DISSERTATION

STUDIES OF TEMPERATURE STRESS IN CULTURED PEAR CELLS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY Min-Tze Wu

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BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

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ABSTRACT OF DISSERTATION

STUDIES OF TEMPERATURE STRESS
IN CULTURED PEAR CELLS

Studies were undertaken to examine the heat stress and freezing stress responses of cultured plant cells. Suspension-cultured pear (Pyrus communis cv. Bartlett) cells were used as the experimental materials.

The response of pear cells to heat stress was studied using three viability tests: regrowth (culture growth during 10 days after stress); triphenyltetrazolium chloride reduction; and electrolyte leakage. Critical temperatures (those causing 50% injury) for a 20 minute exposure were 42°, 52°, and 56°C, respectively, for these viability tests. The measurements of direct response, i.e., TTC reduction and electrolyte leakage, were not adequate substitutes for regrowth tests in assessing heat injury to cultured plant cells.

The pipetting of pear suspension cultures was followed by a substantial but transient decrease in heat sensitivity. During a culture cycle, pear cells were most sensitive to heat at around day three. The influence of

these normal culture variables (handling and age) are potentially serious artefacts and must be well characterized in order to minimize systematic errors in measuring heat tolerance. Beyond this, they may provide clues concerning physiological factors governing the reponses of cells to heat.

Both elevated growth temperature and brief heat shock increased the heat tolerance of pear cells. Several features of heat acclimation induced by these two methods were compared. Based on these comparisons, it was concluded that heat acclimation in response to growth at 30^{o}C and to heat shock occurred via different mechanisms.

The effect of low temperature on heat tolerance and high temperature on freezing tolerance of pear cells was also examined. The heat acclimation induced by elevated growth temperature increased freezing tolerance, but cold acclimation did not increase heat tolerance. It seems likely that some component(s) of cold and heat acclimation may be the same. This indicates that studies of response to different stimuli may help in clarifying mechanisms of temperature acclimation.

The only potential mechanism examined in this study was the role of extracellular polysaccharides in cell response to freezing stress. Extracellular macromolecules from non-acclimated cultures aggregated irreversibly during freezing, while those from acclimated cultures did not. The

aggregation of polysaccharides may be related to the observation that macromolecules from a non-acclimated medium increased the freezing injury of pear cells. Analyses of polysaccharides from acclimated and non-acclimated cultures indicated that the polysaccharides were changed during cold acclimation.

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CHAPTER 1

INTRODUCTION TO TEMPERATURE STRESS

Biological organisms generally can be classified into 3 categories on the basis of their response to temperature: psychrophiles, mesophiles, and thermophiles (43). Psychrophiles grow and develop in a temperature range of 0° to $20^{\circ}C$. The temperature range for mesophiles is about 10° to $30^{\circ}C$, while thermophiles may grow and develop at temperatures between 30° and $100^{\circ}C$. Most crop plants are mesophiles and thrive in the temperature range of 10° to $30^{\circ}C$. At temperatures above $30^{\circ}C$, crop plants may suffer from high temperature stress. On the other hand, as the temperature falls below $10^{\circ}C$, the plants may suffer chilling or freezing injury. The severity of injury in response to temperature extremes depends on how hardy crop plants are. Hardiness is the ability of an organism to survive the direct action of extreme temperatures without suffering permanent damage. There is a tremendous variation in hardiness among different species, within the same species in different seasons, and at different developmental stages during the life cycle.

The variation in hardiness among different species is inherited (40) and in some cases, specific genetic compositions have been associated with resistance to heat or cold (41). However, variation in hardiness involves not only the fixed phenotype produced by the genotype but also

the development of adaptive systems to withstand periods of unfavorable temperatures. Differences in hardiness within the same species in different seasons are induced by climatic conditions such as warmer or cooler weather. Plants acquire their hardiness through the processes of hardening, also termed acclimation. Plants that have been initially subjected to adequate periods of moderate temperature stresses suffer less injury from subsequent heat or cold stresses than do plants not stressed previously. Some developmental stages of plants are known to be more susceptible to extreme temperatures. Examples include seed germination, periods of higher growth activity and reproductive phases (41).

Plant response to temperature stress has been a major focus of physiological research across a broad spectrum of agriculturally important species (53). It is generally believed that there is a close linkage between physiological limitations imposed by temperature stress and potential crop productivity. Although chilling injury commonly occurs in nature, only heat and freezing injury will be considered further in this dissertation.

IMPORTANCE OF HEAT AND FREEZING INJURY IN AGRICULTURE

Heat induced injuries may be quite subtle, so yield reductions caused by heat stress are difficult to estimate. Nevertheless, there are some visible injuries observable in field-grown plants. Wilting, leaf burn, leaf folding and

abscission may be early indicators of damage induced by high-temperature stresses. Other visible heat injuries include bark burn of thin-barked trees, sunburn in fleshy fruits, and girdling death of seedlings due to heat sensitivity of cambium tissue near the soil level (43). Recently, high temperatures have also been reported to induce bud failure symptoms in vegetative buds of almond plants (28) and to reduce tuberization in potatoes (22).

Photosynthesis is one of the most heat sensitive plant processes (11); high temperature causes both a functional and a physical dissociation of the chlorophyll-protein complexes in the thylakoid membranes of the chloroplasts. This results in an inactivation of photosystem II activity and hence the inactivation of overall photosynthesis (10,11). High temperature may also affect some of the soluble enzymes located outside the thylakoid membranes, presumably by some form of denaturation or aggregation of the native protein (10,11).

Phloem translocation may be inhibited at high temperature (69). The translocation of carbohydrates between individual organs in the plant usually has an optimum efficiency between 20^{o}C and 30^{o}C; at temperatures above 40^{o}C, the translocation rate drops sharply. Increased deposition of callose have been observed in experimentally heat-stressed tissues (59), and this buildup is correlated with inhibition of both basipetal and lateral phloem translocation (54). It is known that callose can cause

constriction of plasmodesmata and sieve plate pores, hence increasing resistance to translocation.

The period of meiosis and anthesis is a particularly heat-sensitive developmental stage in many plants. Heat stress during this period results in reduced flower production, pollen production, pollen viability, seed set and fruit set in several tomato species under high temperature ($38^{\circ}/27^{\circ}C$, day/night) (1). Grain set in wheat (73), the amount of pollen shed on the stigma in rice (48), and the percentage of viable pollen in bean (26) were also reduced by exposing plants to high temperatures of 30° to $38^{\circ}C$. Obviously, heat stress during reproductive development can account for substantial yield reductions.

Unlike heat injuries, freezing injuries are usually obvious and have been more intensively studied. Various types of injury to whole plants are caused by late spring and early fall frosts, low midwinter temperature minima, and rapid temperature changes. Freezing injuries include crown kill in winter cereals, biennials, and herbaceous perennials; sunscald on thin-barked trees; blackheart and frost cracking in xylem of trees and shrubs; death of buds and bark in plants which lose hardiness rapidly during transient warm periods in winter; and outright death of tender annuals (14).

MECHANISM OF HEAT INJURY AND RESISTANCE

A. HEAT INJURY

Traditionally considered general mechanisms of heat-induced cellular disruptions include protein denaturation and membrane lipid phase changes (43). Since proteins are denatured at different high temperatures, heat denaturation of a highly sensitive protein may result in primary indirect heat injuries such as starvation due to inactivation of chloroplast enzymes; toxicity due to respiratory disturbances; biochemical lesions due to the inhibition of an intermediate process necessary for growth; and membrane damage due to denaturation and aggregation of membrane proteins.

Besides protein denaturation, lipid liquefaction and damage may be one of the direct causes of heat injuries, especially at very high temperatures (43). The change in lipid phase below the optimum growing temperature is well correlated with chilling injury (53). Although some researchers (68) have detected no phase transition of the membrane lipids above the optimum growing temperature, others (37,38,57) have emphasized that the mobility of the lipid must increase with the rise in temperature until membrane destruction and cell death would occur. With acclimation to elevated temperatures, unsaturated fatty acids have been found to be progressively replaced by the saturated fatty acids which have higher melting points (27,43).

The exposure time to high temperature stress is of considerable importance. This can be expressed by the equation $T = a - b \text{ Log } Z$ (43), which describes the typical relationship between heat killing temperature (T) and time (Z). Indirect heat injury occurs during continued exposure to moderately high temperature. This may include starvation, toxicity effect and biochemical lesions, all of which are metabolic in nature (43). Polyribosomes are disrupted and protein synthesis inhibited by heat stress (43). The failure to replace critical proteins is the likely cause of many indirect or metabolic heat stress injuries.

B. HEAT RESISTANCE

In order to survive high temperature extremes, plants must develop multiple adaptive mechanisms which protect them from the various causes of injury. Heat resistance mechanisms include heat stress avoidance and tolerance (43).

Heat avoidance may be developed by means of insulation between the plant part and a warmer environment above the killing temperature, reduction of radiation absorption and transpirational cooling. Among these, transpirational cooling may be the most effective means to avoid heat injury at high temperature (41), but this depends on a sufficient water supply.

Mechanisms of heat tolerance include the ability of plants to increase protein thermostability, prevent lipid liquefaction and repair metabolic heat injuries

(43). McDaniel (53) pointed out that heat tolerant plants have more thermostable proteins than do their heat sensitive counterparts. Increased thermostability of fraction I protein was also found in heat-hardened beans (36,86). However, these are insufficient to account fully for heat tolerance, as other enzymes have not shown a relation between their thermostability and heat tolerance of plants from which they are extracted (43). Adaptation to elevated temperature is often accompanied by increased saturation of membrane lipid fatty acids (39,47), a change which has been correlated with higher lipid melting points.

Many years ago, Yarwood (96) reported that the heat tolerance of leaves was increased by exposure to 50^{o}C for 15-30 seconds. Since then, there have been numerous reports of similar responses in higher plants, algae, and microorganisms (43). More recently, there has been increasing interest in the effect of brief heat shock on both gene expression and heat tolerance (34). Cells from a diverse array of organisms (50) synthesize heat shock proteins following brief (usually 10 to 30 minutes) exposure to supraoptimal temperatures. The induction of heat shock proteins has been demonstrated for not only plant cells (7), but those of insect (5) and mammalian (80) origin as well. The presence of heat shock proteins has been correlated with increased heat tolerance in soybean seedlings (44) and yeast cells (52), but the mechanism of any thermal protection is unknown. Minton, et al. (56) theorized that heat shock

proteins protect the cell from heat injury by stabilizing other proteins in a non-specific manner. Working with soybean seedlings, Key et al. (34) reported that synthesis of heat shock proteins which occurs at 40°C possibly protects the transcription and/or translation systems at otherwise nonpermissive temperatures.

MECHANISM OF FREEZING INJURY AND RESISTANCE

A. FREEZING INJURY

Freezing injury consists of two main types: (a) primary direct injury due to intracellular freezing and (b) secondary freeze-dehydration injury due to extracellular freezing (42). Intracellular freezing, which is relatively rare in nature, results from rapid freezing and is fatal to the frozen cell. This may cause thousands of tiny ice crystals to form throughout the protoplast and vacuole. As the ice crystals grow, a piercing of the cell membranes is probably the result, although this explanation has not been proved yet (43). Extracellular freezing occurs in temperate climates under normal winter conditions. It results from relatively slow freezing and may or may not injure the cells. Initial ice formation is extracellular because cytoplasmic water has a lower freezing point. During freezing, the vapor pressure gradient between intracellular water and extracellular ice leads to the loss of water from cells to extracellular ice and causes freeze-dehydration. The freeze-dehydration may subsequently produce cell

collapse and damaging solution effects (43). Examples of solution effect include protein aggregation and pH changes due to increased concentration of the salts in the cell sap. Olien (60,61,63) proposed that adhesion of ice to hydrophilic cell surfaces was also an important cause of injury. He suggested that polysaccharides produced during cold acclimation minimize injurious adhesive interactions by interacting with ice crystal growth. The most universal feature of freezing damage appears to be that membranes are the critical sites of cell damage (27,43,46).

B. FREEZING RESISTANCE

Plant cells which freeze under normal conditions cannot tolerate intracellular ice formation. The killing of individual cells leads to plant injury or death, so cold hardiness requires effective prevention of ice formation, i.e. freezing avoidance, in the cell. Supercooling and extracellular freezing of cell water are two major phenomena which prevent intracellular freezing.

As mentioned previously, secondary freeze-dehydration injuries may accompany extracellular freezing. In most plants, tolerance of freeze-dehydration strains is more important for winter survival strategy than is freezing avoidance. Since membranes are critical sites of cell damage, freezing tolerance may be due to changes in membranes <u>per se</u> or in the environment around membrane; the latter may alter the stresses that occur or protect membrane sites directly (46). For example, polysaccharides may act

in both ways. Olien (60,63) suggested that specific polysaccharides in cold-acclimated plants interfere with ice crystal growth, thereby minimizing injury to membrane sites. Santarius (75) reported that dextrans protected chloroplast membranes from freeze-thaw and toxic solute treatments.

STUDIES OF HEAT AND FREEZING RESISTANCE BY TISSUE CULTURE

To elucidate the mechanism of heat or freezing resistance, it is necessary to understand the physiological changes which give rise to the hardy state. The physiological changes during acclimation are complicated and mutually dependent (43,95). Although many investigators have used intact plants as the experimental materials to study these physiological factors, such materials are sometimes unsuitable for studying cellular mechanisms of heat or freezing resistance in plants. Cellular heterogeneity is one of the major limitations. For instance, there may exist variations of heat or freezing resistance between different organs and tissues of the same plant. Tissue culture techniques provide an attractive alternative to the use of whole plants. Cell suspension cultures are especially convenient sources of highly uniform cells. In addition, experimental variables can be conveniently and precisely controlled in the laboratory.

However, there are very few references describing the study of heat resistance using tissue culture. Schroeder

(78) used avocado pericarp disks under sterile conditions to study heat shock-induced heat resistance. Barnett et al. (7) examined heat shock proteins produced after short periods of exposure to temperatures between 35° and 45°C by using tobacco and soybean suspension cells. In contrast to the limited application to heat stress problems, tissue cultures have often been used to study cold hardiness. Examples of success in cold acclimation of callus or suspension cells are plentiful. Tumanov et al. (92) found that the callus derived from cherry twigs may become resistant to -30°C when hardened at 2°C for 10 days. Bannier and Steponkus (6) reported that chrysanthemum callus acclimated for 6 weeks at 4.5°C exhibited complete survival down to -16.1°C, whereas unacclimated callus was able to survive freezing down to only -6.6°C. Chen and Gusta (17) have induced cold acclimation in wheat and smooth brome-grass suspension-cultured cells.

Callus tissues have been used to study mechanisms of freezing resistance. Sugawara and Sakai (85) examined the plasma membrane of unhardened and hardened Jerusalem artichoke calluses by freeze-fracturing method. Chen et al. (15) studied the role of hormones in cold acclimation of potato leaf calluses. Chen et al. (18) used wheat suspension cultured cells to study the changes in membrane permeability following freeze-thaw injury.

Although there is considerable interest in the possibility that resistance to different stresses involves

similar mechanisms (4,43), no attempts to induce heat and cold acclimation in the same tissue culture have been reported.

Suspension cultured pear cells were used in this project to study heat and freezing tolerance. The major objectives of this research were to

(a) study heat stress responses in cultured plant cells using different viability tests.

(b) examine the effect of culture handling and age on heat stress responses in cultured plant cells.

(c) compare the effect of elevated growth temperature and heat shock on the heat tolerance of cultured plant cells.

(d) demonstrate the effect of heat acclimation on freezing tolerance and that of cold acclimation on heat tolerance in cultured plant cells.

(e) study the involvement of extracellular polysaccharides in the freezing tolerance of cultured cells.

CHAPTER 2

HEAT STRESS RESPONSES - DEVELOPMENT AND COMPARISON OF VIABILITY TESTS

INTRODUCTION

Plant responses to supraoptimal temperature include inhibition of photosynthesis (10,43), pollination (48), protein synthesis (9,43,72), translocation (54); promotion of callose synthesis (54,59), and leakage of cell contents (10,16,43). This diversity of responses complicates efforts to study specific mechanisms and to identify meaningful markers of improved heat tolerance by intact plant systems. Tissue culture techniques have the advantages of uniformity, convenience, and simplicity, and may minimize some of the problems associated with the use of intact plants. Although effects of heat stress on photosynthesis are critical to intact plants, the absence of chloroplasts in simple cell cultures may further facilitate close examination of other metabolic responses.

Beyond its usefulness in clarifying intact plant systems, information on cultured cells is important because it may be possible to select for heat tolerance *in vitro* and by regenerating whole plants from single resistant somatic cells, new strains could be produced to increase the productivity of certain crops. Screening for desired characteristics in tissue cultures requires the use of selection pressures and criteria which are effective at the cellular level. Before *in vitro* techniques can be used in

efforts to improve heat tolerance, the basic parameters of cellular response to heat must be understood. It was the objective of this study to examine pear cell culture growth after heat stress treatment and assess the validity of other viability tests.

MATERIALS AND METHODS

PLANT MATERIAL

The suspension culture of pear (Pyrus communis cv. Bartlett) cells was obtained from Dr. Roger Romani, University of California at Davis. The original culture was established in late 1977 from explants of mature Bartlett pear fruit (Romani, personal communication). The cells were grown in MS (58) liquid medium containing 4.52 µM 2,4-D and no cytokinin. Cultures usually consisted of 30 ml of cell suspension in 125 ml Erlenmeyer flasks aerated with gyrotary shaking at 85 to 90 cycles per minute. The normal growing temperature was 22°C. Stock cultures of 110 ml were maintained in 500 ml Erlenmeyer flasks; they were batch-propagated by transferring 10 ml of cell suspension to 100 ml fresh medium at 7 day intervals.

HEAT STRESS TREATMENT

All heat stress treatments were applied to cells from 9 day old cultures. In the case of the electrolyte leakage test, it was necessary to suspend the cells in a solution of low conductivity. Cells were separated from culture medium by filtration through Miracloth and washed with 7 volumes of

a solution containing 0.22 M sucrose and 4.52 µM 2,4-D. Washed cells were then suspended in the same solution; there were approximately 12.5 mg (cell dry weight) per ml. Preliminary experiments (data not shown) indicated that sucrose in the medium had little effect on heat injury.

Heat stress was imposed to 3 ml of suspension cells in 1 x 10 cm test tubes or 2 x 12 cm centrifuge tubes, using a heated water bath. Temperature equilibration in the 3 ml aliquots occurred within 3 minutes. When testing different stress temperatures, the duration of exposure was 20 minutes. In temperature duration experiments, time at a constant temperature was the treatment variable. Cell suspensions were gently stirred at 20 minute intervals during stress treatment. In all experiments, control cells at 22^{o}C were handled and manipulated in the same way.

VIABILITY TESTS

REGROWTH TEST

Following heat stress treatment, the 3 ml samples (three per treatment) were transferred to 20 ml of fresh MS medium in 125 ml Erlenmeyer flasks under aseptic conditions. These were grown at 22^{o}C, with shaking at 85 to 90 cycles per minute. On the basis of initial experiments (Figures 1 and 2), the standard regrowth period was 10 days. Dry weight was determined using lyophilized samples. Injury was expressed as:

Figure 1. Increase in dry weight in pear suspension cultures grown at 22°C.

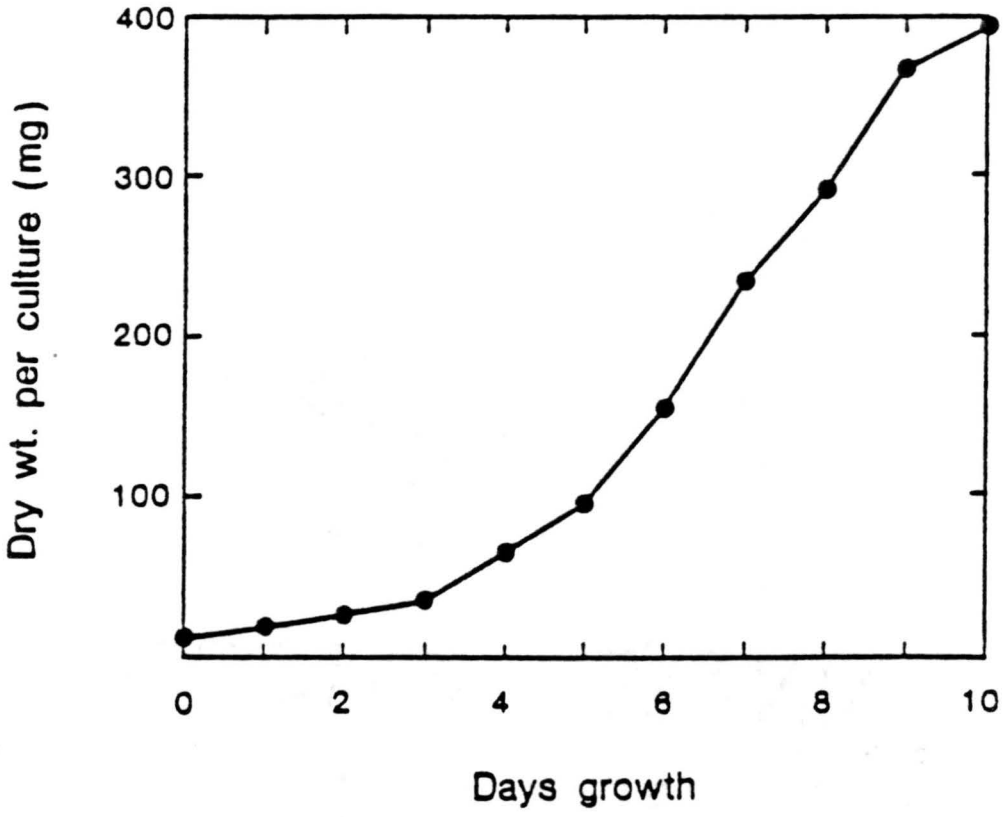
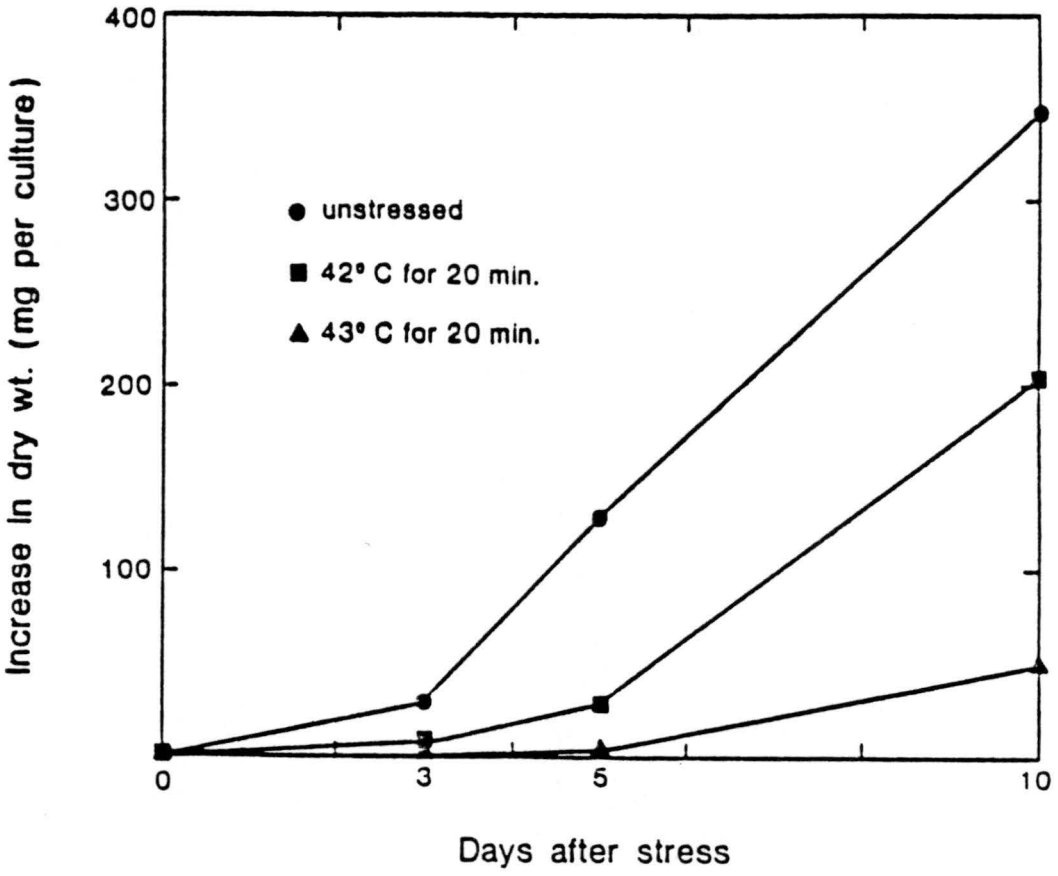

400
300
200
100
Dry wt. per culture (mg)
0
2
4
6
8
10
Days growth

Figure 2. Effect of heat stress on subsequent growth of pear suspension cultures. Cells from 9 day old cultures were stressed for 20 minutes, transferred to fresh medium, and grown at 22°C for 3, 5, and 10 days.


