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Research Article

EFFECT OF SOIL SALINITY ON LEAF ANATOMY AND ANTIOXIDANT DEFENSE IN TWO *LYCIUM* SPECIES

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Summary: A pot experiment was carried out to investigate the effect of salinity on leaf anatomy and antioxidant protection in two *Lycium* species (*Lycium barbarum* L. and *Lycium chinense* Mill.). The reduction in total leaf thickness, palisade and spongy mesophyll thickness, upper and lower epidermis was stronger in *Lycium barbarum* than in *Lycium chinense*. The two investigated *Lycium* species showed different antioxidant defence mechanisms under soil salinity. Based on our results we could suggest that the antioxidant capacity of *Lycium barbarum L.* under salinity condition was determined by increased SOD activity and glutathione content, while in *Lycium chinense* by higher level of SOD and CAT activities as well as increased ascorbate content. *Lycium chinense* demonstrated higher tolerance to salinity because its growth and leaf anatomy were less affected.

Keywords: leaf thickness, antioxidant protection, *Lycium*, salinity.

Abbreviations: Asc – ascorbic acid; APX – ascorbate peroxidase; CAT – catalase; GR – glutathione reductase; MDA – malondialdehyde; POX – guaiacol peroxidase; SOD – superoxide dismutase.

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INTRODUCTION

The genus *Lycium* (Solanaceae) comprises approximately 80 species distributed mainly in arid and semi-arid regions of Asia, America and Africa (Bernardello, 1986; Levin and Miller, 2005). *Lycium* species are long-lived perennial shrubs which produce red or purple, fleshy berries. *Lycium barbarum* and *Lycium chinense* have high nutritional and medicinal value. The plants are used in traditional Chinese medicine for treatment of pneumonia, cough, inflammation and diabetes mellitus. In some Asian countries, the fruits are used as a tonic and invigorating agent, stimulating the immune system. In

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Bulgaria, *L. barbarum* L. is cultivated as an ornamental shrub for making hedges, and also for stabilisation of the soil and landslides. The benefits of the fruits have been widely popularised in Bulgaria during the past few years and the species is strongly recommended for cultivation by some private companies (Goji Berry) (Petrova et al., 2013). The physiological characteristics of drought-tolerance and salt-tolerance make also *Lycium* species ideal plants for preventing soil desertification and alleviating the degree of soil salinity (Zhao and Zeng, 1999). Because of overexploitation and deterioration of *Lycium* natural habitats, the number and individuals of these species have dropped considerably in recent decades and *in situ* conservation strategies should be adopted to protect and restore all existing populations. Micropropagation of these endangered species is also recommended, but the breeding high quality of 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 and Guan, 2012). Some authors have established that the photosynthesis of *Lycium barbarum* is inhibited insignificantly under the soil salt content below 0.5%, which suggests that there is certain salt tolerance of *Lycium barbarum* (Hongxia et al., 2004). According to Dimitrova et al. (2017a), *Lycium chinense* is more salt tolerant in comparison to *Lycium barbarum*. In the leaves of *Lycium chinense* treated with increasing NaCl concentrations, WS-AOC, total DPPH and FRAP antioxidant capacity were greater than in *Lycium barbarum*. Dimitrova et al. (2017b) also established that in hydroponic condition *Lycium chinense* processed higher salt resistant in comparison with *Lycium barbarum* expressed by lower changes in leaf thickness, upper and lower epidermis thickness.

Plants growing in salinity regions of the world need to be adapted to the environment in which salt strongly affects plant growth (Greenway and Munns, 1980). The influence of salinity on key metabolic processes in plants such as photosynthesis, is direct (by limiting stomatal conductivity and decreasing the biochemical capacity of mesophyll cells for photosynthetic fixation of CO_2) or secondary, such as oxidative stress arising from the superimposition of multiple stresses (Chaves et al., 2009). The balance of C under stress conditions is determined by its duration and reaching positive values depends on the rate and degree of photosynthetic recovery.

