



Differential esterase activity in leaves and roots of *Centaurea ragusina* L. as a consequence of salinity

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Abstract

Background and Purpose: *Centaurea ragusina* L. is an endemic Croatian plant species adapted to life in arid and salt-affected environments. Esterase activity (EST) and isoesterase patterns of *C. ragusina* plants cultured in vitro under saline and osmotic conditions were determined with an aim to evaluate the potential value of esterase activity as a biomarker of salt/osmotic stress as well as physiological significance of EST variations.

Material and Methods: Rooted plantlets grown on MS $\frac{1}{2}$ nutrient media supplemented with 150, 300, 450 or 600 mM NaCl or 300 mM mannitol were investigated after 5, 10 and 15 days. Esterase activity was determined using either 1- or 2-naphthylacetate as substrates.

Results and Conclusion: Esterase activities in shoots increased under low saline and mannitol treatments and decreased in response to increased saline treatments. The highest salt and concentration and mannitol stimulated EST activity in roots. In total, twelve and fourteen esterase isoenzymes were resolved in *C. ragusina* leaves and roots, respectively. Both salt and mannitol induced new esterase isoenzyme (EST11) while mannitol specifically induced two more (EST4-5); otherwise some bands were weakly expressed or even disappeared as a result of salinity in *C. ragusina* leaves. Two new isosterases (EST10-11) were resolved in mannitol- and salt-treated roots and four (EST2, EST12-14) only in salt-treated roots. Presented results demonstrate that esterase activities and their isoenzymic patterns could serve as useful bioindicators of salinity.

INTRODUCTION

All organisms that exist within natural environments are subjected to stress at some point of their lives. The two major environmental factors that currently reduce plant productivity are drought and salinity which both cause similar reactions in plants due to water stress. The problem is becoming more prevalent as the intensity of agriculture increases (1). Therefore, it is important to understand how plants respond and adapt to such types of stress. Besides imposing osmotic stress, salinity induces ionic stress driven by toxic action of Na⁺ and Cl⁻ ions. Plants need essential mineral nutrients to grow and develop. However, excessive soluble salts in the soil are harmful to most plants. In fact, no toxic substance restricts plant growth more than salt on a world scale (2). This has led to research into salt tolerance with an aim of improving crop plants. Salt-tolerant plants have higher capacity than salt-sensitive ones to reduce cytosolic Na⁺ by storing it in the vacuole and thus avoiding sodium toxicity. Hyperosmolarity also generates sec-

ondary stress like oxidative stress which is caused by excessive amounts of reactive oxygen species (ROS). ROS can have damaging effect on cellular structures and macromolecules – lipids, proteins and DNA. Besides non-enzymic compounds of low molecular weight, plants have developed numerous antioxidative enzymes against ROS (3).

Change (usually increase) in the level of detoxification enzyme activity, such as peroxidase, has been used as a potential biomarker of many toxic compounds like heavy metals, pesticides, but also of stresses caused by salinity, extreme temperatures, drought, ozone, etc. (3, 4, 5). In our previous study, peroxidase activity of *Centaurea ragusina* L. was markedly stimulated by mannitol and also by NaCl, though only up to 300 mM (6). Two peroxidase isoenzymes were common to both mannitol- and salt-treated *C. ragusina* plants while one isoenzyme was induced by salt only.

Here we investigated esterase activity as a potential biomarker of salt stress using *Centaurea ragusina* L. as a plant model. As this plant grows in the gaps of vertical limestone cliffs along the coast and on some islands of Adriatic Sea, they are primarily affected by drought and high light intensities and indirectly by salinity via seawater aerosol (7). Esterases, a group of hydrolases, catalyze the formation or cleavage of ester bonds of water soluble substrates. Generally, these enzymes have a broad spectrum of substrates and act on a variety of natural and xenobiotic compounds (8). Naphthylacetates (1- and 2-), used for esterase activity and visualization in *C. ragusina* leaves and roots are substrates of arylesterases (EC 3.1.1.2), which are inhibited by sulfhydryl reagents and prefer aromatic substrates, and of carboxylesterases (EC 3.1.1.1) which are inhibited by organophosphates and generally prefer aliphatic esters (9). Esterases have been extensively studied in insects and vertebrates but much less in plants. Since esterases exist in different isoenzymes in plant and animal tissues, their electrophoretic pattern was also analyzed. The relationship between esterase activity and salinity has been investigated in several plant species, irrespective of their tolerance to salt (10, 11, 12). Thus, the aim of the present study was to establish a possible correlation between salt tolerance and esterase activity (EST) of *C. ragusina* plants. Effects of isoosmotic concentrations of NaCl (150 mM) and mannitol (300 mM) were compared in order to discriminate possible differences in *C. ragusina* response to ionic (NaCl) and non-ionic (mannitol) component of salinity.

