Research Article

Understanding Heat Stress Tolerance of Suspended Cells in the Model Plant *Populus euphratica*

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A comprehensive understanding of the physiological responses of plants to extreme temperatures is essential for future strategies for plant improvement. Obvious advantages can result from the study of highly adapted plant species, such as the model tree *Populus euphratica* Olivier that naturally thrives under extreme temperatures, saline soils, and drought. The present paper addresses the issue of *P. euphratica* thermotolerance using a cell suspension model system. *P. euphratica* suspended cells were subjected to a range of temperatures (from 5 up to 75°C) for 20 min, and cultures were evaluated for cell viability and biomass content at specific time points. The results have shown that cell viability was only affected after a temperature stress higher than 40°C, although in these conditions it was observed that a cell growth increases after the recovery period. In contrast, a total decline in cell viability was observed in suspended cells treated at 50°C or higher temperatures, which did not show growth recovery capacity. Therefore, the known natural tolerance of *P. euphratica* to thermal stress was not observable at the cellular level. The greater susceptibility to high temperatures in suspended cells as compared to field plants suggests that high thermotolerance can only be achieved when cells are integrated into a tissue.

1. Introduction

Plants often grow under unfavorable conditions that extensively alter their development and productivity. One such environmental challenge is exposure to adverse temperatures, which can significantly affect many essential metabolic processes and disrupt an extensive range of cellular components. Heat stress can vary in severity, depending upon the intensity and extent of the stress as well as the rate of temperature variation. As sessile organisms, plants have developed several metabolic responses that minimize injuries caused by the constant exposure of plants to daily temperature fluctuations and their association with other abiotic factors [1].

The deeper knowledge on plant abiotic stress resistance has been fundamental in the development of effective engineering strategies leading to enhanced stress tolerance. Plant

transformation with genes conferring thermal tolerance has been successfully achieved [2, 3]. However, many molecular mechanisms involved in thermotolerance are likely still unknown. A successful strategy for the assignment of gene function has been the study of species that are naturally adapted to survive in extreme environments. The advantages of using members of the poplar genus (*Populus*) as genomic models for tree molecular biology have been extensively reported [4, 5]. Among *Populus* species, *P. euphratica* Olivier shows a remarkable survival capability, presenting high growth yields when facing high soil salinity, extreme temperatures (−45°C to +54°C), and drought [6]. Such conditions are typically found in semiarid areas over a longitudinal extent that ranges from China to Spain and from south Kenya to Kazakhstan, where *P. euphratica* is naturally distributed [7]. However, despite being known as a plant tolerant to extreme temperatures in its natural habitat,

the biological mechanisms behind these observations remain largely unknown.

The level of tolerance that a given plant displays depends on the species, tissue, or cell type considered [8]. In multicellular organisms, the elucidation of cell-level thermotolerance mechanisms can be as difficult as it is impractical. The utilization of *in vitro* cell systems has long been a useful strategy to reveal cellular responses that would otherwise be hidden at the whole-plant level. Indeed, several specific features highlight the attractiveness of *in vitro* cultured cells as a system to study stressed-cell responses. Cell cultures rapidly generate a great amount of cell material with reduced cellular complexity. More importantly, studies carried out in this kind of tissue culture allow to completely control the homogeneity of cell response and environmental parameters. Cell cultures also allow a number of quantitative studies in which compounds can be simply added or removed from the medium and cell aliquots can be easily harvested. Moreover, cells in culture are relatively easy to assess, and a large amount of data can be easily obtained for each treatment [9]. A number of protocols to establish stable cell suspensions for studying plant heat stress responses have been reported from several plant species. Therefore, it is well established that cell suspension cultures could be beneficial when studying thermotolerance mechanisms among forest species. The particular use of *P. euphratica* suspension cultures has already been reported in saline and osmotic tolerance studies [10, 11]. However, to our knowledge, no studies concerning thermotolerance in this system have yet been reported. The aim of the present work was to evaluate the heat stress tolerance capacity of *P. euphratica* suspension cell cultures. Suspended cells were subjected to a range of temperatures, and cultures were characterized for cell viability and biomass content at regular time points in the growth cycle.

2. Materials and Methods

2.1. Establishment and Maintenance of *Populus euphratica* Suspension Cell Cultures. *P. euphratica* Olivier calli were kindly provided by Gu et al. [12] and used for the induction of suspension cell cultures. Cell cultures were initiated by transferring 1 to 2 g of 4-week-old callus tissue to 70 mL of Murashige and Skoog (MS) medium [13] supplemented with 0.5 mg/l 1-naphthaleneacetic acid (NAA), 0.25 mg/l benzyladenine (BA), and 2.5% (w/v) sucrose, at pH 5.5. Cultures were maintained in 250 mL flasks at 25°C with continuous shaking (125 rpm) in the dark. Cell-suspension subculturing was performed every 12 days by transferring 10 mL of cell culture into 60 mL of fresh medium. A sustainable culture was obtained after four subculture cycles, when a fine suspension of cells was observed.

