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Studies on thermoprotection induced by heavy metal ions in spring barley seedlings

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Abstract

Barley seedlings (*Hordeum vulgare* L., cv. Fatran) were pretreated with various concentrations of five heavy metal ions (Zn, Cu, Fe, Mg, Mn) for 3 d. When the subsequent heat shock was administered for 2 h, the heavy metal ions had thermoprotective effect against the sub-lethal (40 °C) and lethal (45 °C) temperature stresses, which were otherwise lethal to control (water grown) seedlings. The effectiveness of each of the heavy metal ions was different, the most effective being Cu. The level of protection provided by these heavy metal ions was dependent on both the time and the concentration that plants were exposed to them. The greatest differences were recorded in the thermotolerance mediated by applied metal ions in the shoot and root cells. Thermotolerance was exhibited by both the shoot and root of pretreated seedlings, even though the heavy metal stresses were applied solely to the roots.

Introduction

Exposure to elevated temperature (heat shock) causes a variety of alterations in cellular physiology, including increases in the synthesis of certain proteins (heat shock proteins or hsps) and provokes a similar sequence of events in all living organisms (Neidhardt *et al.* 1984, Craig 1985, Lindquist 1986). Prior exposure to a brief non-lethal heat treatment leads to the development of a transient resistance to an otherwise lethal heat treatment. Thermotolerance is the term generally used to refer to the transient, non-heritable state of resistance (Carper *et al.* 1987, Laszlo 1988). Although the molecular mechanism(s) involved in thermotolerance is not understood, a good correlation has been established between the induction of the hsps and the development of thermotolerance (Lindquist and Craig 1988). Althouth defined as a response to heat, establishing thermotolerance can also be elicited by different agents termed modulators (Lanks 1986). For example, conditioning heat treatments can be replaced by treatments with a wide range of modulators having the common property of inducing hsps at normal temperatures: ethanol, water stress, plant hormones,

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hypoxia, to name a few. Such modulators do not induce hsps in all organisms, but, in those in which they do, they also induce thermotolerance (Czarnecka *et al.* 1984, Plesset *et al.* 1982). In contrast, the pollen tubes of *Tradescantia* (Xiao and Mascarenhas 1985) and rat fibroblasts (Widlitz *et al.* 1986) are capable of mounting a thermotolerance response in the absence of newly synthesized hsps.

The results presented here show the effects of heavy metal pretreatment on the growth and the development of thermotolerance of barley seedlings.

Materials and methods

Plant material: Barley seeds (*Hordeum vulgare* L. cv. Fatran) were surface sterilized and germinated for 3 d on Whatman no. 3MM filter paper soaked in deionized sterile water at 26 ± 1 °C in a dark growth chamber. After treatment (for details see below), the seedlings were planted in plastic pots (25 × 40 cm) containing a mixture of perlite and sand (1:1 by vol.) and grown at 26 °C.

Application of heavy metals and heat stresses: Three-day-old seedlings were incubated on Whatman filter paper, soaked in deionized water (control) or appropriate heavy metal solutions ($ZnSO_4 . 7H_2O$, $CuSO_4 . 5H_2O$, $FeSO_4 . 7H_2O$, $MgSO_4 . 4H_2O$, $MnSO_4 . 4H_2O$) for 3 d at 26 °C. The heavy metal salts of the highest quality were purchased from *Fluka Chemie AG* or from *Merck*. The concentration used ranged from 0.025 up to 1 mM. The coleoptiles were never in direct contact with the stress solutions. Following a 3 d heavy metal treatment, a 2 h temperature stress at 40 °C (sub-lethal) or 45 °C (lethal) was applied to intact seedlings. Heat shock was applied to heavy metal-treated seedlings in shaking water baths in 1 mM potassium phosphate buffer (pH 6.5) plus 1 % sucrose. After treatment, the treated and control seedlings were planted in pots and grown at 26 °C.

