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Are polyamines involved in the heat-shock protection of mung bean seedlings?

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Abstract. Germinating seeds of *Vigna radiata* (Linn) Wilczek cv. ML 311, with a radicle length of 5 mm, were subjected to a heat-shock episode of 50°C for 2 h followed by transfer to the normal temperature (28°C) for 3 days in the dark. Exogenous effects of polyamines (putrescine, spermidine, and spermine) on the recovery growth and membrane integrity of seedling tissues were studied. Application of polyamines, either as a pre-treatment at 28°C for 2 h prior to heat-shock or as a co-treatment (50°C, 2 h) during the heat-shock period itself, enhanced the recovery growth of both roots and hypocotyls but especially the former with the order of effectiveness being putrescine, spermidine, and spermine. Treatment with polyamine biosynthetic inhibitors, i.e. D,L α-diflouromethylarginine (DFMA) and D,L α-diflouromethylornithine (DFMO) resulted in thermosensitization, making seedlings vulnerable to heat-shock. This effect could be ameliorated by putrescine application. An important role of polyamines in heat-shock protection is thus indicated.

Keywords: Heat-shock; Ion leakage; Lipid peroxidation; Polyamines; Root growth; Seedling growth; *Vigna radiata*.

Introduction

The diamine putrescine, the triamine spermidine, and the tetramine spermine are ubiquitous in plant tissues and have been implicated in an overwhelming array of plant growth and developmental processes (Bagni, 1989). There is a growing appreciation of the role of polyamines in plant stress responses (Evans and Malmberg, 1989; Flores, 1991), but their role in heat-shock protection of higher plants is not well understood (Basra et al., 1992).

Heat stress is a major factor limiting the productivity and adaptation of crops, especially when it coincides with critical stages of plant growth and development (McWilliams, 1980; Chen et al., 1982; Paulsen, 1994). Even brief episodes of heat-shock between temperature ranges of 45 to 50°C induce marked changes in plant growth processes. The repair of plant cells exposed to heat stress, after having been returned to an optimal temperature, has been reported (Bauer and Senger, 1979), but the degree of recovery and the time required for recovery depend upon the severity of stress (Berry and Björkman, 1980).

In order to have a better understanding of plant responses to heat-shock, it is important to know the regulatory factors involved in imparting protection and growth recovery processes. In the present study, the polyamine-mediated regulation of the heat-shock response of etiolated mung bean seedlings was investigated using exogenous applications of polyamines and their biosynthetic inhibitors. Changes in electrolyte leakage and lipid peroxidation of seedling tissues were monitored as measures of cellular membrane integrity.

Materials and Methods

Mung bean seeds (*Vigna radiata* (Linn.) Wilczek cv. ML-131) were germinated in the dark for 24 h at 28°C in Petri-dishes (9.0 cm) on filter paper moistened with 5.0 ml of distilled water. Heat-shock at 50°C for 2 h was applied to germinated seeds with a radicle length of 5 mm followed by transfer to 28°C on water for 72 h in the dark to observe recovery growth. For growth measurements, length of the whole seedling (root plus hypocotyl), primary root, and hypocotyl were measured after the 3 day recovery period.

The tested polyamines were: putrescine, spermidine, and spermine. The polyamine biosynthetic inhibitors were: DFMA (D,L α-diflouromethylarginine) and DFMO (D,L α-diflouromethylornithine). These were applied either as a pre-treatment at the normal temperature of 28°C for 2 h before subjecting the seedlings to heat-shock (50°C, 2 h) or as a co-treatment during the heat-shock period itself. Similarly, lysine, an amino acid, was tested at equivalent concentrations to demonstrate that heat-shock protection is really a polyamine related phenomenon and not simply a growth effect from the extra reduced nitrogen and carbon supplied. After each treatment, the seedlings were

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transferred to 28°C and grown in water for 3 days. Seedlings kept entirely at 28°C without subjection to heat-shock but pre-treated in the aforementioned manner were also used as a control to observe the effects of polyamines and their biosynsthetic inhibitors *per se*. Twenty seedlings were used in each experiment. The experiments were repeated on three separate occasions.

