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Heat shock induced lipid changes and solute leakage in germinating seeds of pigeonpea

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Abstract

Heat shock (HS) reduced total lipid and phospholipid contents and their synthesis in germinating seeds of pigeonpea [*Cajanus cajan* (L.) Millspaugh]. Lipid peroxidation was also enhanced with increasing temperature and HS duration. HS influenced lipid metabolism to a higher extent at 45 °C than at 40 °C. This altered lipid metabolism and lipid peroxidation was associated with the loss of various solutes from the germinating seeds, and modification of growth and development. Pretreatment of germinating seeds at 40 °C for 1 h or at 45 °C for 10 min followed by incubation at 28 °C for 3 h prior to 45 °C for 2 h ameliorated solute leakage due to reduced lipid peroxidation and improvement in lipid content and membrane function.

Additional key words: *Cajanus cajan*, lipid peroxidation, phospholipid.

Introduction

Membranes are highly sensitive to alterations caused by temperature. Exposure of germinating seeds to supra-optimal temperatures leads to continuous leakage of cell solutes (Björkman 1980, Levitt 1980, Chen *et al.* 1982, Bhattacharjee and Mukherjee 1998). The regulation of electrolyte leakage has been reported to be a good index of heat stress tolerance (Towill and Mazur 1974, Martineau *et al.* 1979, Sullivan and Ross 1979, Wu and Wallner 1983).

Heat shock (HS) affects many physiological processes including protein and lipid metabolism and enzyme activity (Upadhyaya *et al.* 1991, Kurganova *et al.* 1997, Guo *et al.* 1998). Heat shock proteins (HSPs) synthesized in response to HS are considered to be involved in cellular protection and repair (Schlesinger *et al.* 1990, Howarth and Ougham 1993). Therefore synthesis of HSPs in response to HS and in consequence the control of solute leakage can be viewed as a mechanism of thermotolerance in germinating seeds (Lin *et al.* 1985). Though the studies on HS responses were mainly concentrated on the synthesis of HSPs, effect of HS on other physiological processes also needs attention. Keeping this in view, the present study deals with the impact of HS on membrane permeability based on changes in lipid content and lipid peroxidation, and on solute leakage.

Materials and methods

Plants: Pigeonpea [*Cajanus cajan* (L.) Millspaugh cv. LRG 30] seeds of uniform size were selected and surface sterilized using 0.001 M mercuric chloride for 2 min and were soaked in distilled water for 3 h. The seeds were then germinated in darkness at laboratory temperature (28 ± 2 °C) on *Whatman No.1* filter paper moistened with sterile distilled water.

Heat shock treatment: One-day-old uniform size germinating seeds of pigeonpea kept in buffer medium (1 % sucrose, 1 mM potassium phosphate, pH 6.0) were exposed to different heat shock treatments; 40 or 45 °C for 30, 60, 90 and 120 min. Another two sets of germinating seeds were given pretreatments, one set at 40 °C for 60 min and the other at 45 °C for 10 min

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Abbrevations: HS - heat shock, HSP - heat shock protein.

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followed by intervening incubation of both sets at 28 °C for 3 h prior to the exposure to temperature of 45 °C for 2 h.

Growth: After HS treatments, the germinating seeds were transferred to Petri dishes lined with moist filter papers and were grown at laboratory temperature for 72 h. Simultaneously controls were maintained at laboratory temperature (28 ± 2 °C). Dry mass was measured at 24 h intervals. The relative growth index, RGI [%] was calculated as the ratio: RGI = (average dry mass of embryogenic axis of a treated seedlings/ average dry mass of embryogenic axis of a control seedling) × 100.

Content of total lipids and phospholipids: Immediately after HS treatments, lipid extraction was carried out according to the method of Bligh and Dyer (1959). The total lipid content was determined gravimetrically. The total phospholipid content was determined as the phosphorus content of the phospholipids by the procedure outlined by Bartlett (1959).