Growth measurement and thermotolerance estimation: Seedling growth measurements were made by determining the length [mm] of the shoot and root of seedlings, from the kernel to distal tip. Thermotolerance was determined after a 3 d pretreatment with heavy metals followed by heat stress and was measured every 24 h for the 72 h recovery period. For analysis, 50 seedlings were evaluated and using the mean of these values, thermotolerance was determined as a percentage of control, water grown seedlings. Student's *t*-test and analysis of variance were carried out on each set of data with the aid of computer *ESCOM*.

Results

Effect of selected heavy metal ions on growth seedlings: Application of Zn in any of the concentrations tested had no significant effect on the growth of seedlings. Measured values were very similar to the water-treated control.

In comparison with Zn, Cu considerably stimulated seedling growth, mainly at concentrations ranging from 0.025 to 0.1 mM. A marked stimulation was recorded in the shoots where, at a concentration of 0.1 mM, it reached a value of about 65.9 % higher than that of the control. A different situation was observed in the root, where a concentration of 0.025 mM of Cu was most effective (approx. 40 % increase compared to controls). Seedling growth decreased with increasing the Cu concentration, compared to the control and at the two highest concentrations used it was inhibited (reduced by 15.8 % and 40 %, respectively).

Fe stimulated the growth of both the shoot and the root at all concentrations. For the shoot, the maximum stimulation of growth was at a concentration of 1 mM (increased by 47.3 %). In all other concentrations, the increase in growth was equal and on average represented 36 % over the control. The effect of Fe on the growth of the root was considerable; the maximum stimulation being at 0.1 mM (increased by 53 %). Similarly, Mg ions had a different effect on the growth of the shoot and the root, being more effective in stimulating root growth, mainly at concentration of 0.2 mM. As with Fe or Mg, Mn vigorously stimulated the growth of the root. The most effective concentrations on the root growth were 0.025, 0.1 and 1 mM, where growth exceeded that of the control by 40 %.

Modulation of the seedling response to heat shock by heavy metals: The effect of various concentrations of Zn on the response modulation of seedlings to heat shock at 40 °C and 45 °C is presented in Fig. 1. The maximum protective effect of the shoot to sub-lethal temperature stress was observed at concentration of 1 mM. None of the concentrations tested had a thermoprotective effect upon the shoot against the lethal

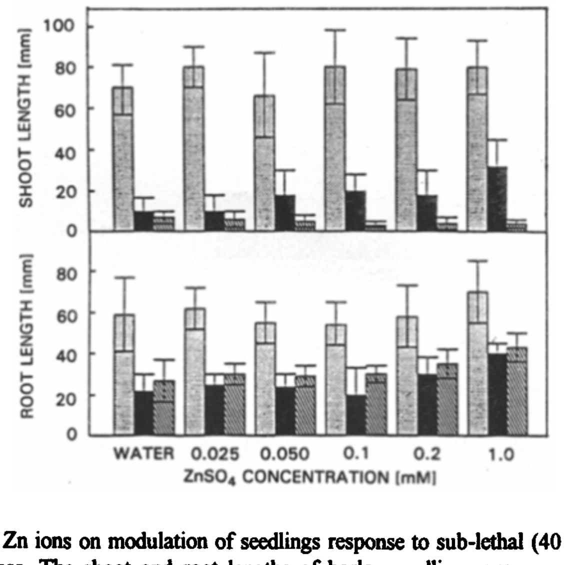

Fig. 1. Effect of Zn ions on modulation of seedlings response to sub-lethal (40 °C) and lethal (45 °C) temperature stress. The shoot and root lengths of barley seedlings grown on water prior to a 3 d treatment with indicated concentrations of $ZnSO_4$ were measured after a total of 4 d growth at 26 °C in the dark (control - *dotted columns*; 40 °C - *full columns*; 45 °C - *hatched columns*). Error bars represent ± SEM ($n = 50$, $P = 0.001$).

temperature. Similarly, in the thermoprotection of the root against both types of stresses the most effective were the concentrations of Zn higher than 0.1 mM. At a concentration of 1 mM the degree of thermoprotection against sublethal shock reached a value of 76.7 % against lethal shock *ca.* 53 % in comparison to control.