Membrane integrity measurements were carried-out in relation to heat-shock control, polyamines, calcium, and EGTA treatments. Measurements were made either immediately upon heat-shock (50°C, 2 h) using excised embryonic axes or after 24 h of recovery growth using sections of primary roots (1.0 cm from the distal tip) or hypocotyls using 1.0 cm portions beneath the point of attachment of cotyledons. All measurements were performed with two replicate samples per treatment and each experiment was repeated twice.

For total electrolyte leakage assay, 10 embryonic axes, primary root tips or hypocotyls were excised and washed briefly with deionized water to remove adhering electrolytes. The tissue sections were then immersed in test tubes containing 20 ml deionised water stirred continuously at 28°C. After 5 h, the electrolyte leakege was estimated by a conductivity meter. The samples were then boiled for 30 min and the conductivity measured again. The percentage leakage was calculated as:

$$\frac{\text{Conductivity before boiling}}{\text{Conductivity after boiling}} \times 100$$

Lipid peroxidation was measured by the thiobarbituric acid (TBA) colour reaction according to Bernheim et al. (1948). For each assay, 10 embryonic axes, primary root tips or hypocotyl sections were homogenized with 10 ml of TBA reagent using a pestle and mortar (TBA reagent was prepared by mixing 18% TCA with 0.45% TBA in a 1:2 ratio). The extracts were kept in a boiling water bath for 15 min and filtered hot through Whatman No. 42 filter paper. Absorbance of the filtrate was read at 532 nm and values were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde was calculated based on its extinction coefficient (Heath and Packer, 1968).

Results and Discussion

The results show that an exposure to 50°C for 2 h was strong enough to cause a 55% decease in primary root length and a 44% decrease in hypocotyl length (Table 1). Whole seedling growth (root + hypocotyl length) could be recovered up to 50% upon return to the normal temperature. We were interested to see whether polyamines could protect the seedling against heat-shock or could enhance the growth recovery.

Certainly, application of polyamines resulted in the enhancement of thermoprotection response (Table 1). Putrescine was most effective, followed by spermidine and spermine. A greater effect was seen at a 1 mM concentration than at 100 μM, and the co-treatment was more effective than the pre-treatment. In the co-treatment, the

Table 1. Influence of polyamines and lysine on the heat-shock response of etiolated mung bean seedlings.

Treatment	Seedling growth (cm)		
	Roots	Hypocotyls	Whole seedlings
28°C (Control)	8.86±1.26 (100)	5.89±0.98 (100)	14.75 (100)
50°C (2 h)	3.97±0.40 (44.80)	3.29±0.29 (55.85)	7.26 (49.22)
28°C+100 μM Putrescine (2 h) -50°C (2 h)	6.12±0.76 (69.07)	3.63±0.28 (61.62)	9.75 (66.10)
28°C+1 mM Putrescine (2 h) -50°C (2 h)	7.72±0.82 (87.13)	4.71±0.61 (79.96)	12.43 (84.27)
28°C+100 μM Spermidine (2 h) -50°C (2 h)	4.78±0.32 (53.95)	3.50±0.36 (59.92)	8.28 (56.13)
28°C+1 mM Spermidine (2 h) -50°C (2 h)	5.50±0.72 (62.07)	3.62±0.28 (61.46)	9.12 (61.83)
28°C+100 μM Spermine (2 h) -50°C (2 h)	4.20±0.55 (47.40)	3.41±0.42 (57.89)	7.61 (51.59)
28°C+1 mM Spermine (2 h) -50°C (2 h)	4.05±0.38 (45.71)	3.52±0.38 (59.76)	7.57 (51.32)
50°C+100 μM Putrescine (2 h)	6.58±0.75 (74.26)	4.0 ±0.26 (67.91)	10.58 (71.72)
50°C+1 mM Putrescine (2 h)	7.80±0.68 (88.03)	4.62±0.52 (78.43)	12.42 (84.20)
50°C+100 μM Spermidine (2 h)	5.54±0.80 (62.52)	3.64±0.31 (61.79)	9.18 (62.23)
50°C+1 mM Spermidine (2 h)	7.02±0.91 (79.23)	4.15±0.44 (70.45)	11.17 (75.72)
50°C+100 μM Spermine (2 h)	4.47±0.30 (50.45)	3.42±0.26 (58.06)	7.89 (54.49)
50°C+1 mM Spermine (2 h)	5.93±0.73 (66.93)	3.80±0.47 (64.51)	9.73 (65.96)
28°C+100 μM Lysine (2 h) -50°C (2 h)	3.65±0.28 (41.20)	3.33±0.28 (56.54)	6.98 (47.32)
28°C+1 mM Lysine (2 h) -50°C (2 h)	3.69±0.19 (41.65)	3.22±0.35 (54.67)	6.91 (46.85)
50°C+100 μM Lysine (2 h)	3.90±0.34 (44.02)	3.34±0.33 (56.71)	7.24 (49.08)
50°C+1 mM Lysine (2 h)	3.70±0.25 (41.76)	3.28±0.29 (55.69)	6.98 (47.32)

