



AMINO ACID AND PROTEIN PROFILES OF *VICIA FABA* SALT-STRESSED SEEDLINGS GROWN FROM THERMALLY-STRESSED SEEDS

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SUMMARY

Seeds of *Vicia faba* L. cv Giza 843 were subjected to 4 °C, 25 °C (control) and 40 °C for 48 h before sowing in clay-loam soil and the seedlings were irrigated with water (control) or 0.1 M NaCl for 35 days (d). Germination percentage, length and fresh and dry weights of 10-d and 35-d old seedlings decreased with the rise of presowing temperature; the decrease was greater when thermal and salinity stresses were combined. In the 10-d-old seedlings, thermal and/or salinity stresses enhanced the assembly of free amino acids into members of the heat shock proteins families Hsp100, Hsp60 and sHsp, which exhausted the free amino acid pool to the point of almost total depletion. The sHsps seem to stabilize the stress-denatured enzymes and structural proteins, thus preparing them for subsequent refolding by members of the Hsp60 and Hsp100 families so that they regain their functional conformations, which might have in turn led to the observed improvement in the germination percentage and in seedling growth. In 35-d-old seedlings, fewer Hsps were synthesized leading to greater accumulation of eight amino acids (proline, glutamic acid, glycine, alanine, methionine, phenylalanine, lysine, arginine) to act as osmoregulators. The strategy for combating abiotic stress seems age-dependent.

Key words: Amino acid, germination, protein pattern, salinity, seed chilling, seed heating, seedling growth, *Vicia faba*

INTRODUCTION

Plants are seldom exposed in nature to only one type of stress. In arid and semi-arid environments, chilling and heat stresses on the seed bank are often coupled with the impact of relatively high salinity levels in the soil and/or irrigation water on emerging embryos and growing seedlings. While there is a plethora of studies on the effect of either thermal or salt stress on plants, investigations of the combined effect of the two stresses are rare. Most of these investigations dealt with grass crops (El-Darier 1999, Kader and Jutzi 2004, Faheed 2005, Song *et al.* 2005), while those concerned with other plants are much fewer (Dell'Aquila 2000, Dowiadar *et al.* 2002).

Thermal stress (whether as chilling or heating) and/or elevated salt concentration are known to induce a number of physiological responses in plants, including changes in protein pattern (Ashraf and O'Leary 1999, Dell'Aquila 2004, Sousa *et al.* 2003, 2004), amino acid profile (El-kholy *et al.* 1997) and water content (Del Zoppo *et al.* 1999, Promila and Kumar 2000, Romero-Aranda *et al.* 2001) of the stressed plants, which ultimately affected their growth. Abiotic stresses cause deleterious changes in many enzymes and structural proteins such as denaturation and aggregation of non-native proteins (Wang *et al.* 2004).

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In the present study a pot experiment was designed to simulate the thermal conditions of arid and semi-arid environments by exposing seeds of an economically important legume crop (*Vicia faba* L. cv. Giza 843) to chilling or heating before germinating them under salinity stress in order to investigate the effects of these stresses on seed germination and on the growth parameters, protein pattern and amino acid profile of the seedlings.

