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Relative NaCl tolerance of rare and endangered coastal plant species in conditions of tissue culture

Ilva Freipica, Gederts Ievinsh*

Department of Plant Physiology, University of Latvia, Kronvalda Bulv. 4, Riga LV-1586, Latvia

*Corresponding author, E-mail: gederts@lanet.lv

Abstract

Three coastal plant species *Glaux maritima*, *Dianthus arenarius* spp. *arenarius* and *Linaria loeselii* were experimentally tested for NaCl tolerance in conditions of tissue culture. NaCl-induced changes in oxidative enzyme activities and ascorbate peroxidase were measured as indicators of salinity-affected processes. Relatively high NaCl tolerance was found for all of the tested species. Growth and development of *G. maritima* explants was stimulated by 100 mM NaCl throughout the cultivation period. The stimulation was not due to osmotic effect, as equiosmotic concentration of polyethylene glycol significantly reduced growth of explants. A relationship between NaCl tolerance and NaCl-induced increase in ascorbate peroxidase activity in explant tissues was found. Salinity-induced changes in peroxidase and polyphenol oxidase activity varied in plants with different NaCl tolerance. The results are discussed in respect to putative salinity tolerance reactions.

Key words: ascorbate peroxidase, coastal plants, peroxidase, polyphenol oxidase, salinity, tissue culture.
Abbreviations: EC, electrical conductivity; PEG, polyethylene glycol.

Introduction

Oxidative stress is believed to be one of the major damaging factors in plants under saline conditions (Hernandez et al. 1995). Thus, cellular protection against reactive oxygen species in conditions of salt stress might be crucial for salinity tolerance. Indeed, NaCl tolerance is usually accompanied by a significant increase in antioxidative capacity (Jitesh et al. 2006). In respect to enzymatic antioxidative protection, ascorbate peroxidase as a major constituent in H_2O_2 scavenging in chloroplasts has been reported (Logan et al. 2006). Other oxidative enzymes, e.a., peroxidase and polyphenol oxidase, are associated with salinity-related responses both at the level of adaptive reactions as well as to changes related to tissue damage (Muthukumarasamy et al. 2000; Demir, Kocaçaliskan 2001).

For plant species native to coastal habitats salinity is one of the most important environmental constraints (Ievinsh 2006). However, in contrast to salt marshes, where sea water inundation results in increased soil salinity, sand dune plants are exposed to salinity mainly in the form of salt spray (Rozema et al. 1985). Physiological investigations of wild plants from coastal habitats are important to understand mechanisms of salinity resistance. In addition, during *ex situ* conservation using tissue culture, in the case of endangered wild plants from salt-affected coastal habitats there is a need to search for a possible specific requirement of NaCl in the cultivation medium.

Tissue culture has been frequently used as a fast and reliable tool for selection of salt tolerance (Smith, McComb 1981; Morabito et al. 1994; Watanabe et al. 2000; Queirós et al. 2007). However, there are contradictory data in the literature on whether whole plant salt tolerance always correlates with that in conditions of tissue culture. At least, the fact that numerous adaptive mechanisms to high NaCl content seem to be based on physiological integration at the whole plant level (Dracup 1991) should be taken into the account.

Three coastal plant species of the Baltic Sea were selected for the present experiments. *Glaux maritima* (Primulaceae) grows in coastal salt marshes and periodically sea-flooded coastal meadows. However, the species can not be designated as an obligate halophyte as its growth has been shown to be slightly inhibited by salt water inundation (Rozema et al. 1985) or slightly stimulated by 150 mM NaCl (Rozema et al. 1978). *G. maritima* exhibits several salt tolerance mechanisms including osmotic adaptation and NaCl secretion (Rozema, Riphagen 1977). *Dianthus arenarius* L. ssp. *arenarius* (Caryophyllaceae) is a typical sand-steppe species (Tyler 2005). In Latvia it is found both in dune forests and on grey dunes. *Linaria loeselii* Schweigg. (Scrophulariaceae) is an endemic species of Eastern Baltics growing on periodically salt-spray affected sand dunes (Schramm 1854). No data on salt tolerance of *D. arenarius*

and *L. loeselii* have been reported so far.