In the case of Cu (Fig. 2) thermoprotection was most effective at lower concentrations in both the shoot and the root. All applied concentrations of Cu to the shoot were equally effective in the thermoprotection against sub-lethal stress, achieving a value of approx. 208.8 %. In the application of the lethal shock, the level of thermoprotection was inversely proportional to concentration of the Cu used. The Cu ions dramatically affected the level of thermoprotection of the root, although in this case the inverse proportion between the concentration used and the degree of thermoprotection was evident. The lower concentration of Cu was the most effective (*e.g.* 0.025 mM) and the thermoprotection to 40 °C and 45 °C reached the value of 217.2 % and 164.3 %, respectively, in comparison with the control.

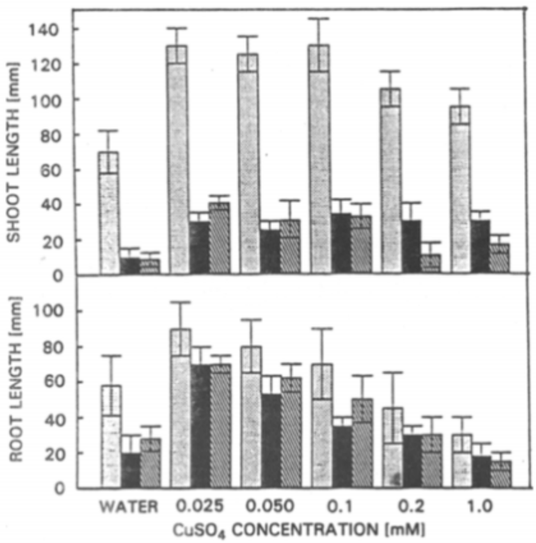

Fig. 2. Effect of Cu ions on modulation of seedlings response to sub-lethal (40 °C) and lethal (45 °C) temperature stress. The shoot and root lengths of barley seedlings grown on water prior to a 3 d treatment with indicated concentrations of $CuSO_4$ were measured after a total of 4 d growth at 26 °C in the dark (control - *dotted columns*; 40 °C - *full columns*; 45 °C - *hatched columns*). Error bars represent ± SEM ($n = 50, P = 0.001$).

After treatment of seedlings by Fe ions and subsequent exposure to the sub-lethal and lethal shock the level of thermoprotection was directly proportional to the Fe concentration (Fig. 3). In the case of the shoot, the amount of thermoprotection rose very slowly with the increasing concentration, and against sub-lethal shock the most effective concentration was 0.2 mM (96 % versus control).

Application of Fe had a minimum effect on the induction of thermoprotection of the shoot cells subjected to 45 °C. In relation to the concentrations of Fe, the induction of thermoprotection in the root was considerably increased against both types of stresses. The lowest concentration induced thermoprotection against 40 °C, by 106.5 % higher than that of the control. The most effective concentration was

