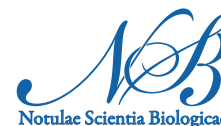


Available online: www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2018, 10(3):430-438. DOI: 10.15835/nsb10310322

**Original Article****Biochemical Markers of Salt Stress in European Larch (*Larix decidua*)**Ioana M. PLESA^{1,2}, Mohamad Al HASSAN^{2,3}, Adriana F. SESTRAS¹,
Oscar VICENTE², Monica BOSCAIU^{4*}, Radu E. SESTRAS¹¹University of Agricultural Sciences and Veterinary Medicine, Faculty of Horticulture, 3-5 Mănăştur Street, 400372 Cluj-Napoca, Romania; ioanaplesa13@gmail.com; adriana.sestras@usamvcluj.ro; rsestras@usamvcluj.ro²Universitat Politècnica de València, Institute of Plant Molecular and Cellular Biology (IBMCP, UPV-CSIC), Camino de Vera s/n, 46022, Valencia, Spain; ovicente@ibmcp.upv.es³Lebanese Canadian University (LCU), Faculty of Engineering, Department of Biotechnology, Aintoura, Lebanon; mohamad_al_hassan@live.com⁴Universitat Politècnica de València, Spain, Mediterranean Agroforestry Institute (IAM, UPV), Camino de Vera s/n, 46022, Valencia, Spain; mobosnea@eaf.upv.es (*corresponding author)**Abstract**

Larix decidua, the European larch, does not grow in natural saline areas, but it can be affected by salinity either by the common practice of winter de-icing of mountain roads with NaCl, or when grown as an ornamental tree in urban areas by the use of low quality, salinised irrigation water. In the present study, the responses to salt stress of young seedlings obtained from seeds of seven Carpathian larch populations were analysed. After 30 days of treatments with 150 mM NaCl, growth and biochemical parameters were determined. Salt stress inhibited growth of all seedlings, as shown by the relative reduction of stem length and fresh weight, and induced significant changes in the needle levels of several biochemical stress markers. Seedlings from all populations showed a marked reduction of photosynthetic pigments contents and an increase of proline and malondialdehyde (MDA) concentrations. Under salt stress, plants accumulated Na⁺ and Cl⁻ in the needles, whereas K⁺ was maintained at a steady level. Responses of seedlings from the different populations were similar, with only small quantitative differences that did not allow the identification of more salt tolerant genotypes. However, the study revealed that several of the biochemical markers mentioned above can be suitable for the rapid and non-destructive assessment of the effects of salinity in European larch.

Keywords: conifer species; ions; proline; photosynthetic pigments; salinity; seedlings**Introduction**

Plants, being sessile organisms, cannot respond to stressful environmental conditions by moving to more favourable places; their responses to stress are based, fundamentally, on the activation of physiological and biochemical defence mechanisms. Abiotic stresses include, among many others, soil alkalinity and acidity, too high or too low temperatures, flooding, heavy metals or UV light irradiation; however, none of them is as damaging to agricultural yields and food production as drought and soil salinity (Boyer, 1982; Bartels and Sunkar, 2005). These two stress conditions are forecasted to become more intense and frequent in the coming decades due to the effects of climate change, including higher average temperatures, reduced rainfall and increasing scarcity of water for irrigation (Koskela *et al.*, 2007; Lindner *et al.*, 2010; Várallyay, 2010).

Soil salinisation induces both osmotic and ionic stress in the affected plants, leading to reduced photosynthesis and inhibition of growth. This is followed by the onset of oxidative stress, causing damage to different cellular components, such as membranes, proteins and nucleic acids (Zhu, 2016). All plant species activate the same general mechanisms as a response to salt stress – based, for example, in the control of ion transport, the maintenance of cellular osmotic equilibrium or the activation of antioxidant systems (Apel and Hirt, 2004; Bartels and Sunkar, 2005; Munns and Tester, 2008). However, the efficiency of these responses varies widely between species, in a continuous range from extremely sensitive to highly salt tolerant plants.

While most crops are generally susceptible to salt, a small percentage of wild species – the halophytes – are adapted to extremely saline natural habitats (Flowers and Colmer, 2008, 2015). This applies to trees as well, whereby fruit trees like avocado and citrus are sensitive even to salt

concentrations at the low millimolar level (Zhu, 2007), while some deciduous tree species seem to be relatively tolerant to salt spraying and contact (Kayama *et al.*, 2003; Goodrich *et al.*, 2009).

The European larch (*Larix decidua* Mill.), a conifer present in the subalpine belt of Central Europe (Geburek, 2010), is an economically important timber species that is not commonly affected by soil salinity in its natural growing areas. This explains why studies on the mechanisms of response of this species to salt stress are scarce and, consequently, little is known about those mechanisms. However, larch trees are widely used in urban landscaping, where they can be affected by salt stress if irrigated with low-quality, reclaimed water. Even in the natural habitats of *L. decidua*, tree stands located in the proximity of mountain roads may suffer the effects of high soil salinity due to the common practice of winter de-icing with large amounts of NaCl. Therefore, elucidating the mechanisms of salt stress responses in larch does not lack academic interest and may have practical relevance.

