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Protein patterns, characterized by computer image analysis, of lentil embryo axes germinating under salt stress

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Abstract

Following 16, 40 and 64 h exposure to 0.33 M NaCl given after 8 h water imbibition, lentil seeds showed a gradual decrease of germination upon their transfer to water. These salt related changes were accompanied by modifications in the protein patterns of embryo axes as revealed by two-dimensional electrophoresis separation and by the computer image analysis of protein spots. In comparison with 8 h water imbibed seeds, prominent proteins comprised between the 5.1 - 7.6 pH isoelectric point in the first dimension and 75 - 50 kDa molecular mass in the second dimension showed a significant increase in their abundance as salt exposure increased. On transfer to water to complete germination, the content of many of these proteins decreased at 24 h in 2 - 3 cm length embryo axes in comparison with the corresponding embryo axes of seeds continuously imbibed in water for 24 h. Some groups of proteins ranging between 15.5 - 17.3 kDa, already present after 8 h water imbibition, were not detectable after 24 h but were expressed in seeds exposed to NaCl and transferred to water for 24 h. Up- and down-regulated proteins in lentil embryo axes, imbibed under non-lethal salt stress conditions, have been tentatively identified by comparison with the protein map of germinating seeds of the model plant *Arabidopsis*.

Additional key words: 2-D electrophoresis, germination, *Lens culinaris.*

Introduction

For most seeds imbibition is generally a triphasic process in which phase I of rapid water uptake and phase II with apparent little change in water content have to be considered essential for germination completion (Bewley 1997). If a non-lethal salt stress is applied during the lag-phase of water uptake, this can be extended by inhibiting radicle protrusion from the seed coat – an event also called 'visible germination'. Upon salt stress removal, germination may be resumed and its completion and subsequent embryo growth depend on NaCl concentration and exposure time (Bliss *et al.* 1986). Gene expression in the embryo may be regulated by prolonged blocking and subsequent recovery of germination. Generally, proteins up- or down-regulated by salinity, as well as by other stressing environmental factors, may be identified first by 2-D PAGE, aided by computer image analysis, and further characterized with microsequencing and mass spectrometry (Zivy and de Vienne 2000). Moreover, the first step of proteome analysis is mainly focused on the comparison of proteins synthesized in phase I and II of imbibition (pre-emergence phases) with those expressed during early radicle protrusion (emergence phase) (Dell'Aquila and Di Turi 2002). In barley embryos, most proteins show a different response to salinity and drought and their accumulation in stressed roots and shoots depends on the genotype (Ramagopal 1988). In polyethylene glycol and NaCl treated wheat embryos, electrophoretic separation of newly synthesized polypeptides shows a delayed synthesis of polypeptides normally expressed at the beginning of radicle emergence phase and an accumulation of novel 'salt stress-induced' proteins in the acid zone of the gel (Dell'Aquila and Spada 1992, 1993). In imbibing lentil embryo axes, highly concentrated salt exposure combined with high temperature results in a lack of most low molecular mass (< 30 kDa) polypeptides (Dell'Aquila 2000). Among proteins induced by NaCl treatment, those with M_r 27 kDa are reported increasing in abundance in

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Abbreviations: 2-D PAGE - two-dimensional polyacylamide gel electrophoresis; M_r - relative molecular mass; DTT - dithiothreitol; IPG - immobilized pH gradient; HSP - heat shock protein.
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somatic embryos of orchardgrass (Dutta Gupta 1999) and in cell cultures of cauliflower (Elavumoottil *et al*. 2003).

The aim of this paper is to study 2-D PAGE protein patterns in lentil seed embryo axes during different highly concentrated NaCl exposure, followed by water germination upon stress removal. In addition, a quantitative analysis of more prominent protein spots, performed by computer image analysis, is provided to describe the effects of salt stress on the inhibition and the resumption of lentil seed germination.

Materials and methods

Plants: Lentil (*Lens culinaris* Medick. cv. Eston) seeds with 98 % germination and 24 h mean germination time were used for the trials.

Germination test: Four replicates of 50 seeds each were imbibed in water on a Petri dish with two sheets of filter paper at 20 °C in the dark. A seed was scored as germinated if the primary root reached 2 - 3 mm length, with no abnormal growth. To characterize cumulative germination curves, the final germination percentage and mean germination time, calculated according to Ellis and Roberts (1981), were determined.