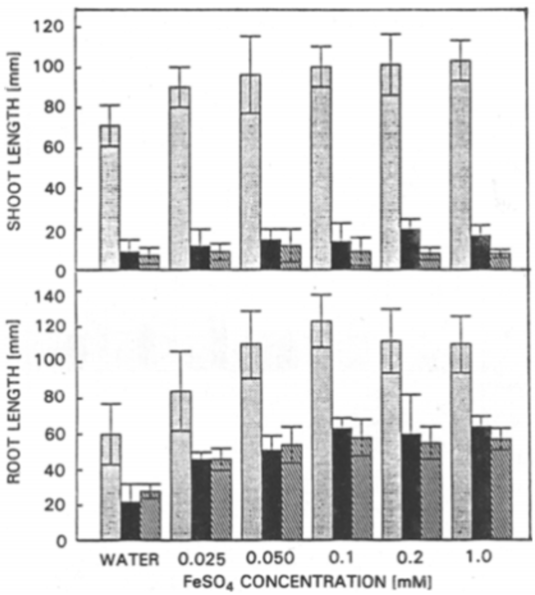

Fig. 3. Effect of Fe ions on modulation of seedlings response to sub-lethal (40 °C) and lethal (45 °C) temperature stress. The shoot and root lengths of barley seedlings grown on water prior to a 3-d treatment with indicated concentrations of $FeSO_4$ were measured after a total of 4 d growth at 26 °C in the dark (control - *dotted columns*; 40 °C - *full columns*; 45 °C - *hatched columns*). Error bars represent ± SEM ($n = 50$, $P = 0.001$).

0.1 mM and this was also the most effective against lethal shock (113.7 % versus control). Mg ions induced seedling protection against both stresses (Fig. 4) at a range of concentrations, but their effectiveness in inducting protection of the shoot subjected to sub-lethal or lethal shock was very low. The most effective concentrations ranged from 0.05 to 0.1 mM (*i.e.* 121.9 % and 144 %, respectively). In the case of the root, all the concentrations of Mg tested were very effective against both of the heat shocks. The protection against 40 °C reached approx. 119 %. At concentrations higher than 0.025 mM Mg ions showed greater thermoprotection against the lethal than the sub-lethal stress.

On the other hand, an application of Mn ions resulted in the differential induction of protection of the shoot against 40 °C and 45 °C (Fig. 5). However, a different protection of the shoot and the root was observed. Concentrations ranging from 0.025 to 0.2 mM stimulated shoot protection (by approx. 100 % at 40 °C). From the concentrations evaluated, neither had a positive effect on induction of thermo-protection against lethal stress. The root when pretreated by various concentrations of Mn responded to both shock differently as did the shoot. The degree of protection induced against the sub-lethal stress was similar in all concentrations applied (average value of 171.4 % *vs.* control). The most obvious difference was found in the response of the shoot and the root subjected to the lethal stress. While no protection in the shoot was recorded, in the main root it achieved a value of *ca.* 93 %. With increasing concentration, the amount of protection decreased.

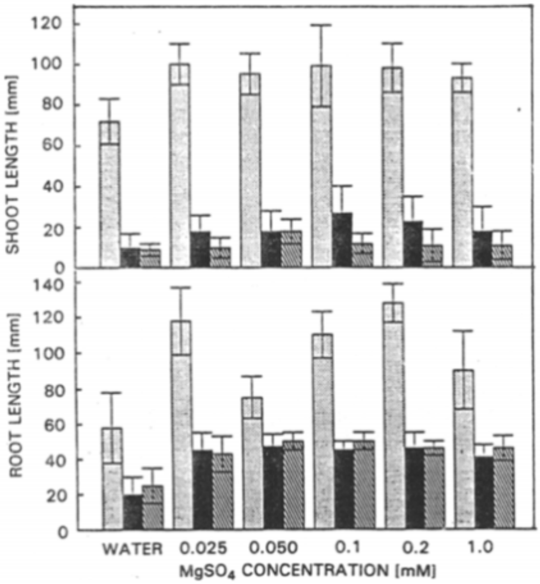

Fig. 4. Effect of Mg ions on modulation of seedlings response to sub-lethal (40 °C) and lethal (45 °C) temperature stress. The shoot and root lengths of barley seedlings grown on water prior to a 3 d treatment with indicated concentrations of $MgSO_4$ were measured after a total of 4 d growth at 26 °C in the dark (control - *dotted columns*; 40 °C - *full columns*; 45 °C - *hatched columns*). Error bars represent ± SEM (n = 50, P = 0.001).