The major aim of the present study was to identify in European larch biochemical markers of salt stress associated to conserved defence mechanisms: control of ion transport, maintenance of cellular osmotic balance and synthesis of antioxidants, as it was previously done with Norway spruce (Schiop *et al.*, 2015). In this regard, the levels of different compounds were determined in needles of salt-treated and control larch seedlings: photosynthetic pigments (chlorophylls a and b, and total carotenoids), monovalent ions (Na⁺, Cl⁻, K⁺), osmolytes (proline and total soluble sugars), malondialdehyde (MDA, an oxidative stress biomarker) and total phenolic compounds and flavonoids (as example of antioxidant metabolites). The experiments were performed with plant material derived from different natural larch provenances in Romania, in an attempt to identify populations with relatively higher salt tolerance, which could eventually be used in reforestation programmes. The same populations have been previously analysed from a genetic point of view, using genomic simple sequence repeat (SSR) markers (Gramazio *et al.*, 2018).

Materials and Methods

Growth conditions and experimental design

Seeds were obtained from the seven Romanian larch populations described in Gramazio *et al.* (2018). Seeds were sown on a mixture of peat (50%), perlite (25%) and vermiculite (25%), and grown in the glasshouses of the Institute for Plant Molecular and Cellular Biology (UPV), Valencia, Spain, under the following controlled conditions:

long-day photoperiod (16 h of light), with light intensity of 130 $\mu\text{E m}^{-2} \text{s}^{-1}$, temperature of 23 °C during the day and 17 °C at night, and relative humidity ranging between 50 and 80%. Hoagland's nutritive solution (Hoagland and Arnon, 1950) was administered twice a week for two months after seed germination. Salt stress was applied to these two-month-old seedlings by watering the pots with 150 mM NaCl solutions, twice a week for one month. In parallel, control plants were grown under the same conditions, but without any salt supplementation in the irrigation solution. Five biological replicates (five individual seedlings) were used per treatment and per provenance.

Soil analysis

Electrical conductivity (EC_{1:5}) of the pot substrate, a parameter reflecting soil salinity level, was measured after the end of the applied treatment to validate the uniformity of the salt treatments applied to all studied populations. Soil samples (five per treatment, per provenance) were collected from each pot, air-dried and then passed through a 2 millimeter sieve. A suspension composed of soil:water (ratio 1:5) was prepared in deionised water and mixed by stirring for one hour at 600 rpm at 21 °C, before being filtered. Electric conductivity was measured with a Crison 522 conductivity meter and expressed in dS m⁻¹.

Analysis of plant growth and dehydration of samples

Seedlings were sampled after one month of salt treatment and the stem length and total weight of shoots were determined. Some of the harvested needles were weighed (fresh weight, FW), dried in an oven at 65 °C for four days and then weighed again (dry weight, DW). Water content percentage (WC%) of the needles was then calculated by the following formula:

$$\text{WC}\% = [(FW - DW) / FW] \times 100.$$

Photosynthetic pigments measurements

Photosynthetic pigments, including total carotenoids (Caro), chlorophyll a (Chl a) and chlorophyll b (Chl b), were quantified following the protocol established by Lichtenthaler and Welburn (1983). Fresh needles were ground and extracted in 80% ice-cold acetone. Absorbance was measured at 663 nm, 646 nm, and 470 nm. The concentration of each photosynthetic pigment was calculated according to the following equations, and then converted into mg g⁻¹ DW:

- Chl a ($\mu\text{g mL}^{-1}$) = $12.21 \times (A_{663}) - 2.81 \times (A_{646})$;
- Chl b ($\mu\text{g mL}^{-1}$) = $20.13 \times (A_{646}) - 5.03 \times (A_{663})$;
- Caro ($\mu\text{g mL}^{-1}$) = $(1000 \times A_{470} - 3.27 \times [\text{Chl a}] - 10 \times [\text{Chl b}]) / 229$

Table 1. Origin of seeds germinated to obtain the seedlings used in the greenhouse experiments

Population	Provenance	Latitude	Longitude	Altitude (m.s.s) (m asl)
1	Anina	45°54'N	21°51'E	680–710
2	Latorita	45°23'N	23°50'E	1.000–1.200
3	Gura Humorului	47°33'N	25°53'E	520–570
4	Brasov Valea Cetatii	45°38'N	25°36'E	800
5	Sacele	45°37'N	25°41'E	870–940
6	Brasov Valea Popii	45°25'N	25°19'E	670–700
7	Sinaia	45°19'N	25°33'E	750

Ion contents measurement

Potassium (K⁺), chloride (Cl⁻) and sodium (Na⁺) contents were measured in needles sampled after one month of salt and control treatments, as described by Weimberg (1987). The extracts were prepared by incubating the samples (50 mg of finely ground, dry material suspended in 15 mL of water), for one h in a water bath at 95 °C, followed by filtration through a nylon filter of 0.45 µm pore. A PFP7 flame photometer (Jenway Inc., Burlington, USA) was used to quantify the levels of Na⁺ and K⁺ cations, while Cl⁻ contents were measured with a chloride analyser.

Osmolyte quantification

Proline (Pro) content was measured in fresh tissue by the ninhydrin-acetic acid method of Bates *et al.* (1973). Briefly, needle extracts prepared in 3% (w/v) sulfosalicylic acid solution were mixed with acid ninhydrin, incubated for one h at 95 °C in a water bath, cooled and extracted with toluene. The absorbance of the organic phase was determined at 520 nm, with toluene as a blank. Pro concentration was calculated in µmol g⁻¹ DW.