Lipid and phospholipid synthesis: Immediately after HS treatments, the seed coats were removed and material (1 g) was incubated in 2 cm^3 of distilled water containing ^{14}C-acetate (2.96 × 10^5 Bq cm^{-3}, specific activity 2.0 × 10^9 Bq $mmol^{-1}$, *BARC*, Bombay, India) and then incubated at 28 ± 2 °C with constant shaking for 6 h. At the end of incubation, labeled seeds were washed three times with distilled water and extraction of lipids was carried out by the method of Bligh and Dyer (1959). Samples (in chloroform) of 0.025 cm^3 each were spotted on *Whatman No. 3* filter paper discs with 5 cm^3 of scintillation fluid (0.5 % 2,5-diphenyloxazole in toluene) and counted by liquid scintillation counter *Rockbeta* (*LKB-1217*, Wallac, Finland).

Phospholipids were separated by two dimensional thin layer chromatography (TLC) on silica gels using chloroform : methanol : 7 M NH_4OH (65:25:4 by vol.) in first direction and chloroform:methanol:acetic acid: water (175:25:25:3 by vol.) as solvent system in second direction. Phospholipids were identified following the method of Skipski and Barclay (1969) using lipid standards. The individual labeled phospholipids were located and scraped off from the TLC plate into scintillation vials (5 cm^3 of scintillation fluid was used per vial).

Lipid peroxidation: The lipid peroxidation was measured as malondialdehyde formed; this was determined by a reaction with thiobarbituric acid (Heath and Packer 1968).

Leachate analysis: Twenty five one-day-old germinating seeds were incubated in 50 cm^3 of distilled water under different HS regimes. At the end of the treatment, the amount of amino acids, soluble sugars, sodium, potassium and conductivity in the incubation medium were estimated. Soluble sugars were estimated by phenol-sulphuric acid method (Dubois *et al.* 1956) using glucose as the standard. Amino acids were estimated by the ninhydrin method (Moore and Stein 1954) using leucine as the standard. Potassium and sodium contents in the leachates were estimated using *Systronics* digital flame photometer (*Burner unit 121*, Hyderabad, India) and conductivity of the leachates was measured with a lock conductivity bridge (*Type CLO1/01A*, *Toshniwal*, Bombay, India).

Results

RGI measured 72 h after HS treatment gradually declined with increasing temperature and duration of treatments. The decline was more pronounced at 45 °C exposed germinating seeds and was 92.7 % after 2-h treatment. However, pretreatment of germinating seeds at 40 °C for 1 h or 45 °C for 10 min followed by a 3 h incubation at 28 °C prior to 2 h HS at 45 °C led to an increase in RGI compared to germinating seeds exposed to 45 °C for 2 h alone indicating induction of thermotolerance (Table 1).

The total lipid content was significantly decreased after HS. Maximum decline was observed after treatment at 45 °C for 2 h (Fig. 1). Less decrease in lipid content was observed after pretreatment at 40 or 45 °C. The rate of decline in lipid synthesis was higher in 45 than in 40 °C. Germinating seeds exposed at the 45 °C treatment did not cause much variation in lipid synthesis in relation to the duration of the HS exposure (Table 2). Pretreatment (1 h and 10 min) of germinating seeds at 40 and 45 °C, respectively, caused a slight increase in lipid synthesis compared to 45 °C for 2 h without pretreatment.

The decline in phospholipid content and its synthesis was greater at 45 °C than at 40 °C (Fig. 1, Table 2). Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine contents continuously declined with increasing temperature and duration of exposure. Though the changes in phosphatidylglycerol and phosphatidylinositol synthesis were not conspicuous at 40 °C, a decline in their synthesis was noted at 45 °C for 2 h. Pretreatment of germinating seeds at 40 and 45 °C caused a considerable increase in phosphatidyl-ethanolamine and phosphatidylcholine. The other phospholipids did not show much variation (Table 2).