All three species have a special conservation status in Latvia. *G. maritima* and *L. loeselii* are nationally endangered species protected by the Regulations of the Cabinet of Ministers of Latvia. *D. arenarius* subsp. *arenarius* and *Linaria loeselii* are listed in Annex II of the European Council Directive 92/43/EEC (Habitats Directive).

The aim of the present experiments was to test for putative NaCl tolerance of these coastal plants in conditions of tissue culture. NaCl-induced changes in oxidative enzyme activities (peroxidase and polyphenol oxidase) and ascorbate peroxidase were investigated as indicators of salinity-affected oxidative processes and antioxidative state, respectively.

Materials and methods

Plant material and establishment of tissue cultures

Tissue cultures were established from seeds collected in wild coastal populations near the Baltic Sea during summer of 2005. *Glaux maritima* seeds were collected in a coastal meadow (57°20'N, 23°08'E) with fluctuating soil Na and Cl concentrations (48.4 to 322.0 mmol kg^{-1} and 37.3 to 191.1 mmol kg^{-1}, respectively), with soil electrical conductivity (EC) 6.4 to 9.0 dS m^{-1}. Seeds of *Linaria loeselii* were collected in embryonic sand dunes (57°36'N, 21°57'E) with soil Na concentration of 0.35 to 0.57 mmol kg^{-1} and Cl concentration of 0.09 to 0.10 mmol kg^{-1}, soil EC 0.15 to 0.16 dS m^{-1}. Seeds of *Dianthus arenarius* ssp. *arenarius* were collected on grey dunes (57°15'N, 21°25'E) with soil Na concentration of 0.35 mmol kg^{-1} and Cl concentration 0.08 mmol kg^{-1}, soil EC 0.25 dS m^{-1}.

Cultures were initially established as described previously (Klavina et al. 2006) on agar-solidified half-diluted Murashige and Skoog medium without growth regulators (Murashige, Skoog 1962) and were further used for obtaining of cultures for the present experiments.

Experimental setup

For establishment of cultures used in the present experiments, nodal and apical explants were excised from established stock cultures. Initial explant length was 10 mm for both *D. arenarius* (apical) and *G. maritima* (nodal with two leaves), and 15 mm for *L. loeselii* (apical). Explants were placed on agar-solidified (0.6%) half-diluted Murashige and Skoog medium containing 0.1 g L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} piridoxine hydrochloride, 0.5 mg L^{-1} thyamine hydrochloride, and 30 g L^{-1} sucrose, pH 5.6 to 5.8. For treatments, NaCl and polyethylene glycol (PEG 4000, only for *G. maritima*) were added to the medium at the final concentrations of 50 to 400 mM and 65 to 130 mM, respectively. Explants were cultivated in 50 mL tissue culture jars closed with aluminium foil, seven to 12 explants per jar. Cultures were kept in a growth chamber under a 16-h photoperiod (white fluorescent lamps; photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}), day/night temperature of 25/20 °C. Morphological measurements were performed in five biological replicates.

Measurement of enzyme activities

For measurement of enzyme activity, explants were rinsed with deionized water, frozen in liquid nitrogen and stored at –80 °C until analysis.

For extraction of ascorbate peroxidase, explant tissues were ground in liquid nitrogen and extracted with 25 mmol L^{-1} HEPES/KOH buffer (pH 7.2) containing 1 mM EDTA, 1 mM sodium ascorbate, and 3 % (w/v) insoluble polyvinylpolypyrrolidone for 15 min at 4 °C. The homogenate was filtered through nylon cloth and centrifuged at 15 000 g_n for 15 min. Ascorbate peroxidase activity was determined in supernatant according to Nakano and Asada (1987) The assay was performed in a final volume of 1 mL, containing 50 mM sodium phosphate buffer (pH 7.0), 1 mM NaEDTA, 1 mM Na ascorbate, and 100 μL of supernatant. The reaction was started by the addition of 0.5 mL 3 mM H_2O_2. The decrease in absorbance at 290 nm was recorded.