Germinating seedlings with the embryonic axis protruding about 5 mm from the seed-coat were subjected to the treatments shown above, applied as either pre-treatments at the normal temperature (28°C, 2 h) or as co-treatments during the heat-shock period itself (50°C, 2 h). After each treatment, the seedling were grown in water in a dark incubation at 28°C and the growth measurements were taken after 72 h. Twenty seedlings were used in each experiment and each one was repeated three times. Values are means ± S.E. Figures in parentheses are relative percentages.

polyamines were applied simultaneously with heat-shock (50°C, 2 h), whereas in the pre-treatment the application was made at 28°C for 2 h immediately prior to the heat-shock treatment. Thermotolerance was induced in both root and hypocotyl tissues, but the roots showed a greater response. Pertinently, the important role of polyamines in root formation and growth has also been demonstrated in other studies (Chatterjee et al., 1983; Jarvis et al., 1983; Palavan-Unsal, 1987).

The maximum response seen with 1 mM putrescine co-treatment resulted in root growth recovery of 88% and hypocotyl growth by 78% (Table 1). At the same concentration, spermidine caused a 79% and 70% recovery of root and hypocotyl growth, respectively. Spermine was ineffective when given as a pre-treatment but showed a small growth-promotory effect as a co-treatment. Its 1 mM co-treatment caused about 67% and 64% recovery growth for roots and hypocotyls, respectively. It may be that polyamines in these experiments act merely as a source of nitrogen when stimulating growth, though this is unlikely because of a marked growth regulatory effect even at 100 µM, particularly in case of putrescine and spermidine (Table 1). The treatment of seedlings with polyamines without a subsequent heat-shock and kept entirely at 28°C revealed only a slight promotory effect up to a maximum of 6%, suggesting a stress adaptive role of polyamines. This point was further proved when lysine, applied at simliar concentrations as polyamines, failed to elicit a protective response (Table 1).

We further tested the role of polyamines using polyamine biosynthetic inhibitors, i.e. DFMA and DFMO, which are specific inhibitors of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively. In plants, polyamines can be synthesized by either ADC or ODC-mediated pathways (Smith, 1985; Evans and Malmberg, 1989). DFMA and DFMO were applied at 2 mM and 4 mM concentrations as a pre-or co-treatment.

Treatment with both the inhibitors rendered the seedlings vulnerable to heat-shock, an effect clearly evident at 4 mM concentration of DFMA or DFMO (Table 2), reinforcing the relationship between polyamines and heat-shock protection. Pertinently, the inhibitory effect was reversed on adding putrescine (Table 2); restoration of root growth being in the range of 76–83% and hypocotyl growth in the range of 67–74%.

In this study, though DFMA had a tendency to be more inhibitory than DFMO, the loss of thermotolerance by both may be interpreted to mean that both ADC and ODC pathways of polyamine biosynthesis are operative and are required for thermoprotection and growth recovery in mung bean seedlings. Friedman et al. (1989) also reported that both ADC and ODC were responsible for salt-induced increase in putrescine biosynthesis of mung bean seedlings.