MATERIALS AND METHODS

C. ragusina seeds were collected from natural habitat on the island of Palagruža. The sterilized seeds were inoculated in test tubes filled with 15 mL of MS ½ medium containing 0.1 g L⁻¹ myo-inositol, 0.1 mg L⁻¹ thiamine×HCl, 0.5 mg L⁻¹ pyridoxine×HCl, 0.5 mg L⁻¹ nicotinic acid, 2.9 mM gibberellic acid (GA₃), 0.5 mM 6-benzylaminopurine (BA), 30 g L⁻¹ sucrose and 8 g L⁻¹ agar (13). The shoots isolated from the seedlings were first

subcultured on the same composition media. After 4 weeks in culture, the shoots were transferred to liquid MS ½ medium containing 2.5 mM indole-3-butyric acid (IBA). Rooted plantlets were transferred to the same composition media supplemented with different concentrations of NaCl (150 mM, 300 mM, 450 mM or 600 mM) and mannitol (300 mM), while control plants were kept in nutrient solution supplemented with IBA during the entire assay.

Enzyme extraction and soluble protein determination

Leaf (250 mg) and root (100 mg) samples were ground at 4 °C using mortar and pestle and tissue extracts were prepared in an ice cold 50 mM potassium phosphate (K₂HPO₄/KH₂PO₄) buffer pH 7.0 containing 1 mM EDTA, with addition of insoluble polyvinylpyrrolidone (PVP-10). The homogenates were centrifuged at 22 000 ×g for 30 min at 4 °C (Sigma 3K18 Centrifuge). Supernatants were used for esterase activity and soluble protein analysis. Soluble protein content was determined by the method of Bradford using bovine serum albumin (BSA, Sigma) as standard (14).

Esterase assays

Esterase activity was determined spectrophotometrically at room temperature (25°C) using either 1- or 2-naphthylacetate as substrates (15). Esterase activity was calculated following the increase in absorbance at 322 nm (for 1-naphthylacetate) or 313 nm (for 2-naphthylacetate), due to the formation of 1-naphthol ($\epsilon_{322\text{nm}} = 2.0 \text{ mM}^{-1}\text{cm}^{-1}$) or 2-naphthol ($\epsilon_{313\text{nm}} = 1.25 \text{ mM}^{-1}\text{cm}^{-1}$) (16). The reaction mixture contained 1.0 ml 100 mM Tris/HCl pH 7.4 and 18 µL 100 mM 1- naphthylacetate or 15 µL 100 mM 2- naphthylacetate, both dissolved in absolute methanol. For each measurement 30 µL of crude extract was used. The enzyme activity was measured every 15 seconds over a three-min-period after a 30 second lag period. The esterase activities were corrected for spontaneous hydrolysis of 1- and 2-naphthylacetate.

The activity was expressed as µmol of hydrolysed substrate per min per mg prot (µmol min⁻¹ mg protein⁻¹).

Statistical analysis

For each analysis, data were compared by analysis of variance (ANOVA), using STATISTICA 7.1 (StatSoft, Inc., USA) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at P < 0.05. Each data point is the average of nine replicates.