In the present study, pot experiments were carried out to investigate the differences in leaf anatomy between two *Lycium* species: *Lycium barbarum* L. and *Lycium chinense* Mill. cultivated *ex vitro* on peat moss and two types of saline soils for a period of two vegetative seasons. The effect of soil salinity on antioxidant defence in the leaves of both plants was traced in order to elucidate its role in the adaptation of *Lycium* species differing in salt tolerance.

MATERIALS AND METHODS

Sampling site, plant materials and pot experiments

Seeds and *in vivo* explants from the species *Lycium barbarum* L. and *Lycium chinense* Mill. were used for developing

of *in vitro* multiplication protocol according to the technology registered by BioTree Ltd., Bulgaria (Dimitrova et al., 2016). Seedlings derived from *in vitro* micropropagation were cultivated in plastic pots filled with 1.5 kg non-saline – peat moss (type 0), non-saline soil (type 1) and saline soil (type 2). The experiment was set as 3 treatments, with 7 replications each and was conducted in a glasshouse (natural sunlight, temperatures 15°C - 35°C, relative humidity 40% - 65%) for two vegetative seasons (from 1April 2015 to 30 July 2016). The basic component of peat moss was *Sphagnum*, supplemented with shavings, ground limestone, sand and moisture holding agent. pH of control was 6.0, while pH values of the soils were 8.0 and 8.9, respectively. The soils used in this study were taken from an area located in the vicinity of the village Belozem, Bulgaria. A sampling strategy was carried out from the surface and at depths of 30-60 cm in two different locations of the areas. The second soil type (2) possessed exchangeable Na content about 5 times higher, Sodium Adsorption Ratio (SAR) about 6 times higher and electrical conductivity 14.0 mS/cm about 2 times higher in comparison with the first soil type (1) (Ivanova et al., 2014).

Leaf anatomy

Samples from the central area of the last fully developed leaf blade were taken and fixed in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2), and embedded in low viscosity Spurr's epoxy resin. Semi thin paradermal sections (ultramicrotome Tesla, Czech Republic) were stained with 0.01% (w/v) toluidine blue and observed under a light microscope (Carl Zeiss, Jena, Germany). Microscopic images of leaf cross sections were captured and saved on a digital image processor (International Micro-Vision Inc., 667 EI Camino Real, Redwood City, CA, USA). For statistical analysis, leaf thickness was evaluated in cross sections, obtained from four leaves per treatment and was measured using 3Ddoctor software (Able Software Corp., Lexington, USA). The thickness of leaf layers (total leaf thickness, palisade and spongy mesophyll, upper and lower epidermis) was examined on transversally fractured planes.

Metabolites assays

For H_2O_2 and MDA determination, 0.3 g fresh mass of fully developed leaves were homogenized in a mortar at 4°C with 3 ml 0.1% trichloroacetic acid (TCA) and centrifuged for 20 min at 15 000 x *g*. The reaction mixture contained 500 µl of the supernatant, 500 µl phosphate buffer, pH 7.4 and after the addition of 1 ml 1 M KI, samples were incubated in the dark for 60 min and absorption was measured at λ=390 nm. The content was calculated using a standard curve of H_2O_2 in the range of 1 – 100 nmol.ml^{-1} H_2O_2 (Jessup et al., 1994). For malondialdehyde (MDA) estimation, 500 µl of the supernatant was mixed with 500 µl phosphate buffer (pH 7.4) and after the addition of 1 ml 0.5 % (w/v) thiobarbituric acid dissolved in 20 % trichloroacetic acid, the samples were boiled for 30 min. After rapid cooling of the samples in an ice-bath, absorption was measured at 532 and 600 nm using the extinction coefficient 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

For the low molecular antioxidant metabolites extraction, 0.2 g of fresh mass of fully developed leaves were ground into fine powder with liquid nitrogen, then