Since exogenous putrescine alone showed the best response (Table 1), and relieved inhibition caused by DFMA or DFMO (Table 2), it is worth noting that exposure of plants to a variety of stress conditions often results in putrescine accumulation (Smith, 1985; Evans and Malmberg, 1989; Foster and Walters, 1991). Heat stress-induced polyamine accumulation has been reported (Das et al., 1987) and linked to higher thermotolerance ability of resistant cells (Shevyakova et al., 1994).

One of the main loci of high temperature injury in plants is membrane damage (Martineau et al., 1979; Ahrens and Ingram, 1988; Upadhyaya et al., 1990). The effect of polyamines on maintenance of membrane integrity under heat-shock was thus investigated. It can be clearly seen that in comparison with the heat-shock control, putrescine and spermine treatments all decreased electrolyte leakage from embryonic axes, root and hypocotyl tissue sections (Figure 1), suggesting protection of membrane integrity. A marked decreased in lipid peroxidation of root tissue was also noticed (Figure 1), which also showed the maximum growth response (Table 1).

Table 2. Influence of polyamine biosynthetic inhibitors (DFMA and DFMO) on the heat-shock response of etiolated mung bean seedlings and reversal of their effect by putrescine.

Treatment	Seedling growth (cm)		
	Roots	Hypocotyls	Whole seedlings
28°C (2 h)	8.91±1.22 (100)	5.90±0.62 (100)	14.81(100)
50°C (2 h)	4.0 ±0.52 (44.89)	3.25±0.41 (55.08)	7.25 (48.95)
28°C+2 mM DFMA (2 h) -50°C (2 h)	3.81±0.40 (42.76)	3.0 ±0.28 (50.84)	6.81 (45.98)
28°C+4 mM DFMA (2 h) -50°C (2 h)	2.80±0.23 (31.42)	2.72±0.33 (46.10)	5.52 (37.27)
28°C+2 mM DFMO (2 h) -50°C (2 h)	3.80±0.51 (42.64)	2.96±0.40 (50.16)	6.76 (45.64)
28°C+4 mM DFMO (2 h) -50°C (2 h)	2.82±0.36 (31.64)	2.85±0.39 (48.30)	5.67 (38.28)
50°C+2 mM DFMA (2 h)	3.63±0.52 (40.74)	2.77±0.36 (46.94)	6.40 (43.21)
50°C+4 mM DFMA (2 h)	2.58±0.24 (28.95)	2.27±0.26 (38.47)	4.85 (32.74)
50°C+2 mM DFMO (2 h)	3.73±0.42 (41.86)	2.92±0.33 (49.49)	6.65 (44.90)
50°C+4 mM DFMO (2 h)	2.71±0.51 (30.41)	2.45±0.40 (41.52)	5.16 (34.84)
28°C, 2 mM DFMA+1 mM Putrescine (2 h) -50°C (2 h)	6.80±0.85 (76.31)	3.96±0.52 (67.11)	10.76 (72.65)
28°C, 2 mM DFMO+1 mM Putrescine (2 h) -50°C (2 h)	6.95±0.90 (78.0)	4.02±0.41 (68.13)	10.92 (74.07)
28°C, 2 mM DFMA+1 mM Putrescine (2 h)	7.20±1.01 (80.80)	4.11±0.37 (69.66)	11.31 (76.36)
28°C, 2 mM DFMO+1 mM Putrescine (2 h)	7.41±0.93 (83.16)	4.39±0.60 (74.40)	11.80 (79.67)

Otherwise as in Table 1.
DFMA=D,L α-Difluoromethylarginine; DFMO=D,L α-Difluoromethylornithine.

Polyamines, being cationic in nature, can associate with anionic components of the membrane such as phospholipids thereby stabilizing the bilayer surface and retarding membrane deterioration (Roberts et al., 1986; Basra et al., 1994). Polyamines also have radical scavenging properties (Drohlert et al., 1986). Protection of membranes from peroxidation by polyamines could involve both their ability to interact with phospholipids and their anti-oxidant activity (Kramer and Wang, 1989). It has also been suggested that under stress conditions, polyamines may partially replace calcium in maintaining membrane integrity by binding to phospholipid components of the membrane (Naik and Srivastva, 1978). It is worth noting that calcium application also decreased electrolyte leakage and chelation of extracellular calcium by EGTA caused extensive leakage (Figure 1).