For polyphenol oxidase and peroxidase measurement, explants were ground in liquid nitrogen and extracted with 25 mmol L^{-1} HEPES/KOH buffer (pH 7.2) containing 1 mmol L^{-1} EDTA and 3 % (w/v) insoluble polyvinylpolypyrrolidone for 15 min at 4 °C. The homogenate was centrifuged at 15 000 g_n for 15 min. The supernatant was used for assays. Peroxidase activity was measured spectrophotometrically at 470 nm in reaction mixture containing 2 mL of 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) with 10 mmol L^{-1} guaicol, 0.5 mL 0.03 mol L^{-1} H_2O_2 and 0.01 mL of enzymatic extract. The reaction mixture without H_2O_2 was used as a reference. The activity of polyphenol oxidase was determined spectrophotometrically in a reaction mixture (3 mL) containing 20 mmol L^{-1} sodium phosphate (pH 6.5) with 25 mmol L^{-1} pyrocatechol and the enzymatic extract (0.01 mL). The change in absorbance was monitored at 410 nm.

Enzyme measurements were performed in three biological replicates.

Results

Effect of NaCl on growth and development of explants

Growth of *G. maritima* explants was stimulated by 100 mM NaCl throughout the cultivation period up to 70 days after initiation (Fig. 1A). At early stages (up to 15 days after initiation) a maximum growth-enhancing effect of 200 mM NaCl was found. However, the growth at this concentration was completely suppressed after 35 days of cultivation. Similarly, leaf and root development was enhanced by 100 mM NaCl in the growth medium (Fig. 1B, C). However 200 mM NaCl had no effect on leaf and root development. No roots were formed at 400 mM NaCl. Formation of

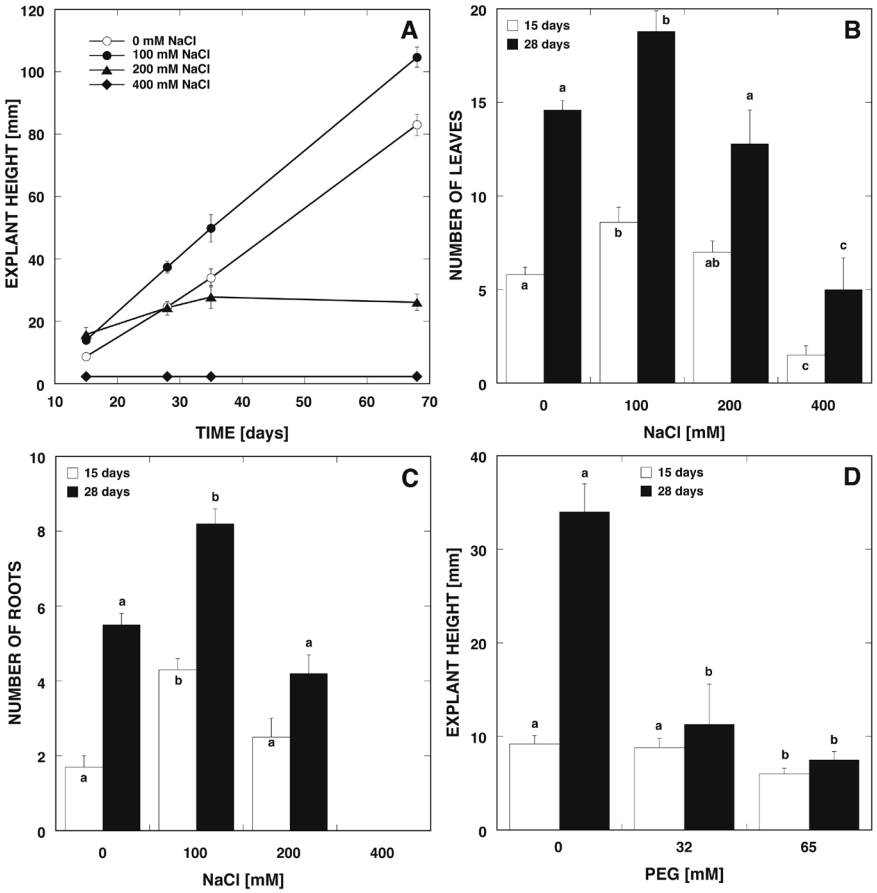

Fig. 1. Effect of different concentrations of NaCl on linear growth of explants (A), number of leaves (B), number of roots (C), and effect of different concentrations of polyethylene glycol on growth (D) of *Glaux maritima* in tissue culture. Data are means ± SE from 5 biological replicates with 7 - 12 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