Thermotolerance induced by heavy metal ions: The effect of sub-lethal or lethal heat shocks on the water- or heavy metal-treated seedlings is presented in Fig. 6. Incubation of seedlings with heavy metal ions at the lowest concentration resulted in the induction of a marked thermotolerance by Cu ions during all of the time intervals examined. After 24, 48 and 72 h from the application of a 2 h sub-lethal stress the increase of thermotolerance induced by Cu ions was, in comparison with the control, 19.3, 28.3 and 42 %, respectively. In the case of other heavy metals, no protective effect was recorded after 24 h. After 48 h, the thermotolerance increased on average by about 10 % (also for Zn and Mn ions). After 72 h, the thermotolerance induced by the respective ions increased by about 20 % as a whole. At a concentration of 0.025 mM the least effective ions was Fe where the difference vs. control corresponded to 43 % and vs. thermotolerance induced by Cu or Zn/Mn ions it amounted to 90 and 70 %, respectively. The situation was similar with the indicated heavy metals concentration during the induction of thermotolerance of the root. The most effective were again Cu ions and the least protective effect was exhibited by Fe ions. From Fig.6A one may conclude that in the case of the shoot the increase in heavy metal concentration caused, in the majority of ions (Mg, Mn and Zn), a rapid increase in the degree of thermotolerance with time. In the case of Cu the difference in the thermotolerance induced between 24 and 72 h represented 40 % and in the case of Mn - as much as 90 %.

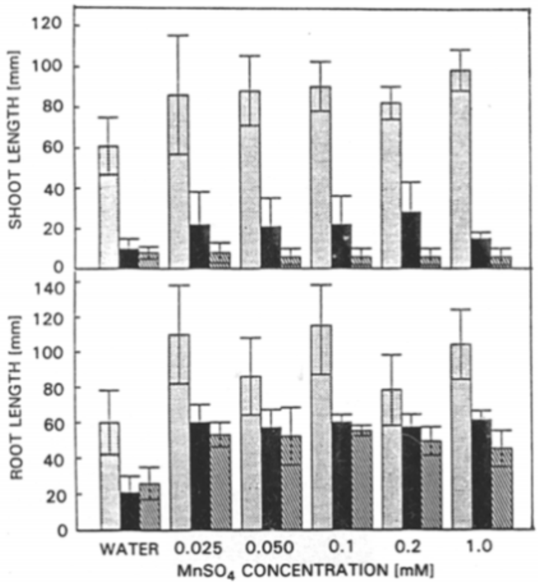

Fig. 5. Effect of Mn ions on modulation of seedlings response to sub-lethal (40 °C) and lethal (45 °C) temperature stress. The shoot and root lengths of barley seedlings grown on water prior to a 3-d treatment with indicated concentrations of $MnSO_4$ were measured after a total of 4 d growth at 26 °C in the dark (control - *dotted columns*; 40 °C - *full columns*; 45 °C - *hatched columns*). Error bars represent ± SEM (n = 50, P = 0.001).

Concentrations ranging from 0.1 to 0.2 mM (mainly for Mg, Mn and Zn) were characterized in the shoot by a steeper increase in induced thermotolerance within the intervals examined. At the highest concentration used, surprisingly, the most effective were Zn ions, when, after 72 h from the application of 40 °C the thermotolerance reached a value of 57 %. The thermotolerance induced in the root subjected to sub-lethal heat shock decreased with increasing metal concentrations.

The treatment of seedlings incubated in water by lethal shock (Fig.6B) resulted in a minimal induced thermotolerance. In the induction of thermotolerance of seedlings subjected to lethal stress Cu ions were again the most effective: in the case of the shoot at concentrations ranging from 0.025 to 0.2 mM and in the case of the root at 0.025-0.1mM. Surprisingly, an increrase of the Cu concentration resulted in a