The amount of malondialdehyde formed was considered as a measure of lipid peroxidation. A significant increase in malondialdehyde content was observed in germinating seeds subjected to heat shock and the magnitude of increase was relatively greater at 45 °C than at 40 °C. Pretreatment of germinating seeds

Table 1. Effect of temperature, HS duration, and pretreatment (PT) at 40 °C for 1 h or 45 °C for 10 min followed by 3 h at 28 °C on RGI [% of controls] of germinating seeds of pigeonpea (means ± SE, $n = 3$). Control: 28 ± 2 °C.

Treatment	Time after HS [h] 24	48	72
40 °C - 30 min	72.92 ± 2.1	90.70 ± 2.4	85.00 ± 3.10
40 °C - 1 h	60.42 ± 2.0	69.77 ± 2.9	71.67 ± 2.80
40 °C - 1.5 h	52.08 ± 2.4	65.12 ± 3.1	66.67 ± 2.50
40 °C - 2 h	47.92 ± 2.1	53.49 ± 3.5	50.00 ± 1.90
45 °C - 30 min	50.00 ± 1.8	56.98 ± 2.9	65.83 ± 2.10
45 °C - 1 h	37.50 ± 1.7	48.84 ± 2.3	47.50 ± 1.60
45 °C - 1.5 h	25.00 ± 1.5	24.42 ± 1.8	24.17 ± 1.10
45 °C - 2 h	16.67 ± 1.9	10.47 ± 0.6	8.33 ± 0.04
Pretreatment			
40 °C (1 h) - 28 °C (3 h)-45 °C (2 h)	31.25 ± 2.0	44.19 ± 2.6	42.50 ± 2.2
45 °C (10 min)-28 °C (3 h)-45 °C (2 h)	25.00 ± 1.7	29.07 ± 1.0	30.00 ± 1.6

Table 2. Total lipid and individual phospholipid synthesis [counts s^{-1} g^{-1}(f.m.)] in germinating seeds of pigeonpea in response to HS. PL - phospholipid (unidentified), PE - phosphatidylethanolamine, PG - phosphatidylglycerol, PC - phosphatidylcholine, PS - phosphatidylserine, PI - phosphatidylinositol

Treatment	Total lipid synthesis	PL	PE	PG	PC	PS	PI
28 ± 2 °C	5540	90.3	213.9	29.9	251.7	19.9	17.5
40 °C - 30 min	4530	87.8	188.5	28.0	218.1	16.5	14.6
40 °C - 1 h	4100	82.7	162.5	27.3	205.3	17.2	15.1
40 °C - 1.5 h	3710	71.0	136.7	24.8	166.9	8.2	11.0
40 °C - 2 h	3500	67.0	115.0	21.5	147.6	5.2	9.9
45 °C - 30 min	3430	70.6	128.0	22.0	164.8	5.3	10.3
45 °C - 1 h	3320	64.5	91.3	17.0	104.7	5.0	6.9
45 °C - 1.5 h	2860	51.0	61.3	18.2	81.5	4.7	5.3
45 °C - 2 h	2850	49.9	40.5	13.0	57.2	4.8	4.8
Pretreatment							
40 °C (1 h) - 28 °C (3 h) - 45 °C (2 h)	3190	59.8	92.9	12.7	109.1	4.9	6.2
45 °C (10 min) - 28 °C (3 h) - 45 °C (2 h)	3040	57.7	63.4	14.6	85.0	4.5	6.4

Table 3. Leakage of sugar, amino acids, sodium, potassium, and conductivity of leachates [% of control] in germinating seeds of pigeonpea in response to HS (means ± SE, n = 3). Control: 28 ± 2 °C.

