

## Variations in Four Genotypes of Lentil under NaCl-Salinity Stress

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**Abstract:** Four lentil cultivars, Eston (E), Rossa of Castelluccio (RC), Pantelleria (P) and Ustica (U) differing in salt sensitivity, were examined for osmolyte contents and activities of  $\alpha$ -amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase enzymes involved in seed germination, in absence as well as in presence of 50, 100, 150 and 200 mM NaCl. The inhibitory effects of NaCl differed, depending on the genotypes tested. In Castelluccio and Eston, cultivars with reduced germination percentage and lower relative water content, the increase in NaCl concentration resulted in the decrease in endogenous levels of proline, total soluble sugars and activities of the main enzymes involved in the germination process. In contrast, Pantelleria and Ustica seeds in response to salt stress accumulated higher proline and total soluble sugar concentrations which improved their water status and the enzyme activities involved in the germination process. Differential response of the different genotypes of lentil to salt stress may be governed by the accumulation of osmolytes in seeds.

**Key words:** Amylases, glucosidase, proline, salt stress, seed germination

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### INTRODUCTION

The progressive salinization of land is one of the most severe world wide problems in agriculture production<sup>[1, 2]</sup>, being the major common abiotic stress for plant in arid or semiarid regions. Salts that generally are a common and necessary component of soil, essential plant nutrient, up to 45 ds m<sup>-1</sup> (50 mM) can cause reduction in water availability, sodium ions accumulation and mineral imbalances that affect seed germination and plant metabolism<sup>[3]</sup>. All these factors manifest themselves by morphological, physiological and metabolic modifications in plant such as decrease in seed germination, decrease in shoot and root length<sup>[4]</sup>, alterations in the integrity of cell membranes, inhibition of different enzymatic activities and photosynthesis<sup>[5]</sup>. Lentil (*Lens culinaris* M.) is considered a strategic crop under agronomic and food point of view, because of its role as possible component of the cropping systems in the Mediterranean areas and as a protein source for human and animal consumption<sup>[6,7,8]</sup>. The species is classified as salt sensitive<sup>[9]</sup> like many other leguminous. Selection for salinity resistance appears as a laborious and hazardous

task and plant breeders are, therefore, seeking for quick, cheap and reliable ways to assess the salt-resistance of selected material. Determination of germination potential of seeds in saline conditions could appear as a simple and useful parameter for several reasons. First, salinity resistance at this stage was shown to be a heritable trait which could be used as an efficient criterion for the selection of salt-resistant populations<sup>[10]</sup>, although it is a polygenic character linked to a complex genetic basis<sup>[11]</sup>. Second, seeds and young seedlings are frequently confronted by much higher salinities than vigorously growing plants because germination usually occurs in surface soils which accumulate soluble salts as a result of evaporation and capillary rise of water. Several studies demonstrated that the evolution of salinity resistance is not the same for all cultivars of a given species<sup>[12, 13]</sup>. Selection of salt tolerant lentil genotypes would allow one to cultivate this crop on saline soils or with saline waters which occur frequently in the Mediterranean areas.

Complex molecular responses including the accumulation of compatible solutes, the production of stress proteins and the expression of different sets of genes are part of the plant signalling and defence

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system against salinity<sup>[5]</sup>. It is well known that one of the most common responses to salinity is the overproduction and accumulation of proline, glycine betaine and total sugars. Solute accumulation by cells is one such mechanism (osmoregulation) which contributes to stabilization of enzyme/protein<sup>[14]</sup> and turgor maintenance<sup>[15]</sup> in growing organs and has been correlated with productivity under stress<sup>[16]</sup>. In the present study, four genotypes of lentil with different salinity tolerance<sup>[17]</sup> were studied to determine factors responsible for failure of germination. To fulfil such an aim, the effects of different NaCl concentrations were evaluated on relevant biochemical processes associated with seed germination.

## MATERIALS AND METHODS

**Plant materials:** The experiment included three lentil landraces, Pantelleria, Ustica and Castelluccio di Norcia and a variety Eston. In particular, Pantelleria and Ustica are native of the homonymous small islands close to Sicily (Southern Italy); Castelluccio di Norcia is a traditional local population of Umbria (Central Italy), whereas Eston is a Canadian commercial variety recently introduced in Italy.