400
300
200
100
0
3
5
10
Increase in dry wt. (mg per culture)
unstressed
42° C for 20 min.
43° C for 20 min.
Days after stress

$$\% \text{ injury} = 1 - \frac{\text{dry wt. increase after treatment}}{\text{dry wt. increase of control}} \times 100$$

ELECTROLYTE LEAKAGE TEST

Following heat stress treatment, the 3 ml suspensions (three per treatment) were allowed to cool, and the conductivity (H_1) of the suspending medium was measured with a Radiometer CDM 3 instrument. Cells were then killed (95°C for 10 minutes), allowed to cool, and conductivity (H_2) measured again. Injury was expressed as:

$$\% \text{ injury} = 1 - \frac{1 - (H_1/H_2)}{1 - (C_1/C_2)} \times 100$$

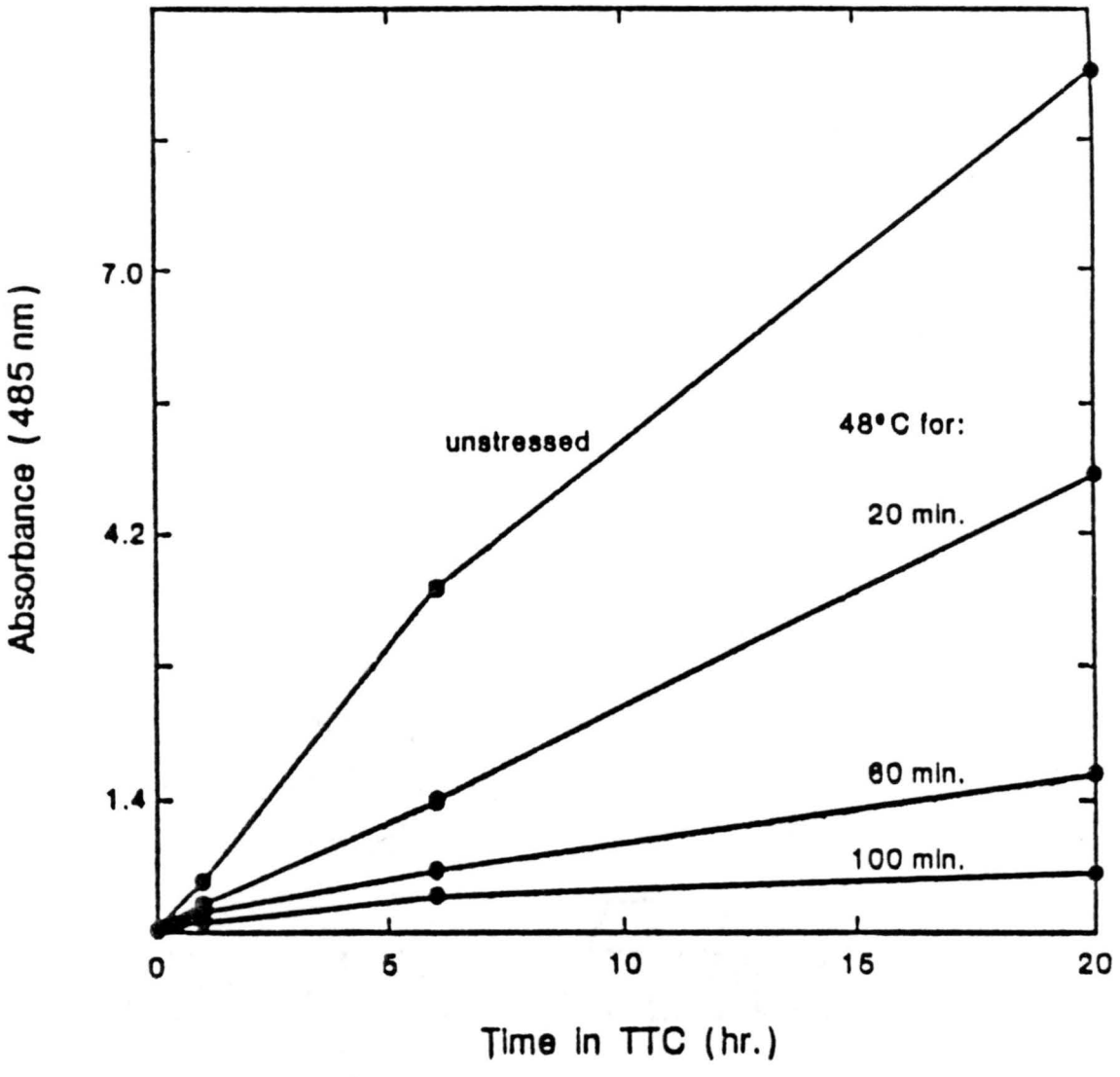
where C_1 was the conductivity reading for unstressed control cells, and C_2 was the value for killed (95°C for 10 minutes) control cells.

TTC REDUCTION TEST

The method of Towill and Mazur (91) was used. The 3 ml suspensions (four per treatment) were centrifuged after heat stress, and then 8 ml of TTC reagent (91) were added to the packed cells. The cells were suspended in TTC solution and kept dark for 20 hours. Preliminary experiments indicated that the rate of TTC reduction was constant over 20 hours in variously treated cells (Figure 3).

The TTC solution was then removed by centrifugation and 10 ml of 95% ethanol were added to extract the formazan product of TTC reduction. The amount of product was

Figure 3. Time course of TTC reduction (formazan production) by unstressed pear cells and pear cells exposed to 48°C 20, 60, and 100 minutes.


Absorbance (485 nm)
7.0
4.2
1.4
0
5
10
15
20
Time in TTC (hr.)
unstressed
48°C for:
20 min.
60 min.
100 min.

determined by reading absorbance at 485 nm. Injury was expressed as:

$$\% \text{ injury} = 1 - \frac{\text{A485 for treated cells}}{\text{A485 for control cells}} \times 100$$

RESULTS

Pear suspension cultures grown at 22°C increased in dry weight most rapidly between 5 and 9 days following transfer to fresh medium (Figure 1). In preliminary stress experiments, exposure to between 42°C and 45°C for 20 minutes substantially reduced post-stress growth capacity. During the 10 days following stress treatment, there were large differences in dry weight accumulation by cultures initiated from control and heat-stressed cells (Figure 2). The 43°C treatment killed or injured cells to the extent that no detectable increase in dry weight occurred during the subsequent 3 days. Even after 10 days, total dry weight in these cultures was much less than in the controls. The effect of 42°C was much less severe. Cells exposed to 42°C for 20 minutes gave rise to cultures which rapidly increased dry weight after 5 days in fresh medium. In all other experiments in which post-stress growth capacity was used as a measure of viability, the period of growth was 10 days. This period allowed near maximum increase in dry weight in control cultures (Figure 1) and the development of large differences between various treatments (Figure 2).

The dependence of regrowth at 22°C on prior stress temperature was exceptionally high (Figure 4A). According to the regrowth test, 41°C caused little stress injury while 44°C resulted in more than 90% injury. In most experiments, the temperature causing 50% injury, measured with the regrowth test, was 42°C but in some cases it was 43°C. However, figure 4A accurately reflects the response and high temperature sensitivity of cultured pear cells. As true for most heat stress responses (43), there was an interaction between the temperature and time of exposure (Figure 4B). For example, 20 minutes at 40°C reduced regrowth by less than 10%, while more prolonged exposure to the same temperature produced severe injury. The time required for 50% injury at 40°C was approximately 85 minutes.

The temperature sensitivity for pear cells as measured by the regrowth test differed greatly from the sensitivity indicated by measurement of electrolyte leakage (Figure 5). According to the electrolyte leakage test, exposure of pear cells to temperatures as high as 52°C to 54°C for 20 minutes resulted in only about 20% injury. Because the regrowth and electrolyte leakage tests provided such different critical temperatures (42°C versus 56°C for 50% injury), TTC reduction was also examined as a viability test.

A comparison of the three viability tests used to measure heat injury is shown in Figure 6. Data points were

Figure 4. Effect of stress temperature during 20 min exposure (A) and the duration of 40°C stress (B) on the regrowth capacity of pear suspension cultures.

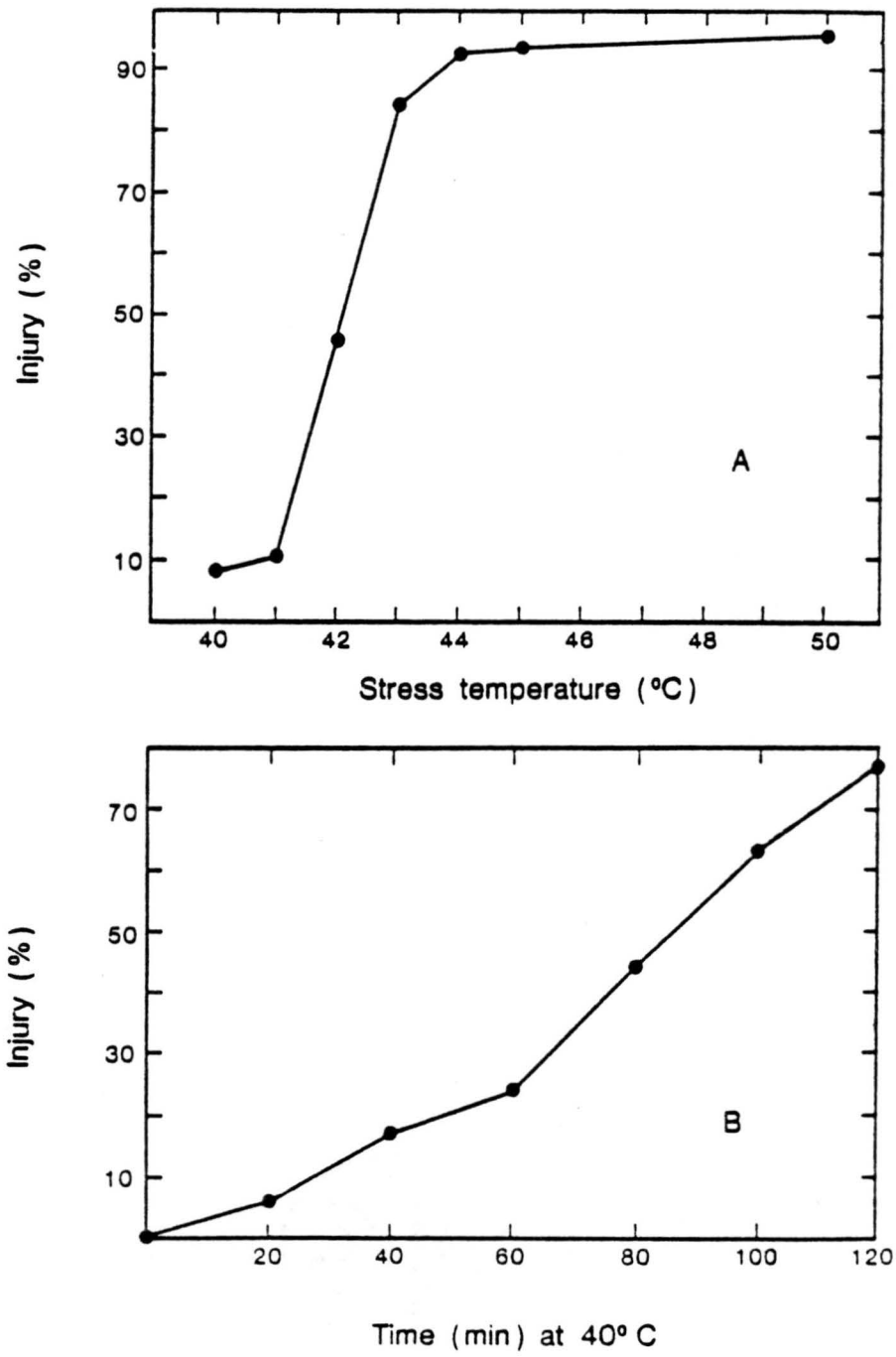

Injury (%)
90
70
50
30
10
40
42
44
46
48
50
Stress temperature (°C)
A
Injury (%)
70
50
30
10
20
40
60
80
100
120
Time (min) at 40° C
B

Figure 5. Effect of stress temperature during 20 min exposure on heat injury as measured with the regrowth and electrolyte leakage viability tests.

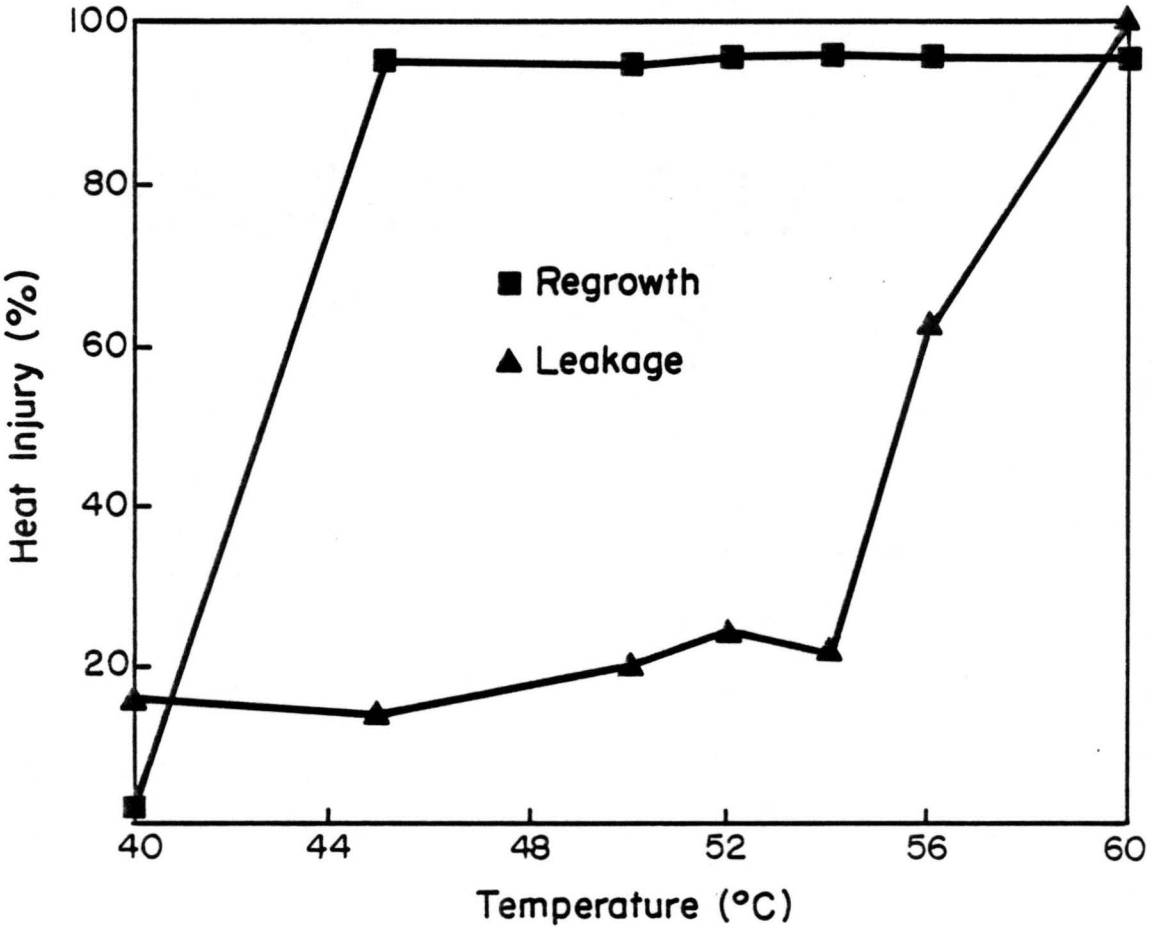
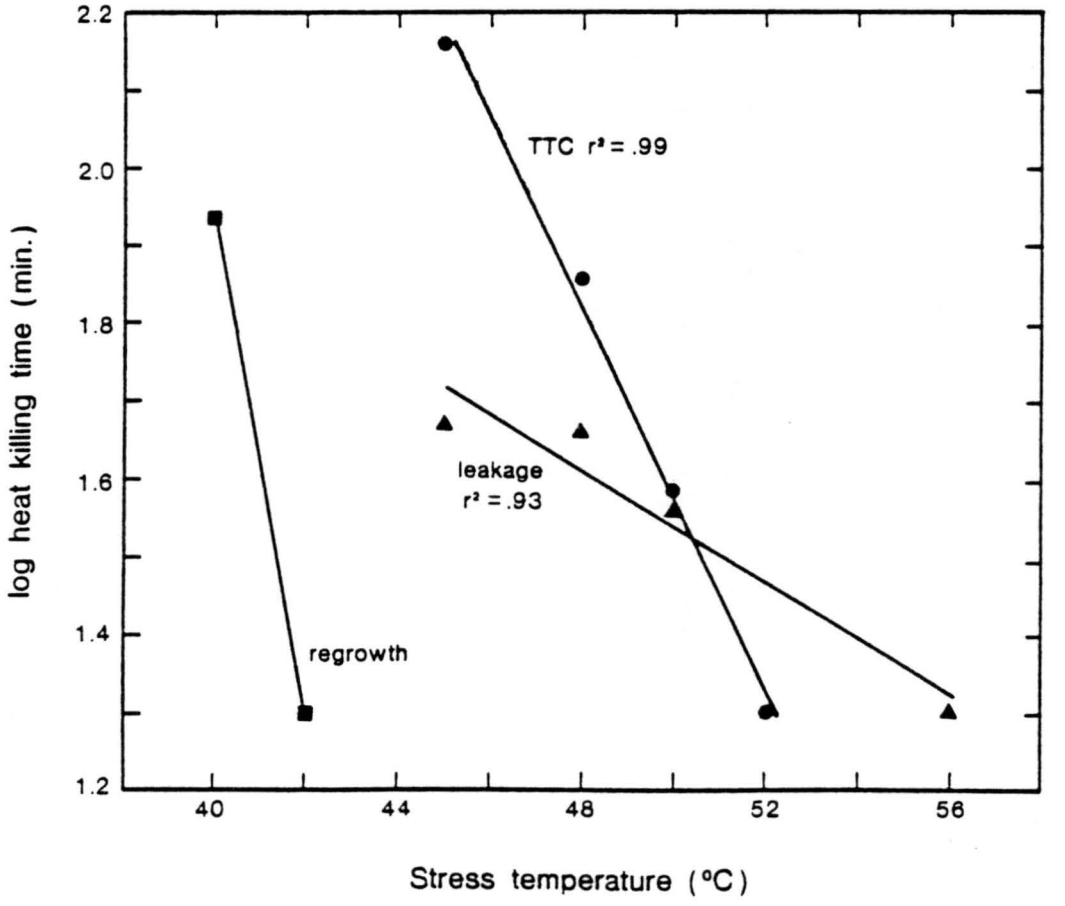

100
80
60
40
20
Heat Injury (%)
Regrowth
Leakage
40
44
48
52
56
60
Temperature (°C)

Figure 6. Relationship between stress temperature and the time required to produce 50 percent injury (heat killing time) as measured by the regrowth (squares), TTC reduction (circles) and electrolyte leakage (triangles) viability tests.


2.2
2.0
1.8
1.6
1.4
1.2
log heat killing time (min.)
TTC r² = .99
leakage
r² = .93
regrowth
40
44
48
52
56
Stress temperature (°C)

plotted and straight lines fitted according to the equation $T = a - b \text{ Log } Z$ (43), which describes the typical relationship between heat killing temperatures (T) and time (Z). Although only two points were available for the regrowth test, it was included in Figure 6 for comparison. Within the 45°C to 56°C temperature range, the TTC and leakage data both fit the straight line equation very well with r^2 values of 0.99 and 0.93, respectively. The slope of these lines indicate that the electrolyte leakage test had the lowest temperature coefficient and regrowth the highest.

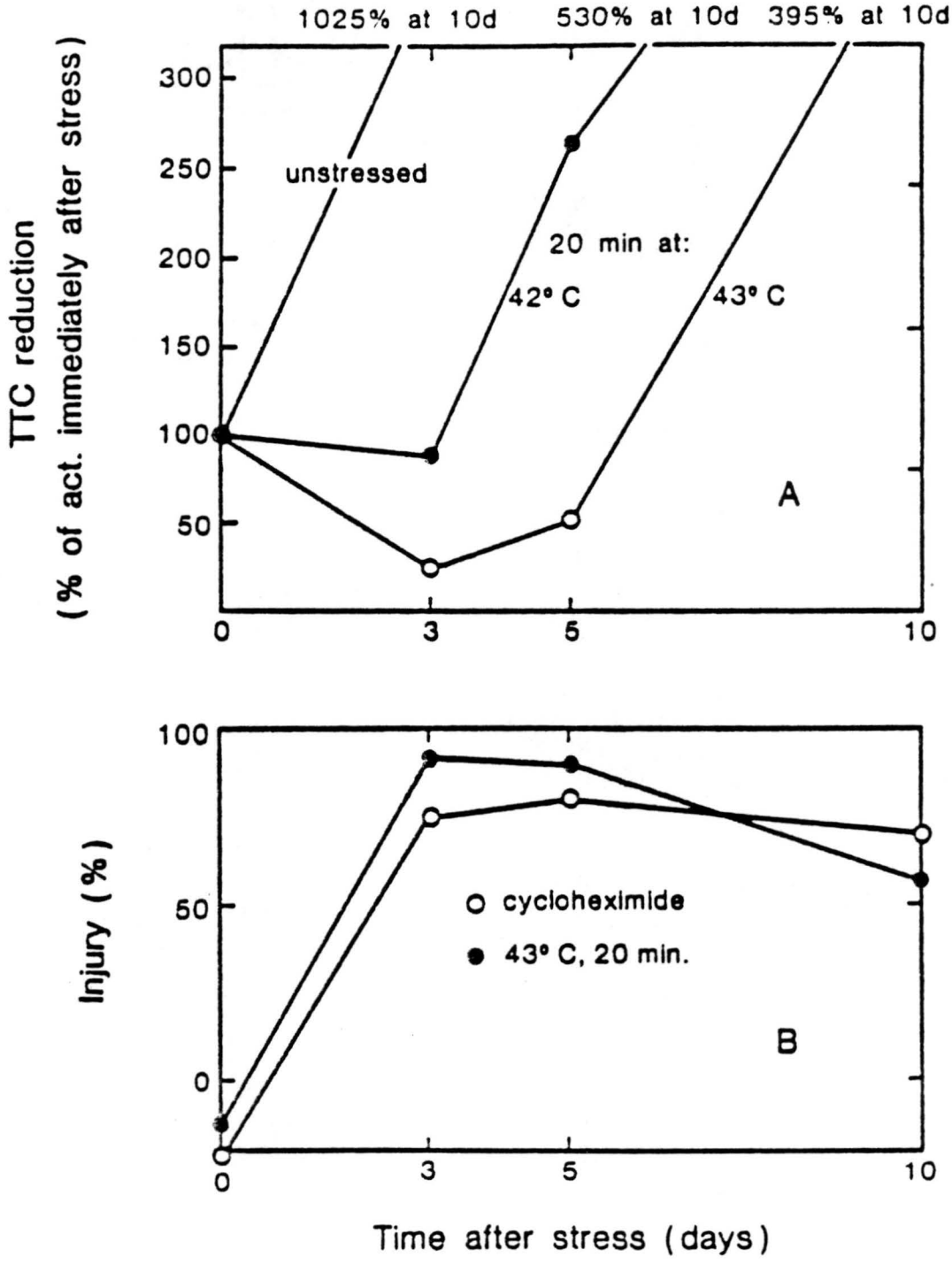
Although the temperature coefficient for the TTC test was higher than that for electrolyte leakage, heat stress at temperatures below 50°C affected leakage more rapidly than it did TTC reduction (Fig. 6). In time course experiments conducted at 45°C and 48°C, electrolyte leakage appeared to be the more sensitive stress response; at 50°C, injury measured by TTC and leakage followed essentially the same time course (Figure 27 in Appendix).