5 ml 1 M $HClO_4$ were added. After 25 min centrifugation at 15 000 x *g* at 4°C, the supernatant was placed on ice and pH was adjusted to pH 7 (for glutathione) and pH 6 (for ascorbate) with 5 M K_2CO_3. The potassium perchlorate was removed by further centrifugation and the clear supernatants were used for the assays (Doulis et al., 1997). The concentration of total (GSH+GSSG) glutathione was determined with an enzyme recycling assay (Griffith, 1980). The assay was based on sequential oxidation of glutathione by 5, 5`-dithiobis (2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of GR. The mixture in 1 ml contained 125 mM potassium phosphate buffer and 6.3 mM EDTA (pH 6.5), 0.3 mM NADPH, 3 mM DTNB and 50 µl of the supernatant. The reaction was initiated by adding 10 µl of GR (5 IU/ml) and the change in absorbance at λ=412 nm was recorded. Standard curves were generated with reduced and oxidized glutathione. The results are expressed per 1 g FW.

The reduced form of ascorbic acid (Asc) was estimated as the decrease in absorbance for 1 min at λ=265 nm, in a reaction mixture, consisting of 100 mM potassium phosphate buffer, pH 5.6, 5 µl ascorbate oxidase and 100 µl supernatant. The reaction was initiated with the addition of 100 µl of the supernatant and the decrease of the absorption of samples was recorded at λ=265 nm. Standard curves were generated with Asc (Foyer et al., 1983). The results are expressed per 1 g FW.

Determination of enzymatic antioxidants

In order to prepare crude extracts for determination of the enzymes superoxide dismutase (SOD), guaiacol peroxidase (POX), catalase (CAT) and glutathione reductase (GR) the plant material was ground with 4 cm^3 of the extraction buffer (100 mM potassium phosphate buffer, pH 7.8; 5 mM ethylenediaminetetraacetic acid (EDTA); 2% polyvinyl pyrrolidone (PVP) that was added to 0.3 g of tissue powder. For the extraction buffer for ascorbate peroxidase (APX) determination 0.15 g of plant sample was added to 50 mM potassium phosphate buffer, pH 7.0; 1 mM ascorbate; 1 mM EDTA; 0.2% PVP. The suspensions were centrifuged (16 000 x *g*, 15 min, 4°C). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27°C using Boeco S-22 UV/VIS spectrophotometer (Germany).

SOD (EC 1.15.1.1) was estimated in a reaction mixture of 50 mM Tris-succinate buffer (pH 8.2), 8 mM pyrogallol, 100 µl extract. The decomposition of pyrogallol was determined by following the increase in absorbance at 412 nm for 3 min (Marklund and Marklund, 1974).

POX (EC 1.11.1.7) was estimated in a reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 200 µl extract, 1 mM H_2O_2. The oxidation of guaiacol was measured by following the increase in absorbance at 470 nm for 2 min (Polle et al., 1994).

CAT (EC 1.11.1.6) was estimated in a reaction mixture of 100 mM potassium phosphate buffer (pH 7.0), 50 µ1 extract, 15 mM H_2O_2. The decomposition of H_2O_2 was determined by following the decline in absorbance at 240 nm for 3 min (Aebi, 1984).

GR (EC 1.6.4.2) was estimated in a reaction mixture of 300 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$,

0.1 mM EDTA, 10 mM GSSG, 200 µl extract, 0.15 mM NADPH. The oxidation of NADPH was determined by following the decline in absorbance at 340 nm for 3 min (Sherwin and Farrant, 1998).

APX (EC 1.11.1.11) was estimated in a reaction mixture of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 200 µl extract, 0.5 mM Na ascorbate. The rate of hydrogen peroxide-dependent oxidation of ascorbate was determined by monitoring the change in absorbance at 290 nm for 3 min (Nakano and Asada, 1981). The protein content was determined after a standard procedure (Lowry et al., 1951).

Statistical data analysis

Data are expressed as means ±SE. Comparison of means was performed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis (Statgraphics Plus, v. 2.1).