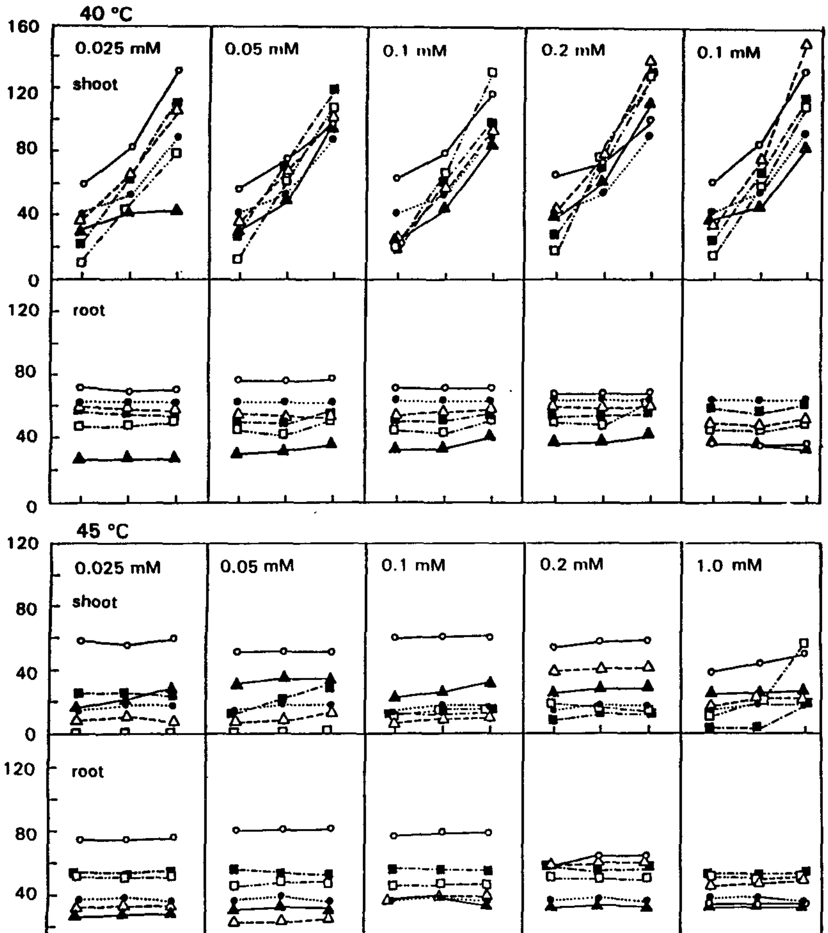

Fig. 6. Effect of a 3-d heavy metal pretreatment (control - *closed circles; Zn - open triangles; Cu - open circles; Fe - closed triangles; Mg - open squares; Mn - closed squares*) on the induction of thermotolerance of seedlings exposed to sub-lethal (40 °C, 2 h -above) and lethal (45 °C, 2 h - below) heat shock. Induction of the thermotolerance was measured as the recovery growth/length of both the shoot and root over a 3 d post-heat shock period. Control seedlings were maintained at 26 °C on water in the dark. Each point is the mean of 50 measurements.

decrease in the percentage induced thermotolerance. The difference between the lowest and highest applied concentration reached a value of about 20 % in the shoot. Fe ions stimulated the induction of thermotolerance of the shoot at concentrations up to 0.2 mM and the difference observed between respective days was minimal. In the induction of thermotolerance of the shoot the Zn and Mn ion was ineffective against lethal temperature stress and the application of Mg in the indicated range of concentrations resulted in inhibition of thermotolerance. In the main root, a relation between both the induction of thermotolerance and the increase of the applied metals concentration was clear. While at a concentration of 0.2 mM thermotolerance was induced by 20-25 % by all the metals applied. Only at a concentration of 1mM were the Mg, Mn and Zn ions able to induce ca 10 % thermotolerance. Fe ions were an exception in this range of concentrations, in having a minimal effect.

Discussion

The results of these experiments demonstrate that the administration of heavy metals to *Hordeum vulgare* seedlings triggers the induction of thermotolerance against both sub-lethal and lethal temperature stresses.

Five different concentrations of heavy metal ions were used. From the point of view of effectiveness on the shoot and root growth the applied heavy metals can be placed in the following sequence: Zn>Mn>Mg>Fe>Cu and Zn>Cu>Mn>Mg>Fe, respectively.