MATERIALS AND METHODS

Seeds of *Vicia faba* L. cv. Giza 843 (broad beans) were supplied by the Ministry of Agriculture (Cairo, Egypt). The seeds were exposed to three temperature treatments for 48 h before sowing: 4 °C, 25 °C, and 40 °C. Plastic pots (25 x 30 cm), each containing 5 kg clay loam soil, were prepared for each treatment with five replications and 20 seeds were sown in each pot. Each pot was irrigated daily for 35 days (d) either with 300 ml distilled water (as control) or with 300 ml 0.1 M NaCl. The seedlings were left to grow in a growth chamber under 16 h light: 8 h dark, at 25 °C ± 1 during the light period and 15 °C ± 1 during the dark period, 62-65% relative humidity, and a photon flux density of 160 μmol m⁻² s⁻¹. The soil was washed weekly with distilled water. The germination percentage was recorded four d after sowing. Growth criteria were recorded for four 10-d and 35-d-old seedlings from each pot. Leaflet area in 35-d-old seedlings was determined as the mean of 80 measurements (2 basal leaves x 2 leaflets x 4 seedlings x 5 pots). The dried shoots (at 50 °C) were used for amino acid analysis by HPLC (Beckman, System Gold, Programmable Solvent Module 126) according to the method of Anderson *et al.* (1977). Thermally untreated dry seeds and four fresh leaves of 10-d and 35-d-old seedlings in each pot were used for the determination of protein patterns by SDS-PAGE according to Laemmli (1970). Molecular mass in kDa of each protein band in the gel was determined using Gel-Pro Analyzer with v 3.1 software.

Results were subjected to 2-way ANOVA to determine the significance of treatment differences for the growth criteria using SPSS v.10 software. Comparison of the main effects was performed using the Least Significant Difference (LSD) from the control.

RESULTS

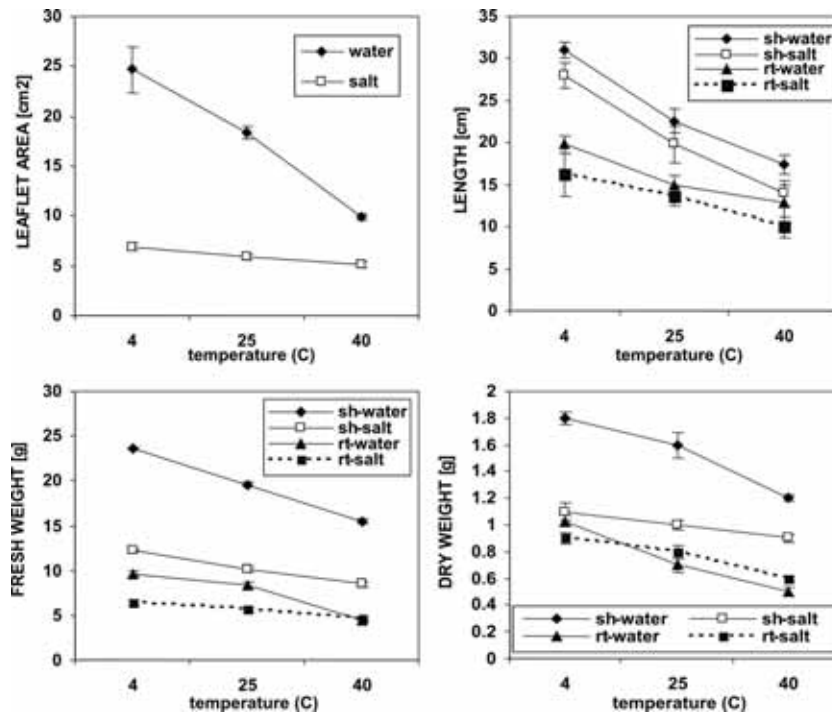
Seed germination: Pre-sowing thermal treatments of seeds which caused reductions in the % germination were in the order: 40 > 25 > 4 °C both with and without the effect of salinity (Fig. 1). Reduction in % germination was consistently greater with salinity.

Seedling growth: Shoot height and root length as well as the fresh and dry weights of 10-d-old seedlings decreased progressively with salinity, the rise of pre-sowing temperature treatment of seeds and their combination (Fig. 1).

In the 35-d-old seedlings, the leaflet area was decreased as the pre-sowing temperature treatment of seeds increased (Fig. 2). Similarly, salinity reduced leaflet area with the increase of temperature. The difference in leaflet area between seedlings irrigated with 0.1 M NaCl and those irrigated with water (i.e. the impact of salinity) was greatest with seed chilling at 4 °C and decreased gradually with the increase of temperature. The effects of thermal treatments of seeds and/or salinity on shoot length and fresh and dry weights of shoots and roots were similar to those on leaflet area (Fig. 2). Except for root fresh and dry weights, salinity caused a significant reduction in growth criteria regardless of the pre-sowing thermal treatment of seeds.