Total soluble sugars (TSS) were quantified according to Dubois *et al.* (1956). Methanol (80%, v/v) extracts of needle samples were mixed with 5% phenol, concentrated H₂SO₄ was added to the samples, which were rapidly mixed and incubated for 20 min at 30 °C, and finally, the absorbance at 490 nm was determined. Glucose was used to prepare the standard curve for the assay, and TSS contents were expressed as 'mg equivalent of glucose' per gram of DW.

Malondialdehyde (MDA) and non-enzymatic antioxidants

Methanol (80% v/v) extracts were prepared from 50 mg needles, by shaking in a rocker shaker at room temperature, for 24-48 h. Supernatants, collected by centrifugation, were stored in a freezer at -20 °C. MDA was quantified following the method described by Hodges *et al.* (1999). Samples were mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA) – or with 20% TCA without TBA for the controls – and then incubated at 95 °C for 20 min. After cooling on ice and a centrifugation step, the absorbance of the supernatants was measured at 532 nm. The non-specific absorbance at 600 and 440 nm was subtracted and the MDA concentration determined using the equations from Hodges *et al.* (1999).

Total phenolic compounds (TPC) were analysed by the method described in Blainski *et al.* (2013), based on the reaction with the Folin-Ciocalteu reagent. Samples were mixed with the reagent and sodium bicarbonate and left in the dark for 90 min. The absorbance was recorded at 765 nm, using gallic acid (GA) as the standard. TPC concentrations were expressed as GA equivalents (mg eq. GA g⁻¹ DW).

Total flavonoids (TF) were determined in the same extracts by the method described by Zhishen *et al.* (1999), based on the reaction of phenolic rings bearing a catechol group with NaNO₂, followed by AlCl₃ in the presence of NaOH. The absorbance was read at 510 nm using catechin as the standard. TF concentration was expressed as equivalents of catechin (mg eq. C g⁻¹ DW).

Statistical analysis

Data were analysed using the programme Statgraphics Centurion v.16. Before the analysis of variance, the Shapiro-Wilk test was used to check for validity of normality assumption and Levene's test for the homogeneity of variance. If ANOVA requirements were accomplished, the significance of the differences among treatments was tested by one-way ANOVA at a 95% confidence level, and post hoc comparisons were made using the Tukey HSD test. All means throughout the text are followed by SE.

Results*Soil electric conductivity (EC_{1.5})*

Soil electric conductivity (EC_{1.5}) reflects soil salinity, as it is closely correlated with the ionic contents of the pot substrates. A significant increase in soil EC_{1.5} was observed in the pots watered with 150 mM NaCl, as compared to the corresponding controls, for all studied larch populations (Fig. 1). The measured conductivities did not show any statistically significant inter-population differences in salt treated soils (Fig. 1). These data confirm that the salt stress treatment was applied uniformly to all seedlings of the different populations.

Analysis of plant growth and dehydration of samples

Stem length was measured at the end of the assays, after 30 days of treatments. In all analysed populations, mean values of salt-stressed seedlings were significant lower than those of the respective controls (Fig. 2A), with similar relative reductions of stem length in seedlings of all populations, except those from Sacele (Population 5), which showed the maximum difference, almost 40% of the control.

A salt-induced inhibition of biomass accumulation, reflected in the relative reduction of the control fresh weight, was also observed for the seven populations (Fig. 2B). The differences between salt-treated and control samples, however, were not significant in seedlings from Anina (Population 1) and Gura Humorului (Population 3). On the other hand, plants from Brasov Valea Popii (Population 6) and Sinaia (Population 7) appeared to be more affected by the NaCl treatments, as they showed more pronounced differences with the controls than the other populations (Fig. 2B).

Water loss is generally a physiological biomarker of high salinity in plants since leaf dehydration is a direct effect of the osmotic component of salt stress.

Water content percentage (WC%) was determined in the harvested needles of the studied populations of Romanian larch (Fig. 2C). Control needles of all seven populations had similar WC%, with small – though statistically significant – variations, ranging from 78 to 82%. As expected, WC% decreased in all samples in the presence of 150 mM NaCl.

Salt-induced loss of needle water, however, was relatively small (although significant, except in seedlings from the Anina provenance), in no case higher than 5% (Fig 2C). Therefore, under the conditions used in the experiments, larch seedlings appear to be relatively resistant to salt-induced dehydration.

