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BRIEF COMMUNICATION

Effect of combined salt and heat treatments on germination and heat-shock protein synthesis in lentil seeds

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Abstract

The germination of lentil seeds was gradually reduced when seeds were exposed to temperature of 30 or 40 °C, either alone or combined with 0.1, 0.2 or 0.3 M NaCl or 34.1 % (m/v) PEG 8000, during 6 - 12 h imbibition. [^{35}S]-methionine incorporation in 12 h imbibed lentil axes also decreased with increasing NaCl concentration at 20 and 40 °C, whereas at 30 °C only 0.3 M NaCl treatment partially inhibited protein synthesis. An analysis of newly synthesized proteins by 1-D SDS PAGE, showed that the expression of most polypeptides decreased following increasing stress. Among these, low molecular mass heat-shock proteins declining, higher in 40 °C treated axes than those treated at 30 °C, supports the hypothesis that at this temperature maximal level of expression of these proteins was achieved.

Additional key words: heat stress, *Lens culinaris*, salt stress.

Combined salt and high temperature shock treatments decrease final germination percentage and rate of higly viable lentil seed proportionally to the imposed stress and to seed vigour degree (Dell'Aquila 1999). In wheat and barley seeds, subjected to heat-shock alone, the expression of most polypeptides normally synthesized during germination was reduced, but specific heat-shock proteins (HSPs) were produced (Dell'Aquila *et al.* 1998, Dell'Aquila and Di Turi 1999). In legume seeds, HSP synthesis increases with increasing temperature and a large heterogeneity of HSP classes has been found. High molecular mass (>30 kDa) HSPs, such as the HSP70 complex, as well as more abundant low molecular mass (<30 kDa) HSPs have been characterized and localized in several cell compartments in pea, soybean, chickpea, bean, *Arabidopsis*, *Brassica* and alfalfa seeds (Vierling 1991, Hernandez and Vierling 1993). Whereas previous reports of thermotolerance in legumes utilized seedlings or vegetative plant tissues, the present study regards the synthesis of HSPs in imbibing lentil seed axes, following a short treatment of combined NaCl and high temperature stress applied before radicle emergence. During early imbibition lentil seeds have substantial high temperature tolerance (Covell *et al.* 1986), but the additional effect of increasing salt stress on the expression of HSPs and its relationship with germination response is unknown at present.

One seed lot of lentil (*Lens culinaris* Medik. cv. Laird, 1997) with 100 % germination was used in the trials. The control germination test was carried out on five replicates of 20 seeds each in 12 cm diameter Petri dishes containing 10 cm^3 distilled water at 20 °C in the dark. The stress integrated germination test was performed by imbibing one hundred seeds for 6 h in distilled water at 20 °C, and then by transferring them to 0.1, 0.2 or 0.3 M NaCl (osmotic potential -0.44, -0.9, -1.38 MPa, respectively) solutions, or alternatively to 34.1 % (m/v) aqueous solution of PEG 8000 giving -1.4 MPa osmotic potential (Michel and Kaufmann 1973, Cochrane 1994), at temperature of 20, 30 or 40 °C. At the end of each treatment, seeds were rinsed several times in distilled water and distributed in five Petri dishes to complete germination in water at 20 °C. Two germination parameters were determined: final germination percentage (G) and time to reach 10 % of germination (T_{10}), as timing of germination onset, calculated

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Abbreviations: 1-D SDS PAGE - one dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis; HSP - heat-shock protein; LMM - low molecular mass; HMM - high molecular mass; PEG - polyethylene glycol.

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according to Tadmor *et al.* (1969). After 6 h imbibition at 20 °C, triplicate samples of twenty axes were dissected from seeds and transferred to the labelling medium (1 cm^3) containing water, 50 μg penicillin G, 50 μg streptomycin sulphate and 1.9 MBq [^{35}S]-methionine (37 MBq $mmol^{-1}$). The axes were incubated at 20, 30 and 40 °C combined with different NaCl or PEG 8000 solutions for an additional 6 h in a rotary shaker, washed with 10 mM L-methionine, blotted dry and stored at -80 °C. Soluble protein extraction and assays, as well as the detection of labelled precursor total uptake and incorporation were carried out as previously described (Dell'Aquila and Spada 1992). For 1-D SDS PAGE, 0.12 cm^3 of soluble protein extract were mixed with 0.06 cm^3 of a buffer containing 30 % (v/v) glycerol, 6 % (m/v) SDS, 15 % (v/v) 2-mercapto-ethanol and 62.5 mM Tris-HCl (pH 6.8). After boiling for 3 min, 0.74 kBq protein samples were loaded per lane for electrophoretic separation. Experimental 1-D SDS-PAGE conditions were: 4 % (m/v) SDS-polyacrylamide for the stacking gel, 12 % (m/v) SDS-polyacrylamide for the separating gel and 25 mA gel^{-1} constant current. Detailed procedures for gel running and fluorography were reported previously (Dell'Aquila and Di Turi 1999).