RESULTS

Leaf anatomy

The leaves of *Lycium* possessed a bifacial and amphistomatic structure with well-developed adaxial and abaxial epidermis and compact arranged in 1-2 layers palisade mesophyll. The spongy mesophyll disposed over the abaxial

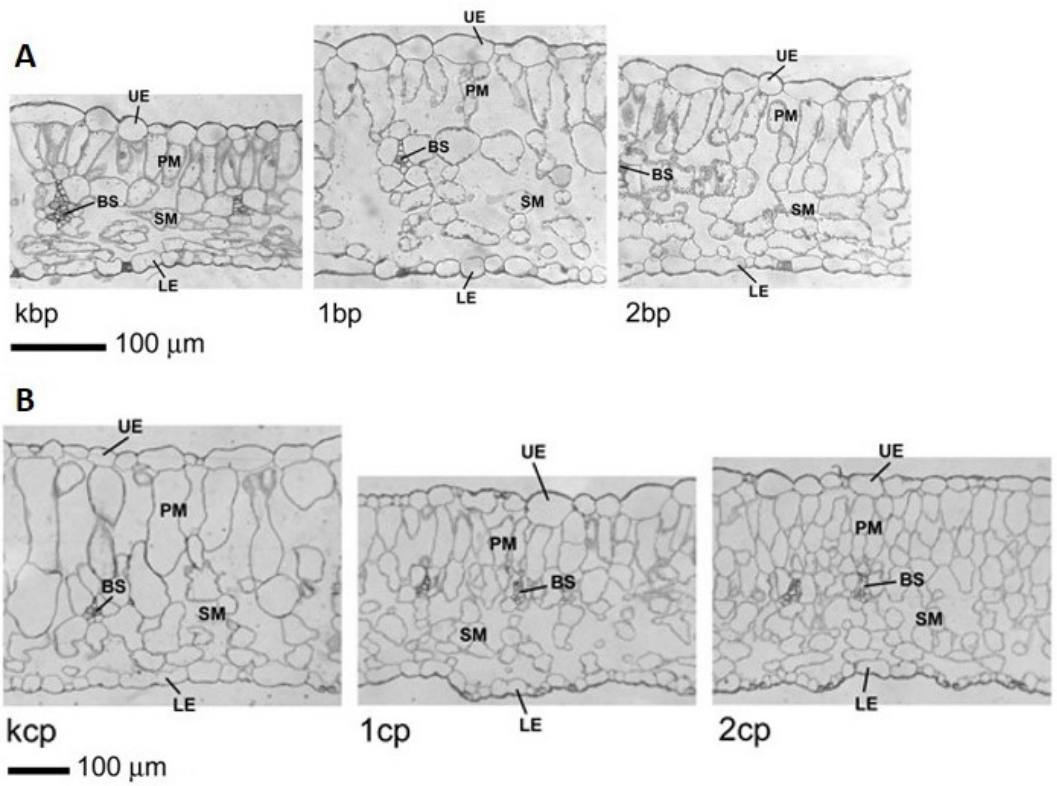

Figure 1. A. Cross-section of a *Lycium barbarum* leaf, prepared in the middle of the second vegetation period from plants grown on: 1/ peat moss - control (kbp), non-saline (1bp) and saline (2bp) soils. **B.** Cross-section of a *Lycium chinense* leaf, prepared in the middle of the second vegetation period from plants grown on: 1/ peat moss - control (kcp), non-saline (1cp) and saline (2cp) soils. Means ± SD, n=4, UE – upper epidermis, PM – palisade mesophyll; BS – bundle sheet; SM – spongy mesophyll, LE – lower epidermis.

epidermis consisted of 2-3 layers shaggy cells surrounding big intercellular spaces (Fig. 1). The leaf thickness of *L. chinense* was approximately twice greater than that of *L. barbarum* after cultivation on peat moss (Tables 1, 2). The leaf, palisade and spongy mesophyll thickness, as well as that of the upper and spongy epidermis, was increased to a higher extent after cultivation of *L. barbarum* on non-saline soil as compared to saline soil (Table 1), while for *L. chinense* the tendency was opposite (Table 2).