Activity gel analysis

Tissue extracts were analyzed electrophoretically under non-denaturing conditions using vertical 10% polyacrylamide slab gels with the buffer system of Laemmli without the SDS (17). A constant voltage of 200 V was applied for 4 h and the temperature maintained at 4°C. Equal amounts of protein (50 µg per well) were loaded

onto each lane. The esterase isozymes were visualized according to modified procedure described by Balen *et al.* (16). One- and 2-naphthyl acetates, used as substrates (40 mg each), were dissolved in 16 mL of 50% (v/v) acetone and mixed with 100 mL of 50 mM Tris/HCl pH 7.1. After staining (30 min), the gels were washed with tap water and incubated in a solution containing 50 mM Tris/HCl pH 7.1 and Fast Blue RR salt until dark brown (1-naphthyl acetate) or purple red (2-naphthyl acetate) bands appeared (20–30 min). The Fast Blue RR salt (200 mg) was dissolved in 10 mL of absolute methanol and filtered into 50 mM Tris/HCl pH 7.1. The gels were once more rinsed with tap water and fixed in 30% (v/v) ethanol.

RESULTS

Morphology of *C. ragusina* plants

Morphological appearance of *C. ragusina* leaves grown in the presence of isoosmotic concentrations of salt (150 mM) or mannitol (300 mM) and without either of them (control) *in vitro* conditions is presented in Figure 1. Compared to control plants, *C. ragusina* stems and leaves become fleshy and succulent under saline conditions. Following hyperosmotic stress induced by mannitol, plantlets lost freshness and turgescence, gradually wilted and became yellow.

Esterase activity of *C. ragusina* plants

Esterase activity was evaluated with two substrates, 1- and 2-naphthylacetate. Generally, esterase activity of *C. ragusina* leaves measured with 1-naphthylacetate (1-EST) decreased with increasing salt concentrations (Figure 1), i.e. 1-EST activity significantly increased in response to

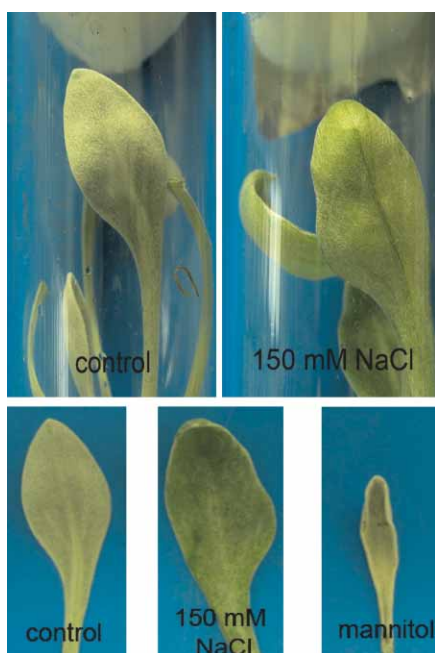


Figure 1. Leaf morphology of tissue cultured control, salt- (150 mM NaCl) and mannitol-affected *C. ragusina* plants.

the lowest salt treatment (150 mM NaCl), showed no change in response to 300 mM NaCl, and decreased in response to higher salt treatments (450 and 600 mM). Mannitol caused considerable increase in 1-EST activity in *C. ragusina* leaves after 10 and 15 days. In roots, 1-EST activity was basically affected only by the highest salt treatment after 10 and 15 days and by mannitol after 15 days; otherwise the activity was similar to control.

The esterase activity of *C. ragusina* leaves assayed with 2-naphthylacetate (2-EST) was almost two times lower compared to the activity assayed with 1-naphthylacetate (Figure 2). After 10 and 15 days, lower (150 and 300 mM) and higher (450 and 600 mM) salt concentrations caused increase and decrease in leaf 2-EST activity, respectively. In roots, 2-EST activity was after 5 days elevated in response to 300 mM NaCl. A significant increase in 2-EST activity of *C. ragusina* roots was obtained at 600 mM NaCl after 10 and 15 days. Osmotic stress caused by mannitol continuously increased 2-EST activity of both leaves and roots.