Stress-induced killing points are typically considered to be those levels of stress which produce 50% injury in a particular test. For a 20 min. exposure, the heat killing temperatures for pear cells were 42°, 52°, and 56°C according to the regrowth, TTC, and electrolyte leakage tests, respectively. Killing temperatures of 43°, 55°, and 65°C are obtained for the same test if the lines in Figure 6 are extrapolated to a killing time of 10 minutes.

When viability tests are conducted immediately following stress treatment (e.g. Figure 27 in Appendix), the measurement obtained reflects direct injury (43). In cultured pear cells, metabolic (indirect) heat injury which affected TTC reducing capacity was also readily detectable when the viability test was conducted several days after stress treatment (Figure 7). Cells exposed to 43°C for 20 minutes and then maintained at 22°C for 3 days lost most of their capacity to reduce TTC (Figure 7A), although temperature up to 50°C for 20 minutes had essentially no direct effect on TTC reduction (Figure 27C in Appendix). Indeed, the immediate post-stress effect of 43°C was to increase slightly the ability of pear cells to reduce TTC (Figure 7B). The delayed response to stress at 43°C for 20 minutes is expressed in terms of percent heat injury in Figure 7B; the decline in this value after 3 days reflects culture recovery and regrowth. Culture growth was responsible for the sharp increase in TTC-reducing capacity in unstressed cells (Figure 7A).

Protein synthesis is a component of metabolic injury which is particularly sensitive to heat stress (9,43). Failure to synthesize new TTC-reducing enzymes together with normal or heat-accelerated turnover of existing enzymes could produce the type of indirect injury shown in Figure 7. Consistent with this idea, treatment of pear cells with cycloheximide (Figure 7B) had an effect on TTC reduction that was similar to the heat-induced injury.

Figure 7. Measurement of indirect (metabolic) injury with the TTC reduction viability test. Effects on TTC reduction were measured immediately and 3, 5, and 10 days after treatment. Treatments were heat stress (A, and B) and exposure to 3.6 µM cycloheximide (B). Effects on TTC reduction 3, 5, and 10 days later were expressed as: percent of that immediately following the stress (A); or percent injury (B) as described in MATERIALS AND METHODS.


1025% at 10d
530% at 10d
395% at 10d
TTC reduction
(% of act. immediately after stress)
300
250
200
150
100
50
unstressed
20 min at:
42° C
43° C
A
0
3
5
10
100
50
0
Injury (%)
cycloheximide
43° C, 20 min.
B
Time after stress (days)

DISCUSSION

Physiological processes of plants often exhibit different threshold temperatures for heat injury. For example, injurious effects on photosynthesis, respiration, and ion leakage were observed at different progressively higher leaf temperatures (10). In cultured pear cells, three viability tests also showed different heat injury temperatures (Figure 5,6).

Regrowth capacity (Figure 2,4,5,6) provided a sensitive measure of heat injury because it integrates the influence of many metabolic strains. One of these is probably disruption of protein synthesis, a process known to be highly sensitive to heat stress. Another process which may be disrupted by heat and affect regrowth is cell division (43,70). Mild heat stress stops mitosis in animal and microbial cells, often with little apparent effect on metabolic integrity (70). Although pear culture regrowth is a sensitive test of injury and can be accurately measured, the results are difficult to interpret in terms of specific cell functions. Part of the reason for this is the complexity of growth kinetics in batch-propagated suspension cultures. The increase in dry weight depends upon both the number and condition of viable cells which are transferred to fresh medium in the regrowth test. Cell number has obvious direct effect on subsequent culture growth, and density-dependent, indirect effects are also known to exist

(84). Unknowns associated with the recovery of injured cells further restrict interpretation of regrowth data. However, the ability to grow is the most direct and valid test of viability (66).

Measurement of TTC reduction by stressed tissue is a rapid, convenient viability test which reflects the activity of succinate dehydrogenase and other respiratory enzymes (83). It has been mostly used to determine low temperature injury, but Li and his coworkers (16,65) have used the TTC test to measure heat injury. In these cases, the assay for TTC reducing capacity was begun immediately following stress treatment and thus measured direct injury only. Effects of freezing stress on TTC activity have been attributed to substrate and cofactor limitations rather than to enzyme inactivation (82) . However, Chen et al. (16) suggested that denaturation of TTC reducing enzymes was the cause of direct heat injury, an explanation which is consistent with the high temperature coefficient shown in Figure 6 for direct injury. The direct effect of 43^{o}C (Figure 7B) was to slightly increase TTC reducing activity, but greatly reduced this function when it was measured 3 days after the stress (Figure 7). In other words, cultured pear cells were highly sensitive to an indirect injury which affected their capacity to reduce TTC. By definition (43), indirect (metabolic) injury requires time, after the stress, to develop.

Inhibition of protein synthesis is a metabolic strain which would indirectly affect TTC reduction. In Bartlett pear fruit slices, high temperature (40°C) induced a marked loss of polysomes and increase in monosomes (72). Subsequent work in the same laboratory (R. Romani, personal communication) showed that cultured pear cells responded to heat stress in a similar manner, i.e. polysomes were dissociated by heat treatment. Bernstam (9) suggested that the influence of superoptimal temperature on protein synthesis is mediated through cell surface membrane alterations which control translation. Regardless of its mechanism, inhibition of protein synthesis would be a major contributing factor to the symptoms of indirect injury. Polanshek (70) reported that protein synthesis and growth of fission yeast were inhibited in the same way by heat stress (15 minutes at 41°C) and cycloheximide. For pear cells, the indirect effect of heat stress (20 minutes at 43°C) on TTC reducing capacity could also be mimiced by cycloheximide treatment (Figure 7B).

Measurement of post-stress electrolyte leakage is a widely accepted method of estimating viability (43,66). Increased leakage (stress-induced injury) has been attributed to lipid phase transitions and to effects on membrane-bound transport proteins (43). For cultured pear cells, leakage data indicated a heat killing point (50 percent injury) of 56° (Figure 5,6), the highest of the three viability tests studied. This was 13° to 14° higher

than the temperature which produced 50 percent reduction in regrowth capacity (Figure 5) . However, disparities between temperatures at which different processes are affected does not necessarily mean that sites of injury differ. For example, plasmalemma perturbation may inhibit protein synthesis (9) before the heat-induced strain becomes severe enough to cause substantial leakage of cell contents.

Furthermore, data showing different critical temperatures (e.g. Figure 5) for different viability tests must be interpreted with caution because killing points are - to a certain extent - arbitrary. That is, damage which affects membrane permeability (and probably other characteristics) occurs before 50 percent leakage is observed. In cultured pear cells membrane leakiness was apparent (Figure 5 and 6) after 20 minutes at between 40^{o} and 45^{o}, the critical range for the metabolic disruptions that influenced culture regrowth. Another factor which is not always adequately considered is the importance of stress duration and its interaction with high temperature (43). Depending upon exposure time, critical (50 percent injury) temperatures for direct effects on TTC reduction and electrolyte leakage in cultured pear cells (Figure 27 in Appendix) was the same (50^{o}C) or different. Thus, it is important to compare viability tests using various combinations of treatment time and temperature. A possible example of inadequate consideration of this aspect is the conclusion of Chen et al. (16) that TTC and electrolyte

leakage tests produced the same results; the only stress temperature used to determine killing times was 50°C. The time factor is less critical in freezing injury (43), and close agreement between critical temperatures for regrowth and direct effect on TTC reduction has been reported for freeze-stressed tissue cultures (85,91).

From the comparison of viability tests described here, subsequent growth is the most useful criterion of heat stress response in cultured cells. The regrowth test is not the most convenient but provides the most sensitive measure of heat injury. Unlike direct measurements of electrolyte leakage and TTC reduction, culture growth integrates the influence of the multiple metabolic strains which contribute to heat injury. The availability of a suitable viability test for cultured cells will facilitate: a) comparison of acclimation by elevated growth temperature to that induced by heat shock (96); b) correlation of biochemical changes (e.g. fatty acid saturation and heat shock proteins) to changes in heat tolerance; and c) development of criteria for selection and characterization of cell lines with increased heat tolerance.

CHAPTER 3

HEAT STRESS RESPONSES -

EFFECT OF CULTURE HANDLING AND AGE

INTRODUCTION

Certain aspects of cultured cell response to heat are similar to those of intact plant tissues. Heat stress disrupts polysomes in cells of intact pear fruit (72) and in cultured pear fruit cells (Romani, personal communication). Heat shock induces acclimation and the synthesis of heat shock proteins in both intact organs (35,43) and tissue cultures (7,78). However, it is clear that the isolation and culture of plant cells alters physiological characteristics which may influence high temperature response. For example, batch-propagated suspension cultures are characterized by stages of relatively high mitotic activity (30). This is relevant to the study of heat stress because certain dividing cells are especially susceptible to heat injury (70,76). Therefore, it is necessary to consider the relationship of culture age to heat sensitivity. Another inherent feature of tissue culture systems is the routine handling and manipulation involved in the transfer and treatment of cells. Tissue handling has been shown to induce subtle but substantial metabolic changes (89,90), some of which could influence heat tolerance. The objective of this study was to examine the effects of suspension culture age and handling on the heat tolerance of pear cells. This knowledge is important

not only in full development of model systems but also as a guide to procedures for *in vitro* selection of heat tolerant mutant cells.

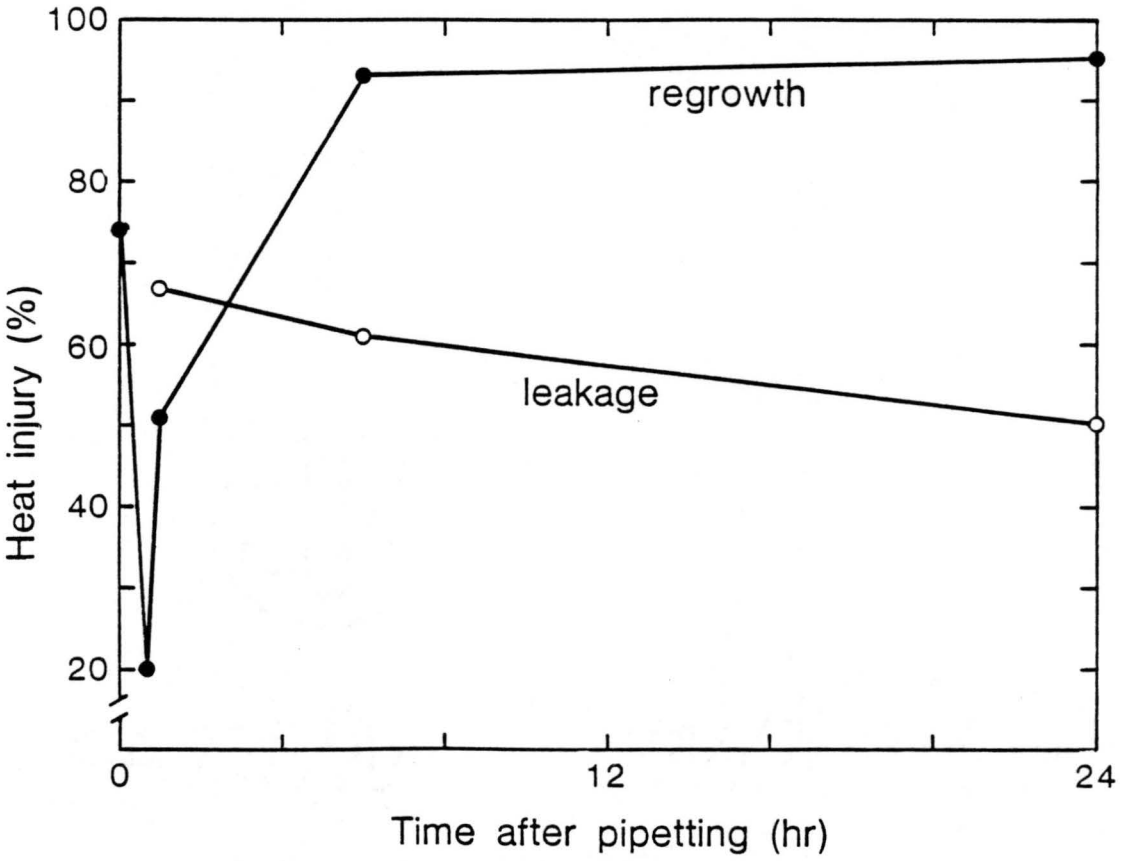
MATERIALS AND METHODS

Plant materials, heat stress treatment and viability tests were as described in Chapter 2. Cell division activity was estimated using aceto-orcein staining to identify mitotic figures. The results were expressed in terms of mitotic index, which was calculated as the percent of cells with stained chromosomes. All the experiments were repeated at least once.

RESULTS AND DISCUSSION

The heat stress treatment described previously involves pipetting aliquots into test tubes for imposition of heat stress. The heat shock experiments which will be described in the next chapter required one more pipetting of samples (i.e., aliquots were pipetted into test tubes for heat shock at sublethal temperature; collected into flasks and shaken at 22^{o}C for certain periods of time; and then pipetted again to test tubes for heat stress treatment). Transfer by pipette influenced the heat sensitivity of non-heat shocked cells (Figure 8). This effect was substantial, but only when heat injury was measured with the culture regrowth test. The pattern of change in heat sensitivity

Figure 8. Effect of time after handling on heat sensitivity of pear cells. The treatment was pipetting 5.0 ml of stirred cell suspension into 1 x 10 cm test tubes. The 0 time samples were heat-stressed immediately. Other samples were aerated with gentle shaking until the time of heat stress. Heat sensitivity was determined by measuring regrowth (after 20 min at 43°C) or electrolyte leakage (after 40 min at 45°C).


100
80
60
40
20
Heat injury (%)
regrowth
leakage
0
12
24
Time after pipetting (hr)

was different when injury was based on electrolyte leakage (Figure 8). This may mean that the heat-induced strains leading to direct (increased leakage) and indirect (reduced regrowth) injuries were separate. During the 30 to 40 minutes after pipetting, pear cells became temporarily very resistant to regrowth-limiting heat injury. Samples stressed immediately after transfer showed 74 percent injury while those stressed 40 minutes later suffered only 20 percent injury. The effect was transient; more than 90 percent injury occurred when heat stress was imposed 6 or 24 hours after pipetting. Stress imposed after 60 minutes resulted in 51 percent injury.

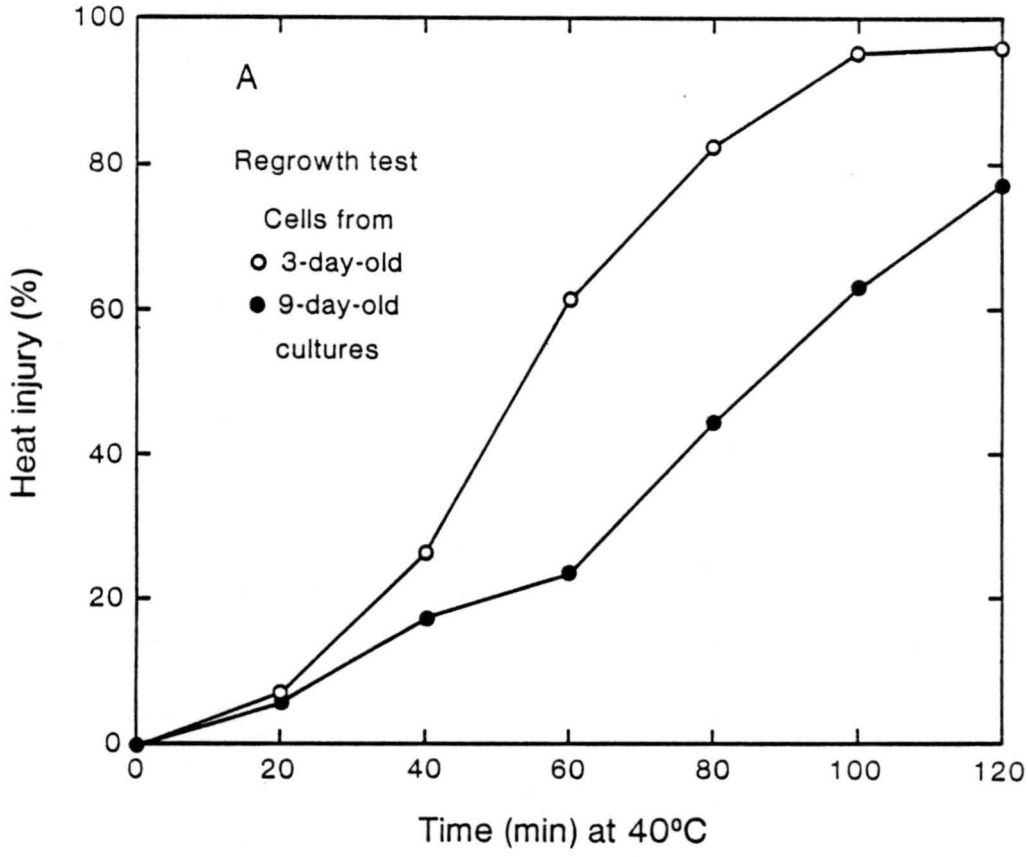
Sampling and harvesting manipulations have been reported to have profound effects on membrane permeability and transport (89,90). The effect of time after handling (Figure 8) emphasizes the importance of using a well-standardized procedure to avoid major errors in measuring heat sensitivity of cell suspensions. Thoiron et al. (90) pointed out the potential artefacts that could result from sampling procedures used with cell suspensions. They called the culture handling effect gas shock because it "occurs when the cells rapidly equilibrate with an atmosphere different from that in the culture flask". Gas shock effects are typically transient ones (90), with normal metabolism restored during gentle agitation in culture medium. Careful monitoring of time-dependent changes is particularly critical in the study of increased heat

tolerance following inductive treatments such as sublethal heat shock.

In addition to handling by pipette, culture age also influenced heat sensitivity. Pear cells were substantially more heat tolerant in the later stages of culture growth (Figure 9,10). Cells from 3 day old cultures showed more rapid and extensive development of injury in response to heat stress than did those from 9 day old cultures (Figure 9). For example, heat injury, measured as the capacity for post-stress growth after a 60 min exposure to 40^{o}C, was 3 times higher in cells from younger cultures (Figure 9A). A comparable difference in heat tolerance was observed when injury was measured using TTC reduction (Figure 9B) and electrolyte leakage (data not shown). Measurements of heat-induced injury to cells during a 12 day culture cycle indicated that maximum sensitivity occurred near the third day after culture initiation (Figure 10). This relationship between age and heat response is similar to that reported for microbial cultures (19). Clearly, culture age is a factor which must be considered in heat stress studies which utilize plant cell suspensions.

The pear cultures showed greatest apparent mitotic activity at three days, which was prior to the phase of most rapid dry weight increase and coincident with maximum heat sensitivity (Figure 10). Mitosis occurred throughout the 12 day culture cycle, typical of the asynchrony of batch-propagated plant suspension cultures (30). In a

Figure 9. Heat sensitivity of pear cells from 3- and 9-day old suspension cultures. Heat injury was determined on the basis of culture regrowth after a 40°C stress (A) or electrolyte leakage after a 45°C stress (B).