The intercellular spaces in palisade and spongy mesophylls decreased in the leaves of *L. barbarum* grown on saline soil in comparison with non-saline soil (Table 1), but these parameters increased in the leaves of *L. chinense* at the same conditions (Table 2).

MDA and H_2O_2 content

The lipid peroxidation level in fully developed leaves measured as the content of MDA raised due to salinity in *L. chinense*, but it decreased in *L. barbarum* (Fig. 2). The content of H_2O_2 increased more strongly in the leaves of *L. barbarum* than *L. chinense* grown on saline soil (Fig. 2).

Table 1. Mean values ± SD (n = 4) of leaf, palisade and spongy parenchyma, upper and spongy epidermis thickness in *Lycium barbarum* grown on peat moss – control (kbp), non-saline (1bp) and saline (2bp) soils. Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P < 0.05$).

Variants	Total leaf thickness [μm]	Palisade mesophyll [μm²/μm]	Spongy mesophyll [μm²/μm]	Intercell. spaces in palis. mes. [μm²/μm]	Intercell. spaces in sp. mes. [μm²/μm]	Upper Epidermis [μm²/μm]	Spongy epidermis [μm²/μm]
kbp	156.0±10^{a}	46.2±4^{a}	74.3±7^{a}	11.3±3^{a}	27.6±4^{a}	20.2±1^{a}	15.4±1^{a}
1bp	245.0±15^{c}	71.0±2^{b}	135.6±10^{c}	31.9±3^{c}	72.4±7^{b}	21.4±4^{a}	17.6±2^{a}
2bp	201.0±9^{b}	67.5±5^{b}	98.7±5^{b}	21.1±2^{b}	32.0±10^{a}	21.0±2^{a}	14.7±2^{a}

Table 2. Mean values ± SD (n = 4) of leaf, palisade and spongy parenchyma, upper and spongy epidermis thickness and in *Lycium chinense*, grown on peat moss – control (kcp), non-saline (1cp) and saline (2cp) soils. Values with the same letter are not significantly different when means are separated by Fisher`s LSD test ($P < 0.05$).

Variants	Total leaf thickness [μm]	Palisade mesophyll [μm²/μm]	Spongy mesophyll [μm²/μm]	Intercell. spaces in palis. mes. [μm²/μm]	Intercell. spaces in sp. mes. [μm²/μm]	Upper epiderm. [μm²/μm]	Spongy epidermis [μm²/μm]
kcp	385.0±12^{b}	169.5±23^{b}	153.7±10^{a}	65.6±14^{c}	72.5±10^{a}	31.6±2^{a}	27.7±2^{b}
1cp	320.0±22^{a}	84.5±15^{a}	172.4±14^{a}	18.7±2^{a}	83.5±5^{a}	33.3±3^{a}	24.5±4^{ab}
2cp	338.0±14^{a}	106.2±10^{a}	190.2±18^{a}	28.9±3^{b}	84.8±12^{a}	27.5±1^{a}	20.2±2^{a}

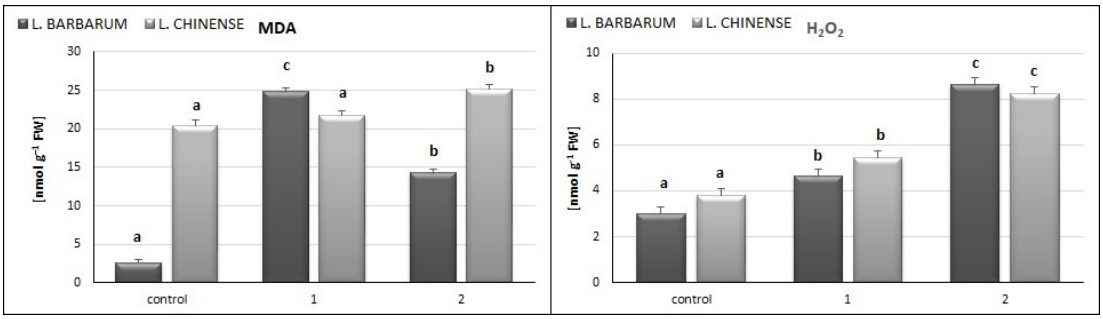

Figure 2. Changes in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content in fully developed leaves of two *Lycium* species (*Lycium barbarum* L. and *Lycium chinense* Mill.), grown on three types of soil: 1/ control - peat moss; 2/ non-saline (1) and 3/ saline (2).