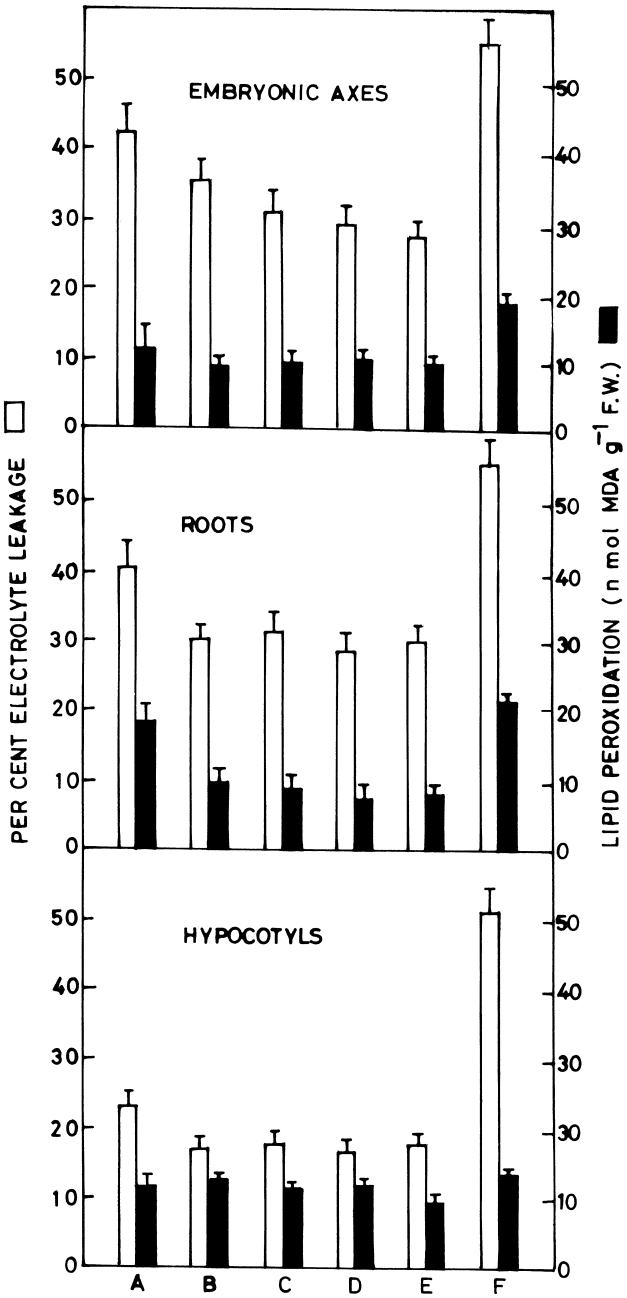

Figure 1. Changes in electrolyte leakage and lipid peroxidation of etiolated mung bean seedlings following exposure to 50°C for 2 h; A, Control; B, 1 mM Putrescine; C, 1 mM Spermidine; D, 1 mM Spermine; E, 1 mM $CaCl_2$; F, 5 mM EGTA. Germinating seedlings with the embryonic axis protruding about 5 mm from the seed-coat were subjected to heat-shock and the growth regulators were applied as co-treatment during the period of heat-shock itself (50°C, 2 h) followed by recovery growth in water at 28°C. Embryonic axes were isolated immediately from the control and treated seedlings while roots and hypocotyls were detached at 24 hours of recovery growth. Values are mean ± S.E.

Overall, it appears that polyamines are important stress factors and are effective in imparting heat-shock protection to mung bean seedlings. Putrescine is particularly effective. Of the various seedling tissues, roots show the maximum protective response. Hence the relationship of polyamines to heat tolerance in crop improvement is worthy of investigation. Our continuing work with mung bean concerns changes in endogenous levels of polyamines, as well as the biosynthesis and catabolism of them.

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