Amino acids: Fig. 3 illustrates the pattern of changes induced by pre-sowing thermal treatments of seeds and salinization of 10-d-old seedling shoots in their content of 17 amino acids. While cysteine was undetectable in seedlings in response to all temperature and salinization treatments, all other 16 amino acids accumulated only in seedlings grown from seeds treated with 25 °C and irrigated with water, but were decreased sharply (mostly to the point of being undetectable) with other thermal and salinity treatments.

Fig. 4 summarizes the changes induced in the amino acid content of the shoots of 35-d-old seedlings grown from pre-sowing thermally treated seeds and irrigated with 0.1 M NaCl. It is evident that the effect of salinity at 4 °C on 11 amino acids (threonine, serine, glycine, alanine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine,



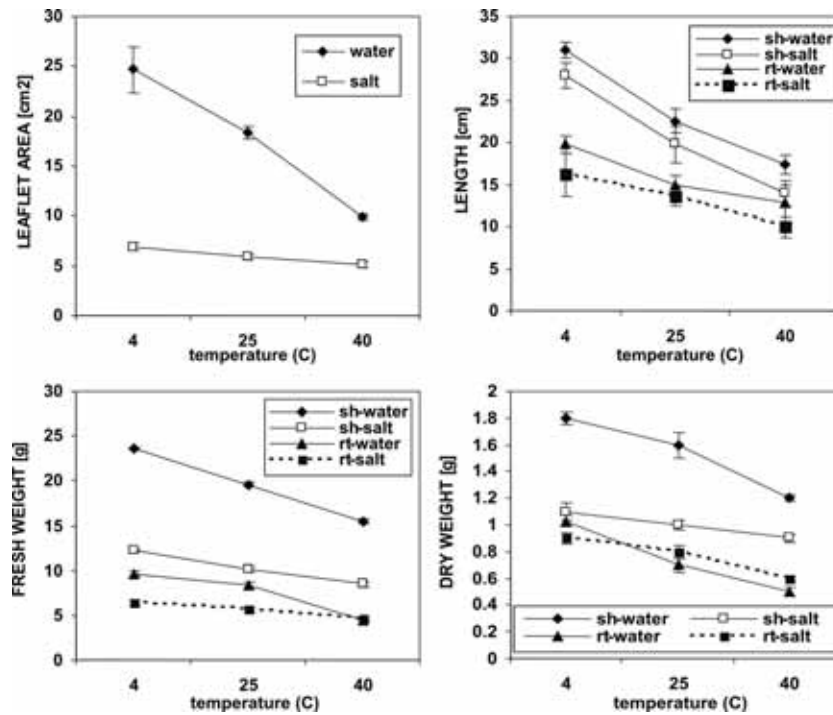
Treatment	% germination	sh. ht	rt. l.	seedl. fw	seedl. dw
temperature	114.8**	199.14**	59.24**	1811.7**	305.8**
salinity	2718.1**	195.76**	11.88**	8798.1**	2581.8**
temperature + salinity	n.s.	25.37**	12.23**	112.4**	112.4**

Fig. 1. Effect of pre-sowing exposure of *Vicia faba* seeds to three thermal treatments (4 °C, 25 °C and 40 °C) for 48 h on % of germination, shoot height, root length, seedling fresh and dry weights of 10-d old seedlings grown under salinity stress (0.1 M Na Cl). Results of 2-way ANOVA (sh.ht = shoot height; rt.l. = root length; seedl. fw = seedling fresh weight; seedl. dw = seedling dry weight). (F values: ** = significant at $p < 0.01$; n.s. = non-significant) are given in tabular form