Salt treatment was carried out by imbibing 200 seeds in 10 cm^3 water for 8 h in a Petri dish with two sheets of filter paper at 20 °C in the dark, and, then, by transferring seeds to a Petri dish with two sheets of filter papers imbibed with 10 cm^3 0.33 M NaCl solution for an additional period of 16, 40 and 64 h. At the end of the salt treatment, seeds were surface rinsed with distilled water and distributed on 4 Petri dishes to complete germination under germination test conditions.

Soluble protein extraction: 200 mg of embryo axes with 2 - 3 mm radicles were excised from lentil seeds, imbibed for 8 or 24 h in water, or for 8 h in water followed by 16, 40 and 64 h in 0.33 M NaCl alone or with additional 24 h water imbibition, and stored at -70 °C up to protein extraction. Excised embryo axes were homogenized in a cold glass homogenizer with 1 cm^3 of 50 mM Tris-HCl pH 7.5, 20 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonylfluoride. Following 10 000 *g* centrifugation, the supernatant, which represents the source of soluble proteins, was divided in small aliquots. One aliquot of 0.02 cm^3 in triplicate was added with 20 % (m/v) trichloroacetic acid, and the resulting pellet of 10 000 *g* centrifugation was resuspended in water and used to assay protein content with a commercial protein assay kit (*Sigma-Aldrich*, St. Louis, USA). Aliquots of 0.15 cm^3 were mixed with 0.075 cm^3 of sample buffer containing 8 M urea, 1 % (v/v) nonidet-P40, 50 mM DTT, 0.2 % (v/v) 3 - 10 pH *Biolyte* ampholine and a drop of 1 % (v/v) bromophenol blue. The final samples were incubated for 5 min at 35 °C and stored at -70 °C.

Two-dimensional polyacrylamide gel electrophoresis: Proteins were separated in the first dimension by active rehydration and focusing using 17 cm length *ReadyStrip* immobilized pH gradient gels (IPG) with 3 - 10 non-linear pH gradient range and a *Protean* isoelectrofocusing cell according to manufacturers instructions (*BioRad Laboratories*, Hercules, USA). Loading was carried out by mixing a soluble protein sample containing 300 μg protein with an amount of sample buffer to reach 0.3 cm^3 final volume, followed by the active rehydration of IPG strips under 50 V and 20 °C for 16 h. Upon rehydration, isoelectric focusing was made under 250 V per 15 min, 10 000 V per 3 h and 60 000 V per hour (50 μA $strip^{-1}$) at 20 °C. Before the proteins were separated in the second dimension, the IPG gel strips were equilibrated for 10 min each in the first equilibration buffer containing 6 M urea, 0.375 M Tris-HCl pH 8.8, 20 % (v/v) glycerol and 2 % (m/v) DTT, followed by the second equilibration buffer in which DTT was substituted by 2.5 % (m/v) iodoacetamide. 4 % (v/v) and 12 % (v/v) sodium dodecyl sulphate polyacrylamide gel for stacking and running gel, respectively, were used for the second dimension separation and running was conducted with 25 mA per gel at 15 °C (Dell'Aquila and Bewley 1989). A mixture of markers (*Sigma-Aldrich*) with known molecular mass - bovine serum albumin (66 kDa), ovoalbumin (45 kDa), trypsinogen (24 kDa) and lysozyme (14.3 kDa) - was loaded next to the IPG gel. Alternatively, a mixture of molecular and isoelectric point (pI) markers (*Sigma-Aldrich*) – myoglobin (17 kDa, 7.6 pI), carbonic anydrase (29 kDa and 7 pI) and ovalbumin (45 kDa and 5.1 pI) – were run separately in five replicates and the related gels were compared with those of other treatments to determine isoelectric point range. Following electrophoresis the gels were fixed, stained with either Coomassie brilliant blue R (Dell'Aquila and Bewley 1989) or silver nitrate salts (*Rapid Silver Stain kit*; *Sigma-Aldrich*) and dried.

Computer analysis of 2-D gels: Silver stained gels were scanned with *Sharp* (Osaka, Japan) *JX-330* scanner and *LabScan v.3* software package, while image analysis was carried out by *ImageMaster 2D Elite v.4.01* software package (*Amersham Pharmacia Biotech*, Uppsala, Sweden). Gels of 8 and 24 h water imbibed embryo axes were chosen as reference gels. After calibration to convert pixel to absorbance, spot detection and background subtraction, treatment gels were matched with the appropriate reference gel. Quantitative comparison among gels was made by estimating normalized value of total spot volume, and reported as absorbance related to a single pixel. Spots were described

as showing qualitative variation when they were either present or absent in comparison with the reference gel, and quantitatively different to corresponding control when their normalized volumes were statistically different (at least two-fold in relative abundance) in the three analyzed silver-stained gels from each treatment (Gallardo *et al*. 2001, Dell'Aquila and Di Turi 2002).