A
Regrowth test
Cells from
3-day-old
9-day-old
cultures
Heat injury (%)
Time (min) at 40°C
0
20
40
60
80
100
120

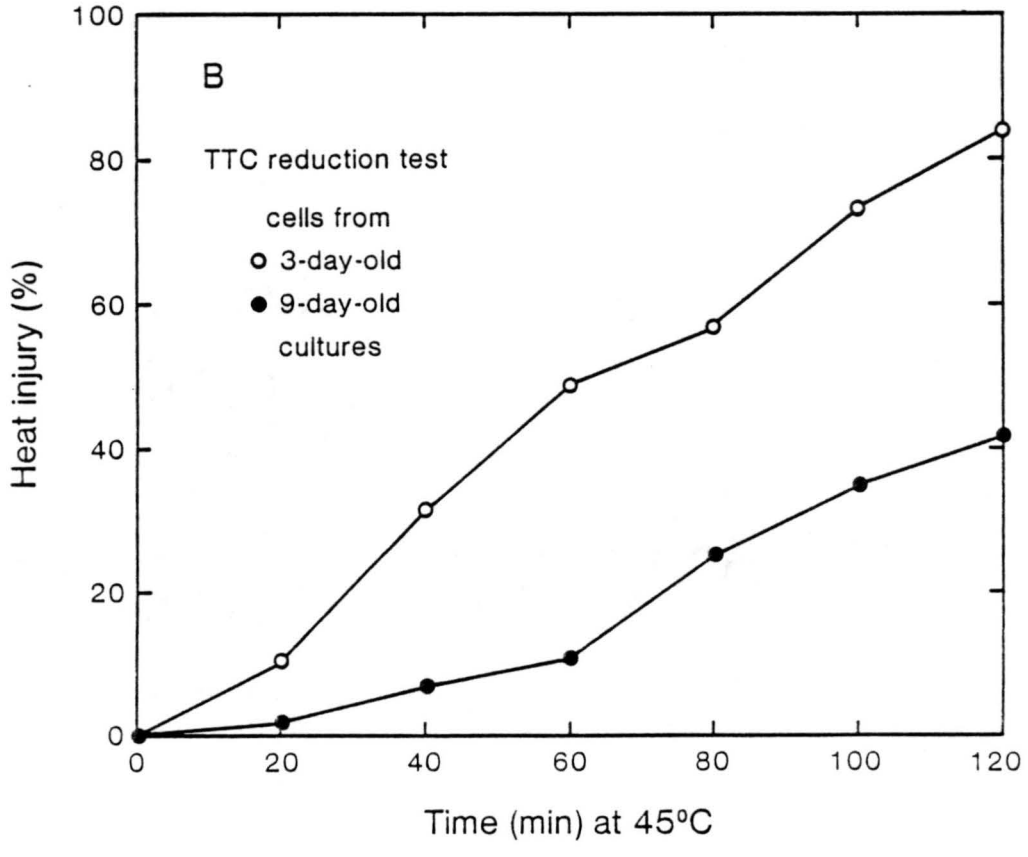
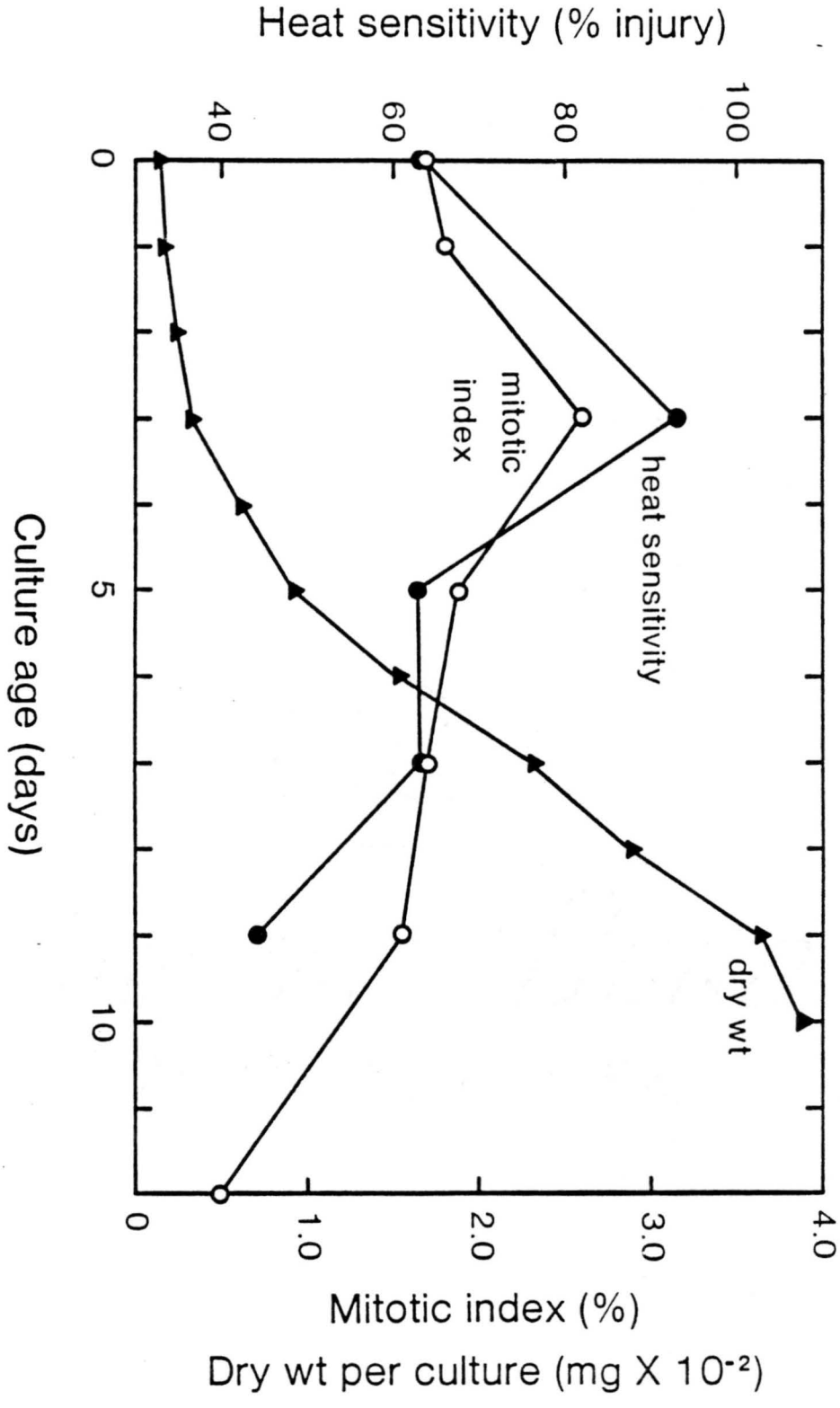

B
TTC reduction test
cells from
3-day-old
9-day-old
cultures
Heat injury (%)
Time (min) at 45°C
0
20
40
60
80
100
120

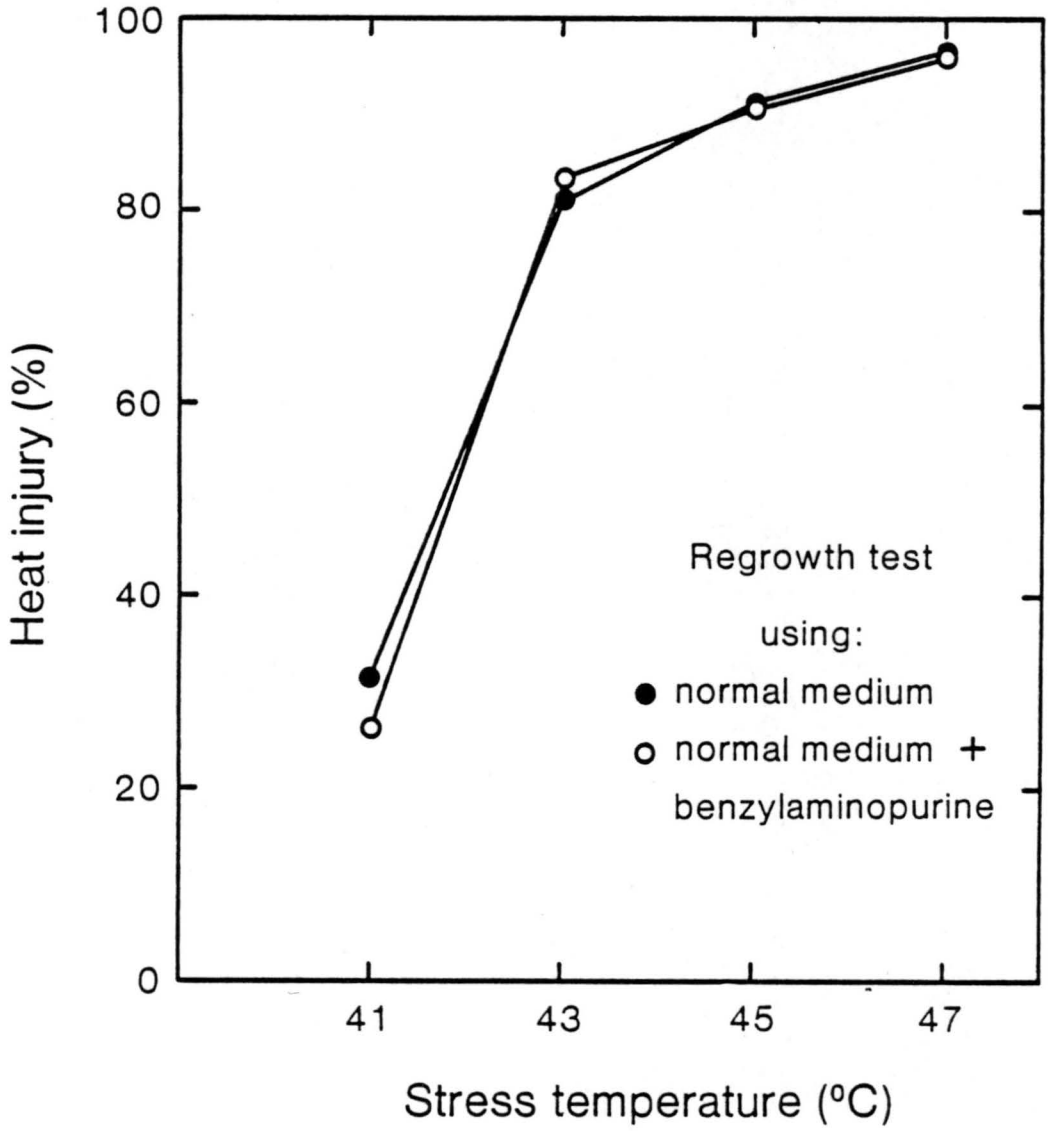
Figure 10. Changes in the heat sensitivity, mitotic activity, and dry weight of pear cells during a 12 day culture cycle. Heat sensitivity (% injury) was determined by measuring TTC reduction after a 52°C, 20 min stress. Mitotic figures were visualized with aceto-orcein stain.


Heat sensitivity (% injury)
100
80
60
40
0
mitotic index
heat sensitivity
dry wt
0
5
10
Culture age (days)
0
1.0
2.0
3.0
4.0
Mitotic index (%)
Dry wt per culture (mg X 10⁻²)

subsequent study (Chapter 4), 30°C-grown cells showed greatest mitotic activity one day after transfer (Figure 28 in Appendix), during the period of minimum heat tolerance (Figure 13 in Chapter 4). The relatively greater mitotic activity in the early stages may be causally related to the greater heat sensitivity of young cultures.

In animal (76) and microbial (19,70) cells, heat stress apparently interrupts mitosis by inhibiting specific stages in the cell cycle. In higher plants, cell division is promoted by cytokinins and there is evidence that brief heat stress reduces cytokinin levels (32). The pear cells used in this study require no exogenous cytokinin, although benzylaminopurine (1.0 ppm) was required during initial development of the cell line (Romani, personal communication). Since examples of specific biochemical lesions in heat stressed plant tissue are well-known (43), it is possible that one effect of heat (e.g. 43°C for 20 minutes) on pear cells might be to eliminate endogenous cytokinin production. This possibility was evaluated by supplying 1.0 ppm BA to heat stressed cells. However, culture regrowth following exposure to high temperature (41° to 47°C) for 20 minutes was not promoted by the presence of BA (Figure 11). Higher concentrations of BA also did not reduce heat injury (data not shown). Thus, although some high temperature effects on detached leaves can be reversed with cytokinin (8,32), heat injury (reduced regrowth) in pear cells probably cannot be attributed to reduced

Figure 11. Effect of benzylaminopurine on the expression of heat injury in pear suspension cultures. Cells were stressed (20 min) immediately after pipetting and then transferred to fresh medium with or without 1 ppm benzylaminopurine. Heat injury was determined using the culture regrowth test.


100
80
60
40
20
0
Heat injury (%)
41
43
45
47
Stress temperature (°C)
Regrowth test
using:
normal medium
normal medium +
benzylaminopurine

cytokinin production. Another cellular feature which has been related to heat tolerance (43) and could change during culture development is fatty acid saturation in membrane lipids. However, in pear cells no change in the relative content of unsaturated fatty acids (Figure 29 in Appendix) could be related to the pattern of change in heat sensitivity (Figure 10). However, the relevant physical property is membrane lipid fluidity and this may not necessarily correlate well with the unsaturation ratio of total fatty acids. More detailed study of the temporal variation in heat sensitivity (Figure 10) should be conducted with cultures induced to undergo synchronous mitosis and should include direct determination of changes in membrane lipid fluidity.

The main purpose of this study was to draw attention to culture variables (age and handling) which can influence heat stress response. Their effects should be characterized as a first step in the study of heat stress response using any plant suspension culture. In addition, large, rapid changes such as that induced by pipetting (Figure 8) may provide useful experimental tools for examining mechanisms of heat resistance and injury. Physical handling leads to rapid changes in the polysome composition of intact tissues (89) and membrane function in cultured cells (90). Similar changes may be involved in heat injury and should be studied in the context of the heat sensitivity response reported here (Figure 8).

CHAPTER 4

HEAT STRESS RESPONSES -

COMPARISON OF HEAT ACCLIMATION INDUCED

BY ELEVATED GROWTH TEMPERATURE AND BRIEF HEAT SHOCK

INTRODUCTION

The heat tolerance of many plants increases when they are growing at moderate, but supraoptimal temperatures. This adaptive response may occur at temperatures below 35°, but the higher the temperature, the shorter the time required to induce heat hardening (27).

Another adaptive response to supraoptimal temperatures known in nature is heat shock at more severe temperature. Heat shock leads to increased heat tolerance in a wide range of organisms (50,77) and is also dependent on a time-temperature interaction (43). How this compares to heat hardening induced by elevated growth temperature is unknown, although some reviews of plant response to high temperature have implied that the two are comparable in their effects on heat tolerance (4,87). The equation $T = a - b \log Z$ (43), used to describe the relationship between heat killing temperature (T) and time (Z), also implies that the response to heat is similar over a wide range of temperatures (since a and b are constants). If so, then heat acclimation induced by elevated growth temperatures and brief heat shock would have similar mechanisms. Results presented in this study, however, indicate that elevated growth temperature and brief heat shock increase heat tolerance of pear cells in clearly different ways.

MATERIALS AND METHODS

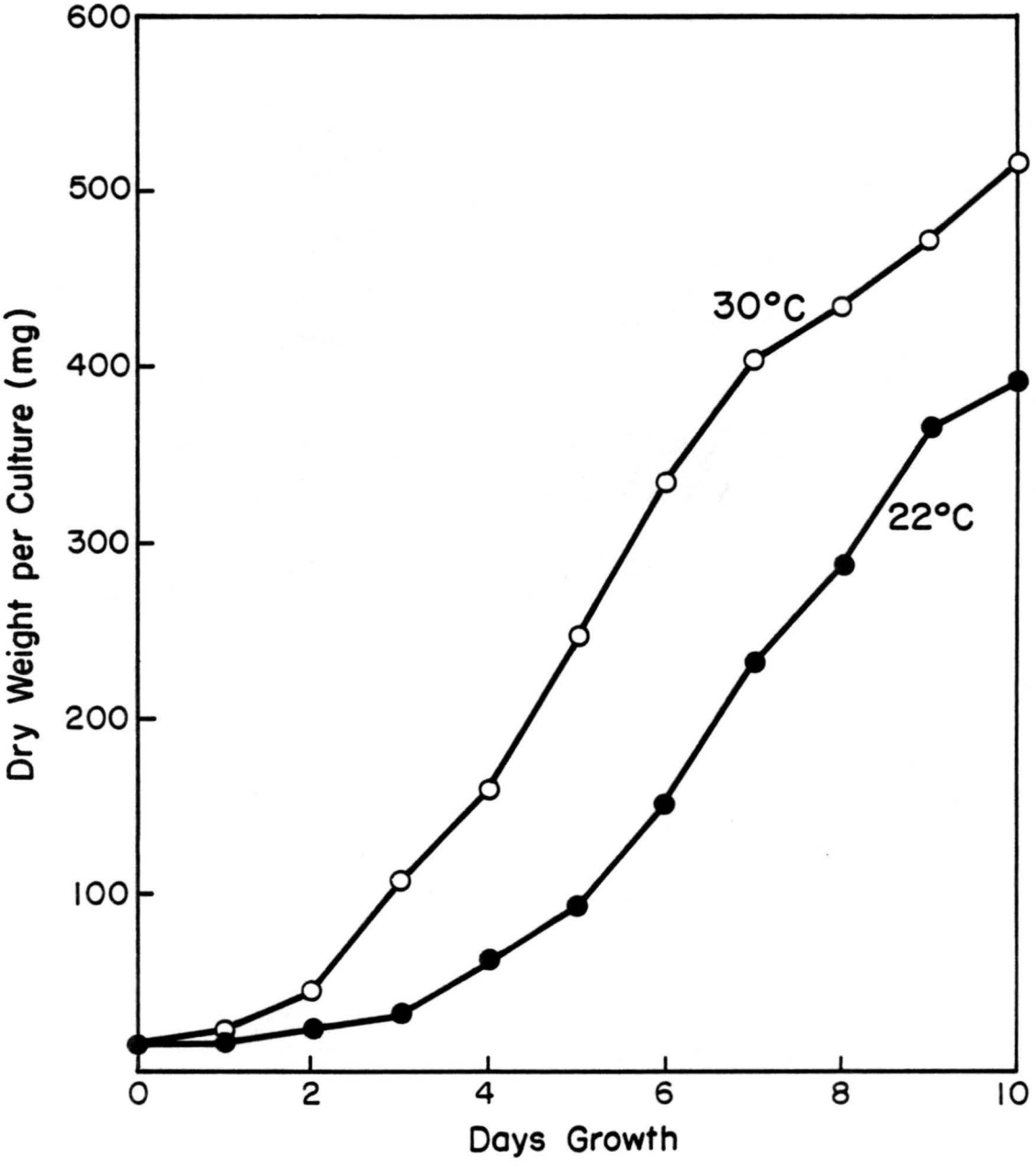
For heat shock treatment, 5 ml of 7 day old pear suspension cells which had been growing at 22°C were pipetted to 1 x 10 cm test tubes and placed in a heated water bath for 20 minutes. Temperature equilibration in the 5 ml aliquots occurred within 3 minutes. The heat-shocked cells (4 aliquots for a total of 20 ml) were then poured into 125 ml Erlenmeyer flasks. The cells were aerated by shaking and kept at 22°C for certain periods of time. Those used as controls were manipulated in the same way except no heat shock treatment was imposed.

Viability tests used in this study were the regrowth, TTC reduction and electrolyte leakage tests described in Chapter 2. All the experiments were repeated at least once.

RESULTS

Pear suspension cultures grown at 30°C increased in dry weight most rapidly between 3 and 7 days following transfer to fresh medium (Figure 12), while those grown at 22°C increased in dry weight most rapidly between 5 and 9 days. Aceto-orcein staining estimates of mitotic index (Figure 28 in Appendix) showed a sharp peak of cell division activity at 1 day and 3 days for cells grown at 30° and 22°C respectively. These data (Figure 12 and Figure 27 in Appendix) indicated that elevated growth temperature, i.e.

Figure 12. Increase in dry weight in pear suspension cultures grown at 30°C and 22°C.


600
500
400
300
200
100
Dry Weight per Culture (mg)
30°C
22°C
0
2
4
6
8
10
Days Growth

30°C, accelerated dry weight increase and mitotic activity of pear cells.

The development of heat acclimation of pear cells at elevated growing temperature was examined during a 10 day time course (Figure 13). The heat tolerance of cells grown at 30°C increased after 3 days and reached its maximum level at day 6. At this time, the hardened cells showed 62 percent survival, while the control cells had less than 10 percent survival after a 43°, 20 min stress based on the regrowth test. This maximum level of heat tolerance of hardened cells was maintained through the tenth day.

When the cultures grown at 30°C for 6 days were transferred to 22°C, they maintained about the same heat tolerance for one day after transfer (Figure 14). However, deacclimation occurred rapidly between the first and second day; by day 2, the hardened cells had lost all of their acquired heat tolerance.

Pear cells exposed to 38°C for 20 min (heat shock) and then incubated for 24 hours at 22°C showed greatly increased tolerance of a subsequent heat stress treatment (Figure 15). The 36° heat shock resulted in considerably less heat tolerance. It is interesting to note that although 20 min at 40° initiated changes leading to heat tolerance, 85 min at 40° or 20 min at 42° killed the pear cells (Figure 4 in Chapter 2).

The increase in pear cell heat tolerance was completed within 6 hours following the 20 min inductive heat shock at

Figure 13. The heat tolerance of pear cells grown at 30°C and 22°C during a 10 day time course. Heat tolerance was determined by regrowth test. 3 - ml pear suspension cells were stressed at 43°C for 20 minutes.

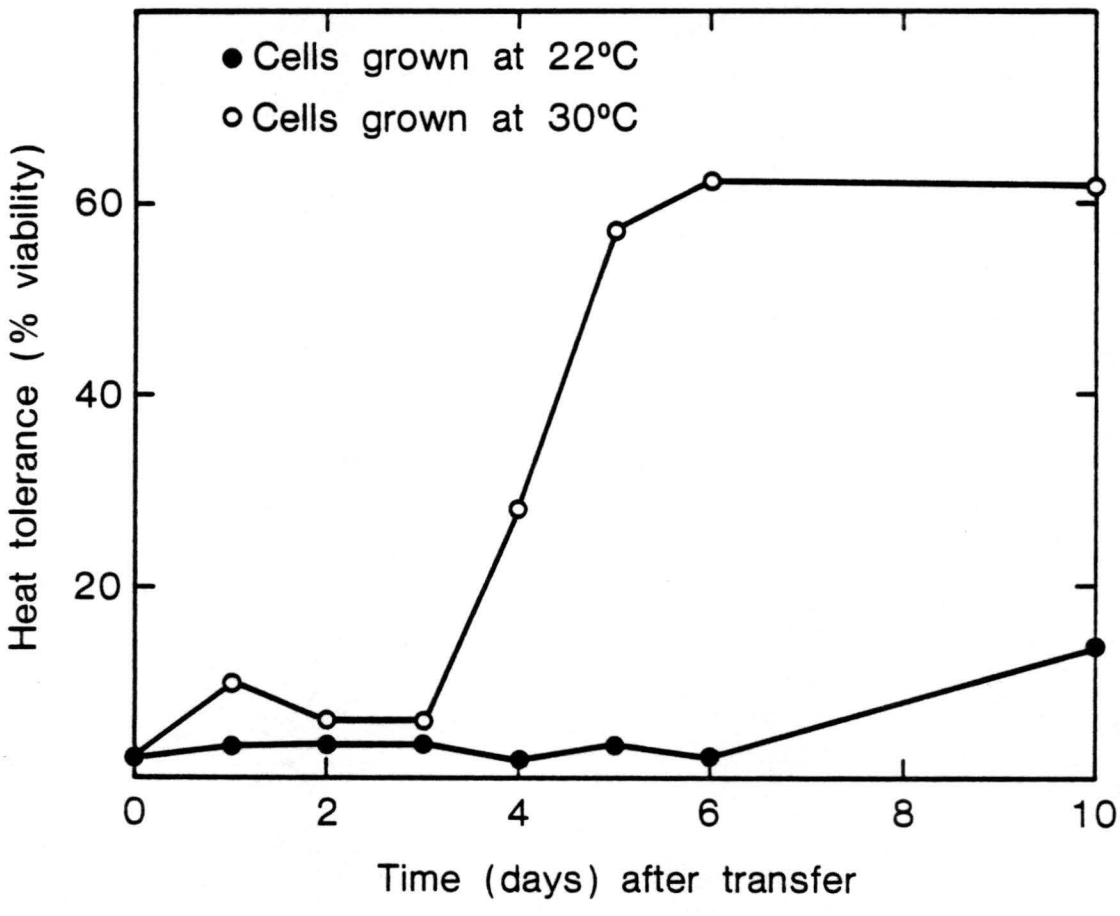

● Cells grown at 22°C
○ Cells grown at 30°C
Heat tolerance (% viability)
60
40
20
0
2
4
6
8
10
Time (days) after transfer

Figure 14. The heat tolerance of pear cells grown at 30°C for 6 days, then transferred to 22°C for days indicated on the abscissa. Controls were the same age as the acclimated cells. Heat tolerance was determined by regrowth test. Cells were stressed at 43°C for 20 minutes.