arginine) was non-significant. With the exception of histidine, these amino acids steadily accumulated with salinity at the higher pre-sowing temperatures. The same trend applied also to total amino acids. The effect of salinity and pre-sowing treatments of seeds on the contents of threonine and serine was almost identical. Glutamic acid and proline accumulated with salinity and with the increase in pre-sowing temperatures; the highest accumulation was recorded in the proline content at 40 °C. While salinity caused significant decrease in the aspartic acid content, the decrease resulting from the rise in pre-sowing temperature was not significant. The content of aspartic acid and methionine in the salinized shoots decreased at all pre-sowing temperatures as compared to their non-salinized counterparts. However, while aspartic acid decreased consistently at all pre-sowing thermal treatments, methionine tended to accumulate at 40 °C.

Pre-sowing chilling and heating of seeds caused sharp decreases in the cysteine and valine contents of salinized and non-salinized shoots. While salinity slightly affected cysteine at 25 °C, it caused appreciable accumulation of valine at the same temperature.

Protein patterns: The leaves of 10-d and 35-d-old seedlings possessed a number of protein bands which seemed highly stable and unaffected by thermal treatments of seeds and salinization of seedlings. Such bands were omitted in Table 1. The three seed proteins with the highest MM (216.8, 192.5 and 152.7 kDa) disappeared directly upon germination. In response to various temperature and salinity treatments, the remaining polypeptide bands of the 10-d-old leaves underwent a number of changes which can be summarized in the following:



Treatment	l.a.	sh.h.	r.l.	sh. fw	r. fw	sh. dw	r. dw
Temperature	98.01**	209.8**	40**	865.6**	200.9**	124.9**	59.3**
Salinity	577**	30.7**	18.6**	8274**	31.9**	655.7**	18.03**
Temperature + salinity	59.4 **	n.s.	n.s.	516.4**	n.s.	33.8**	4.5*

F values (** = significant at p d" 0.01; * = significant at p d" 0.05; n.s. =non-significant)

Fig. 2. Effect of pre-sowing exposure of *Vicia faba* seeds to three thermal treatments (4 °C, 25 °C, 40 °C) for 48 hr on leaflet area, shoot height, root length, shoot and root fresh and dry weights of 35-d old seedlings grown under salinity (0.1 M NaCl). Results of 2-way ANOVA statistical analysis are given in tabular form. l.a. = leaflet area; sh.h. = shoot height; r.l. = root length; sh. fw = shoot fresh weight; r. fw = root fresh weight; sh. dw = shoot dry weight; r. dw = root dry weight.

- three bands with MM 42.9 kDa, 61.9 kDa and 150.7 kDa were synthesized only in response to salinity when seeds were treated with 40 °C, 4 °C and 25 °C, respectively,
- three protein bands with MM 74.6-78.2 kDa, 49.4-49.9 kDa and 36.1-37.2 kDa were synthesized in response to all thermal and salinity treatments except when the seeds were exposed to 25 °C and the seedlings were irrigated with water (the reference control for thermal and salinity treatments),
- three protein bands with MM 50.5 kDa, 39.4-39.9 kDa and 34.5-34.6 kDa appeared in salinized and