Esterase isoenzymes of *C. ragusina* plants

Results presented in Figure 3 show variations in the esterase isoenzyme pattern of *C. ragusina* leaves and roots under mannitol and different salt treatments. In total, twelve esterase isoenzymes were resolved in *C. ragusina*

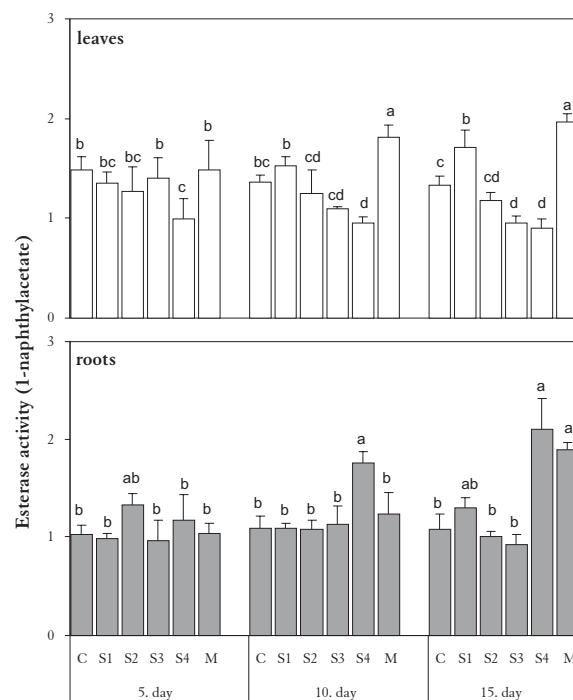


Figure 2. Esterase activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) during a 15-day growth period with 1-naphthylacetate as substrate in *C. ragusina* leaves and roots under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions. Values are mean \pm S.E. based on six replicates. Bars with different letters are significantly different at $P < 0.05$.

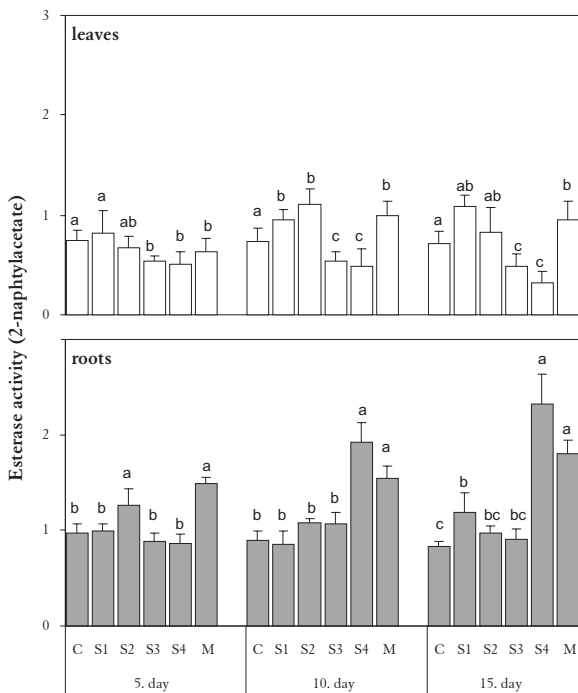


Figure 3. Esterase activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) during a 15-day growth period with 2-naphthylacetate as substrate in *C. ragusina* leaves and roots under control (C) and stress – 150mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions. Values are mean \pm S.E. based on six replicates. Bars with different letters are significantly different at $P < 0.05$.

leaves but only four (EST1, EST6-8) were common to all extracts. Those isoenzymes accumulated less under high salt treatments. Isoesterases EST2 and EST3 were present after five days in all leaf extracts and after 10 and 15 days in control and 150 mM NaCl-treated leaves. Mannitol induced EST4 and EST5 after 10 and 15 days. Isoesterase EST9 was observed as a faint band in all leaf extracts after 5 days and was absent in control and mannitol-treated leaves after 10 and 15 days. Isoesterase EST10 was expressed in control and salt-treated leaves and was absent under mannitol treatment. Its coloration was intensive in response to control after 5 days and in response to lower salt concentrations during all the time. Isoesterase EST11 was induced only after 15 days by higher salt concentrations and mannitol. Isoesterase EST12 was noticed in all extracts after 5 and 10 days. After 15 days it was present only under higher salt and mannitol concentrations. Five leaf EST isozymes (EST2, EST6-9) reacted with 1-naphthyl acetate which gave dark brown coloration, two (EST3, EST11) reacted with 2-naphthyl acetate which gave purple red coloration and four isoenzymes (EST1, EST4-5, EST10) reacted with both substrates (data not shown).