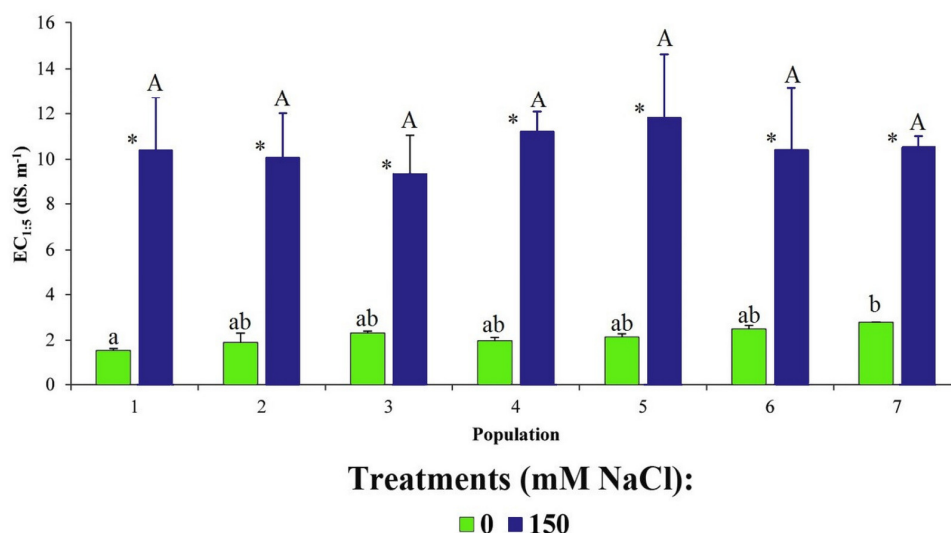


Fig. 1. Soil electric conductivity ($EC_{1.5}$) of the pot substrates, for the seven studied populations of Romanian larch, after 30 days of salt treatment. Values shown are means \pm SE ($n = 5$), expressed in $dS\ m^{-1}$. Different letters over the bars (lower case for controls and capital letters for salt-stressed plants) indicate significant differences between different populations according to Tukey test ($\alpha = 0.05$). Asterisks (*) indicate significant differences between salt and control treatments for the same population. Populations are given number codes according to Table 1

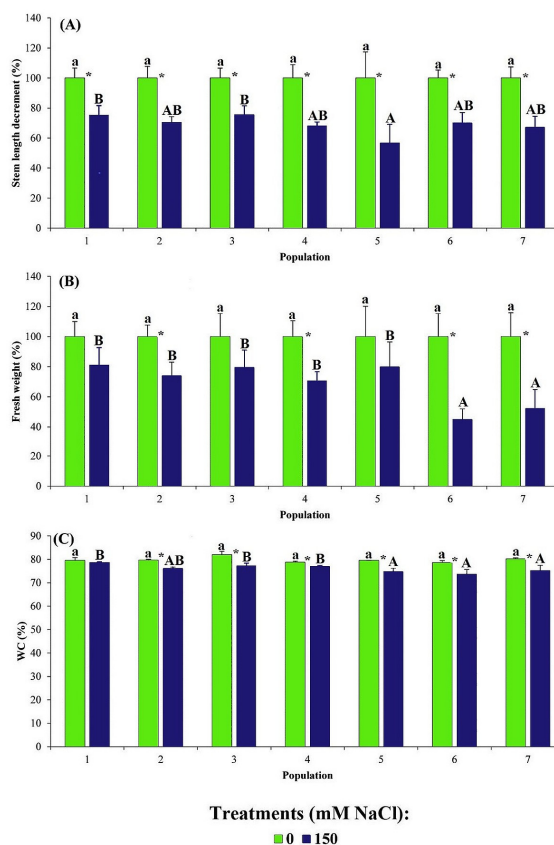


Fig. 2. Stress-induced inhibition of growth in *Larix decidua* after 30 days of treatments (150 mM NaCl and non-stressed controls). (A) Relative stem length reduction; (B) Relative fresh weight reduction; (C) Water content as a percentage of FW (WC%). Values shown are means \pm SE ($n = 5$). Different letters (lower case for controls and capital letters for salt-stressed plants) in a column indicate significant differences between different populations according to Tukey test ($\alpha = 0.05$). Asterisks (*) indicate significant differences between salt-treated and control seedlings of the same population. Populations are given number codes according to Table 1.

Photosynthetic pigments

Salt stress has many deleterious effects on the affected plants, including the oxidation and degradation of photosynthetic pigments; this causes a reduction in photosynthetic yield, which is one of the reasons for the salt-induced inhibition of plant growth. The contents of photosynthetic pigments were measured in the needles of control and salt-treated seedling of the seven studied larch populations (Fig. 3). In control, non-treated seedlings, Chl a, Chl b and Caro levels did not vary significantly when comparing the different larch populations, except for population 6 (Brasov Valea Popii), which showed lower contents of the three pigments. As expected, watering the plants for one month with 150 mM NaCl led to a reduction in the levels of the three measured photosynthetic pigments, in all seven larch populations; the salt-dependent reduction of chlorophylls (a and b) contents was statistically significant in all cases (Fig. 3A, B), whereas total carotenoids showed a significant decrease only in populations 1 (Anina), 4 (Brasov Valea Cetatii), 5 (Sacele) and 6 (Brasov Valea Popii), but not in the other three (Fig. 3C). Some quantitative inter-population differences were observed, regarding the degree of pigment contents reduction, with populations 4 and 5 registering the most significant decrease, while population 7 recorded the smallest change with respect to its control levels.

Ionic contents

Ionic contents were measured in the needles of larch seedlings from the seven studied populations, showing a significant increase in Na^+ and Cl^- ions in all salt-treated plants, in comparison with their respective controls (Table 2). Nonetheless, this relative increment varied widely, quantitatively, between the different populations. For example, Na^+ needle contents increased from about 2-fold in population 1 (Anina) to almost 7-fold in population 3 (Gura Humorului), with intermediate values for the other provenances. The relative increase of Cl^- in needles of NaCl-treated seedlings was more pronounced, ranging between 3.5-fold in population 1 and more than 20-fold in population 7 (Sinaia) (Table 2).