2.2. Characterization of Cell Growth. Cell growth was monitored by the estimation of cell suspension dry weight. Periodically, 3 mL aliquots of suspended cells were harvested and filtered using a piece of preweighted filter paper (Whatman). Dry weight was determined after oven drying at 60°C for 24 h. This procedure was repeated every 2 days over a total

period of 16 days. Biomass values were transformed into their natural logarithms for calculations of specific growth rate (μ) [14].

2.3. Imposition of Heat Stress. To determine the temperature that results in cell death, the response of suspended *P. euphratica* cells to temperature stress was evaluated by subjecting suspension cultures to a range of temperature treatments (from 5 up to 75°C). Flasks containing mid-log phase cell suspensions (day 6 of growth) were immersed into a water bath at one of several different temperatures (5, 15, 25, 35, 40, 45, 50, 55, 65, and 75°C) for 20 min, with continuous shaking. After temperature stress, the cells were transferred into another water bath at 25°C for 10 min to reestablish the typical growth temperature. Suspension cultures were then returned to the previous incubation conditions (25°C, with 125 rpm continuous shaking, in the dark). At regular intervals, cell aliquots were collected for quantification of cell viability and dry weight.

2.4. Determination of Cell Viability. Analysis of cell viability was performed before the imposition of the 20 min temperature stress, after recovery at 25°C for 10 min and at regular intervals. The viability of suspended cells was measured using the trypan blue exclusion method [15]. Aliquots containing 150 μ L of cell culture were gently mixed with an identical volume of 0.4% (w/v) trypan blue (Sigma-Aldrich) and incubated in the dark for 10 min. A 50 μ L sample was observed under a Leica ATC 2000 light microscope, and the number of viable (unstained) and dead (stained) cells was subsequently determined. Cell viability was calculated as the percentage of viable cells out of the total number of cells observed. Results are represented as mean values of twenty independent cell counts (>50 cells per count). Whenever cell death was observed, viability values were converted into their natural logarithm for specific death rate determination.

3. Results

Fine, stable cell suspension cultures with small cell aggregates and single cells are required for controlling the uniformity of environmental and physiological parameters. A cell suspension culture was established from *P. euphratica* callus tissue, which was subcultured every 12 days in fresh MS medium. The suspended cells grew vigorously with each subculture, maintaining small clusters made up of a few cells. After a short adaptation period during the first two days of subculture, cell suspensions started an exponential growth phase (specific growth rate of 0.21/day) that lasted for 8 more days. The biomass continued to increase until day 12, after which the growth rate leveled off, and cell degeneration and necrosis became apparent. These results indicate that cell suspensions should be subcultured at intervals no longer than 12 days for optimal growth. For subsequent thermotolerance studies, in which uniformity in the physiological state of the cells is required, cells in mid-exponential phase (day 6) were used.

The response of suspended *P. euphratica* cells to the imposition of a temperature stress was evaluated by treating the

FIGURE 1: *P. euphratica* cell viability after 20 min exposure to different temperatures (5 to 75°C). Cell viability was determined by the trypan blue exclusion method immediately before heat treatment (●), after recovery at 25°C for 10 min (○) and for 20 h (◆). Results represented mean values of twenty independent cell counts (>50 cells per count).

suspension cultures to a range of temperatures comprised between 5 and 75°C. The analysis of cell viability corresponding to the two initial time points (before the imposition of the 20 min temperature stress and after recovery at 25°C for 10 min) revealed that, during this period, cell viability was essentially unaffected for temperatures ranging from 5 up to 45°C (Figure 1). Incubation at 50°C resulted in slightly decreased cell viability (89%), declining thereafter with increased temperature of the heat shock and reaching values near 25% for the highest temperature tested (75°C). A null cell viability value was not achieved, which was probably due to the natural aggregation of plant cells, resulting in an inaccurate trypan blue viability measurement. When cell viability was evaluated 20 h after heat treatment, different levels of cell viability were obtained. A decrease in cell viability was observed for the 40°C heat treatment. Also, for heat treatments at higher temperatures, cell viability after 20 h of recovery was lower than after 10 min of recovery. Altogether, these results suggest that heat treatment not only has an immediate effect on cells but also produced a long-term effect on cell viability. While the percentage of cell viability obtained immediately after heat treatment reflects the instant effect on cells, provoked by membrane damage or necrosis that instantly destroy the cells, the results obtained after 20 h reflect the activation of cell-defence mechanisms against temperature, leading or not to cell recovery, and therefore are seen as long-term effects.

Cell viability was further analysed upon stress treatment and during recovery at 25°C as a function of time over a maximum period of 80 h (Figure 2). Cell viability was significantly affected after heating at 55°C. Although cell viability was still as high as 72% after heat shock (0.5 h), it rapidly declined to negligible levels (~16%) after 10 h at recovery temperature (25°C). A more moderate cell death induction was observed after treatment at 50°C. Cell viability after heat shock (0.5 h) remained high (82%) but

TABLE 1: Specific death rates of *P. euphratica* cell suspensions after incubation for 20 min at different temperatures (25 to 75°C) followed by recovery at 25°C.