Although fourteen esterase isoenzymes were resolved in *C. ragusina* roots, only six (EST1, EST3-4, EST6-8) were observed in all extracts during the entire observation period. Isoesterases EST3 and EST4 accumulated

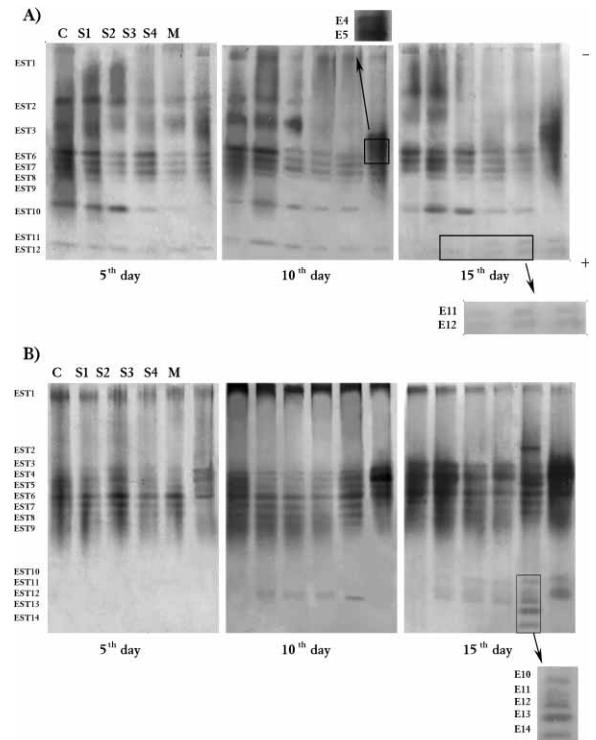


Figure 4. Electrophoretic patterns of leaf and root esterase isoenzymes under control and stress conditions during a 15-day growth period. Gels were incubated in a solution of both substrates together. Equal amount of proteins (50 μg per well) was loaded onto each gel lane. C-control, S1-150 mM NaCl, S2-300 mM NaCl, S3-450 mM NaCl, S4-600 mM NaCl, M-300 mM mannitol.

more in response to mannitol. Isoesterase EST5 was visible after 5 and 10 days in all extracts whereas after 15 days it appeared only in response to 600 mM NaCl. Salt induced isoenzymes EST9 and EST12. Isoenzyme EST9 appeared as a faint band after 5 days under lower salt concentrations and after 10 and 15 days at 600 mM. Isoenzyme EST12 was noticed as a faint band under all salt treatments except the highest one under which it was more intensely expressed. Both salt and mannitol induced isoenzymes EST10 and EST11 at 15 days but the isoenzymes were more pronounced under mannitol stress. Root isoenzymes EST2, EST13 and EST14 were specifically induced by 600 mM NaCl after 15 days of growth. Under that treatment, very intensive coloration of isoenzymes EST5-9 was noticed. Almost half of root EST bands were dark brown (EST2, EST5-9) and the other half purple red (EST3-4, EST10-11, EST14) depending on the reaction with 1-naphthyl acetate and 2-naphthyl acetate, respectively. Three isoenzymes (EST1, EST12-13) reacted with both substrates (data not shown).

DISCUSSION

The response of plants to salt stress is based on the action of many defense proteins/enzymes (18). Plant isoenzymes and esterase activity have been related to heavy