**Germination condition:** Seven months old well stored ( $20\pm 1^\circ\text{C}$  and  $\pm 5\%$  RU) seeds of each lentil genotype used in the experiment were surface-sterilized for 20 min in 30% (v/v)  $\text{H}_2\text{O}_2$ , rinsed and soaked in distilled water for 1 h. Fifty representative seeds per cultivar were placed on a filter paper in 9 cm Petri dishes containing 3  $\text{cm}^3$  of distilled water (control), or 50, 100, 150 and 200 mM NaCl. The Petri dishes were hermetically sealed with parafilm to prevent evaporation and then care kept in a humidity chamber at a temperature of  $25\pm 1^\circ\text{C}$  in the dark. The seeds were considered germinated when there was radicle protrusion through the seed coat. In order to determine the dry weight, twenty-five seeds of each cultivar, were taken out and were dried at  $70^\circ\text{C}$  in an oven till there is no decrease in weight.

**Enzyme assays and osmolytes content:** Alpha-, beta-amylase and alpha-glucosidase activities in the crude extracts of each cultivar were determined. The seeds of each cultivar, in deionised water (control) and treated with different concentrations of NaCl (50, 100, 150, 200 mM) were homogenised in a chilled mortar with distilled water 1:4 (w/v) and centrifuged at 14000 g for 30 min. The supernatants were filtered through a single layer of muslin cloth and were used for  $\alpha$ -amylase (EC 3.2.1.1)<sup>[18]</sup>,  $\beta$ -amylase (EC 3.2.1.2)<sup>[19]</sup>,  $\alpha$ -glucosidase (EC 3.2.1.20)<sup>[19]</sup> estimation.

For determination of proline contents seeds were hand-homogenized in 3% of sulfosalicylic acid and

centrifuged at 3000g at  $4^\circ\text{C}$  for 10 min. The supernatants were used for proline estimation<sup>[20]</sup>.

The total soluble sugars were determined with the anthrone method<sup>[21]</sup>.

**Experimental design and statistical analysis:** Data were analyzed separately for each genotype by one way procedure of ANOVA ( $p\leq 0.05$ ) according to a completely randomized design with five replicates. Treatment means were compared using the Student-Newman-Keul test ( $p\leq 0.05$ )<sup>[22]</sup>.

## RESULTS

Germination of all cultivars began from 24 h after sowing, reaching germination percentage higher than 98% in control seeds. Salinity caused a delay in germination that was different among the cultivars. At 48 and 72 h the germination percentage of Castelluccio and Eston under salinity was significantly lower compared to Pantelleria and Ustica. However, at 200 mM NaCl, final germination percentage was significantly reduced in Eston (50%) and even more in Castelluccio (10%) cultivars, while under control condition 100 and 98% germination occurred for both cultivars, respectively. The cultivars Pantelleria and Ustica germinated more than the other ones (74 and 82%), exhibiting a fair degree of salt tolerance. Increasing salinity a gradual decrease in the RWC in all cultivars was observed. The lower water content was detected in Castelluccio and Eston genotypes<sup>[17]</sup>.

The proline content of Pantelleria and Ustica cultivars was enhanced at different salt concentrations (Fig. 1). The highest amount of proline was observed at 150 and 200 mM NaCl for both cultivars. Interestingly, Pantelleria and Ustica accumulated two times more proline than Castelluccio and Eston (Fig. 1).

The level of total soluble sugars increased to a smaller extent over stressed seeds of Castelluccio and Eston cultivars. On the contrary, as a consequence of stress, in Pantelleria and Ustica genotypes, increasing salinity a gradual increase in total soluble sugars was observed.

Salinity had pronounced effects on  $\alpha$ -amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase activities. The activity of these enzymes decreased in a dose-dependent manner, differing among the cultivars. The effect of salinity was more pronounced in seeds of cultivars Eston and even more Castelluccio.

$\beta$ -amylase and  $\alpha$ -glucosidase activities showed also a decreasing trend with the increase in NaCl concentration. The activities of  $\beta$ -amylase and  $\alpha$ -glucosidase in stressed seeds of Pantelleria and Ustica were higher compared to those detected in Castelluccio and Eston genotypes (Table 1-3).