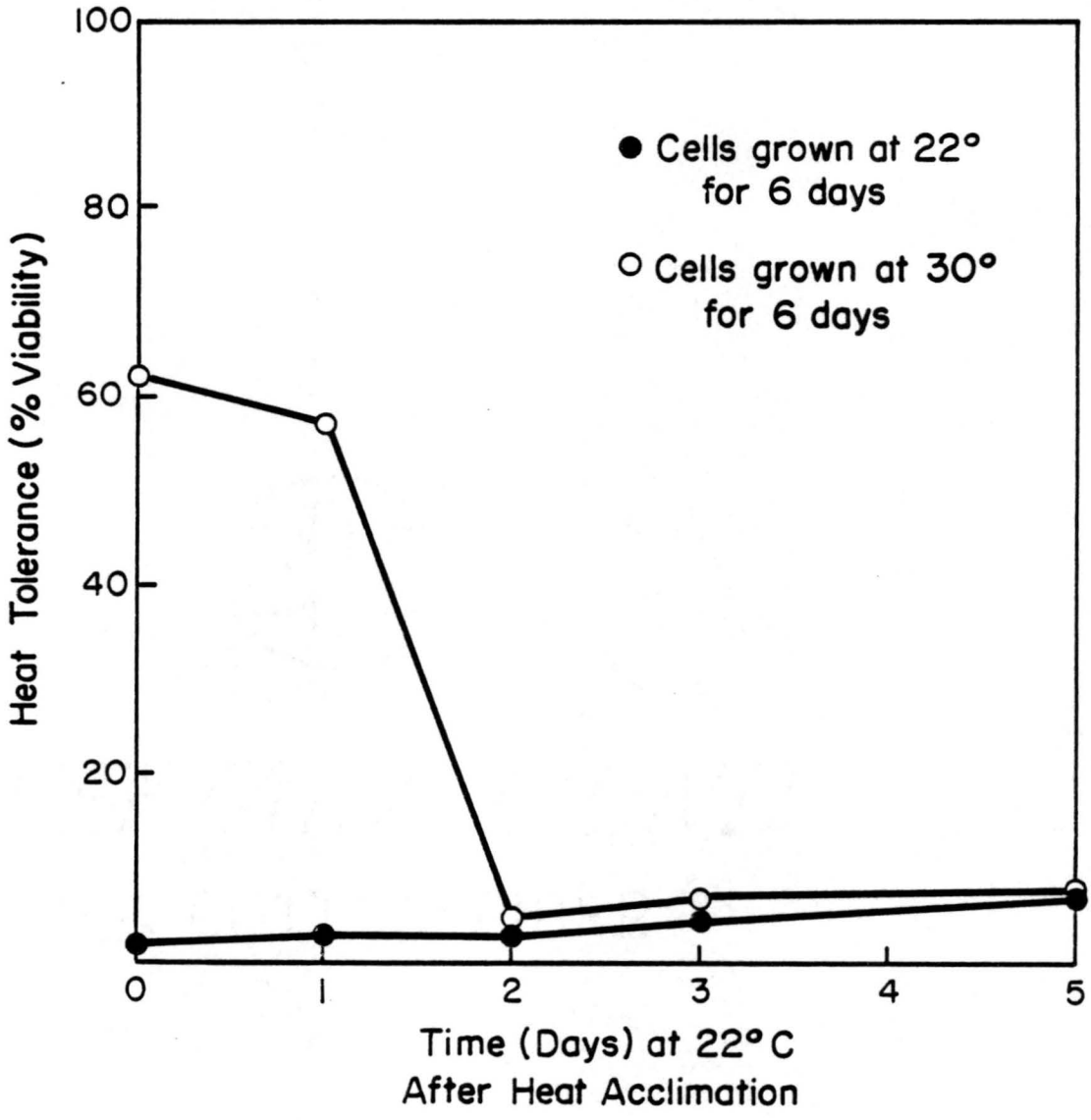
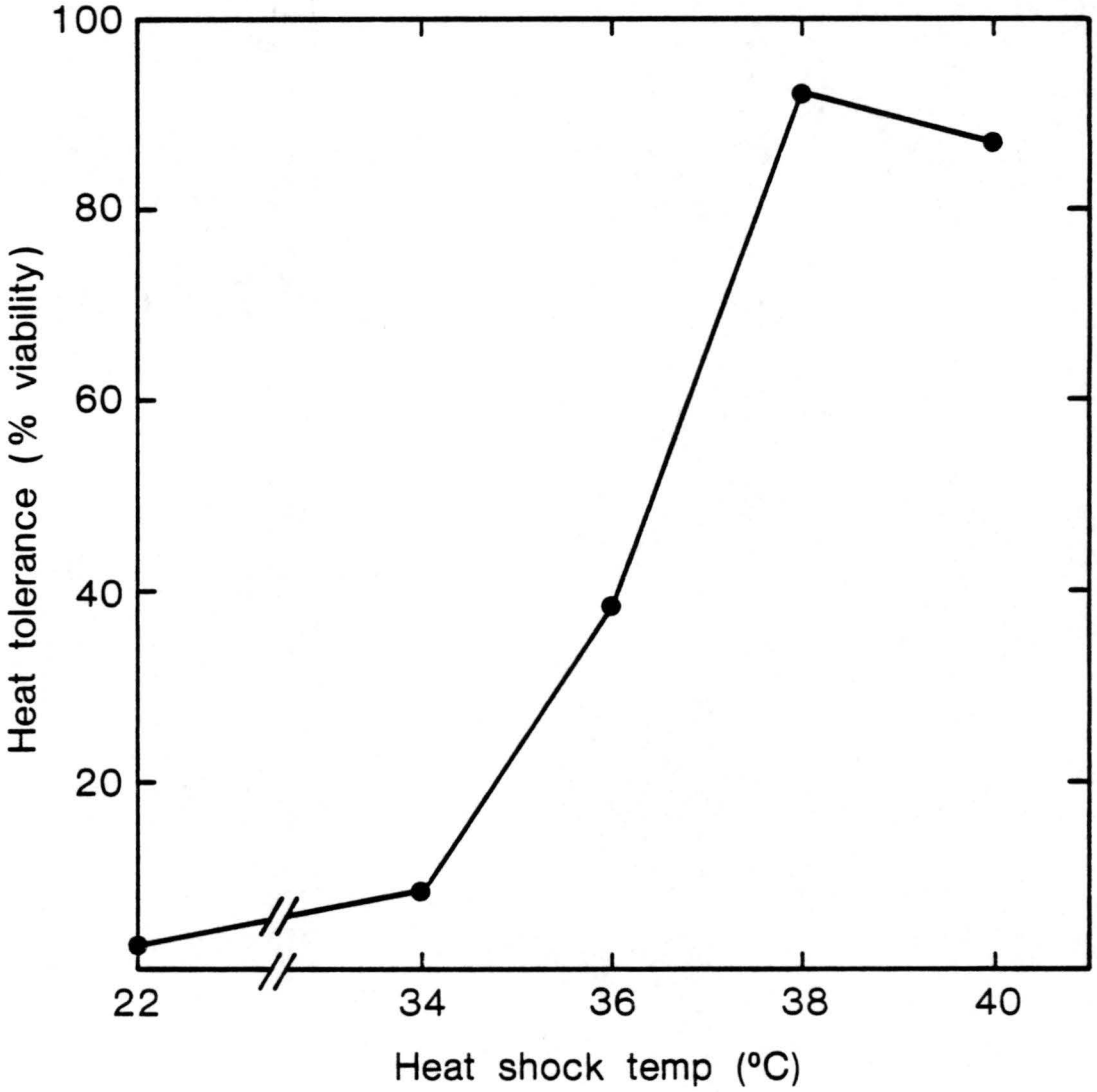

100
80
60
40
20
Heat Tolerance (% Viability)
● Cells grown at 22° for 6 days
○ Cells grown at 30° for 6 days
0
1
2
3
4
5
Time (Days) at 22° C
After Heat Acclimation

Figure 15. The heat tolerance of pear cells exposed to 34°, 36°, 38°, and 40° for 20 min, and then incubated for 24 hours at 22°C. Heat tolerance was detected by measuring regrowth following a 43°C, 20 minute stress.


100
80
60
40
20
Heat tolerance (% viability)
22
34
36
38
40
Heat shock temp (°C)

38°C (Figure 16). The tolerance of heat-shocked cells was much greater than that of controls during the days following heat shock (Figure 16). In the experiments which produced the data for Figure 16, zero time measurements could not be obtained due to the manipulation of samples. As discussed previously (Chapter 3), the high percent survival of control cells one hour after manipulation was due to a transient tolerance induced by culture handling. The apparent decline in control cell tolerance during the first day probably reflects return to a normal condition. However, tolerance of heat-shocked cells was fully maintained for 2 days at 22°C and at day 4 was still considerably higher than that of controls.

Heat shock (39°C for 20 min) also increased the tolerance of 30°-grown cells (Figure 17). Because the initial level of tolerance was higher than for cells grown at 22°C (Figure 13), higher heat stress temperature (45°) was used for the experiment shown in Figure 17. The heat shock response was comparable to that obtained with cells grown at 22°C. The cells grown at 30°C which received a 39° heat shock rapidly developed tolerance which allowed a high survival of a subsequent 45°, 20 min stress. As in Figure 16, control (non-heat shock) cells received the same handling (pipetting) treatment as did the heat-shocked cells, and responded to this handling with a rapid, transient rise in tolerance (control in Figure 17). As a result, the most appropriate comparison of heat tolerance

Figure 16. The effect of 38°, 20 min heat shock on the development of heat tolerance of 22°-grown pear cells. Cells were kept at 22°C after heat shock for time indicated on the abscissa. Controls were 22°-grown cells which were manipulated in the same way as heat-shocked cells but no heat shock was imposed. Heat tolerance was detected with the regrowth test. 3-ml of either heat-shocked or control cells were stressed at 43°C for 20 min.

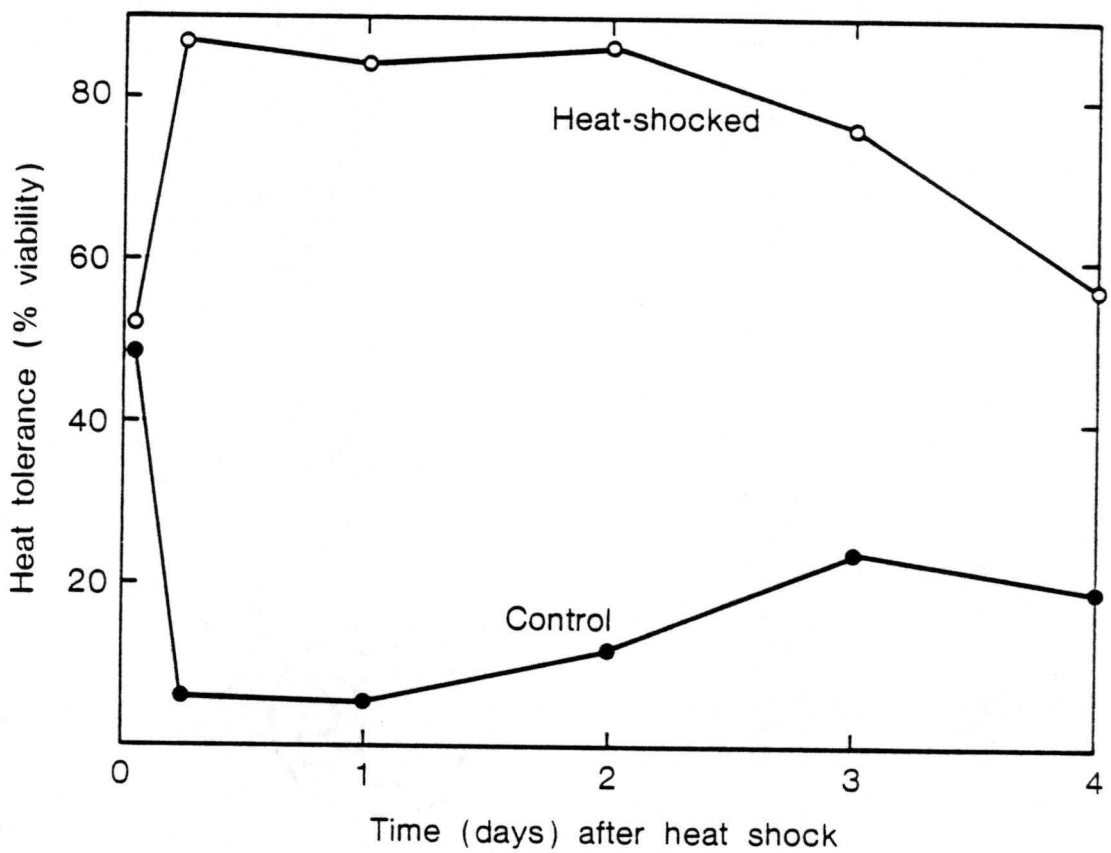
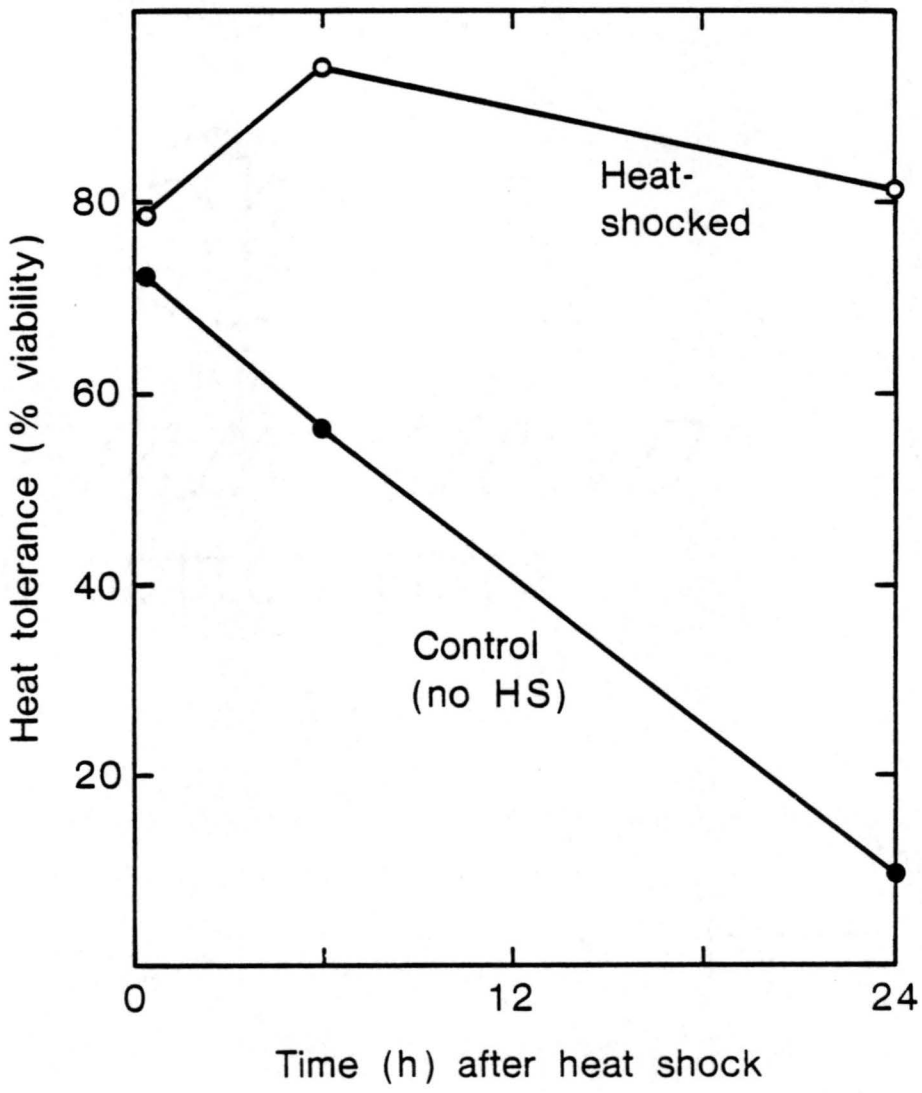

Heat tolerance (% viability)
80
60
40
20
Heat-shocked
Control
0
1
2
3
4
Time (days) after heat shock

Figure 17. The effect of 39°, 20 min heat shock on the development of heat tolerance of 30°-grown pear cells. Cells were kept at 30°C after heat shock for time indicated on the abscissa. Controls were 30°-grown cells which were manipulated in the same way as heat-shocked cells but no heat shock was imposed. Heat tolerance was detected with regrowth test. 3-ml of either heat-shocked or control cells were stressed at 45°C for 20 min.


Heat-
shocked
Control
(no HS)
80
60
40
20
0
12
24
Heat tolerance (% viability)
Time (h) after heat shock

is 24 hours after heat shock; at this time, control and heat-shocked cells showed 9% and 81% survival, respectively, of the 45°, 20 min stress.

Besides the regrowth test, electrolyte leakage and TTC reduction tests were used to measure the increase in hardiness induced by heat shock and growth at 30°C (Table 1). These three tests are commonly used to assess heat stress injury (43) and were previously characterized as indices of plant cell heat injury (Chapter 2). The increase in hardiness was expressed as percent injury to control cells divided by that to hardened cells. Significant increase in heat tolerance induced by both heat shock and growth at 30°C were again detected with the regrowth test (Table 1). In addition, acclimation during growth at 30°C led to a 2 to 5-fold increase in heat tolerance as measured with electrolyte leakage and TTC reduction tests. Table 2 shows that growth at 32°C also produced a general increase in heat tolerance of pear cells. However, no significant heat shock-induced tolerance was detected with these two tests (Table 1).

DISCUSSION

The 38° optimum for heat shock-induced heat tolerance of cultured pear cells (Figure 15) is close to the optimum for heat shock protein synthesis in other systems. Comassie blue staining of electrophoresed proteins from pear cells indicated that heat shock proteins were produced (Figure 30

Table 1. Increase in hardiness by elevated growth temperature and 38°C, 20 min heat shock based on 3 viability tests.

Viability Test	Stress Condition	Increase in hardiness (Injury to control ÷ Injury to acclimated)	
		6 d at 30°C	20 min at 38°C
TTC red.	52°C, 20 min	4.5	1.3
Leakage	48°C, 40 min	2.6	1.1
Regrowth	43°C, 20 min	3.0	11.7

Table 2. The 50 percent heat killing temperature and 50 percent heat killing time of pear cells[a] grown at 22°C and 32°C based on 3 viability tests.

Viability Test	Heat killing temp. (°C)[b]		Heat killing time (min.)[c]	
	Control	Hardened[d]	Control	Hardened[d]
Regrowth	42	45	105	162
TTC reduction	50	54	51	88
Electrolyte leakage	54	56	30	82

[a]Cells were from 6-day-old cultures.

[b]Heat stress imposed for 20 min.

[c]Heat stress was 40°C for regrowth, 48°C for TTC reduction and electrolyte leakage tests.

[d]Control cells were grown at 22°C, hardened cells at 32°C.

in Appendix). However, definite identification of unique protein synthesis requires the use of labelled amino acids to confirm that such synthesis was induced by heat shock. Using such techniques, Barnett et al. (7) reported that 39°C was optimal for heat shock protein synthesis in cultured tobacco and soybean cells. Temperature optima for heat shock responses have not been precisely identified in most cases, but 36° to 40° has been found effective for heat shock protein synthesis in maize roots (20), soybean hypocotyls (35), and drosophila cells (5), and for the development of heat tolerance in soybean seedlings (44) and yeast cells (52). Correlations consistent with a causal relationship between heat shock protein synthesis and acquired heat tolerance have been described for yeast cells (52) and soybean seedlings (44). Minton, et al. (56) theorized that heat shock proteins protect the cell from heat injury by stabilizing other proteins in a non-specific manner.

The heat tolerance of pear suspension cells induced by 38°C (Figure 15, 16) required only a 20 min heat treatment and was completed within 6 hours. However, pear cultures grown at 30°C began to show increased heat tolerance only after 3 days (Figure 13) and the maximum tolerance was not reached until cultures had been at 30°C for 6 days. Furthermore, the maximum tolerance achieved by cells grown at 30° was between 60 and 65 (% viability after a 43°, 20 min stress, based on the regrowth test, Figure 13), while

that induced by heat shock was between 90 and 95 (Figure 15,16).

In addition to the difference in maximum heat tolerance, cells hardened by 38°C heat shock and by 30°C did not lose tolerance in the same way upon return to 22°C (Figure 14,16). The persistence of heat tolerance in heat-shocked cells agrees well with the limited reports for intact plants. Heat shock-induced tolerance was fully maintained in cabbage leaves for 2 days (87) and in beans for at least 3 days (96). If heat shock proteins confer heat tolerance directly, they must be relatively stable because their synthesis ceases soon after return to normal temperature (7,20,35). Declining heat tolerance in a suspension culture could be due to loss of the acquired physiological condition in existing cells and/or to growth, i.e. the production of new, non-hardy cells at 22°. The growth of heat-acclimated cultures at 22°C was not measured in this study, but large differences between 38° heat-shocked and 30°-grown cells would not be expected.

Another observation which suggests that acclimation by heat shock and by growth at 30°C are fundamentally different is that heat shock greatly increased the tolerance of the cells grown at 30°C (Figure 17). In this case, the increase in tolerance induced by heat shock was nearly as great as that induced by heat shock treatment of 22°-grown cells (Figure 16). If the heat hardening during 8 days at 30°C

were similar in nature to heat shock, the subsequent heat shock response would likely have been less striking.

The most important difference between 38° heat shock and growth at 30° of pear cells is the different effects on heat tolerance as measured with 3 viability tests (Table 1). Acclimation during growth at 30° lead to a general increase in the heat tolerance of all measured cellular functions. The capacity for TTC reduction and culture regrowth potential after heat stress were increased 4.5 and 3-fold, respectively. Resistance to membrane injury (indicated by electrolyte leakage) was also substantially increased by growth at 30°. In contrast to the general heat hardening developed at 30°, significant heat shock-induced tolerance was detected only with the regrowth viability test. The capacity for post-stress culture growth was greatly enhanced by heat shock (Figure 15, 16; Table 1), but heat-shocked cells were not resistant to the direct injuries which TTC reduction and electrolyte leakage tests measure (Table 1).

Recognition of differences between heat shock and elevated growth temperature effects on heat tolerance is critical to the study of plant performance at high temperatures. Results presented in this study show that heat acclimation in response to heat shock and to growth at 30° occur via different mechanisms. That is, $T = a - b \log Z$ (43) does not describe the relationship between time and temperature for heat acclimation over part of the range of supraoptimal temperature.

CHAPTER 5

EFFECT OF TEMPERATURE ON FREEZING AND HEAT STRESS TOLERANCE IN CULTURED PEAR CELLS

INTRODUCTION

One approach to the study of biological acclimation mechanisms is to compare the influence of diverse environmental stimuli on the adaptive response (4,25,43,65). Such comparisons have been prompted by actual and conceptual similarities between the effects on plants of different stresses (4,43). For example, Lin et al. (44) reported that arsenite treatment mimiced the heat shock response and was a useful probe with which to study the mechanism involved. A wide variety of environmental conditions have been reported to increase both freezing and heat tolerance in certain plant tissues (4,43). In nature, cold acclimation which confers freezing tolerance to plants is often accompanied by increased heat tolerance (4,33,43,65). The many examples of plants which develop heat tolerance in winter led Kappen (33) to suggest that heat and freezing tolerance are "... linked to each other", but Li and his coworkers (16,65) found no such systematic relationship among Solanum species.

Plant tissue cultures offer convenient experimental systems for investigating questions regarding cellular mechanisms of temperature acclimation. Cell suspension cultures of appropriate species origin are known to cold acclimate at low temperatures (17,29). In previous work,

suspension-cultured pear cells were shown to develop heat tolerance in response to brief heat shock and elevated growing temperatures (Chapter 4). If freezing and heat tolerance were closely correlated in a cultured cell system, then the parameters of cold hardening, including the molecular changes which accompany it, could provide new approaches to and understanding of heat tolerance, and vice-versa. In this study, it is shown that the same pear cells also cold acclimate and the effect of low and high temperature on both adaptive responses is compared.

MATERIALS AND METHODS

The suspension cultures were as described in Chapter 2. Temperature acclimating treatments were imposed by maintaining cultures at 2°C or 30°C and by heat shock treatment. For heat shock, 5 ml aliquots of cell suspension were held at 38°C for 20 min and then at 22°C for 24 hours (see Chapter 4).

Heat stress tolerance was measured using stress conditions and viability tests as previously described (Chapter 2). Heat stress involved various combinations of exposure temperatures and duration; injury was measured with the electrolyte leakage or TTC reduction tests.