Results

Under optimal conditions lentil seeds reached 98 % final germination with a 24 h mean germination time (Fig. 1). When seeds were exposed to 0.33 M NaCl for 16 or 40 h, following a pre-imbibition of 8 h in water, final germination percentage decreased to 56 and 38 %, respectively, with a corresponding increase of mean germination time to 64 and 83 h, respectively. After 64 h NaCl treatment, only few seeds germinated (2 %) with abnormal growth.

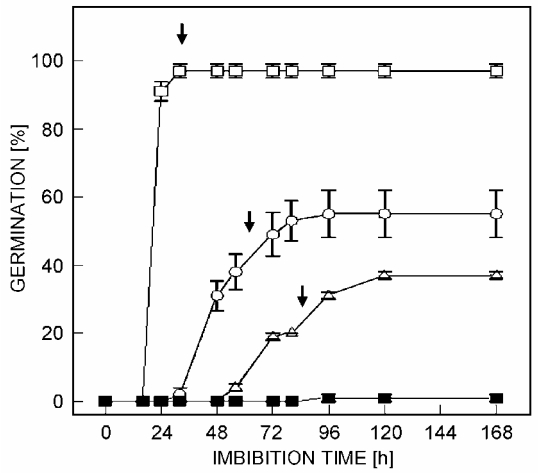

Fig. 1. Germination of lentil seeds for 8 h in water (W) and then for different periods in 0.33 M NaCl (S): *open squares* - continuous water imbibition (control), *open circles* - 8 h W + 16 h S, *open triangles* - 8 h W + 40 h S, *solid squares* - 8 h W + 64 h S. *Arrows* indicate mean germination time for each treatment. *Vertical bars* represent SE of the mean of four replicates of 50 seeds each.

A computer imaging analysis enabled the identification of 141 and 138 individual protein spots in gels of 8 h (pre-emergence phase) and 24 h (emergence phase) water imbibed embryos, respectively (Fig. 2). In a recent review on plant proteomics, Kersten *et al*. (2002) reported that the number of spots resolved by 2-D PAGE ranges widely, depending on tissue and species, between few hundred and 2000 spots. This experimental variability could be due mainly to a poor amount of proteins and the presence of many metabolic compounds which negatively affect protein extraction in plant tissues. In the case of lentil embryo axes, the number of revealed spots detected by image analysis of 2-D gels through different treatments did not differ significantly from the reference gels, except for the 64 h NaCl treatment without or with an additional 24 h water imbibition, where 242 and 73 spots, respectively, were detected. To