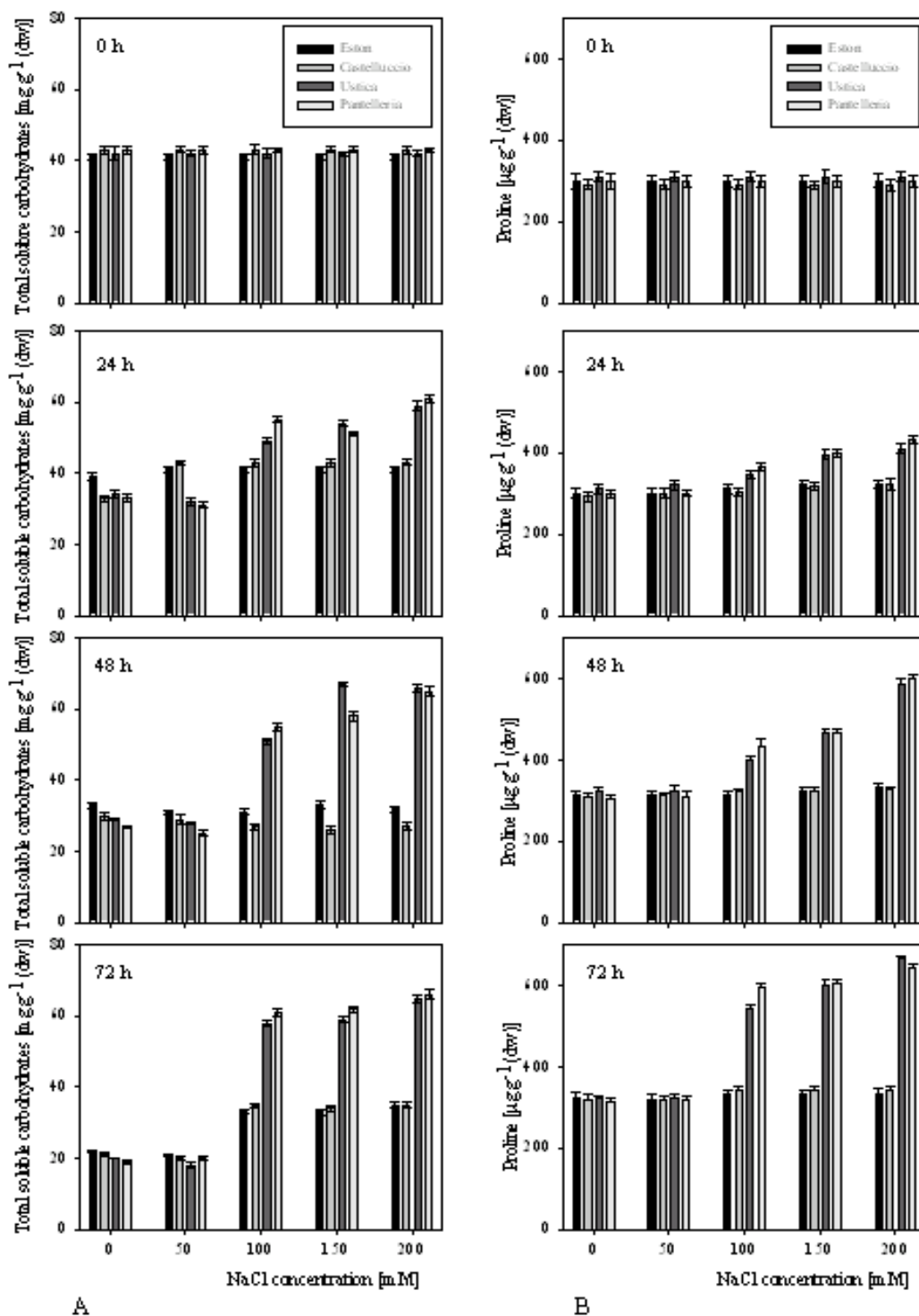


Fig. 1: Total soluble carbohydrates (A) and proline content (B) in Eston, Castelluccio di Norcia, Ustica and Pantelleria lentil cultivar seeds in 0, 50, 100, 150 and 200 mM NaCl at 0, 24, 48 and 72 hours. Vertical bars indicate SD (n=5)

Table 1:  $\alpha$ -Amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase activities in control and in lentil cultivars: Eston (Canadian commercial variety), Castelluccio di Norcia (traditional local population of Central Italy), Pantelleria and Ustica (native of the homonymous small islands in Southern Italy) that had been exposed for 24 h to different salt concentrations. Enzyme activities are expressed as  $\mu$ moles of reducing sugars formed  $\text{min}^{-1}\text{g}^{-1}\text{fw}$