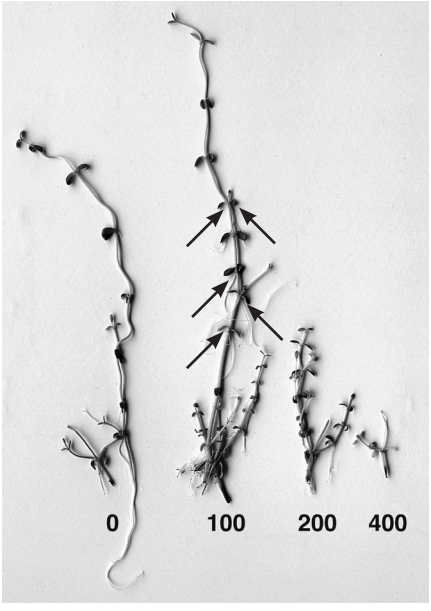

Fig. 2. Characteristic morphological changes of *Glaux maritima* explants after 70-day-long cultivation at different concentrations of NaCl in the medium. Formation of adventitious roots is indicated by arrows. NaCl concentration (mM) is given below.

adventitious roots at the nodes was induced by 100 mM NaCl (Fig. 2). In addition, new shoots were formed at the base of the explants. In contrast to NaCl treatment, a concentration of polyethylene glycol (32 mM) equiosmotic to that of 100 mM NaCl resulted in extremely arrested growth of *G. maritima* within 28 days of cultivation (Fig. 1D).

Explants of *D. arenarius* were relatively tolerant to 50 mM NaCl at the level of growth and leaf formation (Fig. 3). Growth and leaf formation of *D. arenarius* was only moderately depressed by 100 mM NaCl, especially 14 days after the start of cultivation (Fig. 3A, B). However both root and shoot formation was significantly inhibited by increasing concentrations of NaCl (Fig. 3C, D).

In contrast, explants of *L. loeselii* cultivated on medium with 50 mM NaCl showed significant growth inhibition (Fig. 4A). Already 18 days after transplanting 33 % of explants cultivated on 200 mM NaCl became necrotic. Leaf formation was less sensitive to NaCl up to 100 mM (Fig. 4B). However, root formation was inhibited by more than 50 % at 50 mM NaCl, and no roots were formed at higher concentrations (data not shown).