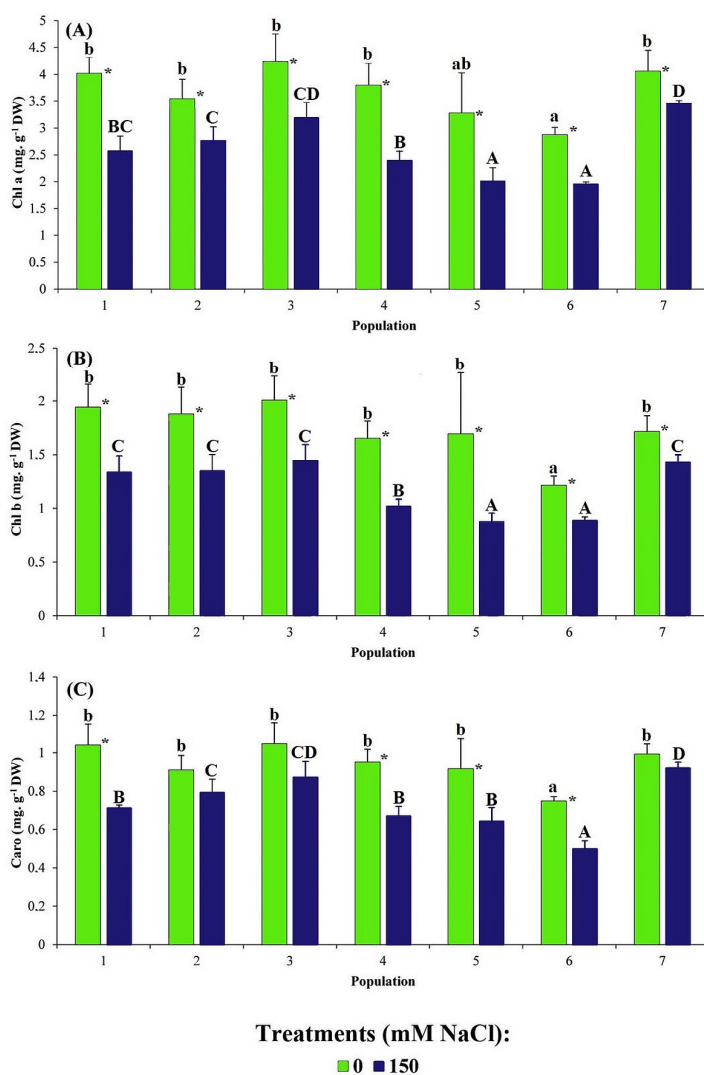


Fig. 3. Photosynthetic pigments contents in seedling needles of the seven studied populations of Romanian larch, expressed in mg g⁻¹ DW. (A) chlorophyll a (Chl a); (B) chlorophyll b (Chl b); (C) carotenoids (Caro). Values shown are means ± SE (n = 5). Different letters over the bars (lower case for controls and capital letters for salt-stressed plants) indicate significant differences between different populations according to Tukey test (α = 0.05). Asterisks (*) indicate significant differences between salt-treated and control seedlings of the same population. Populations are given number codes according to Table 1

Table 2. Ion contents (Na⁺, K⁺, Cl⁻) and K⁺/Na⁺ ratios in seedling needles of the seven populations of Romanian larch. Values shown are means ± SE (n = 5). Different letters (lower case for controls and capital letters for salt-stressed plants) in a column indicate significant differences between different populations according to Tukey test (α = 0.05). Asterisks (*) indicate significant differences between salt-treated and control seedlings of the same population

Population	Na ⁺ (μmol g ⁻¹ DW)		K ⁺ (μmol g ⁻¹ DW)		Cl ⁻ (μmol g ⁻¹ DW)		K ⁺ /Na ⁺	
	NaCl treatment		NaCl treatment		NaCl treatment		NaCl treatment	
	(mM)		(mM)		(mM)		(mM)	
	0	150	0	150	0	150	0	150
1	212.5 ± 3.7c*	414.7 ± 59.6A*	438.4 ± 13.6b*	566.2 ± 71.4C*	193.8 ± 8.3c*	706.6 ± 78.2A*	2.06	1.36
2	173.6 ± 11.4b*	564.1 ± 116.1B*	431.3 ± 25.1b	466.9 ± 19.9B	83.7 ± 9.5ab*	750.5 ± 173.9A*	2.48	0.82
3	123.7 ± 5.8a*	836.7 ± 96.6C*	368.4 ± 15.9a*	466.4 ± 32.8B*	102.8 ± 16.5b*	740.5 ± 111.8A*	2.97	0.55
4	156.0 ± 10.4b*	651.6 ± 54.2B*	427.8 ± 26.9b	493.4 ± 26.7B	103.6 ± 8.7b*	938.2 ± 31.6B*	2.74	0.75
5	161.3 ± 25.1b*	724.2 ± 188.4BC*	393.4 ± 45.6ab	419.1 ± 9.4A	71.0 ± 28.2a*	798.1 ± 241.18A*	2.43	0.57
6	174.1 ± 7.0b*	944.9 ± 48.7C*	358.8 ± 43.6a	417.6 ± 19.5A	64.3 ± 0.7a*	1008.1 ± 213.6B*	2.06	0.44
7	212.3 ± 11.8c*	1080.2 ± 47.8D*	447.2 ± 11.1b*	549.3 ± 27.7C*	63.5 ± 17.3a*	1295.8 ± 64.8C*	2.11	0.51