Enzymes	cv.	Treatments				
		Control	50 mM	100 mM	150 mM	200 mM
$\alpha$ -amylase	Eston	16.10a	15.43ab	14.01b	10.50b	8.10c
	Castelluccio	17.20a	4.50b	4.05b	1.91d	2.04c
	Ustica	26.11a	26.51a	22.93b	19.04c	17.96d
	Pantelleria	26.43a	26.25a	24.39b	18.18c	16.00d
$\beta$ -amylase	Eston	0.75a	0.41b	0.44b	0.21c	0.18d
	Castelluccio	0.70a	0.21b	0.21b	0.11c	0.10c
	Ustica	1.00b	1.15a	1.01b	1.11a	1.14a
	Pantelleria	0.98c	1.40a	1.11b	1.10b	0.84d
$\alpha$ -glucosidase	Eston	0.271a	0.280a	0.190b	0.091c	0.081c
	Castelluccio	0.220a	0.091b	0.041c	0.031d	0.011e
	Ustica	0.301a	0.294b	0.291b	0.190c	0.100d
	Pantelleria	0.310a	0.281b	0.290b	0.189c	0.095d

Means in the same row with the same letter are not significantly different at  $p \leq 0.05$

Table 2:  $\alpha$ -Amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase activities in control and in lentil cultivars: Eston (Canadian commercial variety), Castelluccio di Norcia (traditional local population of Central Italy), Pantelleria and Ustica (native of the homonymous small islands in Southern Italy) that had been exposed for 48 h to different salt concentrations. Enzyme activities are expressed as  $\mu$ moles of reducing sugars formed  $\text{min}^{-1}\text{g}^{-1}\text{fw}$

Enzymes	cv.	Treatments				
		Control	50 mM	100 mM	150 mM	200 mM
$\alpha$ -amylase	Eston	16.40a	16.30a	15.01b	13.25c	6.10d
	Castelluccio	15.50a	4.50b	4.55b	2.01c	2.04c
	Ustica	27.11a	26.90a	21.03b	19.04c	15.66d
	Pantelleria	26.10a	27.25b	23.39c	20.18d	13.95e
$\beta$ -amylase	Eston	0.92a	0.49b	0.51b	0.29c	0.21d
	Castelluccio	0.81a	0.29b	0.27c	0.15d	0.12e
	Ustica	1.41a	1.21b	1.19b	1.01d	1.15c
	Pantelleria	1.30b	1.61a	1.21c	1.24bc	0.92d
$\alpha$ -glucosidase	Eston	0.281a	0.296a	0.193b	0.095c	0.090c
	Castelluccio	0.230a	0.091b	0.049c	0.039d	0.016e
	Ustica	0.321a	0.300b	0.296b	0.200c	0.101d
	Pantelleria	0.318a	0.290b	0.294b	0.192c	0.096d

Means in the same row with the same letter are not significantly different at  $p \leq 0.05$

Table 3:  $\alpha$ -Amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase activities in control and in lentil cultivars: Eston (Canadian commercial variety), Castelluccio di Norcia (traditional local population of Central Italy), Pantelleria and Ustica (native of the homonymous small islands in Southern Italy) that had been exposed for 72 h to different salt concentrations. Enzyme activities are expressed as  $\mu$ moles of reducing sugars formed  $\text{min}^{-1}\text{g}^{-1}\text{fw}$

Enzymes	cv.	Treatments				
		Control	50 mM	100 mM	150 mM	200 mM
$\alpha$ -amylase	Eston	16.43a	17.43a	15.43b	13.7c	7.80d
	Castelluccio	12.083a	1.085b	1.054b	1.045b	1.049b
	Ustica	24.97a	24.58a	21.43b	14.84c	16.76d
	Pantelleria	26.03a	25.03b	25.39b	21.86c	15.90d
$\beta$ -amylase	Eston	1.16a	0.58b	0.59b	0.31c	0.27d
	Castelluccio	0.98a	0.35b	0.32c	0.21d	0.16e
	Ustica	1.74a	1.45b	1.35c	1.16d	1.16d
	Pantelleria	1.45b	1.74a	1.36c	1.32c	0.98d
$\alpha$ -glucosidase	Eston	0.298a	0.301a	0.211b	0.101c	0.098c
	Castelluccio	0.133a	0.102b	0.054c	0.043d	0.021e
	Ustica	0.345a	0.302b	0.300b	0.211c	0.110d
	Pantelleria	0.320a	0.298b	0.298b	0.201c	0.102d

Means in the same row with the same letter are not significantly different at  $p \leq 0.05$ .