In both cases studied it was shown that Zn ions had a minimal effect on growth. The Fe, Mg and Mn ions in all the applied concentrations had a very positive effect. The stimulation by Cu was mainly on the root and strongly dependent on the concentration.

The question of the concentreation dependence is very interesting. When Bonham-Smith *et. al.* (1987) examined the heavy metals accumulation (at concen-trations of 0.05 or 0.2 mM) by root cells, they observed with the aid of atomic absorption measurements that metal uptake of Zn, Cu or Cd was very similar. However, their effects on seedling growth were considerably different. The higher concentrations of Cu (0.2 mM and above) dramatically inhibited the growth activity of the main root. These findings are in good agreement with our results. Conversely, the lower concentrations of Zn had a minimum effect on growth activity.

Heavy metals induce synthesis of one or only a few proteins, but not heat shock proteins (Kapoor 1986; Bonham-Smith *et al.* 1987). When sub-lethal or lethal heat shock was applied to the seedlings pretreated with five concentrations heavy metals there was a divergence in the protective efficiency between the heavy metal used and the individual concentrations. The lower concentrations of Zn had no protective effect against both shocks. A vigorous protective effect of Zn ions was evaluated only after application of the highest concentrations in both treated tissues.

In the case of the thermoprotective effect of Cu ions it was observed, as with Bonham-Smith *et al.* (1987) using maize seedlings, that the lowest concentrations were the most effective. In relation to the shoot, all concentrations of Cu used seemed to be thermoprotective against sub-lethal stress. In the case of lethal stress an inverse proportion exists between the concentration of Cu and the percentage induced thermotolerance. This is again in very good agreement with the findings of Bonham-Smith *et al.* (1987) obtained in maize. The Fe ions had a reverse effect on the induction of thermotolerance compared with Cu. The level of protection was directly proportional to the concentration used. A marked difference in thermotolerance mediated by Fe in the shoot and root was exhibited against sub-lethal as well as lethal stress. A similar situation was observed in the case of Mg and Mn ions.

Surprisingly, the thermotolerance against 40 °C or 45 °C is not only characteristic of heavy metals. The exogenous application of abscisic acid (ABA) or triadimefon (a fungicide) demonstrated an enhanced ability to withstand the effects of a 3 h sub-lethal or lethal heat shock (Bonham-Smith *et al.* 1988).

Another very important question is understanding the mechanism(s) of activation of the thermotolerant response. We have examined an induction of thermotolerance during the recovery (3 d) after application of both the sub-lethal and lethal shocks. As the most effective was again the Cu ion against both stresses. The increased level of thermotolerance acquired by Zn and Mn against the sub-lethal stress was observed after 2 d recovery period, but only for the shoot. The percentage induced thermotolerance of the root reached the same values as in the water-treated control. A 3 d pretreatment of barley seedlings with various metal concentrations resulted in the stimulation of thermotolerance against lethal stress. The most effective, at least in lower concentrations, were the Cu ions, which has been independently described by other authors (Bonham-Smith *et al.* 1987).

Although heavy metal treatments were applied solely to the roots of the barley seedlings, increased thermotolerance was also acquired by the shoot. As suggested by Bonham-Smith *et al.* (1987), in the absence of Cu, Cd or Zn from the shoot, increased thermotolerance must be due to a relayed effect from the root. As far as we know, a similar type of response has been observed in wounded potato (Walker-Simmons *et al.* 1984) and pea plants (Davies and Schuster 1981) where tissue away from the wounding site, both basipetally and acropetally, showed wound-induced changes in cell properties, mediated by a putative wound signal called PIIF (Proteinase inhibitor inducing factor, Plunkett *et al.* 1982).

Furthemore, it is interesting that not only heavy metals may promote thermotolerance. The converse is also true. It was observed that heat shock induces tolerance to some heavy metals (Orzech and Burke 1988), ethanol (Lindquist 1986) and other forms of stress.

To better understanding of mechanism(s) included in the heavy metal acquired thermotolerance further studies on the level of hsps synthesis need to be undertaken. These experiments start in our laboratory now.

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