Accumulation of Na^+ in the leaves is normally accompanied by a reduction of K^+ contents, as both cations compete for the same transport systems. Larch, however, appears to possess efficient mechanisms to maintain K^+ concentrations under salt stress, as its levels in needles did only not decrease in the salt-treated plants, but rather increased in all tested populations – even if only slightly and, in several cases, not significantly (Table 2). The combination of these salt-induced changes in Na^+ and K^+ levels led to a reduction in K^+/Na^+ ratios, down to 20-30% of the non-stressed control values in all populations, except for provenance 1 – ca. 70% of the control (Table 2).

Osmolytes quantification

Stressed plants ubiquitously produce osmolytes to adjust their osmotic potential. Thus, the levels of two common plant osmolytes were measured in the needles of the studied larch plants. Proline (Pro) showed a significant increase in salt-stressed seedlings of the seven analysed populations, varying from 4- to 7-fold over the control values determined in non-stressed plants (Fig. 4A). Absolute Pro concentrations reached values of 30-40 $\mu\text{mol g}^{-1}$ DW, which, assuming the accumulation of the osmolyte predominantly in the cell cytoplasm, would contribute to osmotic adjustment under salt stress.

Total soluble sugars (TSS) were also quantified in the larch seedlings (Fig. 4B).

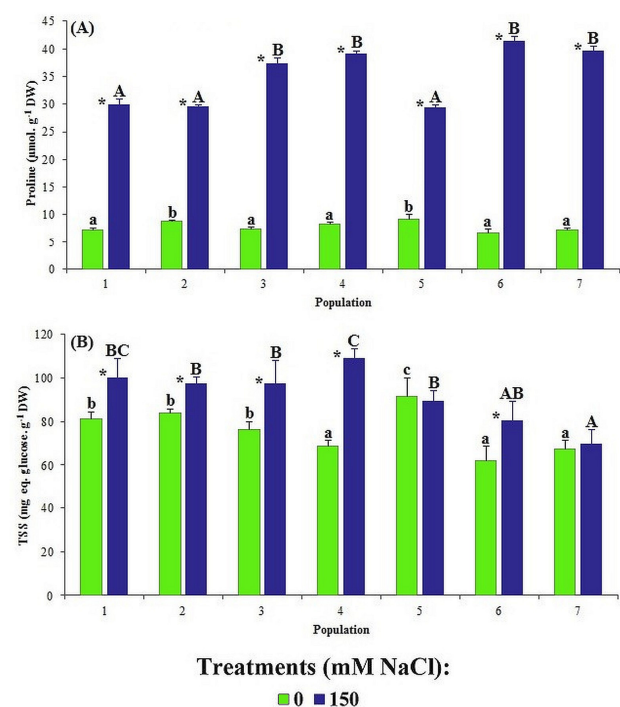


Fig. 4. Measured osmolytes of the seven studied populations of Romanian larch. (A) Proline (Pro) contents expressed in $\mu\text{mol g}^{-1}$ DW; (B) Total Soluble Sugars (TSS) contents expressed as mg eq. of glucose g^{-1} DW. Values shown are means \pm SE ($n = 5$). Different letters over the bars (lower case for controls and capital letters for salt stressed plants) indicate significant differences between different populations according to Tukey test ($\alpha = 0.05$). Asterisks (*) indicate significant differences between salt-treated and control seedlings of the same population. Populations are given number codes according to Table 1.

TSS contents also increased significantly in response to the NaCl treatment, except in populations 5 and 7, although the changes recorded were much smaller than those observed for Pro in the same samples (Fig. 4B).

Malondialdehyde (MDA) and non-enzymatic antioxidants

MDA is considered as an excellent indicator of oxidative stress (Del Rio *et al.*, 2005) as increased levels indicate membrane degradation due to ROS accumulation. MDA contents increased significantly with respect to the corresponding controls, in needles of salt-treated seedlings from the seven larch populations analysed (Fig. 5A). The relative increment in MDA level varied slightly between provenances, but the absolute MDA concentration reached in the presence of salt was the same in all of them (Fig. 5A).

Contrary to MDA, Total Phenolic Compounds (TPC) needle contents decreased in response to salt, even though the changes with respect to the corresponding controls were generally non-significant or very small (Fig. 5B).