Ascorbate and glutathione content

The content of low molecular antioxidants - ascorbate and glutathione changed in a different manner (Fig. 3). Ascorbate content was highest in the leaves of both species grown on peat moss, while glutathione prevailed only in the leaves of *L. barbarum* at the same growth conditions. Ascorbate increased significantly in the leaves of *L. chinense* with increasing salinity level, while glutathione increased only in the leaves of *L. barbarum*.

Activities of antioxidant enzymes

Antioxidant enzymes in fully developed leaves of both *L. species* showed differential responses in dependence of soil salinity (Fig. 4). SOD activity increased significantly in the leaves of *L. barbarum*, while in *L. chinense* it increased slightly (Fig. 4). The same tendency was observed for CAT activity in *L. chinense*, but the change of the activity in *L. barbarum* was more significant after cultivation on non-saline soil (Fig. 4). POX activity changed negligibly in *L. barbarum*, but in *L. chinense* it decreased with increasing soil salinity (Fig. 4). APX activity decreased in the same manner in *L. chinense*, while in *L. barbarum* it was highest after cultivation on non-saline soil (Fig. 5). GR activity increased significantly with increasing salinity level in *L. barbarum*, but in *L. chinense* it decreased slightly (Fig. 5).

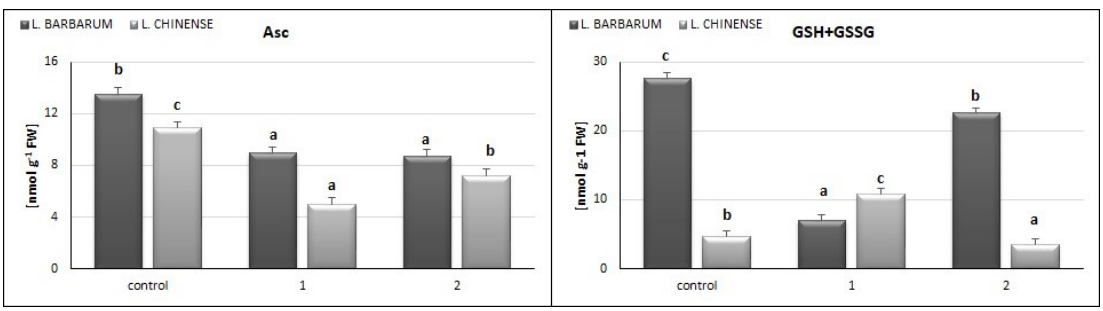

Figure 3. Changes in ascorbate (Asc) and total glutathione (GSH+GSSG) content in fully developed leaves of two *Lycium* species (*Lycium barbarum* L. and *Lycium chinense* Mill.), grown on three types of soil: 1/ control- peat moss; 2/ non-saline (1) and 3/ saline (2).

