} s^{-1} on the upper leaf surface, 25/17±1 ºC day/night temperature, relative humidity 54/45%). Each vessel contained two plants which represented one replication. After 21 days of cultivation the plants were transferred to 1/2 Hoagland solution. 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), 50, 100, and 200 mM NaCl was added. The solutions were aerated for 1-h every day and were changed in every 3 days to prevent depletion of nutrients and NaCl. Plants were harvested after 10 days of treatment.

Measurement of plant growth. At the end of the experiment the plant samples were collected, washed with tap water and rinsed with distilled water before being separated into shoots and roots and fresh mass of each plant sample were measured gravimetrically. Dry mass of shoots and roots were determined after oven-drying (60ºC) for 2 days until constant weight was obtained.

Pigment determination. Samples were collected from the fully developed leaves of 5-7 plants grown at varying concentrations of NaCl. Samples of the control plants of two *Lycium* varieties were also collected at this time. For pigment extraction, 100 mg of fresh material from the middle part of the fully developed leaves of each plant was extracted with 5 ml 80% acetone and filtered through a glass filter G4. The pigment content was determined spectrophotometrically (Lichtenthaler, 1987)

Proline determination. 500 mg of fresh material of the fully developed leaves of each plant were homogenized with 5 ml of 3% aqueous sulfosalicylic acid. The suspensions were centrifuged (16 000g, 15 min, 4ºC). Then 2 ml of the supernatants was mixed with 2 ml of glacial acetic acid and 2 ml acid-ninhydrin for 60 min at 100ºC, and the reaction was terminated in an ice bath. After cooling the reaction mixtures were extracted with 4 ml toluene. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was measured at λ=520 nm. The proline content was determined using a standard curve and calculated on a fresh weight basis (Bates et al.,1973).

Statistical analysis. All values reported in this work were mean of at least five - seven independent experiments. The mean values ±SD and exact number of experiments are given in the tables. The significance of differences between

control and each treatment was analyzed by Fisher`s LSD test (P≤0.05) after performing ANOVA multifactor analysis (Statgraphics Plus, V.2.1).

RESULTS AND DISCUSSION

Seedlings growth is normally limited by increasing concentration of NaCl (Sun et al., 2011). In our study, the root and shoot length of *Lycium barbarum* is reduced more than that of *Lycium chinense* (data not shown). With increasing salinity levels, the root and shoot fresh/dry mass ratios of *L. barbarum* declined more than that of *L. chinense* as compared to the control (Table 1). The highest values were established at 200 mM NaCl for root and shoot ratios of *L. chinense*. At the same concentration of NaCl decreasing of these ratios for *L. barbarum* were observed as compared to the control (Table 1. Lower and middle salinity level (50 and 100 mM NaCl) reduced the chlorophyll a and chlorophyll b contents in the leaves of *L. barbarum*, but not in the leaves of *L. chinense* (Fig. 1 and Fig. 2). The highest concentration of NaCl – 200 mM lead to increasing in chlorophyll a and chlorophyll b, namely in the leaves of *L. chinense.* The ratios of chlorophyll a/chlorophyll b decreased in *L. barbarum,* but increased in *L. chinense* under salt stress as compared to the control (Table 2).The investigations showed that high salinity decreases the chlorophyll content, but the decrease depends on the salt tolerance of the plant species (Ashraf and Haris, 2013). The chlorophyll a is more susceptible than chlorophyll b and decreased in the leaves of three *Paulownia* hybrid clones exposed to the same salt stress (Miladinova et al., 2013).

Table 1. Mean values ± SD (n = 5-7) of root and shoot fresh/ dry mass of *Lycium barbarum* and *Lycium chinense*, grown in hydroponic in response to salt stress

Treatments	Root fresh/ dry mass	Shoot fresh/ dry mass
	[g/g]	[g/g]
Lycium barbarum		
Control	13.84±2.43a	8.48±1.84a
50 mM NaCl	12.85±1.65a	9.20±0.84a
100 mM NaCl	13.44±1.92a	7.60±0.69a
200 mM NaCl	9.29±0.87b	4.85±0.36b
Lycium chinense		
Control	9.94±0.79a	7.32±0.63a
50 mM NaCl	10.38±1.25a	7.98±0.59a
100 mM NaCl	9.83±0.89b	8.30±0.77a
200 mM NaCl	12.61±1.37c	8.72±0.74b

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test (P<0.05).

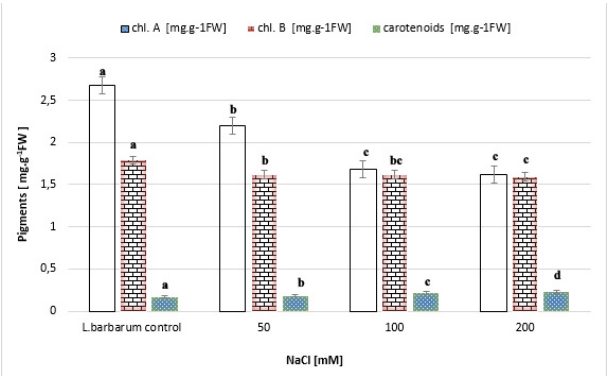

Figure 1. Changes in chlorophyll a, chlorophyll b and carotenoid contents in the leaves of *Lycium barbarum* grown in hydroponic at three levels of salinity: **1/** control; **2/** 50 mM NaCl; **3/** 100 mM NaCl; **4/** 200 mM NaCl.