Increasing temperature alone from 10 to 40 °C did not affect G (Fig. 1*A*), whereas it significantly increased T_{10} in 40 °C treated seeds (Fig. 1*B*). In addition, within the range of applied salt stress conditions, T_{10} was more sensitive than G, as confirmed also by highly significant correlation coefficients ($P < 0.01$) calculated between T_{10} and NaCl concentration for each tested temperature (data not shown). When 0.3 M NaCl was substituted by iso-osmotic solution (-1.4 MPa) of PEG 8000, G did not change in comparison with the control, and the osmotic effect was highest in 40 °C treated seeds. Similar results have been reported in spring wheat seeds continuously imbibed at increasing concentration of chloride and sulphate salts and PEG 8000 (Hampson and Simpson 1990). It is noteworthy that PEG 8000 treatment induced different germination response with respect to similar iso-osmotic NaCl treatment, whose inhibiting effect can be also due to a toxic influence of Na and Cl ions on cell mebrane integrity (Bliss *et al.* 1986).

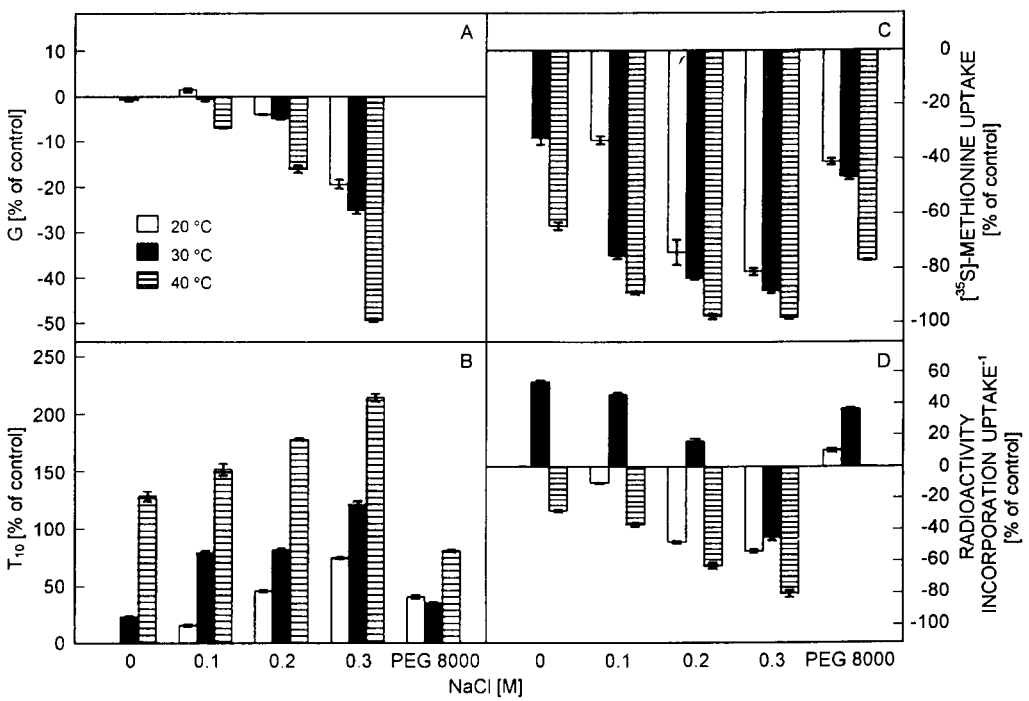

Fig. 1. Changes of germination percentage, G (*A*), time to reach 10 % of germination, T_{10} (*B*), [^{35}S]-methionine uptake (*C*) and ratio between [^{35}S]-methionine incorporation and uptake into 12 h imbibed axes (*D*) of lentil seeds treated by 0.1, 0.2, 0.3 M NaCl or 34.1 % (m/v) PEG 8000 at temperature of 20, 30 and 40 °C. Values were reported as percentage of control seeds (20 °C, 0 M NaCl). Vertical bars represent standard error of the mean of 5 replicates of 20 seeds each for G and T_{10} and of 3 replicates of 20 axes each for radioactive uptake and incorporation.