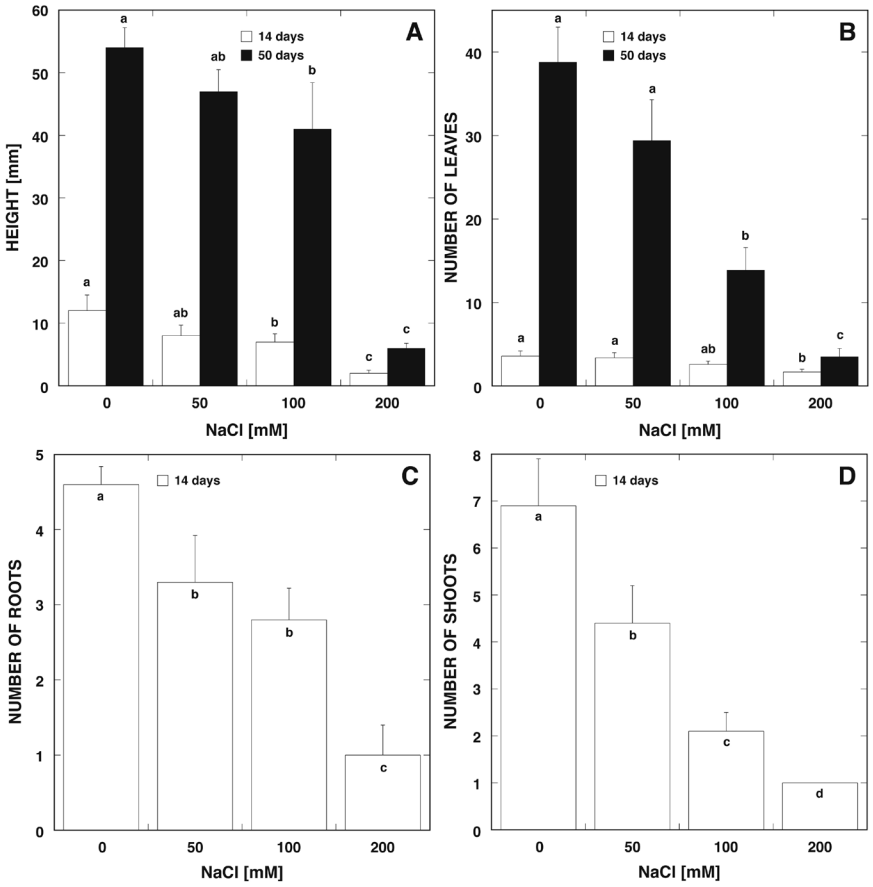

Fig. 3. Effect of different concentrations of NaCl on growth (A), number of leaves (B), number of roots (C), and number of shoots (D) of *Dianthus arenarius* spp. *arenarius* explants in tissue culture. Data are means ± SE from 5 biological replicates with 7 - 12 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

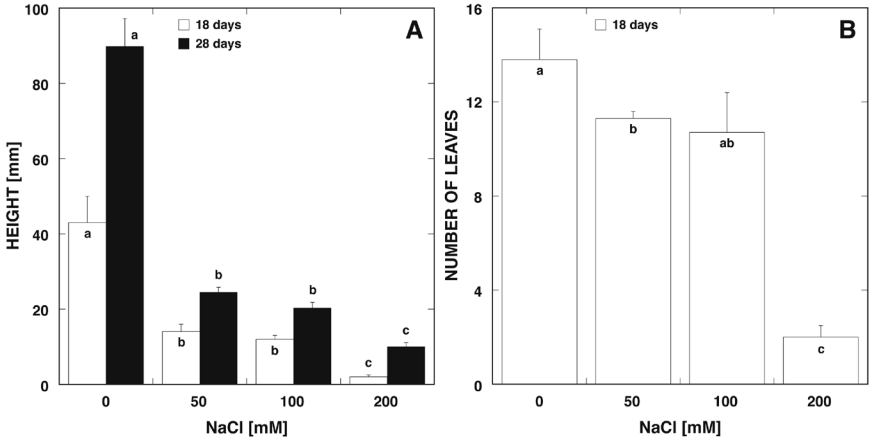

Fig. 4. Effect of different concentrations of NaCl on growth (A) and number of leaves (B) of *Linaria loeselii* explants in tissue culture. Data are means ± SE from 5 biological replicates with 7 - 12 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

Effect of NaCl on oxidative enzymes and ascorbate peroxidase

In general, peroxidase activity increased for all the tested plant species in explant tissues cultivated in the presence of elevated NaCl levels (Fig. 5 to 7). The increase was more pronounced with increased time of cultivation. However in *D. arenarius* there was a decrease of peroxidase activity in NaCl-treated explants at the early stages of cultivation (14 days, Fig. 5A).