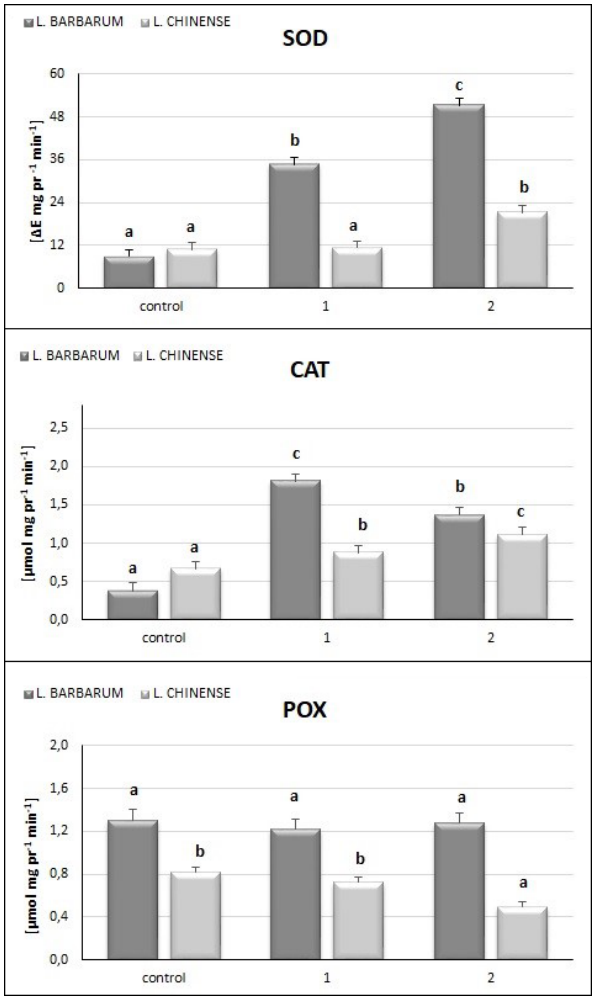

Figure 4. Changes in superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POX) activities in fully developed leaves of two *Lycium* species (*Lycium barbarum* L. and *Lycium chinense* Mill.), grown on three types of soil: 1/ control- peat moss; 2/ non-saline (1) and 3/ saline (2).

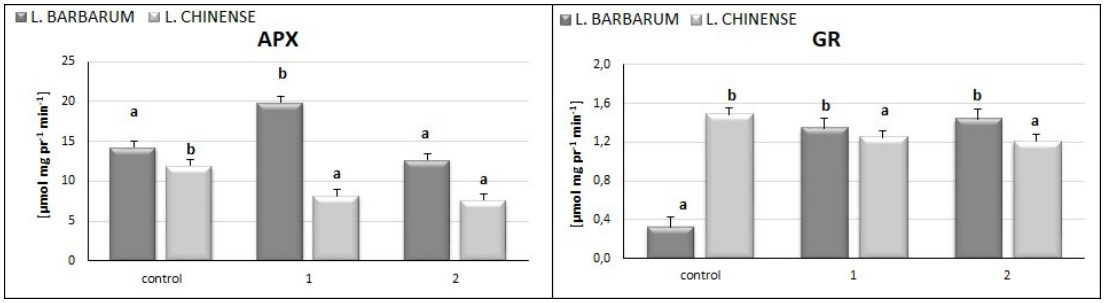

Figure 5. Changes in ascorbate peroxidase (APX) and glutathione reductase (GR) activities in fully developed leaves of two *Lycium* species (*Lycium barbarum* L. and *Lycium chinense* Mill.), grown on three types of soil: 1/ control- peat moss; 2/ non-saline (1) and 3/ saline (2).

DISCUSSION

The present study showed that the two-year old *Lycium* plants possessed different potential for growth. The results showed that with increasing salinity level root and shoot dry mass of *L. chinense* increased, while root and shoot dry mass of *L. barbarum* decreased. The root and shoot ratios FM/DM were highest after cultivation of both plants on peat moss, but this ratio was enhanced for shoots of *L. chinense* after cultivation on saline soil (unpublished data). Time-dependent changes of growth and development of plants exposed to salt stress have been reviewed (Munns, 2002). In the first few minutes, cells lose water and shrink, whereas over hours they regain their volume, but the expansion rates are limited. Over days and weeks, reduced cell elongation and cell division results in slower leaf appearance and inhibition of shoot growth. Salt stress caused swelling in chloroplast volume and as a consequence increasing of the size of mesophyll cells (Mitsuya et al., 2000; Koyro 2002). Our results showed that leaf morphology of the two species changed in a different manner after cultivation on peat moss, non-saline and saline soils (Fig. 1A, B). The leaf, palisade and spongy mesophyll thickness, as well as that of the upper and spongy epidermis was greatest in *L. barbarum* grown on non-saline soil, while these parameters showed the highest values in *L. chinense* grown on peat moss (Tables 1, 2). It is well known that besides leaf area, leaf thickness is characteristic of plant species (Gratani et al., 2003), but it changes depending on the treatment and environmental conditions (Di Baccio et al., 2009) or developmental stages (Marchi et al., 2008). Salt stress enhanced the size of all leaf parameters in *L. chinense* except for upper and lower epidermis, while in *L. barbarum* the opposite trend was observed (Tables 1, 2).