Germination impairment by combined salt-temperature treatments was associated to protein synthesis alteration in 12 h treated lentil axes. A progressive decrease of [^{35}S]-methionine uptake with increasing temperature and NaCl treatment was noted (Fig. 1*C*). The ratio between incorporation and uptake of radioactive precursor into 12 h imbibed lentil axes (Fig. 1*D*) showed that the reduction of protein synthesis

was proportional to increasing NaCl concentration only at 20 and 40 °C, whereas at 30 °C protein synthesis was higher than the control up to 0.2 M NaCl. Using 0.3 M NaCl, the ratio between [^{35}S]-methionine incorporation and uptake was -45 % of control. PEG 8000 treatment reduced the radioactive precursor uptake less than 0.3 M NaCl, and increased rate of protein synthesis at 20 - 30 °C. Similar effect on [^{35}S]-methionine incorporation has been already described in wheat embryos treated with -1.4 MPa NaCl or PEG 8000 (Dell'Aquila and Spada 1992).

The combined effects of increasing NaCl concentration and temperature were also studied on 1-D SDS PAGE fluorographs of total soluble protein extracts from lentil axes (Fig. 2). The analysis of newly synthesized protein patterns can be summarized as follows: *a*) at each temperature, the radioactiviy of polypeptide bands decreased with increasing salt concentration. At 40 °C most polypeptides were faintly discernible in 0.1 - 0.2 M NaCl and their lack in those treated by 0.3 M NaCl could be due by the lost of most polypeptides, possibly with a molecular mass below 14.3 kDa, from the bottom of the gel. On the contrary, PEG 8000 treated axes showed normal patterns of protein bands at 20 °C, 30 °C, and partially at 40 °C; *b*) the main change in qualitative and quantitative expression of HSPs (more prominent polypeptides were selected and numbered 1 - 9) was found in 30 and 40 °C treated axes with or not the additional salt stress. High molecular mass (HMM) HSPs were represented mostly by HSP_1 (89.5 kDa) and HSP_2 (70.7 kDa), and their slight presence was noted also at 20 °C. These polypeptides were massively produced in all other samples, except for 40 °C - 0.3 M NaCl. Low molecular mass (LMM) HSPs represented by HSP_3 (29.4kDa), HSP_4 (28.7 kDa), HSP_5 (24 kDa), HSP_7 (17.6 kDa), HSP_8 (16.4 kDa), and HSP_9 (15.5 kDa) were synthesized in 30 °C treated axes and generally decreased with increasing NaCl concentration. HSP_3, HSP_4 and HSP_5 were expressed also in PEG 8000 - 20 °C treated axes, whereas the other LMM HSPs may be considered as novel proteins. All selected LMM HSPs were produced in 30 °C - PEG 8000 treated axes, but disappeared or were present in traces in 0.3 M NaCl axes. It is noteworthy that in NaCl - 30 °C treated axes the LMM HSP synthesis was higher than in corresponding NaCl - 40°C axes, except for HSP_6 (19.3 kDa; novel protein) and HSP_9.