Relatively low polyphenol oxidase activity was noted

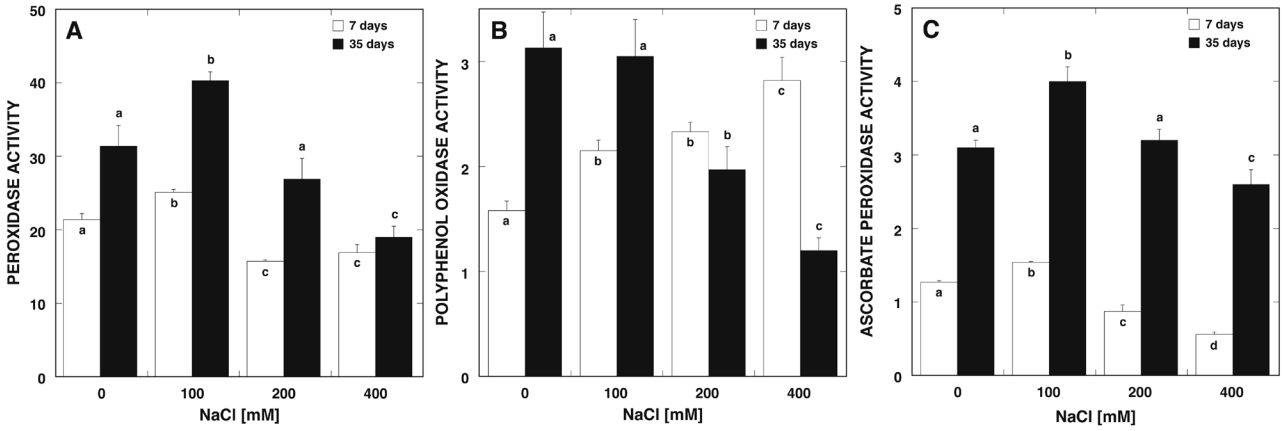

Fig. 5. Effect of different concentrations of NaCl on peroxidase (A), polyphenol oxidase (B), and ascorbate peroxidase activity in explants of *Glaux maritima*. Data are means ± SE from 3 biological replicates with 3 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

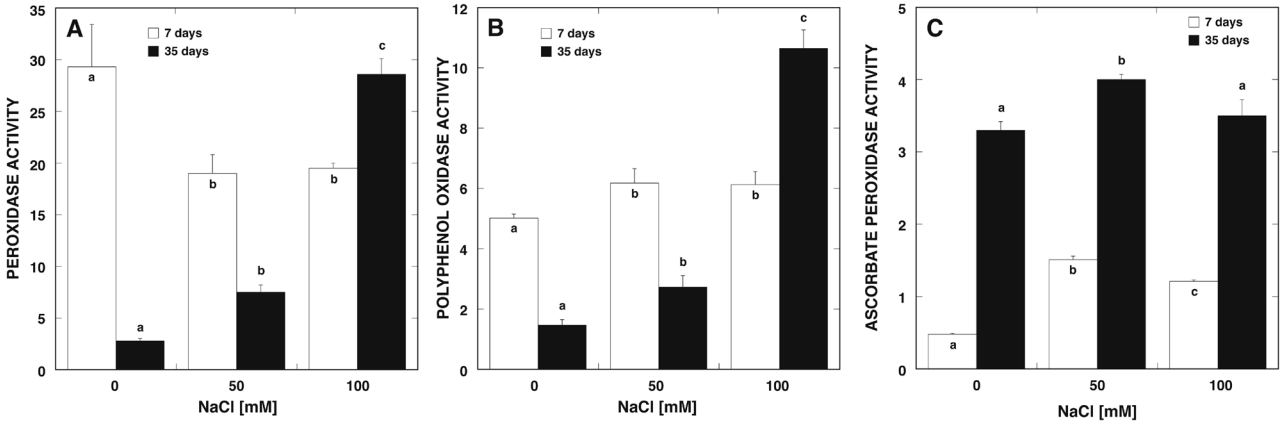

Fig. 6. Effect of different concentrations of NaCl on peroxidase (A), polyphenol oxidase (B), and ascorbate peroxidase activity in explants of *Dianthus arenarius* spp. *arenarius*. Data are means ± SE from 3 biological replicates with 3 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

for *G. maritima* explants throughout the growth period both in control conditions and under the effect of 100 to 300 mM NaCl (Fig. 5B). While polyphenol oxidase activity in the explants in the presence of elevated NaCl showed considerable increase at 7 days after start of cultivation, prolonged incubation with increased levels of NaCl in the growth medium (both 200 and 400 mM) caused decrease of the activity. Polyphenol oxidase activity decreased with time of growth in control explants of *L. loeselii* and *D. arenarius* (Fig. 6B, 7B). However, NaCl-treated explants of these species exhibited increased polyphenol oxidase activity during prolonged incubation (35 days), in comparison with control explants.

Ascorbate peroxidase activity significantly increased during cultivation of plant tissues for all the species studied. Ascorbate peroxidase activity increased by NaCl treatment in explants of *G. maritima* (100 mM; Fig. 5C) and D. arenarius (50 mM; Fig. 6C). In contrast, no effect of NaCl was evident for explants of *L. loeselii* (Fig. 7C).