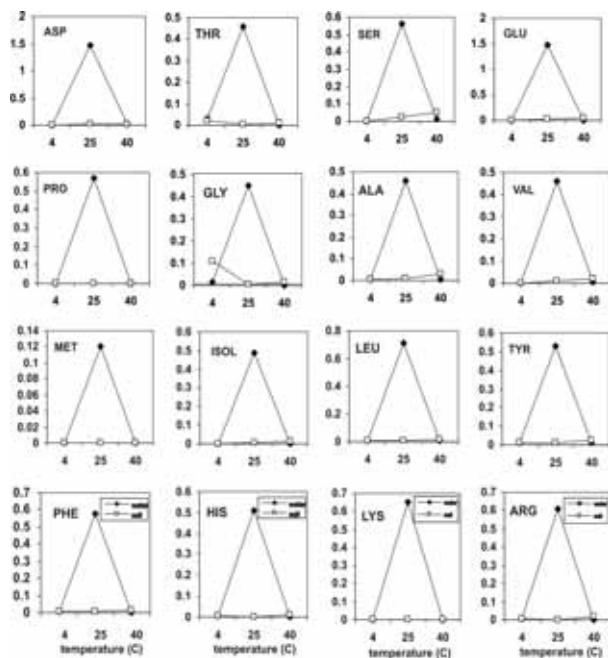


Fig. 3. Effect of pre-sowing exposure of *Vicia faba* seeds to three thermal treatments (4 °C, 25 °C and 40 °C) for 48 h on amino acid content (g 100g⁻¹ dry weight) of 10-d-old seedling shoots irrigated with water or 0.1 M NaCl.

unsalinized seedlings grown only from chilled or heated seeds,

- a protein with MM 25.3 kDa was formed in salinized and unsalinized seedlings only in response to seed chilling,
- a seed protein with MM 21.6 kDa disappeared only in salinized and unsalinized seedlings grown from chilled and heated seeds,
- a protein with MM 27.6 kDa disappeared in salinized seedlings only when salinization was combined with seed chilling or heating at 40 °C,

The changes induced by seed chilling, seed heating and seedling salinization in 35-d old seedlings were much fewer. The most noteworthy change concerns the two protein bands with MM 30.2-30.4 kDa and 25.7 kDa, which disappeared under the effects of seed chilling and seed heating whether or not the seedlings were exposed to salinity. Only one protein (MM 57.9 kDa) was

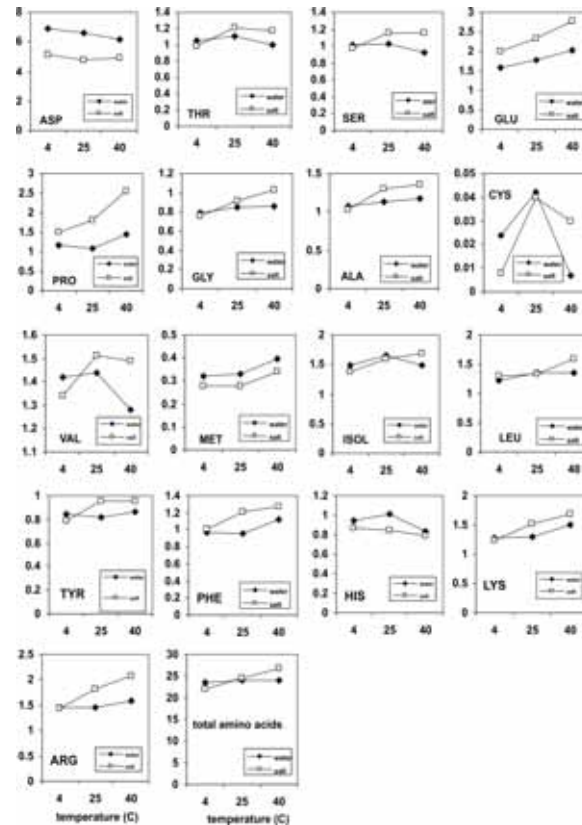


Fig 4. Effect of pre-sowing exposure of *Vicia faba* seeds to three thermal treatments (4 °C, 25 °C and 40 °C) for 48 h on amino acid content (in g 100g⁻¹ dry weight) of 35-d-old seedling shoots irrigated with water or 0.1 M NaCl.

synthesized in response to seed chilling when combined with salinity and to seed heating with and without salinity.