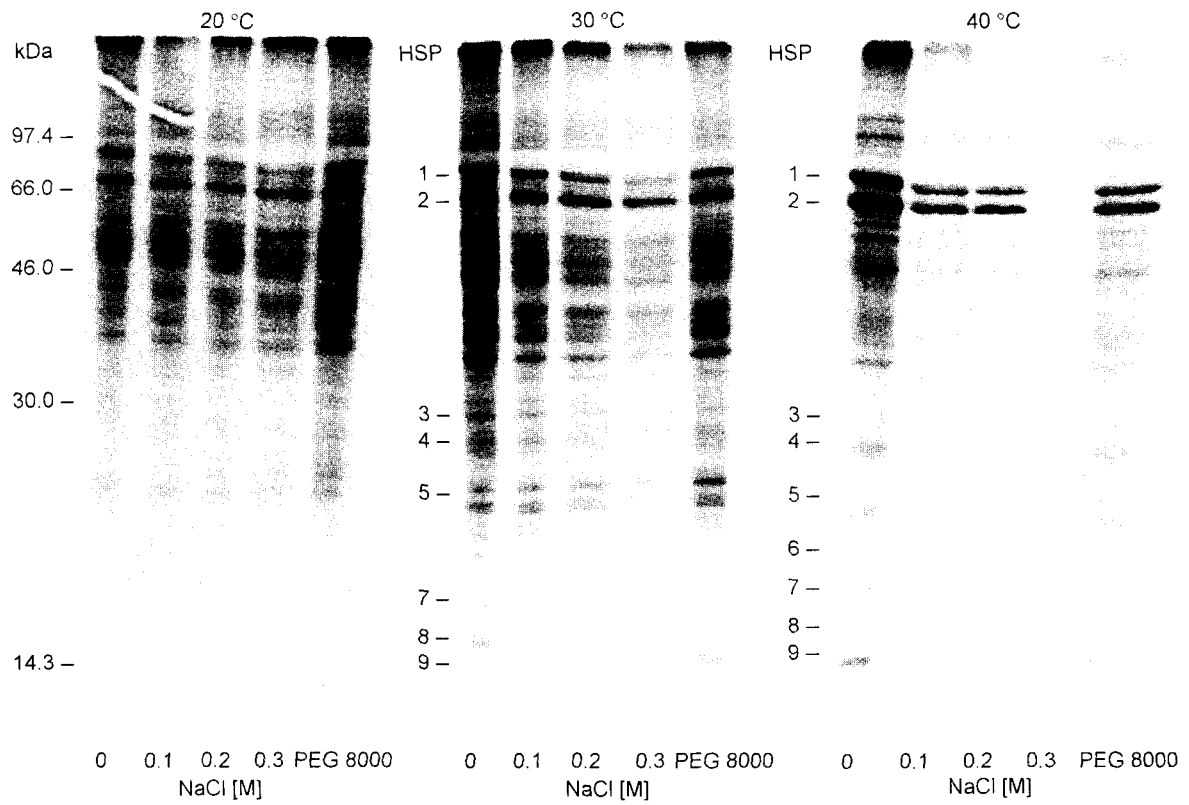

Fig. 2. Fluorographs od 1-D SDS-PAGE of total soluble proteins synthesized at 12-h imbibition in lentil axes, following various NaCl, PEG 8000, and temperature treatments as in Fig. 1. Molecular mass markers are on the left side of the 20 °C gel and HSPs are indicated by numbers 1 - 11 on 30 °C and 40 °C gel, respectively.

Generally, in studies on heat-shock induced proteins in seeds, only one environment stress factor, high temperature, is considered. Our preliminary data on lentil axes show that the interaction between osmotic and salt

stress and increasing temperature can differently affect the quantitative protein synthesis rate and the expression of different classes of HSPs. HSP70 is representative of a protein complex family which is responsive to cold stress in mung bean seeds (Wu *et al.* 1993) or is synthesized in germinating low viability wheat seeds, where most proteins associated to germination disappeared (Dell'Aquila 1994). In germinating lentil axes treated at 30 - 40 °C only, HSP_3 and HSP_4 are abbundant components of HMM HSPs, and their massive presence over several salt treatments may reflect increased synthesis of one or more HSP isoforms. In contrast, LMM HSPs are expressed during various phases of seed development and found at maturity in cytosol, chloropasts, endoplasmic reticulum, and mitochondria (Waters *et al.* 1996). The hypothesis that the accumulation of LMM HSPs leads to increased thermotolerance (Waters *et al.* 1996) is in agreement with our findings on salt stressed lentil axes. Maximal synthesis of LMM HSPs was obtained with treatment at 30 °C only, which induced the full complement of LMM HSPs. Quantitative and qualitative changes of these polypeptides are progressively shown either under salt influence or under 40 °C. In conclusion, LMM HSP patterns may be considered as good indicators of lentil seed surviving under hostile conditions of germination.

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