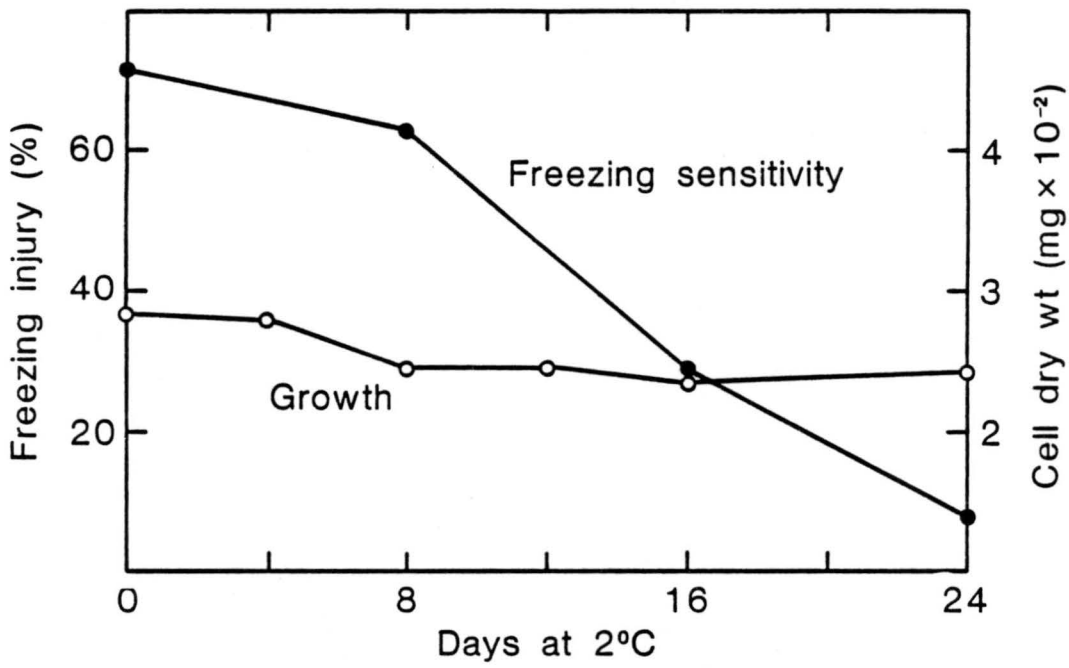
Freezing stress was imposed and injury measured essentially as described by Towill and Mazur (91). Aliquots (3 ml) of cell suspension were exposed to freezing temperatures in a methanol bath; nucleation was accomplished

by seeding with ice at -2°C. The frozen cell suspensions were held at -2°C for 18 hours after which the bath temperature was reduced by 5°C per hour. At indicated test temperatures, samples were removed and held at 2°C for slow thawing. Freezing injury was determined using the TTC-reducing assay as described for pear cell heat injury (Chapter 2).

RESULTS AND DISCUSSION

Suspension-cultured pear cells showed considerable capacity for both cold (Figure 18) and heat acclimation (Chapter 4). To induce cold acclimation, pear cultures were subjected to constant 2°C when they were seven days old, in a phase of rapid growth (Figure 1 in Chapter 2). At 2°C, growth stopped and cold acclimation was observed (Figure 18); after 24 days in the cold, exposure to -6°C produced only 7 to 8% injury compared to more than 70% injury suffered by non-acclimated pear cells. The development of freezing resistance after growth cessation in pear cell suspensions is consistent with the idea that growth prevents cold hardening (43). In contrast to cold acclimation, the greatest decline in pear cell heat sensitivity occurred during the phase of most rapid culture growth at 30°C (Figure 12,13 in Chapter 4). Brief heat shock (38°C for 20 min) induced cellular changes leading to more substantial heat tolerance (Figure 15,16 in Chapter 4) than that developed at 30°C. Figure 18 and Chapter 4 show that the

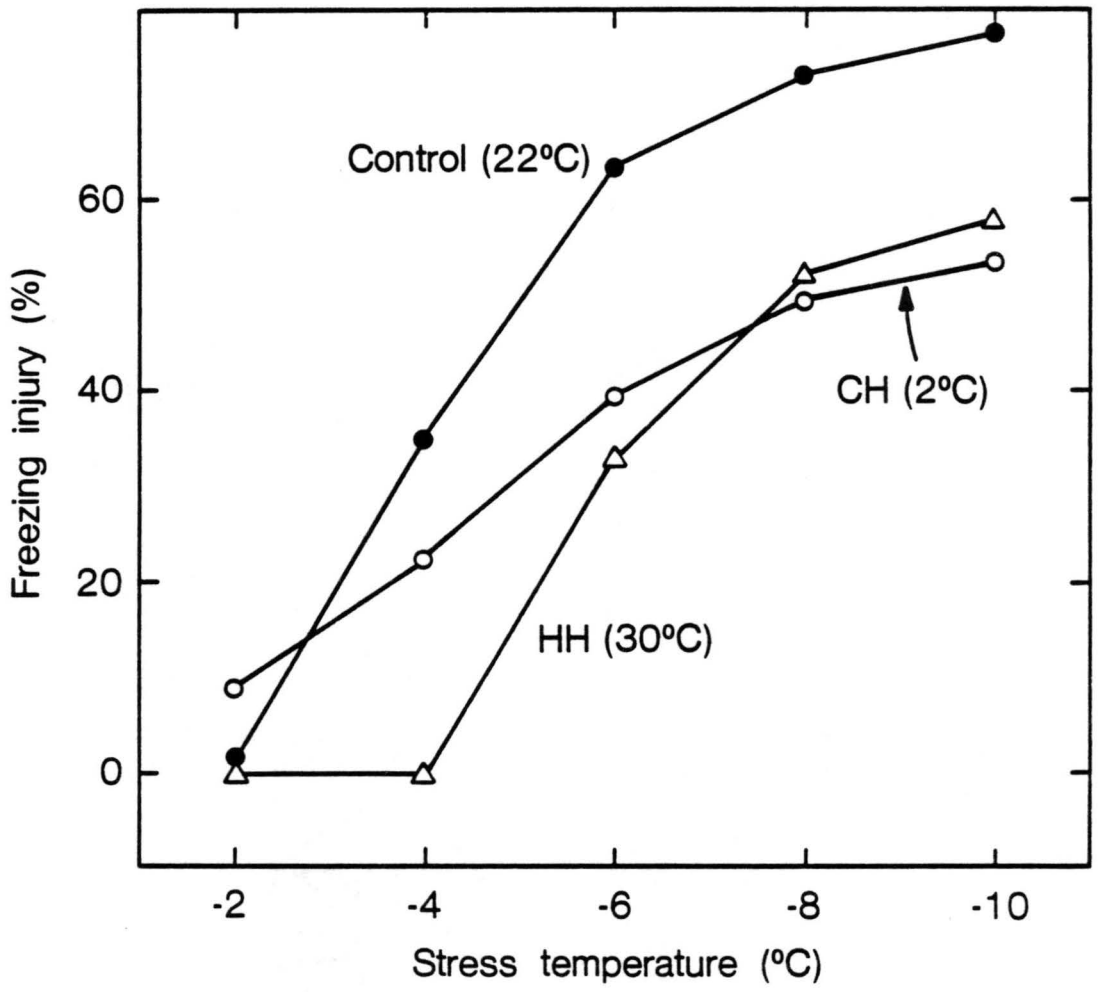
Figure 18. Freezing sensitivity and growth in pear suspension cells grown at 2°C following initial 7 days growth at 22°C. Freezing sensitivity was measured by TTC reduction test and the testing temperature was -6°C. Growth was detected by measuring the cell dry weight.


Freezing injury (%)
60
40
20
Freezing sensitivity
Growth
4
3
2
Cell dry wt (mg x 10⁻²)
0
8
16
24
Days at 2°C

physiological mechanism(s) for both heat and cold acclimation exist in the same population of cells and can be conveniently triggered *in vitro*.

The freezing tolerance of cells was increased after incubation at 2°C for 10 days, or 30°C for 6 days (Figure 19). Differences in freezing injury between control cells and those grown at either 2°C or 30°C were statistically significant (5% level) at all stress temperatures except -2°C. Over the range of freezing temperatures tested, the heat-hardened cells were at least as cold hardy as those held at 2°C for 10 days. However, with additional time at 2°C, pear cells became substantially more cold hardy (Figure 18). Reports of freezing tolerance development coincident with heat hardening are rare. Alexandrov *et al*. (4) studied many plants but found no increase in freezing tolerance during heat hardening, and Levitt (43) concluded that "... high-temperature tolerance as a result of heat hardening does not carry with it a tolerance of chilling or freezing low temperatures". In light of this apparent inconsistency with other's (4) observations, the data of Figure 19 must be interpreted with caution. However, one consideration is that temperature stress tolerances are multi-faceted phenomena having components which could develop independently (43). In addition, the method of measuring stress injury can influence estimates of temperature tolerance (Chapter 2). What does seem clear is that, in the restricted case of pear cell injury as measured via

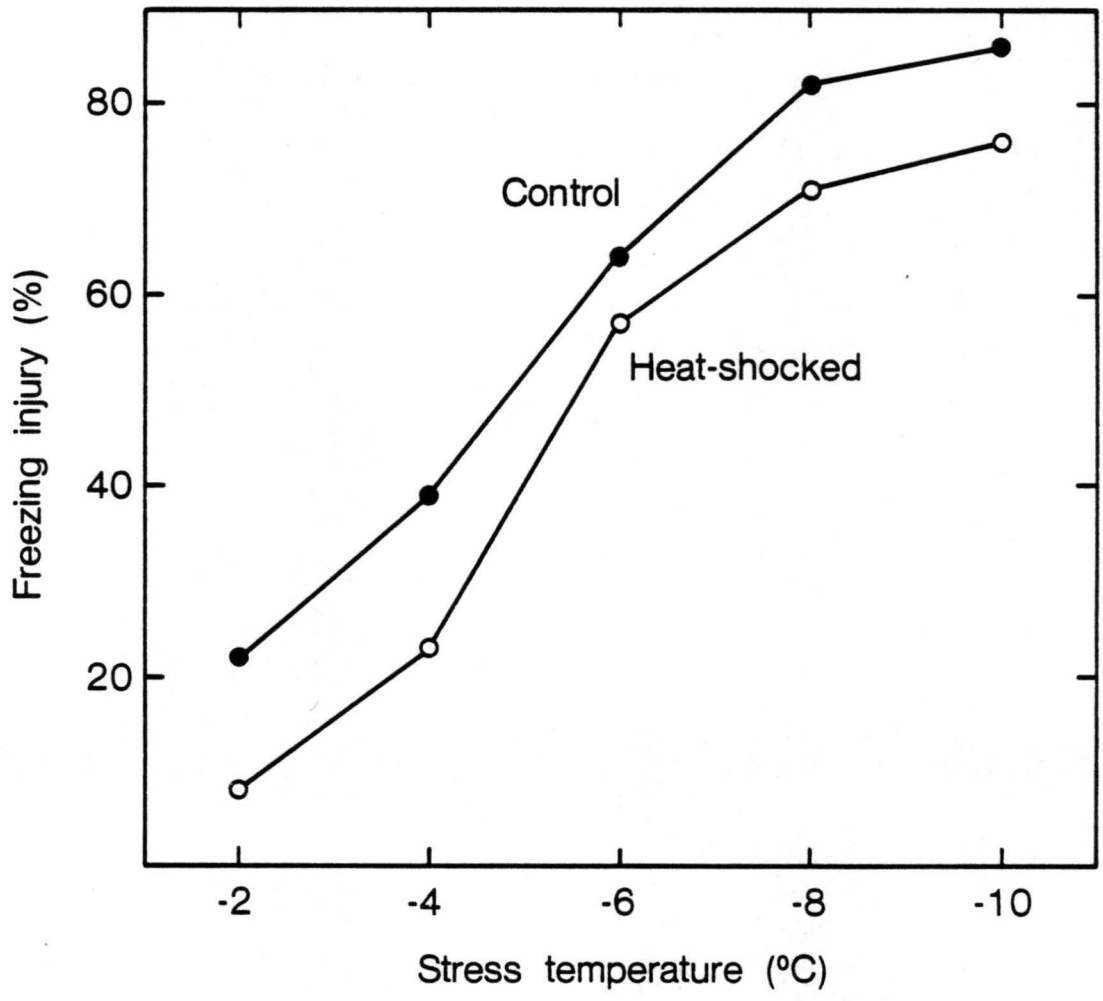
Figure 19. The freezing tolerance of cold acclimated, control and heat acclimated pear cells based on TTC reduction test. The heat acclimated cells were grown at 22°C for 3 days and then transferred to 30°C for heat hardening for 6 days. The cold acclimated cells were grown at 22°C for 6 days and then transferred to 2°C for cold hardening for 10 days. Those grown at 22°C for 9 days were the controls. 3-ml of pear suspension cells were nucleated by ice at -2°C. The duration of freezing stress was 30 minutes at each freezing temperature.


Control (22°C)
CH (2°C)
HH (30°C)
Freezing injury (%)
0
20
40
60
-2
-4
-6
-8
-10
Stress temperature (°C)

TTC-reducing ability, there were changes at 30°C (i.e., during heat hardening) which made the cells more resistant to freezing stress. This raises the possibility that some component of acclimation (i.e. that responsible for thermostability of TTC-reducing systems in pear cells) may be the same for both cold and heat-hardening.

The concept of an adaptive response common to various stresses is evident in recent discussions and studies of the heat shock phenomenon (44). A wide range of stresses induce the synthesis of heat shock-like proteins which may confer resistance to the conditioned cells. However, in contrast to growth at 30°C, pear cell heat-hardening induced by heat shock was not accompanied by a significant increase in freezing tolerance (Figure 20). There was a slight but consistent difference in the means for freezing injury between control and heat-shocked cells, but no statistically significant (5% level) pattern was established in three separate experiments. Similarly, the effect of heat shock on the heat stability of pear cell TTC-reducing systems was only very slight (Table 1 in Chapter 4). Thus, although growth at 30°C involved change(s) which conferred both heat and freezing resistance, heat shock had little effect on either when resistance was determined with the TTC test. This is consistent with the conclusion in the last chapter that the two treatments evoke different adaptive mechanisms. Clearly, heat shock did not produce the freezing resistance-related change that growth at 30°C did.

Figure 20. The freezing tolerance of heat shocked and control pear cells. The heat shocked samples were 8 day old cells which were heat shocked at 38°C for 20 minutes and then shaken at 22°C for 24 hours. The cold stress treatment was the same as that in Figure 18.


80
60
40
20
Freezing injury (%)
Control
Heat-shocked
-2
-4
-6
-8
-10
Stress temperature (°C)

Heat shock did greatly increase thermostability as measured using a culture regrowth test (Chapter 4). Whether heat shock can increase freezing tolerance as measured using the regrowth test remains unknown, because the technique for freezing test under sterilized condition has not been successful in the pear suspension culture system.

Cold acclimation at low temperature has often been observed to also increase the heat tolerance of plants (4,33,43). However, pear cells which were exposed to 2°C for 10 days lost rather than gained heat tolerance based on the TTC test (Figure 21). Unlike the similar freezing stress response of cold and heat-acclimated pear cells (Figure 19), there was a large difference in their heat sensitivity (Figure 21). Heat injury to cold-acclimated cells was also significantly greater than to the 22°C-grown controls (Figure 21). The same result was obtained when heat injury was measured with the TTC (Figure 21) and electrolyte leakage (Figure 22) viability tests.

One difference between plants grown at different temperatures is membrane fatty acid composition and related physical properties (43). Temperature affects the phase transition point of membranes, and cultured cells grown at 15°, 25° and 35°C contained membranes with progressively more saturated fatty acids (24,47). The electrolyte leakage test assesses cellular membrane thermostability (49), and the TTC test can also detect membrane injury because of the nature of TTC reducing enzyme systems (83). The pattern of

Figure 21. The heat tolerance of cold acclimated, control and heat acclimated pear cells based on TTC reduction test. The three materials were the same as those used for study of freezing tolerance in Figure 18. 3-ml of pear suspension cells were stressed at each testing temperature for 20 minutes.

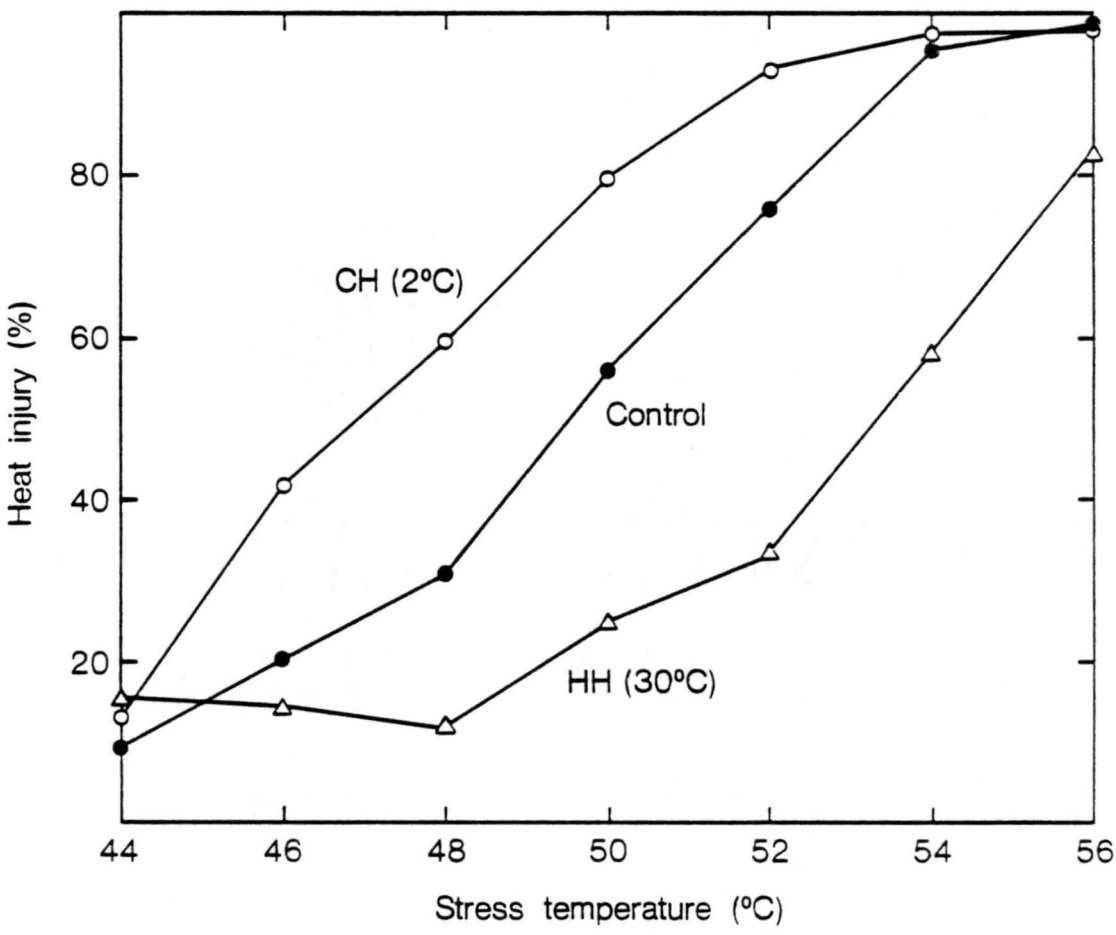
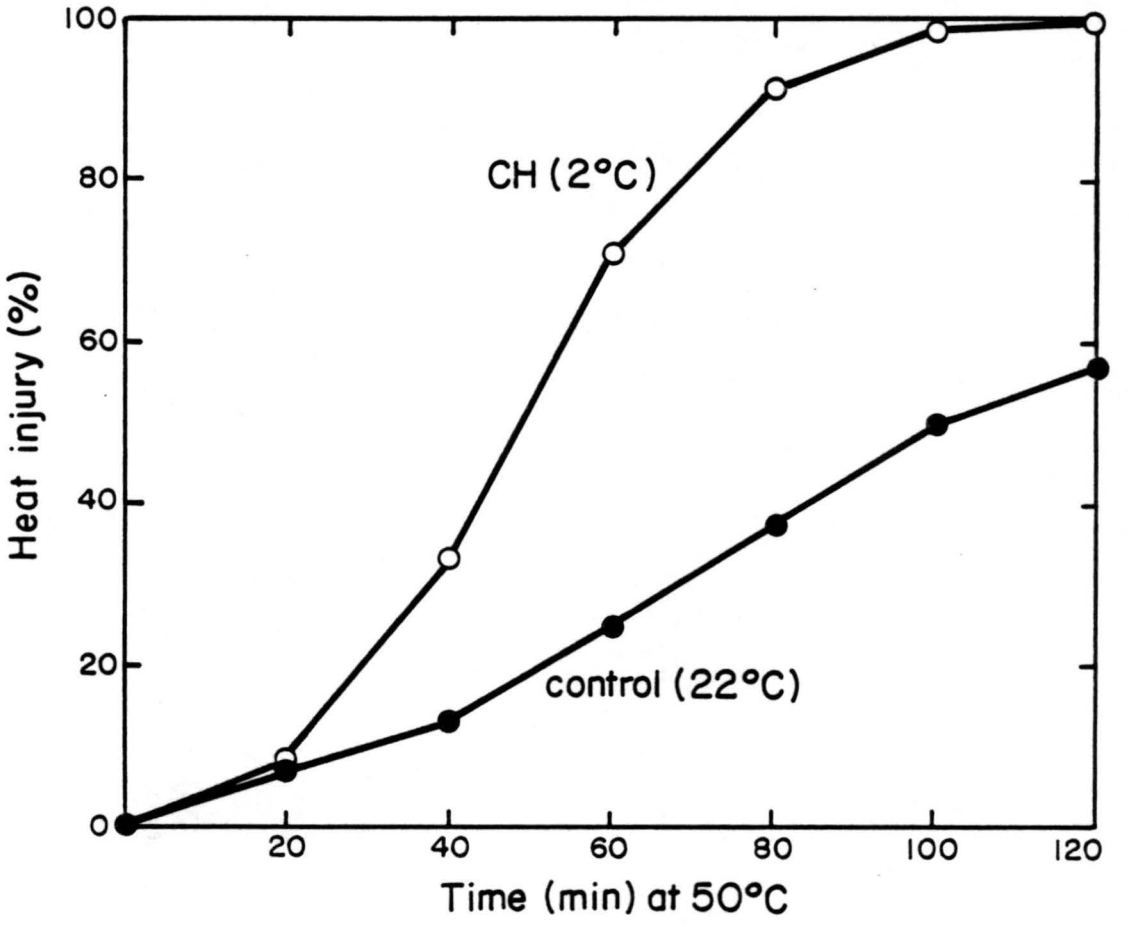

Heat injury (%)
80
60
40
20
CH (2°C)
Control
HH (30°C)
44
46
48
50
52
54
56
Stress temperature (°C)

Figure 22. The heat tolerance of cold acclimated and control pear cells based on electrolyte leakage test. The cold acclimated cells were grown at 22°C for 6 days and then transferred to 2°C for cold hardening for 10 days. Those grown at 22°C for 9 days were the controls. 3- ml pear suspension cells were stressed at 45°C for time indicated on the abscissa.


100
80
60
40
20
0
Heat injury (%)
CH (2°C)
control (22°C)
20
40
60
80
100
120
Time (min) at 50°C

differences evident in Figure 21 and Figure 22 is consistent with the idea that membrane disruption is a prime determinant of the measured heat injury. However, despite probable differences in membrane characteristics, cold- and heat-acclimated pear cells responded to freezing stress in a similar way (Figure 19).

At least one component of both cold and heat acclimation appears to minimize some of the effects of freezing stress. Also, heat acclimation has component(s) which stabilize the cell against both heat and freezing stress. Whether any of these components are the same, i.e. whether cold and heat acclimation have any common elements, is a question which might be answered in future research using suspension-cultured pear cells.

CHAPTER 6

EXTRACELLULAR POLYSACCHARIDES AND FREEZING INJURY IN CULTURED PEAR CELLS

INTRODUCTION

Visual observations made during initial freezing experiments (Chapter 5) along with the work of Tao, et al. (88) led to this preliminary investigation of the role of cell wall polysaccharides in freezing injury and cold hardiness. There are two general ways in which extracellular polysaccharides could influence freezing injury and cold hardiness. These are to: a) cause injury by damaging the membrane during the freeze-thaw cycle (43). In this case, changes during cold acclimation might produce polysaccharides with reduced ability for injurious interaction with the membrane; b) minimize injury by protecting membranes directly (46) or interfering with ice crystal growth (60,61,62,63). The production of protective polysaccharides would be one component of the cold acclimation process.