DISCUSSION

While pre-sowing chilling of *Vicia faba* seeds at 4 °C increased the percentage of germination, length and fresh and dry weights of shoots and roots of 10-d and 35-d-old seedlings as compared to the effect of the reference control temperature (25 °C), seed heating at 40 °C caused a marked decrease in all these parameters (Figs. 1-2). The same trend was noticed in the effect of salinity on these parameters and the reductions were consistently greater than those recorded under the non-saline condition. Seed chilling tended to alleviate the deleterious effects of salinity especially on seed germination, leaflet area and shoot fresh and dry weights. Similar results were reported by Sharma and Kumar (1999) in *Brassica juncea*,

EFFECT OF THERMAL AND SALINITY STRESSES ON *VICIA FABA*

Table 1. SDS-PAGE of protein extract of dry seeds and fresh leaves of *Vicia faba* L. cv Giza 843. Leaves were taken from 10-d and 35-d-old seedlings grown from seeds exposed to three thermal treatments (4 °C, 25 °C, 40°C) and irrigated with water (w) or 0.1 M NaCl (s). M = marker; MM = molecular mass; + = present; - = absent.

M (kDa)	MM of bands (kDa)	Dry seed	10-d-old leaves						35-d-old leaves						
			4°C		25°C		40°C		4°C		25°C		40°C		
			w	s	w	s	w	s	w	s	w	s	w	s	
214	216.8	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	192.5	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	152.7	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	150.7	-	-	-	-	+	-	-	-	-	-	-	-	-	-
118	102.4	-	-	-	-	+	-	-	-	-	-	-	-	-	-
92	74.6-78.2	-	+	+	-	+	+	+	-	-	-	-	-	-	-
	61.9	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	57.9	-	-	-	-	-	-	-	-	+	-	-	+	+	
52.2	50.5-50.8	-	+	+	-	-	+	+	-	-	-	-	-	-	-
	49.4-49.9	-	+	+	-	+	+	+	-	-	-	-	-	-	-
	42.9	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	39.4-39.9	-	+	+	-	-	+	+	-	-	-	-	-	-	-
	36.1-37.2	-	+	+	-	+	+	+	-	-	-	-	-	-	-
35.7	34.5-34.6	-	+	+	-	-	+	+	-	-	-	-	-	-	-
	30.2-30.4	-	-	-	-	-	-	-	-	-	+	+	-	-	
28.9	27.6	-	+	-	+	+	+	-	-	-	-	-	-	-	-
	25.7	-	-	-	-	-	-	-	-	-	+	+	-	-	
	25.3	-	+	+	-	-	-	-	-	-	-	-	-	-	
	21.6	+	-	-	+	+	-	-	-	-	-	-	-	-	
20.8	19.9-20.5	+	+	+	+	+	+	+	+	+	+	+	+	+	

Dell'aquila (2000) in lentil, Kasim and Hamada (2003) in *Eruca sativa* and Faheed (2005) and Song *et al.* (2005) in wheat. The inhibitory effect of NaCl stress on seed germination and seedling growth may be attributed to the combined effect of low osmotic potential of the growing medium, specific ion toxicity and nutrient ion deficiency (Gulzar *et al.* 2001, Faheed 2005). Furthermore, prolonged or even transitory exposure of barley seeds to high temperatures was attributed to altered metabolic functions

in seedlings, and the most conspicuous alterations took place in cellular membranes and enzymes (Abdul Wahid and Shabbir 2005). Similarly, it was shown that the response of germination of *Vicia faba* seeds to short-term exposure to pre-sowing high temperature could be attributed to the activation of seed enzymes and the fast breaking of dormancy (El-Darier 1999). Germination of *Bromus auleticus* increased by either pre-sowing chilling the grains at 7 °C for 7-d or soaking in 0.05% gibberellic

acid (Ruiz *et al.* 2006), which seems to indicate that seed chilling might enhance the activity of endogenous hormones involved in seed germination. Pre-sowing soaking of seeds in gibberellic acid also alleviated the detrimental effect of salinity on seed germination and seedling growth in radish (Kasim and Dowiadar 2000, 2006). Heat, salinity and their combination seem to play a suppressive role on the activity of some germination and growth regulators.