In many studies it has been reported that salinity affects plant nutrients uptake (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-), growth, membrane integrity, osmotic adjustment, photosynthetic activity, and antioxidant activity (Yang et al., 2008; Dogan et al., 2010; Erdal and Çakirlar, 2014). The capacity and activity of the antioxidant protective system are important to limit the production of reactive oxygen species (ROS), which cause damage of proteins, lipids, carbohydrates and DNA (Arora et al., 2002). Our results showed differences in the observed changes of two indicators for oxidative stress, MDA and H_2O_2. The MDA content was highest in the leaves of *L. barbarum* grown on non-saline soil in spite of the highest morphological parameters measured, while in *L. chinense* it was highest in the leaves plants grown on saline soil (Fig. 2A). Hydrogen peroxide content increased gradually in the leaves of *L. barbarum* and *L. chinense* grown on peat moss, non-saline and saline soils (Fig. 2). Peroxidation of membrane lipids, known as MDA, caused by salt stress has been reported in different species such as *Arabidopsis thaliana* (M`rah et al., 2006), *Vigna radiata* (Hayat et al., 2010) and *Periploca sepium* Bunge (Sun et al., 2011). *L. chinense* showed a stronger increase in MDA content, but H_2O_2 content was less increased due to soil salinity (Fig. 2). We established higher accumulation of Na^+ in the leaves of *L. chinense* than in *L. barbarum* grown on control, non-saline and saline soils at the end of the second vegetative season. The K^+ content

prevailed in the shoots of *L. barbarum* (unpublished data). Changes in K^+ and Na^+ concentrations affect the K^+/Na^+ ratio, which is an important selection criterion for salt tolerance (Morant-Manceau et al., 2004; Ashraf and Orooj, 2006). The K^+/Na^+ ratio decreased with increasing salt concentration in safflower cultivars grown in hydroponics, but the highest values were characteristic for plant species more tolerant to salt stress (Erdal and Çakirlar, 2014).

The level of the antioxidant enzymes SOD, CAT and POX may determine the sensitivity of plants to lipid peroxidation (Kanazawa et al., 2000). In our study, with increasing salinity level, the activities of SOD and CAT were enhanced, but POX activity remained either unchanged in *L. barbarum* or decreased in *L. chinense* (Fig. 4), thus indicating that the ability of this enzyme to eliminate ROS was limited. One of the antioxidative defence systems which protects plants against oxidative stress is the ascorbate-glutathione cycle (Valderrama et al., 2006). Our results showed that APX activity declined in both species grown under salinity conditions, while GR activity was enhanced only in *L. barbarum* (Fig. 5). The quantities of low molecular antioxidants - GSH increased drastically in the leaves of *L. barbarum* plants grown on saline soil (Fig. 3), whereas Asc content decreased slightly (Fig. 3). Asc was enhanced in the leaves of *L. chinense* (Fig. 3).

In conclusion, the results of this study showed that salt stress affected more negatively leaf morphology in *L. barbarum* than in *L. chinense* at the vegetative stage. However, *L. barbarum* was able to withstand higher salinity conditions by up-regulating protective mechanisms. Although *L. barbarum* and *L. chinense* showed similar values for the content of MDA and H_2O_2, *L. barbarum* exhibited much better responses in terms of antioxidant enzyme activities.

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