Tao, et al. (88) recently reported that injury to cultured potato cells was more severe if the membranes were appressed to the cell wall prior to freezing. Their results supported the classical mechanical stress concept of freezing injury first proposed by Iljin (31). Iljin suggested that membrane-cell wall contact/adhesion points produce strain on the membrane, especially during thawing. Contemporary ideas of freezing injury emphasize the role of

injurious solution effects (43), and some authors disregard any role of the cell wall (88). However, it seems clear that both solution effects (e.g. pH, solute concentration, protein precipitation) and mechanical stress may damage membranes.

Cold acclimation is a physiological phenomenon which reduces the impact of injury-causing strains, and thereby increases cell tolerance of extracellular ice and cell dehydration (i.e. freezing stress). Changes in the amount of many protoplasmic constituents such as sugars, sorbitol, starch, soluble proteins and amino acids have been correlated to cold acclimation (27,43), but little is known about the related specific functions of these intracellular components. On the other hand, there has been very little consideration of extraprotoplasmic (e.g. cell wall) changes during cold acclimation.

There have been many reports on the protective effects of externally supplied cryoprotectants (51,55). In plant cells and tissues, cryoprotectants with small molecular weight (e.g. glucose, sucrose, glycerol, DMSO and proline) have been widely studied (43). However, there have been very few studies concerning large molecular weight cryoprotectants. One reason for this may be an assumption that cryoprotection depends upon entry into the cell. However, there is evidence of cryoprotection by macromolecules, including proteins (93) and polysaccharides (71,75). Protection at the cell surface could involve

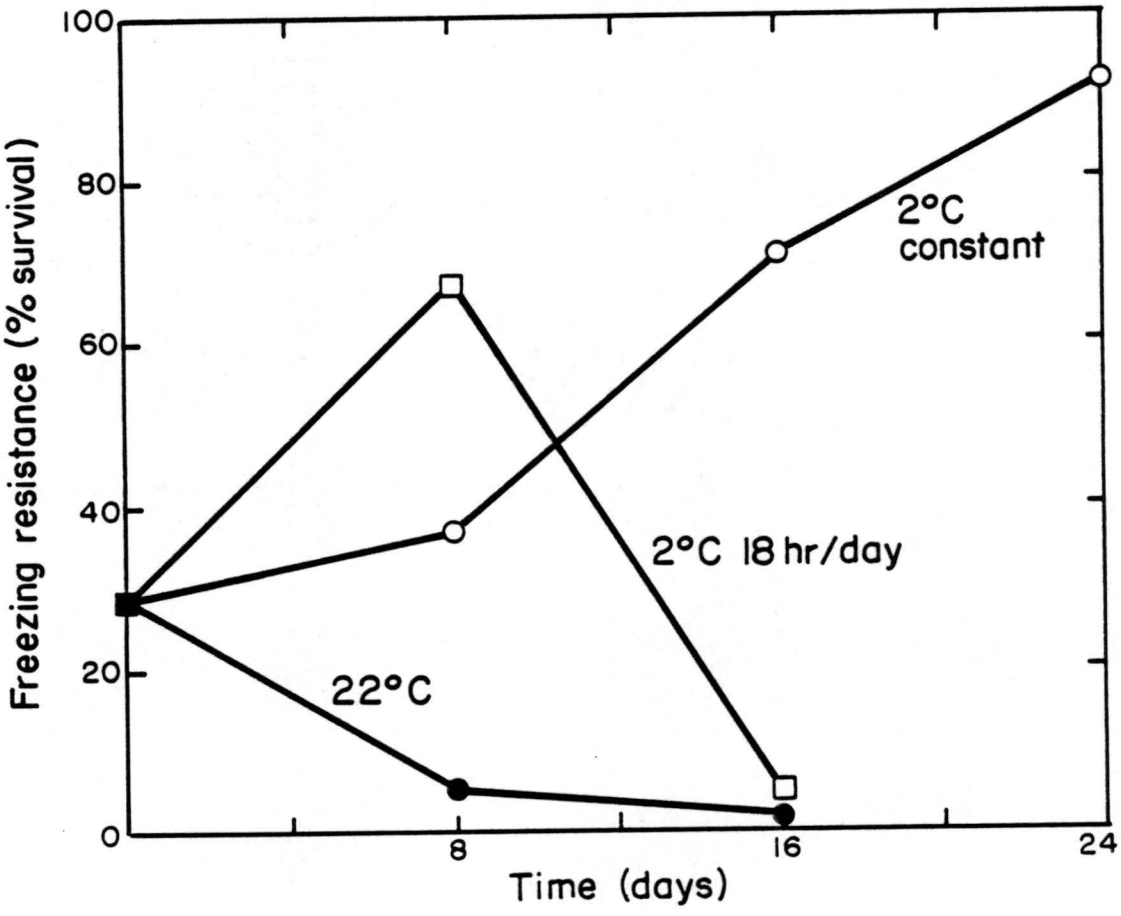
direct interaction at the membrane or interaction with water and ice to reduce the severity of freezing stress (63,75).

The purpose of this study was to conduct preliminary investigations on the effect of: a) extracellular macromolecules on freezing injury to cultured pear cells and, b) cold acclimation on patterns of extracellular polysaccharide production. Cell suspension cultures are especially useful for such studies because extracellular polysaccharides can be easily recovered from the culture medium. Polysaccharides secreted into the liquid medium of suspended cells are often assumed to at least partly represent - in a qualitative way - those present in the cell wall per se (3,94).

MATERIALS AND METHODS

Pear cell suspension cultures, grown and maintained as described in Chapter 2, were used in these experiments. In routine experiments, cold acclimation was induced by placing 7-day old cultures at 2°C. However, seedlings and tissue cultures are often cold acclimated by exposing to different day and night temperatures (e.g. 5°, 18 hr/0°, 6 hr day/night). Therefore, an experiment was conducted to compare the effect of a treatment of 18 hours at 2°C followed by 6 hours at 22°C to a treatment of continuous 2°C exposure. As shown in Figure 23, the constant low temperature treatment was best for producing cold hardy pear cells. Although the 18 hr treatment appeared to initiate

Figure 23. The freezing resistance of cold acclimated and non-acclimated pear cells. Following 7 days' growth at 22°C, cells were acclimated at 2° constantly or for 18 hr/day. Those which were kept at 22°C were the controls. The testing temperature was -6°C and the duration of freezing stress was 30 minutes. TTC reduction test was used to measure freezing resistance. Each point represents 2 cultures; the experiment was repeated with the same results.

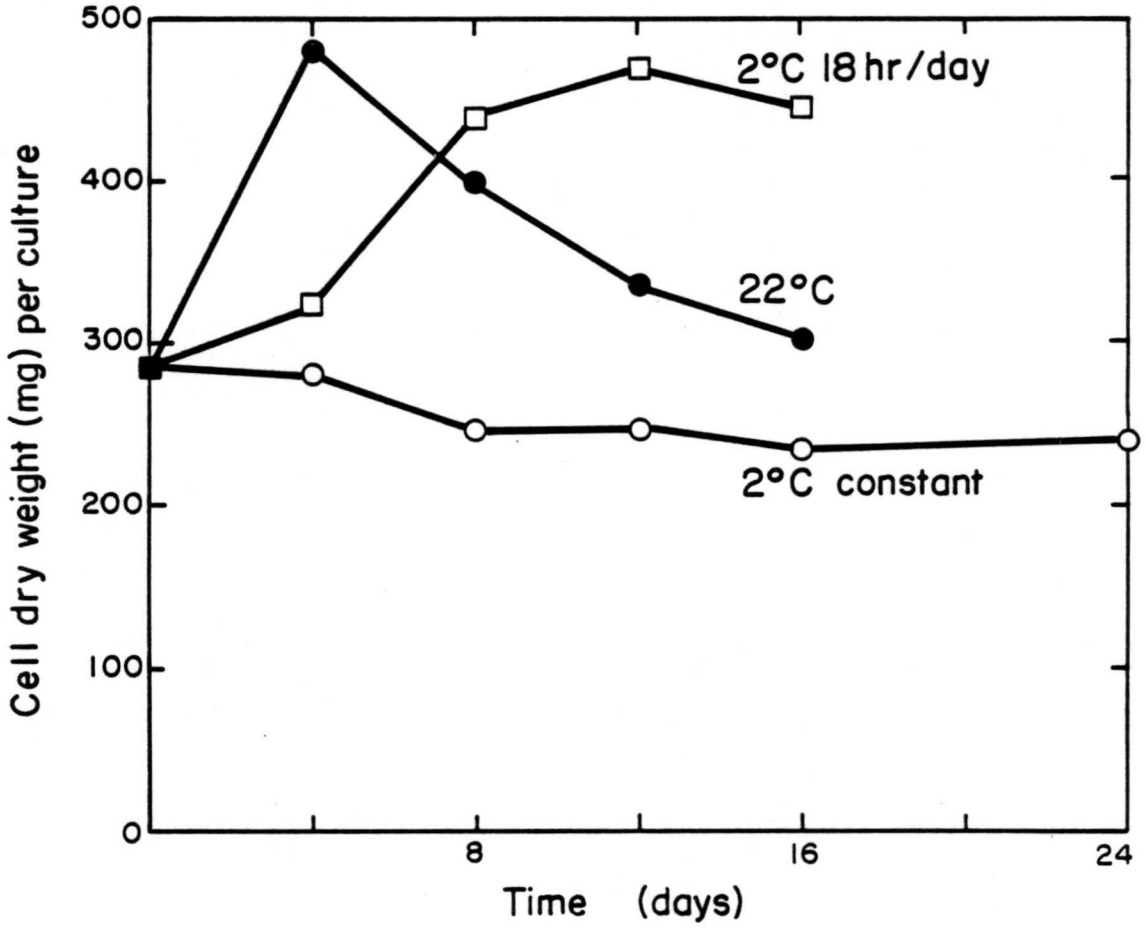

100
80
60
40
20
0
Freezing resistance (% survival)
2°C
constant
2°C 18 hr/day
22°C
8
16
24
Time (days)

acclimation, this condition was rapidly lost (Figure 23), perhaps due to senescence, which might have followed achievement of the stationary phase of culture growth (Figure 24).

To obtain extracellular macromolecules, cells were separated from their medium by filtration through Miracloth and washed with a small volume of distilled water. The culture filtrates were dialyzed against distilled water, using membranes with a 6000-8000 molecular weight cut-off. After dialysis, the macromolecule solutions were concentrated (approximately 5-fold) under reduced pressure at 50^{o}C. The osmolarity of concentrated samples was measured using an Osmette freezing point depression instrument.

The effect of medium macromolecules on freezing injury was determined using 7-day old, non-acclimated pear cells. Cultures were passed through Miracloth and the cells were washed with distilled water. Washed cells were then suspended in equimolar solutions of dialyzed concentrated culture media (from non-acclimated and acclimated cultures) or sucrose. Equimolar solutions were used and sucrose included as a treatment in order to eliminate or minimize the influence of colligative solution effects which are known to contribute to both freezing injury and cryoprotection (43). Freezing stress treatment and measurement of injury (i.e. viability testing) were as described in Chapter 5.

Figure 24. Dry weight changes in pear suspension cultures grown at 22°C, 2° constantly and 2°C for 18 hr/day following initial 7 days growth at 22°C. Each point represents 2 cultures; the experiment was repeated with the same results.


500
400
300
200
100
0
Cell dry weight (mg) per culture
2°C 18 hr/day
22°C
2°C constant
8
16
24
Time (days)

The effect of cold-acclimating treatments on extracellular polysaccharides was determined using colorimetric carbohydrate analyses and gel filtration chromatography. Since plant cells in suspension culture secrete large amounts of polysaccharide during growth, 7-day old culture media contained considerable amounts of polysaccharides and quantitative changes after cold treatment were difficult to detect. Therefore, in these experiments (Figure 25,26), cells were removed from 7-day media, washed, and resuspended in sterile fresh medium. Pear cells in fresh medium were then immediately placed at 2°C for cold acclimation or 22°C. At indicated intervals, culture medium was harvested by filtration through Miracloth and dialyzed to remove sugar and other small solutes. Dialyzed medium preparations were then analyzed for neutral sugar using the anthrone test (81) and for uronic acid using the method of Ahmed and Labavitch (2). Gel filtration chromatography was accomplished using concentrated medium samples and Bio-gel P-100 as described in the legend for Figure 26.

RESULTS AND DISCUSSION

During earlier freezing experiments, observations suggested consideration of extracellular polysaccharide involvement in freeze-thaw effects on pear cells. One of these observations was a pronounced post-thaw clumping of non-acclimated cells. Pear cells in suspension

Figure 25. Changes in neutral sugar (A) and uronic acid (B) in polysaccharides released by pear cells grown at 22°C, 2°C constantly, and 2°C for 18 hr/day. Cells were obtained from 7-day old cultures and washed with distilled water. Washed cells were then resuspended in fresh medium and exposed to the indicated temperature treatments.

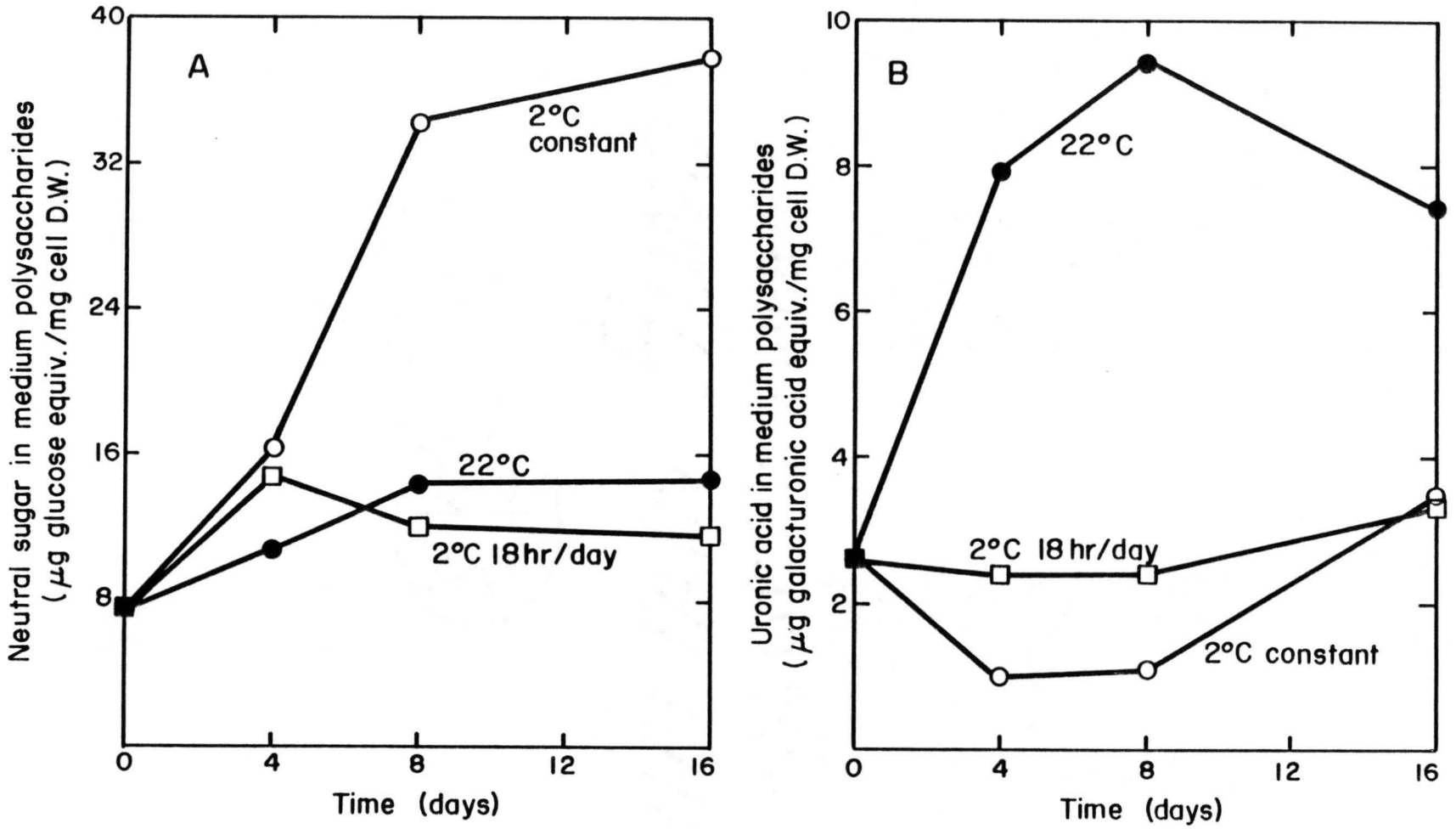
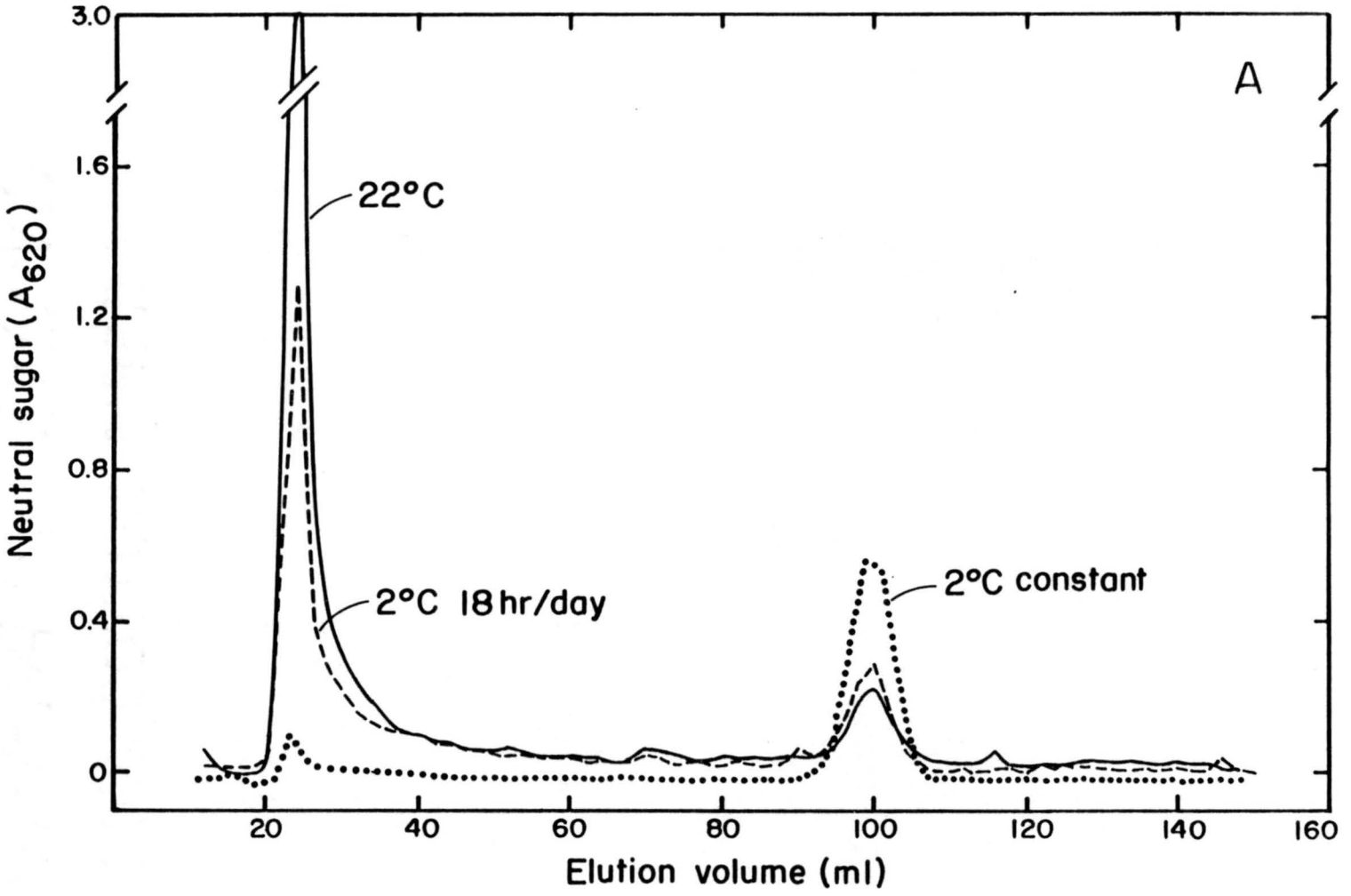
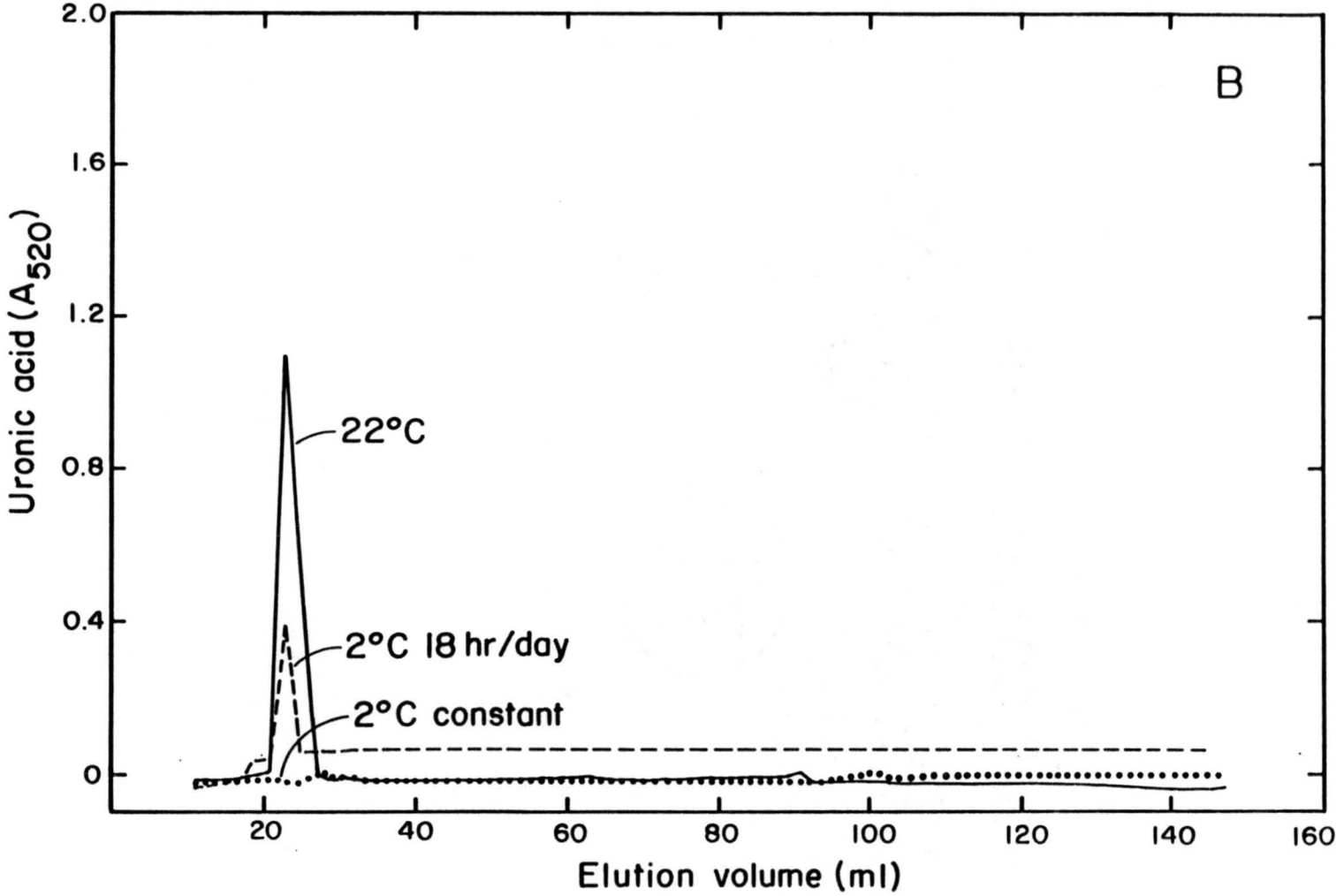

A
Neutral sugar in medium polysaccharides
(μg glucose equiv./mg cell D.W.)
40
32
24
16
8
0
4
8
12
16
Time (days)
2°C
constant
22°C
2°C 18hr/day
B
Uronic acid in medium polysaccharides
(μg galacturonic acid equiv./mg cell D.W.)
10
8
6
4
2
0
4
8
12
16
Time (days)
22°C
2°C 18 hr/day
2°C constant

Figure 26. Gel-filtration pattern of polysaccharides released by pear cells at 22°C, 2°C constant, and 2°C for 18 hr/day. Cells from 7-day old cultures were filtered and washed with distilled water. Washed cells were then resuspended in fresh medium and cold acclimation was induced. Sixteen days after resuspension in fresh medium, cells were removed by filtration and the medium was dialyzed and concentrated. Samples (1.5ml) were passed through a Bio-Gel P-100 column; fractions (1.0 ml) were tested for neutral sugar (A) and uronic acid (B). The void volume of the column was about 20 ml.