The role of proline accumulation in combating abiotic stress is variously interpreted. Its accumulation is suggested to increase thermotolerance in *Nicotiana sylvestris* (Kuznetsov and Shevyakova 1997), mulberry (Chaitanya *et al.* 2001) and wheat (Faheed 2005). According to Delauney and Verma (1993), proline is an osmoregulatory solute which functions as a protector of macromolecules in plants subjected to hyper-osmotic stress. Under salinity stress, proline enhanced the ability of *Zea mays* seedlings for water absorption (El-Darier 1999), and its accumulation was assumed to be a metabolic adaptation which confers survival value by relieving stress. According to Elabsy (2006), proline and glutamic acid accumulation in the two halophytes *Nitraria retusa* and *Arthrocnemum macrostachyum* might be regarded as the adaptive mechanism to saline habitat. In the present study, the response of 35-d-old *Vicia faba* seedlings to heat and/or salinity was not limited to the accumulation of proline and glutamic acid but included six other amino acids (glycine, alanine, methionine, phenylalanine, lysine and argenine).

Thermal and salinity treatments, whether separately or in combination, caused major changes in the protein patterns of *Vicia faba* seeds and seedlings, especially in the earlier stages (10-d) of seedling growth. In 10-d-old seedlings, the *de novo* synthesis of two groups of polypeptide bands (group 1: MM 74.6-78.2 kDa, 49.4-49.9 kDa and 36.1-37.2 kDa; group 2: MM 50.5-50.8 kDa, 39.4-39.9 kDa and 34.5-34.6 kDa) in response to thermal and salinity treatments coincides with the consumption of the amino acid pool under those treatments (Fig. 3). A similar correlation between the decrease in total soluble protein and an increase in total amino acid content of mulberry leaves in response to high temperature (40 °C) was reported by Chaitanya *et al.* (2001). The degradation of the two proteins with low MM (27.6 and 21.6 kDa)

seems to have only a slight effect on the amino acid budget of 10-d-old seedlings. Therefore, synthesis of stress-related proteins takes place at the expense of the free amino acid pool of the seedlings. Consequently, the much greater protein stability of 35-d old seedlings (Table 1) might explain the relatively limited changes in their amino acid profiles under various thermal and salinization treatments (Fig. 4).

When combined with salinization of seedlings, each thermal treatment of seeds induced the synthesis of characteristic temperature-specific high MM protein(s) in the leaves (61.9 kDa for seed chilling, 150.7 kDa and 102.4 kDa for the reference control temperature, and 42.9 kDa for seed heating). Each of these proteins might be regarded as an indicator of the type of stress to which the seeds and seedlings were exposed. The 61.9 kDa and 102.4 kDa proteins might be members of the two complex heat shock protein families Hsp60 (or chaperonins) and Hsp100, respectively, which are found in the mitochondria and plastids of eukaryotes and play a crucial role in cellular homeostasis by assisting a wide range of newly synthesized and newly translocated proteins to achieve their native forms (Wang *et al.* 2004). Another category of temperature-specific proteins (with MM of 50.5 kDa, 39.4-39.9 kDa and 34.5-34.6 kDa) was synthesized in the leaves in response to both seed heating and chilling; synthesis of these polypeptides was not induced by salinity when the seeds were exposed to the reference control temperature (25 °C). It could be speculated that proteins with MM 39.9 kDa or less in Table 1 might be representatives of the small heat shock protein (sHsp) family with MM 12-40 kDa (Wang *et al.* 2004). sHsps are not themselves able to refold non-native proteins but they have a high capacity to bind and stabilize them, thus providing a reservoir of substrates for their subsequent refolding by members of the Hsp60 and Hsp100 families so that the stress-denatured enzymes and structural proteins can regain their functional conformations (Lee and Vierling 2000), which might in turn lead to improved seed germination and seedling growth.

It seems reasonable to assume that the strategy adopted by the *Vicia faba* seedlings to combat abiotic stress is age-dependent. In their early stages of growth, the seedlings resorted to the assembly of members of

various Hsp families capable of repairing the stress-induced changes in their native proteins, whereas in the later stages they switched to the use of free amino acids as osmoregulators in conjunction with fewer Hsps.

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