Discussion

Relatively high NaCl tolerance was found in the present experiments for three coastal plant species *G. maritima*, *D. arenarius* and *L. loeselii* in conditions of tissue culture. For *G. maritima*, a potential halophyte, a stimulative effect of 100 mM NaCl was evident both at the level of development as well as linear growth of shoots. This influence on growth was not related to osmotic effect as equiosmotic concentration of polyethylene glycol (32 mM) resulted in significant growth inhibition. It was argued that cell proliferation and cell differentiation has different sensitivity to NaCl, as 400 mM stimulated formation of adventitious tissues on leaf calluses of mangrove *Bruguiera sexangula* while inhibiting callus growth (Mimura et al. 1997). The above could explain the various effect of NaCl on growth and development found in the present experiments, where development of *D. arenarius* was more sensitive to NaCl in comparison to linear growth, in contrast to *L. loeselii*

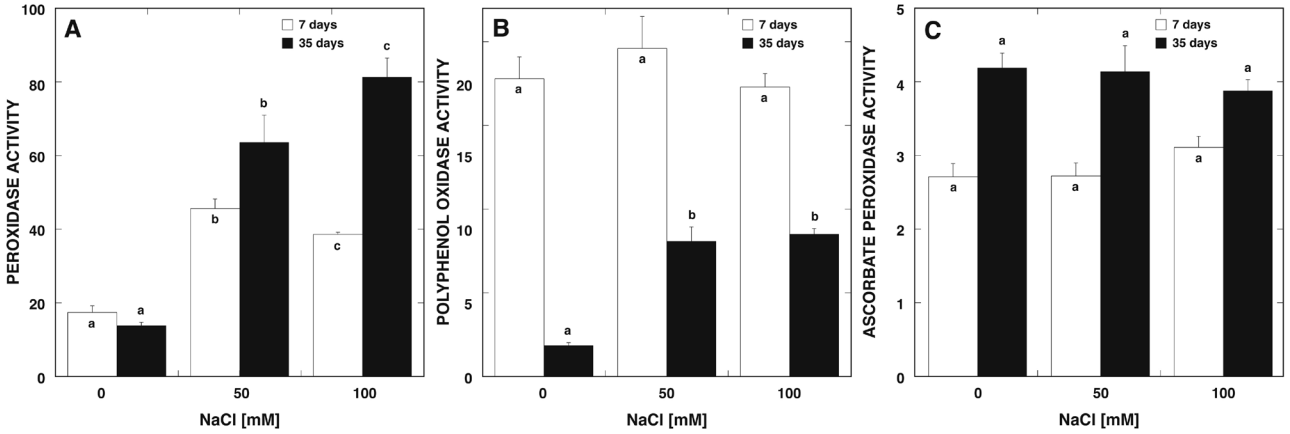

Fig. 7. Effect of different concentrations of NaCl on peroxidase (A), polyphenol oxidase (B), and ascorbate peroxidase activity in explants of *Linaria loeselii*. Data are means ± SE from 3 biological replicates with 3 measurements per replicate at every time point for each treatment. Identical letters indicate no statistically significant differences between the means for a particualr time point ($p < 0.05$).

where the opposite effect was found. The differences in NaCl tolerance between the investigated spaces most probably reflects differences in growth conditions in native habitats, i.e. where *G. maritima* is periodically affected by sea water inundation, but *D. arenarius* and *L. loeselii* are only occasionally influenced by salt water spray.

Contradictory results have been found in literature concerning salt tolerance of whole plants vs. cultivated tissues. In general salt tolerance of cultivated tissues of halophytes mainly depends on cellular salt tolerance mechanisms e.g. compartmentation of ions in vacuoles and osmotic adjustment of cytoplasm by compatible solutes (Flowers 1985). Thus dry mass increase of unadapted callus of halophyte *Spartina patens* at 510 mM NaCl was identical to that at control conditions, indicating the existence of a cellular salt tolerance mechanism (Li et al. 1995). Similarly studies with callus cultures of halophyte *Atriplex halimus* demonstrated the existence of a cellular basis for salinity tolerance (Bajji et al. 1998). However, when different explant tissues are used for cultivation, various levels of physiological integration can be found. For a species with adaptive mechanisms at the high level of physiological integration between particular organs lower NaCl tolerance in conditions of tissue culture should be expected. It was shown that the growth of callus cultures of wild halophytes *Atriplex uncunlata* and *Suaeda australis* was significantly inhibited by NaCl at concentrations optimal for growth of intact plants, 62.5 mM and 125 mM, respectively (Smith, McComb 1981).