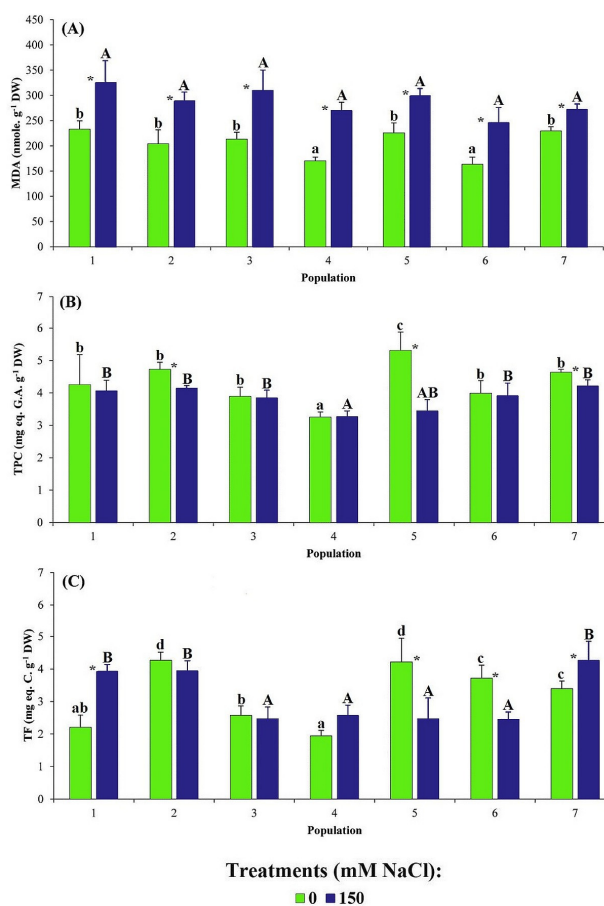


Fig. 5. Oxidative stress and antioxidant responses in the seven studied populations of Romanian larch. (A) Malondialdehyde (MDA) contents expressed in nmol g^{-1} DW; (B) Total Phenolic Compounds (TPC) expressed as mg eq. of gallic acid g^{-1} DW; (C) Total Flavonoids (TF) expressed as mg. eq. of catechin g^{-1} DW. Values shown are means \pm SE ($n = 5$). Different letters over the bars (lower case for controls and capital letters for salt stressed plants) indicate significant differences between different populations according to Tukey test ($\alpha = 0.05$). Asterisks (*) indicate significant differences between salt-treated and control seedlings of the same population. Populations are given number codes according to Table 1.

Total Flavonoids (TF) needle contents, on the other hand, did not show any clear pattern of variation in the salt-stressed plants: significant increases, significant reductions and no significant changes in TF concentration were observed in the different larch populations (Fig. 5C).

Discussion

Growth inhibition is the first and most general reaction of plants to stress, as all resources are redirected from primary metabolism and biomass accumulation to the activation of defence reactions against stress (Zhu, 2001). Therefore, deleterious effects of stress are usually assessed in terms of growth reduction, by determining different growth parameters, such as stem length, foliar area, fresh weight or dry weight, etc. (e.g., Al Hassan *et al.*, 2016a, b; Kumar *et al.*, 2017). However, such indicators of stress based exclusively on growth parameters are not sufficient in the case of slow-growing species, such as many woody species including conifers. In such species, the use of biochemical stress markers represents a rapid and reliable alternative for the detection of early effects of environmental stress, as it has been recently proposed in spruce (Schiop *et al.*, 2015, 2017). All plants activate a series of basic, conserved mechanisms as a response to environmental stress and several metabolites involved in these responses – mono and divalent cations, osmolytes, photosynthetic pigments, antioxidant compounds, etc. – can be used, *a priori*, as stress biomarkers. However, the significance of these responses in the mechanisms of stress tolerance largely varies among taxa. The analysis of stress responses in species of the same genus (Cicevan *et al.*, 2015; Al Hassan *et al.*, 2016a,b; Koźmińska *et al.*, 2018, 2019) or in different cultivars of the same species (Al Hassan *et al.*, 2016c) allowed us to establish which of those responses were relevant for tolerance, through the identification of suitable biochemical markers for each investigated species or group of related taxa.

Except for the authors' previous study (Plesa *et al.*, 2018), there are no other published reports dealing with the responses of *Larix decidua* to salt stress, and only a few on other species of this genus (e.g. Renault *et al.*, 2005).

Among coniferous species, the European larch is considered as a relatively fast-growing tree (Matras and Paque, 2008). This was confirmed by the present experimental approach, since after one month of treatment a reduction of growth could be measured in salt-stressed plants as compared to their respective controls. The relative reduction of fresh weight was mostly due to growth inhibition and not to dehydration, as the water loss in needles from all populations was less than 5%. Therefore, exposure of young seedlings to 150 mM NaCl has a deleterious effect on growth. There were some variations among the analysed populations, but they could not clearly separate populations that responded better to salinity.

Growth inhibition was associated with a reduction in the leaf contents of photosynthetic pigments. This reduction occurs in many plant species under diverse abiotic stresses, as the result of a combination of factors, such as the inhibition of chlorophyll biosynthesis, the activation of its degradation by chlorophyllase, and the inactivation of primary enzymes involved in photosynthesis (Santos, 2004). The reduction of photosynthetic pigments concentration under salt stress has been previously reported in larch (Plesa

et al., 2018) and in other conifer species (Crosser, 2001; Schiop *et al.*, 2015).