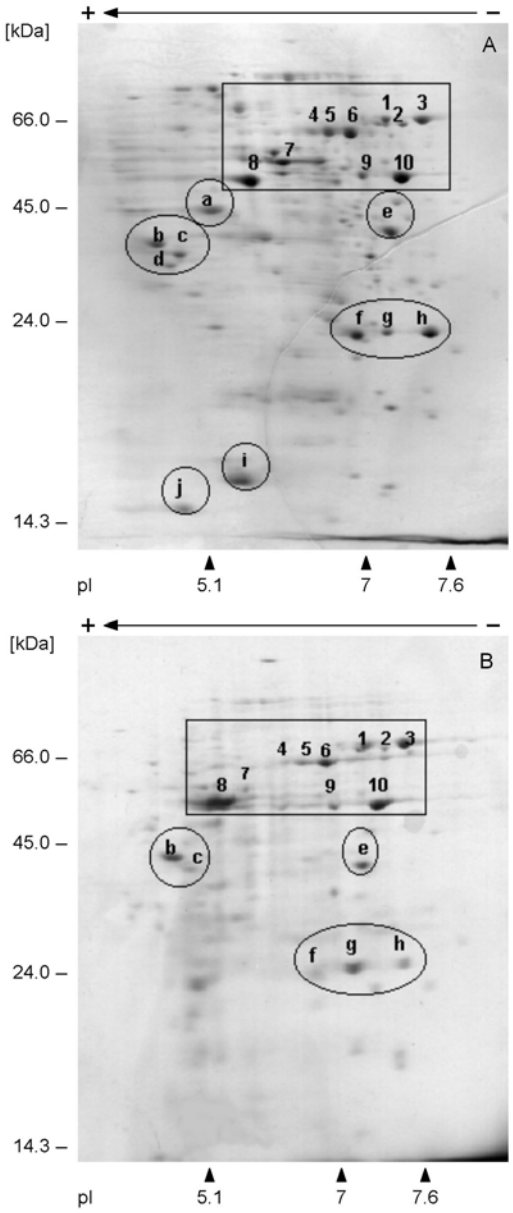

Fig. 2. 2-D gels of soluble proteins extracted from lentil embryo axes imbibed as follows: *A* - 8 h water, *B* - 24 h water. Polypetides labelled a - j or 1 - 10 have been selected in the entire gel or in the box delimited from 5.1- 7.6 pI and 75 - 50 kDa, respectively. Molecular mass and isoelectric point (pI) markers are reported on the left and bottom side, respectively.

study protein regulation, more prominent proteins were selected in a particular area of the gel, comprised between the isoelectric point range 5.1 - 7.6 pH in the first dimension and M_r range 75 - 50 kDa in the second dimension, and numbered 1 - 10. In this gel zone proteins associated to later germination and embryo growth have been well documented in *Arabidopsis* and wheat seeds (Gallardo *et al.* 2001, Dell'Aquila and Di Turi 2002). In addition, some large protein spots displayed outside the selected area were chosen as representative of the changes which occurred through the salt treatments and labelled a - j. The comparison between 8 and 24 h water gels showed that polypeptides 1 - 10 were present in both gels, whereas polypeptides a (46.3 kDa, 5.1 pI), d (32.8 kDa, 4.9 pI), i (17.3 kDa, 5.4 pI) and j (15.5 kDa, 4.9 pI) were not detectable in growing embryo axes (Fig. 2*B*). In these, polypeptides 1 (73.4 kDa, 6.9 pI), 3 (74.16 kDa, 7.2 pI), b (36.14 kDa, 4.9 pI) and g (25.2 kDa, 6.9 pI) were more abundant, while those labeled 4 (68.6 kDa, 6.5-6 pI), 7 (60.6 kDa, 6.1 pI), 9 (57.3 kDa, 6.9 pI), c (34.2 kDa, 4.9 pI), f (24.5 kDa, 7 pI) and h (24.5 kDa, 7.3 pI) were less abundant in comparison with 8 h embryo axes (Table 1).

The gel of 8 h water imbibed embryos was chosen as the reference for treatments in which embryo axes were exposed for different periods to NaCl alone, whereas gel of 24 h water imbibed embryos was chosen as the reference gel for embryo axes (2 - 3 mm) obtained from seeds transferred to water for 24 h following NaCl imbibition (Fig. 3, Table 1). Generally a prolonged salt imbibition induced a gradual increase in protein abundance, as it was shown in 3, 7 and 10 (57.3 kDa, 7.1 pI) polypeptides of 40 h NaCl treated seeds and 1 - 7 and 10 polypeptides of seeds exposed for 64 h in NaCl (Fig. 3, Table 1). This trend was confirmed also for polypeptides a, b and c, while polypeptides f and g disappeared under salt stress. When NaCl treated seeds germinated upon their transfer to water for 24 h, many polypeptides comprised in the selected gel area decreased in comparison with reference gel, *i.e.*, polypeptides numbered 1, 2 (70.5 kDa, 6.9 pI), 4, 5 (67.6 kDa, 6-5-6 pI), 6 (67.7 kDa, 6.5-6 pI), 8 (55.6 kDa, 5.4 pI) and 10 (Fig. 3, Table 1). The decrease was the highest in 64 h NaCl treated seeds, where germination was inhibited to the lowest level. Major changes occurred in the proteins selected in the rest of the gel: protein b and e (37.2 kDa, 7.1 pI) decreased in abundance, whereas those marked c, d, h and j showed an increased abundance. It is noteworthy that there is a group of new polypeptides (19 - 18 kDa, 5.4 pI) which together with proteins i and j were expressed in germinating seeds following 40 and 64 h NaCl treatment.

Table 1. Quantitative changes, as measured by a computer image analysis, of 1 - 10 and a - j polypeptides due to different treatments. Assessments of protein spot intensity are reported as average of three gels and are expressed as normalized total volume (absorbance per single pixel). W - water; S - 0.33 M NaCl; ndt - no detectable trace.