Treatment	Sugars	Amino acids	Sodium	Potassium	Conductivity
40 °C - 30 min	151.42 ± 3.2	129.16 ± 2.7	0.00 ± 0.0	300.00 ± 4.7	123.21± 1.7
40 °C - 1 h	193.06 ± 2.0	147.63 ± 3.8	200.00 ± 5.8	300.00 ± 5.6	128.33 ± 3.2
40 °C - 1.5 h	203.07 ± 1.8	153.44 ± 4.3	250.00 ± 3.6	375.00 ± 5.2	157.25 ± 2.6
40 °C - 2 h	219.04 ± 2.1	164.58 ± 2.5	250.00 ± 6.6	380.00 ± 2.8	171.65 ± 1.5
45 °C - 30 min	304.28 ± 3.2	206.25 ± 4.4	300.00 ± 2.6	500.00 ± 6.5	172.32 ± 2.7
45 °C - 1 h	306.35 ± 3.2	251.81 ± 4.1	330.00 ± 5.0	525.00 ± 7.6	180.83 ± 2.1
45 °C - 1.5 h	338.97 ± 4.5	254.59 ± 5.2	350.00 ± 5.9	700.00 ± 9.6	195.96 ± 2.6
45 °C - 2 h	367.61 ± 3.5	260.30 ± 5.0	450.00 ± 5.0	740.00 ± 7.1	218.11 ± 2.7
Pretreatment					
40 °C (1 h) - 28 °C (3 h)-45 °C (2 h)	257.14 ± 3.9	177.40 ± 4.1	250.00 ± 3.6	420.00 ± 5.4	170.86 ± 3.2
45 °C (10 min)-28 °C (3 h)-45 °C (2 h)	280.00 ± 2.3	204.27 ± 4.9	300.00 ± 3.2	500.00 ± 5.6	187.40 ± 3.8

both at 40 and 45 °C caused a decline in malondialdehyde content following 45 °C for 2 h exposure (Fig. 1).

The leaching experiments indicated an increased solute leakage in response to increasing HS temperature and duration (Table 3). At 45 °C maximum amount of amino acids, soluble sugars, sodium and potassium ions leaked out from germinating seeds but preincubation reduces the amount of solute leakage.

Discussion

The decline in RGI of embryonic axes in response to HS (Table 1) may be attributed to altered metabolism of germinating seeds which affected the supply of nutritional reserves from the cotyledons to the embryonic axes. Decline in embryonic axis growth in response to HS and considerable improvement in its growth under pretreatment followed by lethal HS was also reported in soybean (Lin *et al.* 1984), mung bean (Chen *et al.* 1986), sorghum (Ougham and Stoddart 1986), barley (Marmiroli *et al.* 1989) and pearl millet (Howarth 1990) seedlings. The greater RGI at 40 °C as well as in pretreated seeds might indicated the synthesis and accumulation of