Potassium is considered as the 'physiological' cation, essential for plant growth and development, whose deficiency has negative effects on photosynthesis, osmoregulation, protein biosynthesis and other fundamental processes (Gierth and Mäser, 2007). On the contrary, high concentrations of sodium have negative effects in many physiological processes, by inhibiting enzymatic activities and interfering with K⁺ uptake and homeostasis systems (Adams and Shin, 2014; Rodriguez-Navarro and Rubio, 2006). Glycophytes generally respond to high soil salinity by limiting the uptake of toxic ions, Na⁺ and Cl⁻, either by exclusion at the root level or by blocking their transport to the leaves (Munns *et al.*, 2015; Mbarki *et al.*, 2018). Several studies in *Larix laricina* (Renault, 2005), in *Picea abies* (Schiop *et al.*, 2015) and recently in *Larix decidua* (Plesa *et al.*, 2018) reported the maintenance of relatively high K⁺ concentrations in the needles under saline conditions, as a mechanism to partly counteract the deleterious effects of salt stress. This pattern, which seems to be common in conifers, is rather unusual, as Na⁺ accumulation is generally accompanied by a reduction of K⁺ levels, as the two ions compete for the same transport systems (Greenway and Munns, 1980). Avoiding an excessive reduction of cytoplasmic K⁺/Na⁺ ratios appears to be an important mechanism of defence against high salinity in European larch, as in all populations salt-stressed plants maintained or even increased slightly the needle K⁺ concentrations measured in control seedlings.

Another general mechanism of response to all types of environmental conditions, inducing osmotic stress, is the synthesis of compatible solutes or osmolytes, which are diverse compounds that do not interfere with cellular metabolism, even at high concentrations and help to avoid cell dehydration and to maintain osmotic balance under stress (Zhu, 2016). Proline is a common compatible solute in plants (Verbruggen and Hermans, 2008; Szabados and Savouré, 2010) and it has been reported to significantly increase in many plant species, both halophytes and glycophytes, in response to salt treatments. An increase in Pro has been described in many conifer species under salinity and/or drought conditions, for example, in pine (Newton *et al.*, 1986; Zamani *et al.*, 2014; Taibi *et al.*, 2017) and spruce (Ditmarová *et al.*, 2010; Schiop *et al.*, 2015, 2017). In larch, a significant increase in Pro over control values was shown in the presence of cadmium, and were reported in the hybrid *L. × eurolepis* Henry (*L. decidua* × *L. kaempferi*) and in *L. decidua* under saline stress (Bonet *et al.*, 2016). In the present study, Pro values in the presence of salt varied in similar ranges in the seven larch populations, from 4- to 7-fold higher concentrations than in the corresponding controls. In addition to its possible contribution to cellular osmotic adjustment, Pro accumulation may have a significant role in the mechanisms of stress tolerance as a low-molecular-weight chaperon, ROS scavenger, or as a signalling molecule (Szabados and Savouré, 2010).

Soluble sugars also belong to the category of osmolytes in many plant species, including conifers (Clancy *et al.*, 1995), but do not appear to play this functional role in European larch. A significant increase in TSS contents was observed in

the stressed plants, but only in five of the seven populations analysed, not in all. Also, this variation was too small to consider that they may play an important osmoregulatory function in this species. In any case, it is always difficult to demonstrate the specific contribution of soluble sugars, acting as osmolytes, to the responses to stress, due to their multiple functional roles in plants, as metabolic precursors, energy sources, or signalling molecules.

MDA is a reactive aldehyde generated by the ROS-induced oxidation of membrane lipids and is used as a suitable biomarker of cellular oxidative stress (Del Rio *et al.*, 2005). In the seven populations analysed, measured MDA concentrations were significantly higher in the seedlings exposed to salt stress than in the non-stressed controls, although this increment is smaller than that observed in other glycophytes under similar conditions (Al Hassan *et al.*, 2017; Kumar *et al.*, 2017; Nxele *et al.*, 2017). In the present study, a clear pattern of variation in the concentrations of non-enzymatic antioxidants was not detected among the studied populations; when differences between the values registered in control and salt-stressed seedlings were observed, they were generally very low. These data are in agreement of those from our previous work (Plesa *et al.*, 2018), which suggested that one month of exposure to 150 mm NaCl, generated only a weak oxidative stress in larch seedlings, due to the efficiency of the antioxidant enzymatic systems activated in response to the salt treatment. These antioxidant enzymes have been defined as the first line of defense against oxidative stress (Fini *et al.*, 2011).

Conclusions

The detected biochemical responses to salt stress were similar in the seven Romanian populations of *Larix decidua* analysed in the present work. Several biochemical markers could be associated, for all populations, with the observed salt-induced inhibition of growth: decreasing levels of photosynthetic pigments, increasing concentrations of Na⁺ and Cl⁻, maintenance of K⁺ levels, Pro and MDA accumulation in needles of European larch seedlings subjected to salt stress treatments. These parameters could be optimal for the rapid analysis of the degree of stress affecting the plants, at least at the seedling stage; further studies will be required to establish if these results can be extended to other developmental phases. The selected biochemical stress markers can be easily and rapidly quantified by non-destructive assays, requiring simple equipment and small amounts of plant material. Despite some quantitative variations in the responses of the different Romanian larch populations, it was not possible to identify any of them showing a higher degree of tolerance to salinity. The genetic analysis using SSR markers of the same populations revealed a large genetic diversity of highly heterozygous individuals, as well as limited genetic differentiation of those populations. Therefore, it was not likely that the studied populations could be differentiated by their biochemical responses to salt, as shown by the present results.

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