metal and pesticide toxicity, pathogenesis, morphogenesis and embryogenic potential (19, 20, 21). In the present study, esterase activity and isoesterase pattern were studied in leaves and roots of *C. ragusina* subjected to NaCl- and mannitol-induced stress. In this plant species, esterase activity differed less with regard to substrates than to plant organs, as with both 1- and 2-naphthylacetate the activity in leaves was much more affected than that in roots. Stimulation of leaf esterase activity was evident under lower (150 and 300 mM NaCl) salt concentrations (Figure 1, 2). Similarly, mung bean (*Vigna radiata*) and *Sueda maritima* plants, cultured in *in vitro* conditions, exhibited the highest esterase activity between 150 and 400 mM NaCl (12). Increase of esterase activity was also observed in *Lemna minor* plants (duckweed) exposed to lead, cadmium, chromium, zinc, copper and mercury (22). In the study, authors imply esterase variation as well as the induction of several new esterase isoenzymes to be adaptive mechanism of duckweed to metal contamination. In agreement with that view, stimulation of *C. ragusina* esterase activity with increasing salt treatments could be related to salt tolerance of the species achieved through ion accumulation (6). However, as higher salt concentrations (450 and 600 mM NaCl) suppressed both 1- and 2-EST of *C. ragusina* leaves, positive correlation between esterase activity and salt concentration seems to exist only up to as high as 300 mM NaCl. It has been demonstrated that salt concentrations higher than 400 mM NaCl inhibit most enzymes because of the perturbation of the hydrophobic-electrostatic balance between the forces maintaining protein structure (18). Opposite to that, esterase activities of salt-treated roots showed almost no difference in comparison to control apart from the highest salt (600 mM NaCl) concentration which caused considerable increase in 1- and 2-EST activities. Comparison of isoosmotic NaCl (150 mM) and mannitol (300 mM) concentrations, it is evident that mannitol caused much greater stimulation of esterase activity in both *C. ragusina* leaves and roots. The result might reflect different osmoregulation of *C. ragusina* to osmotic stress caused by NaCl and mannitol. Succulent and enlarged leaves and stems of *C. ragusina* observed on saline media, suggests that *C. ragusina* uses salt ions as relatively cheaper compatible solute in comparison to organic solutes such as proline or sucrose (6). Thus, by much greater esterase activity detected in *C. ragusina* plants exposed to mannitol-induced osmotic stress, the cells would be provided with enough components and/or energy needed for synthesis of organic solutes. Indeed, the metabolic costs i.e. the number of moles of ATP for osmotic adjustment achieved by accumulation of synthesized organic solutes, proved to be much higher than using NaCl instead (23). Mannitol-induced growth inhibition of *C. ragusina* plants noticed in our previous study (24) might be explained by higher production of organic solutes.

The esterase pattern in *C. ragusina* displayed tissue specificity. The number of isoenzymes in roots was higher than in leaves of control plants. Another example of

the variability observed between leaf and root esterases is their difference in substrate preference. Esterase activity measured with 1-naphthyl acetate (1-EST) in *C. ragusina* leaves was two times higher than the activity measured with 2-naphthyl acetate (2-EST). This corresponded to isoesterase pattern of *C. ragusina* leaves where the majority of bands were dark brown and only few were purple red after gel coloration with 1-naphthyl acetate and/or 2-naphthyl acetate, respectively. Oppositely, preference of esterase toward particular substrate was not observed in *C. ragusina* roots.

Variability between leaf and root esterases was also observed under salt stress. Generally, the number of isoesterases in *C. ragusina* leaves was the same as in control while that of roots increased under the influence of NaCl and mannitol. Salt induced one new isoenzyme in leaves and seven in roots of *C. ragusina* plants. The esterase patterns of peanut (*Arachis hypogaea* L.) roots exposed to salinity were also more affected compared to those in leaves (11). In the study, salt stress induced 4–5 isoenzymes in peanut roots versus three new isoenzymes in its leaves after two-week period. However, some of salt-induced esterase isoenzymes in *C. ragusina* roots (EST 10 and 11) were also induced by mannitol which points to some differences in *C. ragusina* response to ionic and osmotic component of salinity. In addition, two esterase isoenzymes (EST4, EST5) in *C. ragusina* leaves resulted only from mannitol-treatment.

Majority of new isoesterases appeared as late as at the end of the two-week period (only few appeared after 10 days), suggesting *de novo* synthesis of new esterase isoenzymes. In confirmation with the postulate, induction of esterase activity caused by mannitol and salt was noticed after 10 and especially after 15 days. However, some saline treatments also induced new isoenzymes although esterase activities were decreased or similar to control.

The wilting of *C. ragusina* plants observed upon mannitol treatment and development of succulence in response to isoosmotic concentration of salt implies tolerance of *C. ragusina* to NaCl-induced osmotic stress.

In conclusion, isoesterases proved to be more suitable biomarkers of salt/osmotic stress than isoperoxidases as a greater number of specifically induced isoenzymes was observed in *C. ragusina* esterase pattern (ten in total) than in its peroxidase pattern (six in total). Thus, the presented results demonstrate that esterase activities and their isoenzymic patterns could serve as useful bioindicators of salinity.

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