## DISCUSSION

Genetic variability within a species offers a valuable tool for studying mechanism of salt tolerance. Our results show a decrease in germination and vigour of seeds in all studied genotypes, with increasing salinity. In the cultivars Castelluccio and, in minor extent, in Eston the magnitudes of such decrease were more as compared to that of cultivars Ustica and Pantelleria, in particular way when high salinity concentrations were used. The germination process can be considered in terms of three sequential steps: imbibition, metabolism leading to initiation of radicle growth and radicle growth leading to radicle emergence. A threshold level of hydration is required for the synthesis of hydrolytic enzymes which are responsible for the hydrolysis of stored substrates. The hydrolyzed products are utilized in seedling tissue synthesis and radicle elongation<sup>[23]</sup>. In lentil seeds, the role of providing utilizable substrates is taken over mainly by amylases. Inhibition of germination due to salinity as suggested in previous reports<sup>[24,25,17]</sup> is attributed to a decrease water content, that affect the synthesis of hydrolytic enzymes limiting the hydrolysis of food reserves from storage tissues as well as to impaired translocation of food reserves from storage tissue to developing embryo axis<sup>[26,27]</sup>. It can be also hypothesized that the presence of NaCl at low concentrations, which is penetrating ions, could have contributed to a decrease in the internal osmotic potential of germinating structures, as suggested by Dodd and Donovan<sup>[28]</sup> and Almasouri *et al.*<sup>[13]</sup> leading to water uptake and initiation of germination processes. Our results indicate a lower content of total soluble sugar and proline in presence of the highest salt concentration in Castelluccio and Eston compared to Ustica and Pantelleria cultivars, suggesting that salt tolerance ability of these two last landraces appears to be associated to the accumulation of osmolytes which improved their water status. Salt stress has been reported to limit the mobilization of starchy endosperm reserves in several species, as a result of inhibition of different enzymatic activities<sup>[29,30]</sup>. Starch mobilization results from simultaneous activities of  $\alpha$ -amylase,  $\beta$ -amylase and  $\alpha$ -glucosidase. In germinating seeds, starch degradation is initiated by  $\alpha$ -amylase<sup>[31]</sup>, that produces soluble oligosaccharides from starch and these are then hydrolyzed by  $\beta$ -amylase to liberate maltose. Finally,  $\alpha$ -glucosidase breaks down maltose into glucose, the main respiratory substrate<sup>[32]</sup>. Numerous works correlated germination performance with  $\alpha$ -amylase, but in a study of the comparative importance of  $\alpha$  and  $\beta$ -amylase in

determining germination ability, Das and Sen-Mandi<sup>[33]</sup> demonstrated greater importance of  $\beta$ -amylase compared to  $\alpha$ -amylase, during the early hours of germination in wheat scutella. In addition, Nandi *et al.*<sup>[34]</sup> showed that  $\beta$ -amylase activity becomes detectable immediately before visible germination becomes evident, whereas  $\alpha$ -amylase activity is initiated at later stage of germination, suggesting that  $\alpha$ -amylase affects rate of seedling growth while  $\beta$ -amylase activity is associated with initiation of germination. Therefore,  $\beta$ -amylase is a crucial and essential enzyme for germination. Our results show that high salt concentrations adversely affected each enzyme in all genotypes examined. Nevertheless in this work  $\beta$ -amylase activity was always significantly lower in Castelluccio and Eston compared to their own control and salt treated Pantelleria and Ustica at every concentration used. In addition also when Castelluccio and Eston were treated with low salt concentration (50 mM),  $\beta$ -amylase activity at 72 h was lower than that detected at 24h in their own control or in Pantelleria and Ustica salt treated seeds at every concentration used, suggesting that this enzyme could be salt sensitive related. The variation in stress sensitivity of contrasting lentil genotypes may be linked to their ability to osmoregulate under stress, which cause a strong decrease in water content affecting the hydrolytic enzyme activities, particularly  $\beta$ -amylase levels.  $\beta$ -amylase is probably synthesized during imbibition, in fact a seed hydration pre-treatment in rice or other cereals species, that enhances seed vigour was found also to bring about an enhancement in  $\beta$ -amylase activity<sup>[34]</sup>. Thus, data presented in all these reports provide support for the view that the higher  $\beta$ -amylase activity in Pantelleria and Ustica cv. might be the cause for the major seed germination in presence of NaCl salinity.

Some important conclusions can be drawn from the results achieved during this experiment. Although lentil is considered a very sensitive specie to salinity, much more than other legumes such as broadbean and soybean, we have identified two cultivars, Ustica and Pantelleria, found in a southern Italian semiarid environment, that could be utilized not only in breeding programs to improve the saline resistance of the species but also to be cultivated in environments where salinity of the soils is a frequent constraint.

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