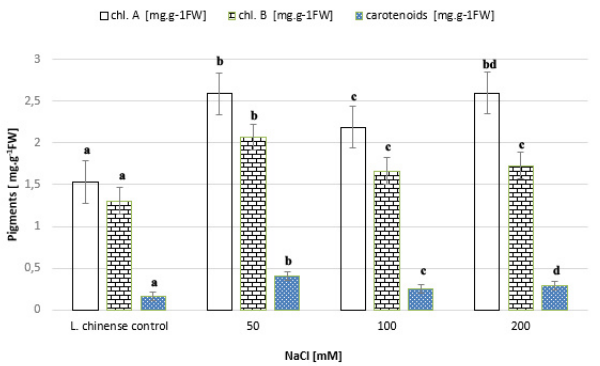

Figure 2. Changes in chlorophyll a, chlorophyll b and carotenoid contents in the leaves of *Lycium chinense* grown in hydroponic at three levels of salinity: **1/** control; **2/** 50 mM NaCl; **3/** 100 mM NaCl; **4/** 200 mM NaCl.

The carotenoid content significantly increased in the leaves of both *Lycium* plants grown in different concentrations of NaCl. The manner of the carotenoid changes in both varieties is different (Fig.1 and Fig. 2). The ratios of chlorophylls a+b/ carotenoids are reduced more in the leaves of *L. barbarum* (Table 2). In sweet cherry (*Prunuscerasus* x *canescens*) a decrease in chlorophyll is observed as a result of NaCl exposure (0, 50, 100 and 150 mM) (Erturk et al., 2007) and in beans reduction of green and yellow pigments is shown with increasing salinity (100 mM) (Stoeva et al., 2008). The carotenoid content remained relatively invariable in the leaves of three *Paulownia* hybrid clones exposed to salt stress (Miladinova et al., 2013). The changes in carotenoid content are considered as a reliable criterion for characterization of

plant tolerance to salt stress because it is known that these pigments participated as a precursor in signalling during plant development under stress conditions (Gomathi and Rakkiyapan, 2011).

Table 2. Mean values ± SD (n = 5-7) of ratios chlorophyll a/ chlorophyll b and chlorophylls/ carotenoids in the leaves of *Lycium barbarum* and *Lycium chinense*, grown in hydroponic in response to salt stress

Treatments	Chl a/ Chl b	Chlorophyll a+b/ carotenoids
Lycium barbarum		
Control	1.49±0.12a	26.61±2.85a
50mM NaCl	1.36±0.11b	21.12±2.44b
100mM NaCl	1.04±0.09c	15.28±1.69c
200mM NaCl	1.02±0.11d	14.11±1.06d
Lycium chinense		
Control	1.17±0.09a	17.82±1.62a
50mM NaCl	1.24±0.08b	11.43±1.19b
100mM NaCl	1.31±0.09c	15.27±1.27c
200mM NaCl	1.51±0.12d	14.56±1.54d

Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P<0.05$).

Proline accumulation is found to be one of the common physiological responses of higher plants when they are exposed to a number of environmental stresses (Verbruggen and Hermans, 2008).This amino acid is important osmolyte and may protect plant cells against oxidative damage by stabilizing key cellular detoxification mechanisms (Szekely et al., 2008). Its enhanced biosynthesis is suggested to stabilize redox potential and $NAD(P)^+$/ NAD(P)H ratios during stress conditions (Hare and Cress, 1997). Our results showed that proline content in the leaves of control plants of *L. chinense* is approximately twice higher than in the leaves of *L. barbarum*. With increasing salinity level this content rise by about 3-fold in the leaves of *L. barbarum* and only 0.6-fold in the leaves of *L. chinense*, measured at 100 mM NaCl (Fig. 3).

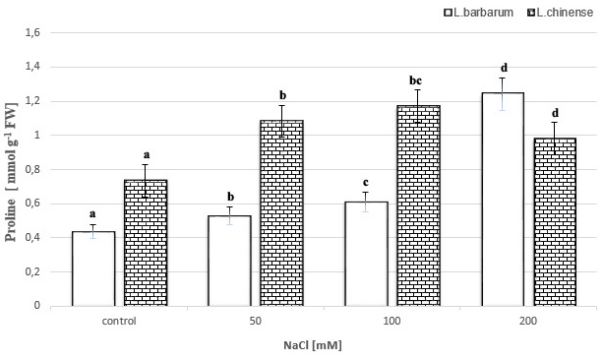

Figure 3. Changes in proline contents in the leaves of *Lycium barbarum* and *Lycium chinense* grown in hydroponic at three levels of salinity:
1/ control; **2/** 50 mM NaCl; **3/** 100 mM NaCl; **4/** 200 mM NaCl.

The investigations showed that free proline level is higher in halophyte species, such as *Thellungiella halophila* than in closely related glycophyte species (Ghars et al., 2008). The results indicated that the high proline accumulation in halophytes can contribute to adaptation to saline environment. Our results showed that *L. barbarum* is more sensitive to salt stress at the salinity conditions tested than *L. chinense* because proline content rises gradually and significantly. At the same time proline content in the leaves of *L. chinense* is highest at the middle salinity level (100 mM NaCl), while at high salinity level (200 mM NaCl) – decreased.

CONCLUSIONS

We examined the sensitive physiological indicators for characterization of two *Lycium* varieties, which are micropropaged by BioTree Ltd., Bulgaria. The ratios of root and shoot fresh/ dry mass and chlorophyll a/ chlorophyll b, but not chlorophyll a+b/ carotenoids are enhanced during high salinity level (200 mM) in *Lycium chinense*. The leaf content of proline is higher in control plants of *Lycium chinense* as compared to *Lycium barbarum*, but it enhanced significantly with increasing NaCl concentration only in *Lycium barbarum*. Our results showed that selected markers are sensible, but insufficient to characterization of salt tolerance of both *Lycium* varieties investigated.

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