A
22°C
2°C 18 hr/day
2°C constant
Neutral sugar (A_{620})
Elution volume (ml)
3.0
1.6
1.2
0.8
0.4
0
20
40
60
80
100
120
140
160


B
Uronic acid (A_{520})
2.0
1.6
1.2
0.8
0.4
0
22°C
2°C 18 hr/day
2°C constant
20
40
60
80
100
120
140
160
Elution volume (ml)

culture normally occur in cell aggregates, but after thawing aggregates were associated in larger cell masses. The cell surface interactions responsible for this clumping did not occur with cold-acclimated cells; freeze-thaw induced clumping was not observed with cold-acclimated cells.

The clumping noted above was probably cell wall-mediated and may be related to a second observation. The second observation was freeze-thaw induced gelation in solutions of medium macromolecules from non-acclimated cultures. Component(s) of non-acclimated culture medium preparations (dialyzed as described above) underwent irreversible gel-forming aggregation during freezing. Similar aggregation has been described for various polysaccharides (21) and is due to the freeze-reduction in solution volume which brings potential aggregating molecules close enough to interact. Macromolecules from the medium of cold-acclimated pear cultures were never observed to form gels as a result of freezing.

The freeze-induced molecular interactions involving cell surfaces and extracellular macromolecules are of interest here because they may be one source of Iljin's (31) mechanical stress injury. Aggregation involving cell wall and/or plasmalemma components could produce injurious strain. As noted above, Tao, et al. (88) found that freezing injury was lessened if the cell wall and membrane were not in physical contact when cultured potato cells were frozen.

The possibility that extracellular polysaccharides may contribute to freezing injury was tested by exposing washed pear cells to medium macromolecules during freezing. Cells for these experiments were from 7-day old, non-acclimated cultures. Numerous experiments were conducted, and the data of Table 3 are representative of these. The main result was that more freezing injury was observed when pear cells were frozen in solutions of macromolecules from non-acclimated cultures than in those from cold-acclimated cultures. This can be interpreted as evidence for an injurious effect of macromolecules from non-acclimated cultures. Analyses indicated that the medium preparations contained both polysaccharides and proteins (data not shown); no effort was made to distinguish the specific contribution of either to the observed effect. However, polysaccharides were present in greater amount and comprise most of the plant cell wall.

The differences (visual observations and Table 3) between culture media of acclimated and non-acclimated pear cells prompted comparative analyses of polysaccharide components. The most striking effect of the cold-acclimating treatments (i.e. low temperature) was to inhibit release of large molecular weight pectic polysaccharides and to increase that of a relatively small neutral polysaccharide. At $2^{\circ}C$, pear cells released much more neutral sugar (on a cell dry weight basis) into the culture medium than they did at $22^{\circ}C$ (Figure 25A). After 8 days, this difference was more than two-fold. When the $2^{\circ}C$

Table 3. Effect of medium macromolecules secreted by non-acclimated and cold-acclimated pear cells on freezing injury. Media from 7-day old non-acclimated cultures and cultures exposed to 2°C for 8 days were dialyzed and concentrated to about 5-fold original concentration. Test cells (from 7-day old, non-acclimated cultures) were washed with distilled water and suspended in equimolar (8.3 mosm) solutions of sucrose and medium macromolecules. Freezing and viability testing were as described in Chapter 5.

Treatment	Freezing (-6°C) injury (%; TTC reduction test)
Sucrose	59.4
Non-acclimated medium	82.8
Acclimated medium	61.6

exposure was interrupted for 6 hr/day (i.e. 2°C for 18 hr/day), the effect on neutral sugar content of extracellular polysaccharides was similar to the control (22°) treatment. In contrast to the effect on neutral sugars, 2°C inhibited the release of uronic acid-containing polymers, i.e. pectic polysaccharides (Figure 25B). Exposure to 2°C for 18hr/day inhibited pectic polysaccharide release nearly as much as did the constant 2°C treatment. The effects of constant 2°C (Figure 25A,B) were similar to that of chilling (0°C storage) on cell wall composition in cucumber fruits (23). Cell wall composition was not determined in this study, but soluble extracellular polysaccharides are representative of cultured plant cell walls (3,94).

Essentially all extracellular uronic acid occurred in polysaccharides which exceeded 100,000 molecular weight (Figure 26B). The large molecular weight fraction also contained large amounts of neutral sugar (Figure 26A). Initial efforts to study this fraction by filtration through agarose A 0.5 M (Bio-Rad, separation range up to 500,000 molecular weight) indicated a range of molecular weights (results not shown). The release of a discrete, relatively small (approximately 15,000 dalton) polysaccharide (Figure 26A) accounted for most of the increased neutral sugar (Figure 25A) released by pear cells at 2°C. It contained little or no uronic acid and was the

predominant polysaccharide in the medium of cells grown at $2^{o}C$.

The gelling characteristics of pectins are well known, and medium pectic polysaccharides probably account for the freeze-induced gel formation described earlier. The medium from $2^{o}C$ cultures contained essentially no pectic polysaccharides (Figure 26B) and did not gel during freezing. The large molecular weight polysaccharides may also have been responsible for the clumping of non-acclimated cells via freeze-induced cell surface cross-linking.

These interpretations are speculative and relationships to freezing injury and cold hardiness are not clear. However, the following summary points permit hypothetical discussion of ideas to be examined in subsequent research:

1. There is evidence that extracellular polysaccharides reflect part of the structure of cultured plant cell walls (3,94).
2. Polysaccharides from non-acclimated cultures aggregate irreversibly during freezing; cold acclimation (Figure 23) produces changes (Figure 25,26) which apparently prevent this aggregation.
3. Polysaccharides from non-acclimated cultures increase the freezing injury of pear cells (Table 3).
4. Minimizing contact between the cell wall and membranes reduces freezing injury (88).

The hypothesis based on these observations is that freeze-induced aggregation of cell wall polysaccharides is one component of cellular freezing injury. There is considerable evidence that similar aggregation of proteins is one element of freezing injury (43). Irreversible aggregation of polysaccharides within the wall or between the cell wall and membrane components may damage the membrane, especially as the cell re-expands during thawing. In other words, polysaccharide aggregation may be the cause of the mechanical injury proposed by Iljin many years ago (31).

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APPENDIX

Figure 27. TTC reduction and electrolyte leakage - heat injury during exposure to 45, 48, and 50°C. The methods for these 2 tests are described in Chapter 2.

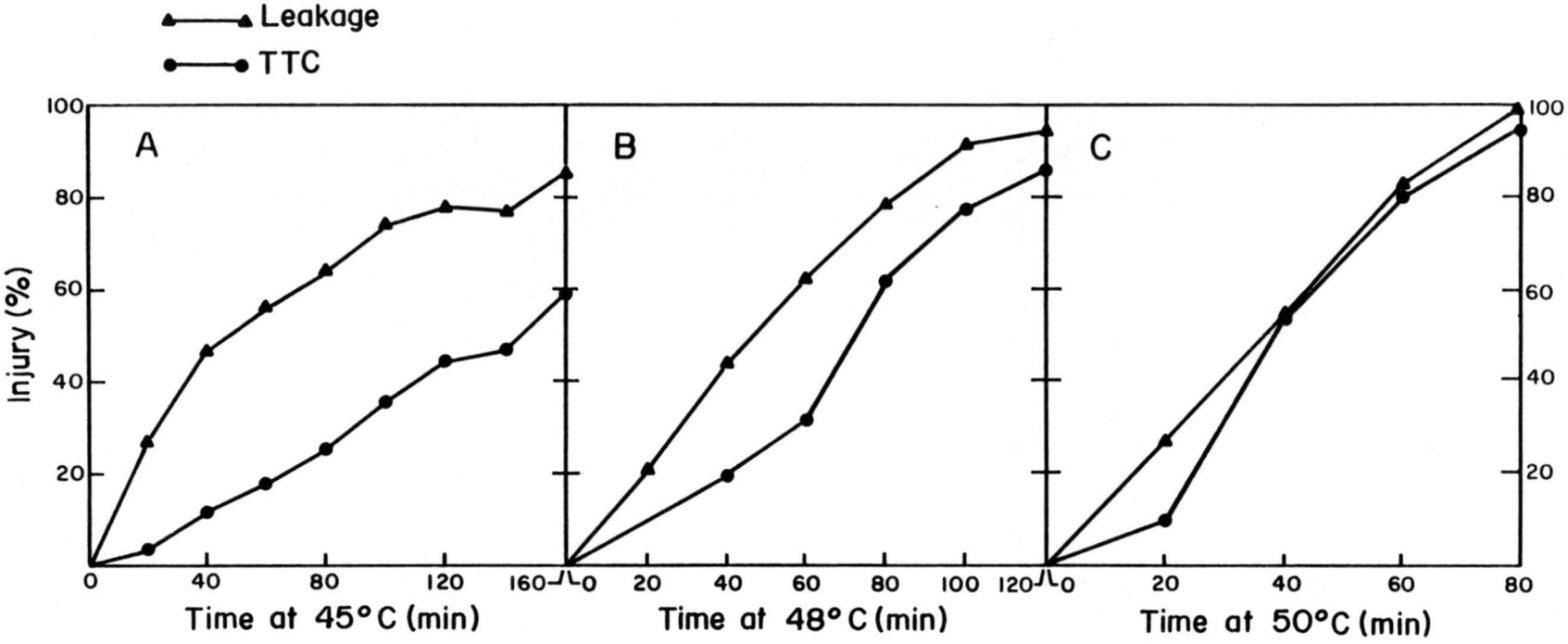

Leakage
TTC
A
B
C
Injury (%)
100
80
60
40
20
0
40
80
120
160
0
20
40
60
80
100
120
0
20
40
60
80
Time at 45°C (min)
Time at 48°C (min)
Time at 50°C (min)

Figure 28. Mitotic activity of pear cells grown at 30°C and 22°C during a 12-day culture cycle. Mitotic figures were visualized with aceto-orcein stain.

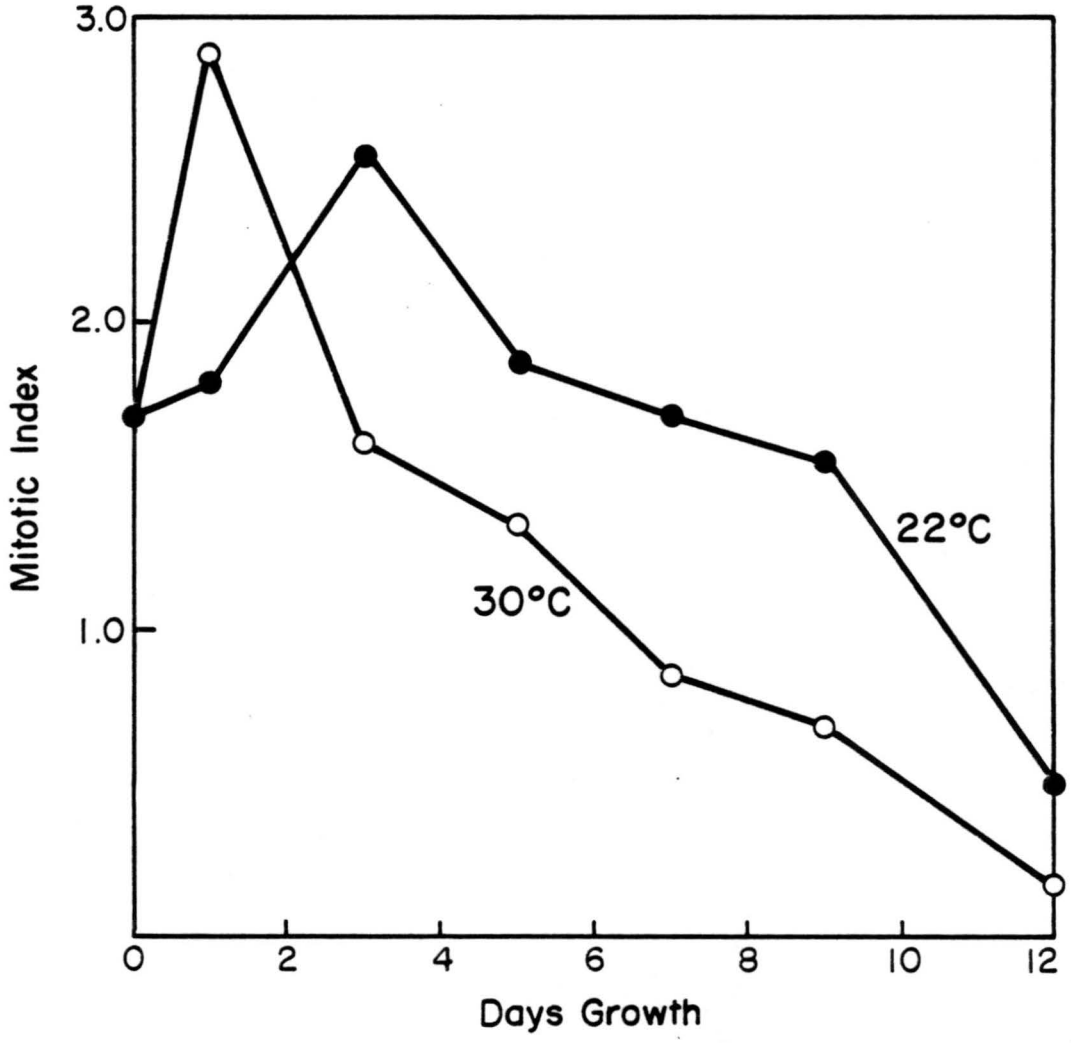
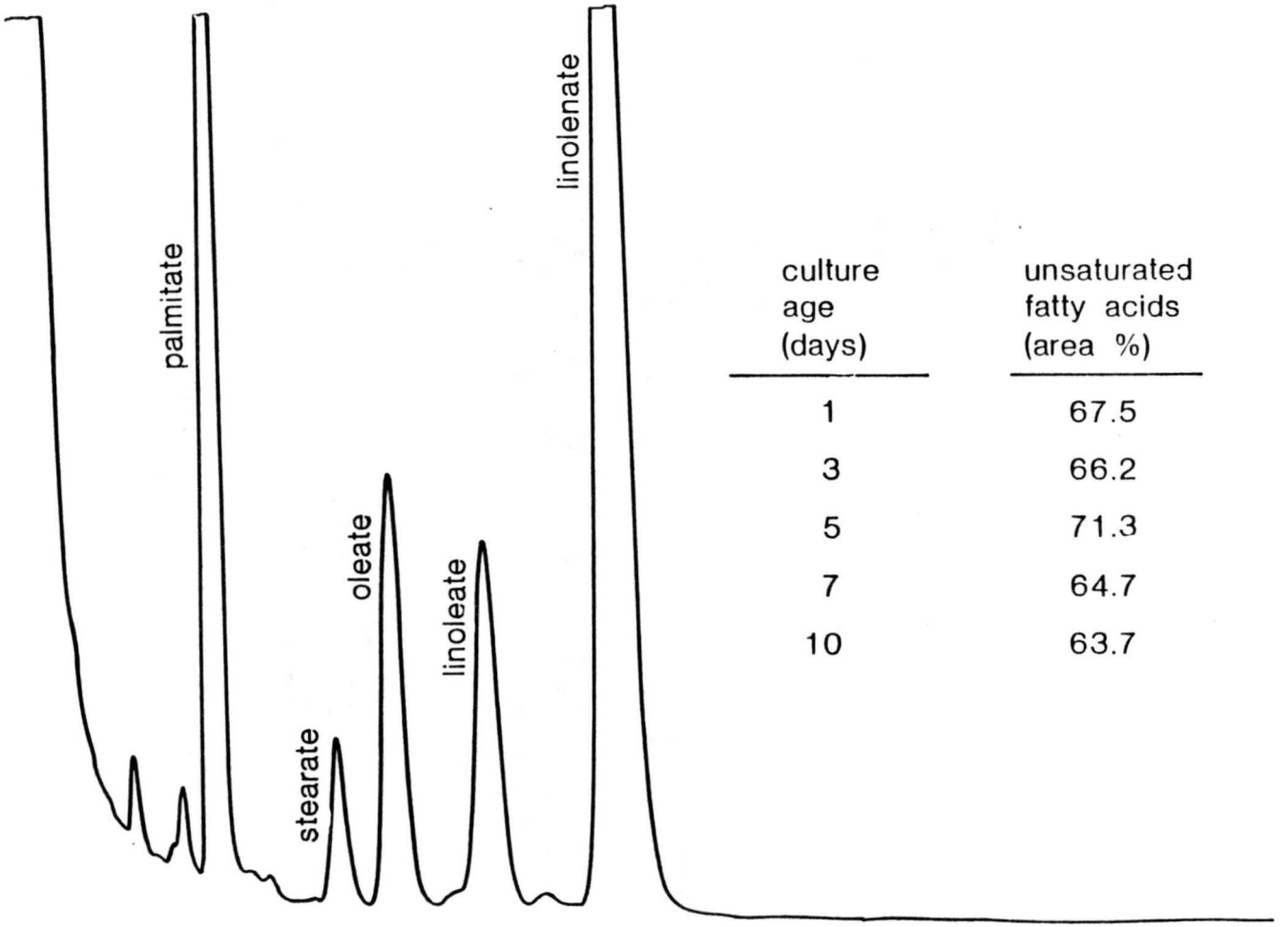
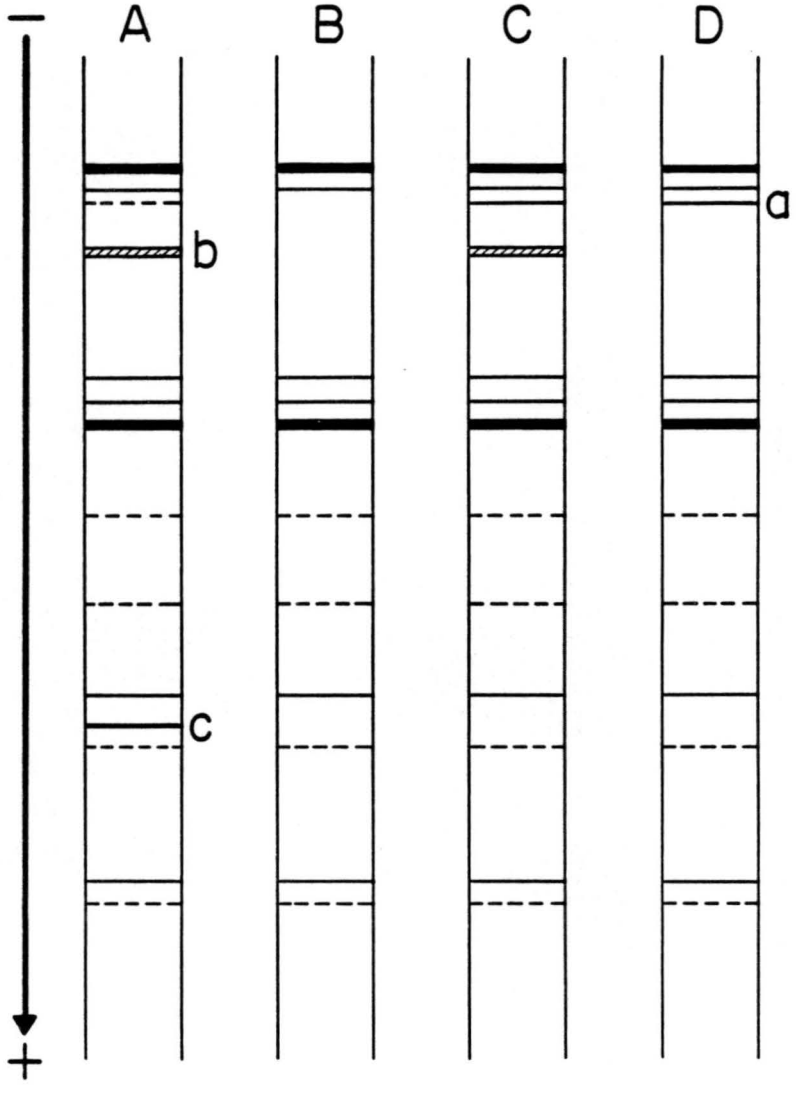

3.0
2.0
1.0
Mitotic Index
22°C
30°C
0
2
4
6
8
10
12
Days Growth

Figure 29. Gas chromatograph trace of methyl esterifed fatty acids from pear cells (8-day old suspension culture) and the relative proportion of unsaturated fatty acids in cells from cultures of various ages. Relative amounts were estimated by summing the peak areas for saturated and unsaturated fatty acids. Lipid extraction and determation of fatty acid composition were accomplished using the methods of Bligh and Dyer (12). For each sample, 3 to 5 g fresh weight of pear cells were extracted. Fatty acid methyl esters were separated and relative amounts determined using a Hewlett-Packard 5840A gas chromatograph. The column (2.4 m stainless steel) was packed with 2.7 g of 10% SP-2330 cyanosilicone on 100/120 chromsorb WAW (Supelco, Inc., Bellefonte, PA). The oven temperature was 200°C, the injector and detector were at 225°C, andthe nitrogen carrier flow rate was 20 ml min^{-1}. The percent of total fatty acid peak areas represented by oleate, linoleate, and linolenate was used to estimate fatty acid unsaturation.



culture age (days)	unsaturated fatty acids (area %)
1	67.5
3	66.2
5	71.3
7	64.7
10	63.7

Figure 30. The diagrammatic representation of heat shock protein synthesis in (A) heat shocked 22°C-grown (B) 22°C-grown (C) heat shocked 30°C-grown (D) 30°C-grown pear suspension cells. 7 day old cells were heat shocked at 38°C for 20 min, and then incubated at 22°C (A) or 30°C (C) for 24 hours. 30 mg of lyophilized cells were placed in 1 ml of 0.35 M KH_2PO_4 + 3% PEG + 3% PVP-40, pH 7.2 buffer at 4°C for 12-16 hours. These were centrifuged at 40,000 g at 4°C for 20 min. The sample size was adjusted to deliver 10 µg of protein per sample well. Protein content of the sample was determined by the method of Bradford (13). The proteins were separated in a pH 8.9, 7% acrylamide gel. The gel and buffer system were those of Ornstein and Davis (64). The electrophoresis was carried out using a Hoefer SE-500 vertical slab gel electrophoresis unit (Hoefer Scientific Instruments Inc., San Francisco, CA) connected to a Hoefer PS 500 power supply at a constant current of 40 mA. The gel was fixed with 12% tricloroacetic acid (TCA) and stained with Comassie blue G-250 0.25% w/v in 7% acetic acid.


−
A
B
C
D
a
b
c
+