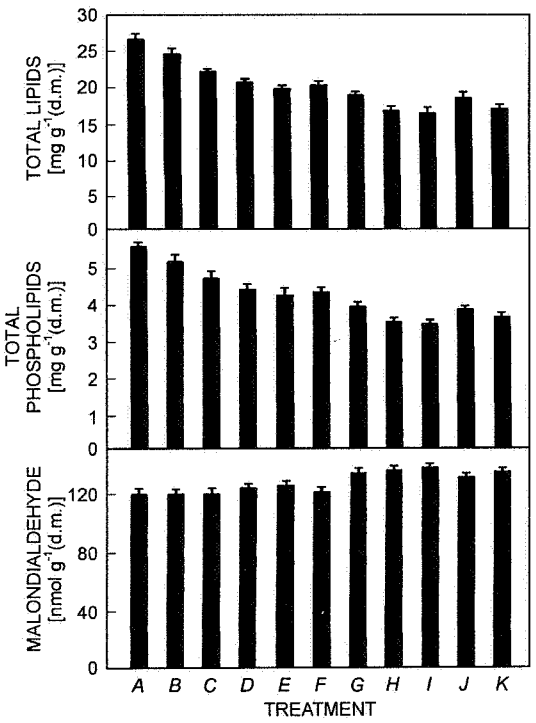

Fig. 1. The content of total lipids, phospholipids and malondialdehyde in the germinating seeds of the pigeonpea in response to HS. *A* - control, *B* - 40 °C for 30 min, *C* - 40 °C for 1 h, *D* - 40 °C for 1.5 h, *E* - 40 °C for 2 h, *F* - 45 °C for 30 min, *G* - 45 °C for 1 h, *H* - 45 °C for 1.5 h, *I* - 45 °C for 2 h, *J* - pretreatment at 40 °C forl h followed by intervening incubation at 28 °C for 3 h prior to 45 °C for 2 h, *K* - pretreatment at 45 °C for 10 min followed by intervening incubation at 28 °C for 3 h prior to 45 °C for 2 h. *Vertical bars* represent SE, $n = 3$.

HSPs which are considered to play an important role in the acquisition of thermotolerance in germinating seeds of pigeonpea (Sridevi *et al.* 1999).

Lipid content and lipid synthesis in 40 °C treated germinating seeds were less effected than in 45 °C treated ones (Fig. 1, Table 2). The reduction of lipid synthesis in response to HS was also demonstrated in bean leaves (Ordin *et al.* 1974), *Cyanidium caldorium* (Kleinschmidt and McMohan 1970), and rape leaves (Aid *et al.* 1995, 1998).

The decline in phospholipid content and increase in malondialdehyde content in respone to HS (Fig. 1) suggests that lipid peroxidation occurred leading to degradation of phospholipids. Thus the reduction in total phospholipid content of germinating seeds of pigeonpea may probably involve both peroxidative degradation and decreased synthesis of phospholipids (Table 2). It may also be presumed that HS causes a decline in the activity or synthesis of enzymes that are involved in the phospholipid synthesis. It was noted that loss of membrane integrity is associated with decline in the rate of incorporation of ^{32}P into the phospholipids of tobacco leaves (Benzioni *et al.* 1973). High temperature caused membrane damage through lipid peroxidation in *Amaranthus* seeds (Bhattacharjee and Mukherjee 1998), *Vigna* seedlings (Updhayaya *et al.* 1991), and wheat root and leaves (Guo *et al.* 1998). Malondialdehyde formed as a consequence of lipid peroxidation can also affect protein synthesis (Dhindsa 1982, Manwaring and Csalleny 1988). Thus the build up of products of lipid peroxidation may lead to the decline in the rate of protein synthesis in germinating pigeonpea seeds in response to 45 °C HS. In the 40 °C and pretreated seeds, the less lipid peroxidation may be connected with higher protein synthesis (Sridevi *et al.* 1999).

In response to altered lipid metabolism the increased quantities of sugars, amino acids, sodium, potassium and other electrolytes were detected in the leachates of germinating pigeonpea seeds exposed to HS (Table 3). Increase in the amount of solutes in leachates with HS suggested considerable membrane disruption. Loss of membrane integrity in response to HS leading to greater leaching of solutes was also found in soybean cotyledons (Leopold 1980), radish seeds, pine embryos (Murphy and Noland 1982), soybean seedlings (Lin *et al.* 1985), and *Vigna* seedlings (Upadhyaya *et al.* 1991).

It is suggested that increased lipid peroxidation and associated decline in phospholipid contents and their synthesis at 45 °C may cause greater destabilization of membranes leading to greater solute leakage and poor seedling growth of pigeonpea. The 24 h germinating

seeds both treated with 40 °C and pretreated showed better seedling growth due to relatively lower lipid peroxidation, higher phospholipid contents and their synthesis, and improved membrane function as reflected by lower leakage of solutes. Heat shock proteins formed as observed in our previous findings (Sridevi *et al.* 1999) might have been also involved in membrane protection and repair.

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