Polypeptides	8 h W	8 h W + 16 h S	8 h W + 40 h S	8 h W + 64 h S	24 h W	8 h W + 16 h S + 24 h W	8 h W + 40 h S + 24 h W	8 h W + 64 h S + 24 h W
1	390	484	467	1067	838	260	214	243
2	477	577	565	1091	321	211	123	60
3	1363	1557	2887	4055	2807	2145	1856	2468
4	274	345	294	641	113	176	109	45
5	919	939	807	1940	803	376	347	323
6	1824	1715	1649	3973	2110	1882	621	706
7	512	432	1047	2099	29	48	751	612
8	1949	1960	1794	1208	2633	1021	1285	524
9	669	546	783	798	30	23	45	38
10	3514	3625	7460	7925	4078	2017	2001	1945
a	1302	1867	1972	2624	ndt	1660	1461	1569
b	576	51	1649	2335	2328	97	386	382
c	496	42	1092	1775	35	ndt	375	173
d	195	ndt	641	449	ndt	20	308	303
e	1707	785	1436	1521	2105	2131	702	1031
f	1844	ndt	ndt	ndt	124	ndt	ndt	ndt
g	552	ndt	ndt	ndt	2655	429	185	ndt
h	2132	917	1056	1897	1026	1166	2562	2607
i	835	ndt	649	697	ndt	ndt	403	183
j	693	819	677	293	ndt	671	1381	1559