However some cultivated plant tissues have been shown to possess higher tolerance against NaCl in comparison to intact plants. Seedling growth of *Nicotiana tabacum* was inhibited by NaCl above 50 mM (Niknam et al. 2004). In contrast, for *N. tabacum* explants in conditions of tissue culture, 100 mM NaCl significantly increased both fresh and dry mass. In addition, several *Medicago* species exhibiting *in vitro* NaCl tolerance at 85 mM were sensitive to NaCl at the whole plant level (McCoy 1987).

As salinity leads to increased production of active oxygen species, it is commonly believed that adaptation to saline conditions is inevitably associated with increased activity of antioxidative enzymes. Regarding intact glycophytes, salt-tolerant varieties are often characterized by increased activity of antioxidant enzymes in conditions of salt stress (Meloni et al. 2003). In conditions of tissue culture, the existence of a similar relationship has been found. Salt tolerant callus culture of *Citrus limon* showed an increase in antioxidant enzyme activity (Piqueras et al. 1996). However, increased antioxidative capacity in tissue culture under the effect of NaCl not always correlates with the level of salt tolerance. Thus a relatively salt tolerant cultivar of *Morus alba* showed increased ascorbate peroxidase activity in tissue culture on medium with 150 mM NaCl in parallel with inhibited growth, reduced chlorophyll content and increased proline level (Harinasut et al. 2000).

In NaCl-tolerant *G. maritima*, optimal concentrations of NaCl resulted in increased ascorbate peroxidase activity. Similarly moderately tolerant *D. arenarius* exhibited increased ascorbate peroxidase activity at 50 mM NaCl. In contrast, in *L. loeselii* a less NaCl-tolerant plant, no increase in ascorbate peroxidase activity was found. Thus the relationship between NaCl tolerance and NaCl-induced increase in ascorbate peroxidase activity in explants of coastal plants in conditions of tissue culture is evident.

Relatively high peroxidase activity in plant tissues often has been regarded as an indication of general defense response. Both in seedlings and callus cultures of *Trigonella foenum-graecum*, moderate NaCl levels induced increase in peroxidase activity (Niknam et al. 2006). However, further increase of salinity (150 mM and more) led to decreased peroxidase activity in the seedlings, while in calli the activity remained at a high level (three times that of control calli). However increased peroxidase activity in NaCl-treated explants could be regarded also as an indication of morphological disturbance or physiological senescence. In the present experiments, peroxidase activity

increased in NaCl-treated explants of *D. arenarius* and *L. loeselii* during prolonged cultivation in a concentration-dependent manner, suggesting general NaCl toxicity effect. In contrast, a significant increase of peroxidase activity in *G. maritima* was found only at optimal NaCl concentration (100 mM), most probably related to biochemical adaptation mechanisms. Thus increased peroxidase activity may have different physiological meaning, depending on relative salt tolerance and NaCl concentration used.

Relatively high polyphenol oxidase activity during early stages of cultivation is a result of explant wounding-induced increase of the enzyme. Wounding-dependent increase in polyphenol oxidase activity is a common characteristic of different plant tissues (Constabel et al. 2000; Kruzmane et al. 2002). During prolonged cultivation with high doses of NaCl, increase of the enzyme activity in explants of *L. loeselii* and *D. arenarius* possibly reflects salt-stress associated metabolic disturbances. No such increase in polyphenol oxidase activity was noticed for the potential halophyte *G. maritima*.

In theory, salt tolerance/susceptibility in tissue culture vs. intact plants should be more similar for glycophytes than salt tolerance of halophytes. However, here we demonstrated that a potential halophyte *Glaux maritima,* from a periodically sea-water inundated coastal meadow ,has typical features of an obligate halophyte in conditions of tissue culture. Therefore, it can be concluded that, for successful ex situ conservation of wild plant species from salinity-affected habitats by means of tissue culture, a medium with elevated NaCl content should be used for the best results.

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