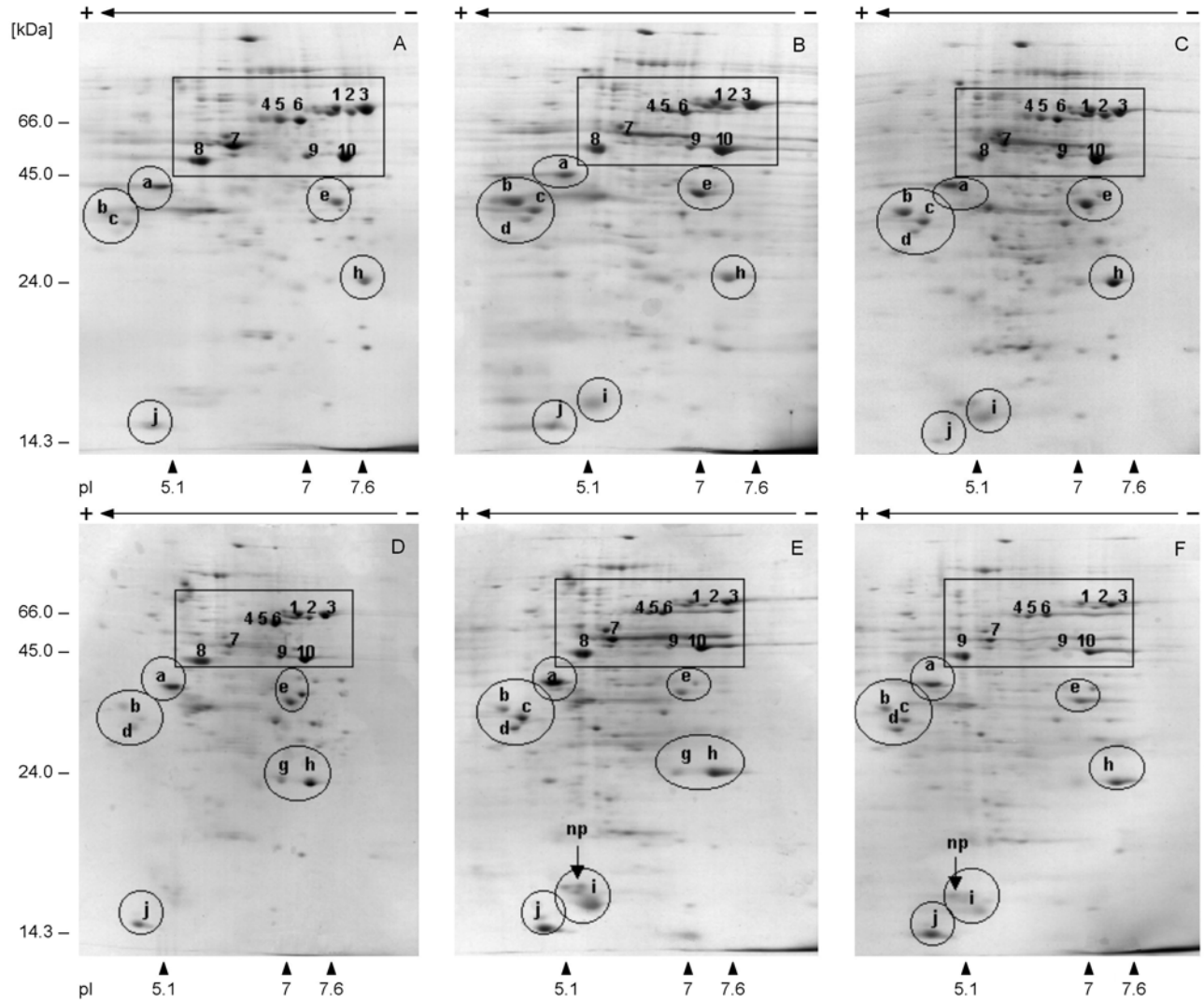

Fig. 3. 2-D gels of soluble proteins extracted from lentil embryo axes imbibed as follows: 8 h in water followed by 0.33 M NaCl exposure for 16 (*A*), 40 (*B*) and 64 h (*C*) alone or with an additional transfer to water for 24 h (*D* - 16 h NaCl, *E* - 40 h NaCl, *F* - 64 h NaCl). Molecular mass and isoelectric point markers and selected polypeptides as in Fig. 2. *Arrows* in Fig. 2*E* and *F* indicates the expression of novel proteins with M_r 19 - 18 kDa and pI 5.4.

Discussion

The identification of proteins revealed on 2-D gels is a useful approach to study the regulation of protein synthesis during germination phases under non-lethal abiotic stresses, such as wounding, heat and cold shock, osmotic and salt treatments (Thiellement *et al*. 1999).

Changes in gene expression occur in 0.33 M NaCl stressed lentil embryo, as shown by the patterns of more prominent proteins delimited in the pI range 5.1 - 7.6 and Mr 75 - 50 kDa. The nature of these polypeptides cannot be deduced using 2-D gel analysis alone. Electrophoresis features of well resolved protein spots may be compared with those reported in the reference map of germinating *Arabidopsis* seeds, obtained by combining 2-D PAGE and mass spectrometry analysis (Gallardo *et al*. 2001). In this zone of the gel some proteins associated with the later stage of germination have been characterized, such as late embryogenesis-abundant (LEA) proteins, HSP70 family, elongation factors (EF-1 and EF-2), enzymes involved in oxidative stress and α- and β-tubulin classes. These proteins, with protective function, are regulated not only during seed maturation but also during the late phase of germination. Such a behaviour was observed also in imbibing wheat embryos subjected to heat-shock, polyethylene glycol and salt stress and controlled conditions of aging (Dell'Aquila and Di Turi 2002). In this case, high M_r HSPs were identified during heat

treatments and β-tubulin subunits are accumulated in germinating wheat embryos following advancing deterioration. In addition, a group of novel polypeptides with low M_r has been identified at 24 h in water imbibed lentil embryo axes, following 40 - 64 h NaCl treatments, and could represent salt-induced proteins. This varying proteome response to salt stress suggests that a large number of proteins are quantitatively or qualitatively affected, probably due to the accumulation of mRNAs and related proteins, as reported for roots of salt-tolerant barley (Ramagopal 1987) and wheat (Gulick and Dvorak 1987) genotypes and tomato (Chen and Tabaeizadeh 1991). The presence of low M_r proteins in lentil axes at 24 h water following salt treatment seems to confirm the expression of proteins like low M_r HSPs with molecular chaperon function (Waters *et al*. 1996). These proteins were observed also in aged wheat and barley genotypes, deteriorated under controlled conditions and, then exposed to heat and salt shock (Dell'Aquila *et al*. 1998, Dell'Aquila and Di Turi 1999). Abundance of low M_r HSPs, of 17.7 - 17.4 kDa and 5.2 - 5.36 pI, was found in polyethylene glycol primed *Arabidopsis* seeds, declining quickly during germination (Gallardo *et al*. 2001). The detection of such proteins in 2 - 3 mm embryo axes of germinating seeds pre-treated with NaCl seems to confirm that the accumulation of low M_r HSPs may be possibly involved to overcome hostile environment conditions and, so they might represent biochemical markers of germination.

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