Temperature	Specific death rate (/h)
25°C	0
40°C	0.007
45°C	0.012
50°C	0.026
55°C	0.179
65°C	0.543
75°C	0.669

FIGURE 2: *P. euphratica* suspended cells viability after heat shock for 20 min at 25°C (◆), 40°C (□), 45°C (●), 50°C (■), and 55°C (○) and subsequent recovery at 25°C, for up to 80 h. Cell viability was determined by the trypan blue exclusion method. Results represented mean values of twenty independent cell counts (>50 cells per count).

progressively declined to very low values (~5%) after 75 h at 25°C. For those heat-treated suspension cultures displaying signs of cell death (from 40 up to 75°C), the specific death rates were determined (Table 1). Results also indicate the cell death induction in 55°C and 50°C heat-treated suspension cultures.

Resumption of cell growth was only observed after treatment at 40°C and 45°C (Figure 2). Immediately after heat shock (0.5 h), cells were not strongly affected, displaying ~90% cell viability. However, an induction of cell death was evident after 45 h at recovery temperature, attaining cell viability values of ~75% and ~60%, respectively. After 45 h and until the end of the experiment (80 h), both cell suspensions were able to recover their growth ability, reaching initial cell viability levels. Interestingly, a 50°C treatment resulted in total cell viability loss (no recovery), despite the significantly slow death rate over a long (80 h) recovery period, which might be indicative of cell fate reprogramming. The ability of cell suspensions to recover was also evident when determining culture growth parameters after treatment at different temperatures (from 25 up to 65°C) (Figure 3). By following the time course of biomass content,

FIGURE 3: Dry weight of *P. euphratica* suspension cell cultures after incubation for 20 min at 25°C (◇), 35°C (○), 45°C (■), 55°C (□), and 65°C (●) and recovery at 25°C, over a total period of 18 days. Heat treatment (HT) was performed in the middle of exponential growth phase (day 5).

it was observed that treatment at 35°C did not affect the exponential growth phase of the suspension cultures. The recovery capacity of cell suspensions given a 45°C treatment was evident. After a period of decline corresponding to the first 45 h after heat treatment, the biomass content showed a progressive increase and reached maximum levels similar to those obtained with control suspensions (25°C) but with a delay. In contrast, in suspension cultures incubated at 55°C and 65°C, cell growth was completely impaired after treatment.

In summary, the incubation of suspended *P. euphratica* cells at 35°C did not affect their viability or growth, presenting a similar behavior as compared to that of control cells (25°C). Suspension cultures only displayed susceptibility at temperatures higher than 40°C. A heat-induced decline in cell viability was observable at 40°C, but it was reversed during the recovery period. Although an extended decrease in viability was found in 45°C treated cells, cell growth was still able to recover. In contrast, suspended cells treated at 50°C or 55°C were not able to recover growth, and a complete decline in cell viability was registered. In more extreme heat treatments (>55°C), growth cessation and accelerated cell death were observed.

4. Discussion

Incubation of suspended *P. euphratica* cells at temperatures above 55°C resulted in growth cessation and accelerated cell death, probably due to cell membrane damage and necrosis. This result is in agreement with previous reports on heat-treated *P. euphratica* leaf discs (50°C and 55°C for 30 min), which have shown membrane damage as evaluated by electrolyte leakage [16]. These authors also suggested that *P. euphratica* plants can tolerate temperatures of 42°C for 54 h without adverse effects on survival. Using *P. euphratica* cell suspensions that were heat shocked for 20 min, the

temperature of 45°C corresponded to the highest sublethal temperature that was tolerated by cells.

Results from other culture systems in nontolerant species exhibited quite similar responses. Although the results cannot be strictly comparable because the heat treatments were applied just for 10 min, in the well-documented *Arabidopsis* and tobacco species, suspension cultures can easily tolerate heat stresses lower than 45°C for 10 and 20 min, respectively [9, 17]. The strongest induction of carrot and *Arabidopsis* cell death occurred at temperatures above 45°C, attaining maximum levels at 55°C [9, 18]. The same results were also registered using suspended tobacco cells that were heat treated for 20 min [17]. In another study performed with tobacco cell suspensions treated for 10 min, a time-dependent effect on cell viability was found after incubation at 55°C [19]. This response was very similar to that observed in *P. euphratica* cultures subjected to temperatures of 50°C for 20 min.

Heat stress has been reported as having a highly detrimental effect on the growth and metabolism of plants in their natural habitats. For *in vitro* *P. euphratica* plants, although no effects in survival were verified after prolonged exposure to temperatures of 42°C, growth arrest was evident which can result from a reduction in the ability to photosynthesize [16]. In the present work, the high natural thermotolerance of the model tree *P. euphratica* (up to 54°C) was not clearly evidenced by viability assays performed in suspended cells. In *P. euphratica* cell suspensions, heat treatments above 40°C for 20 min not only have an immediate effect on cell viability, but also induce subsequent cell death. Similar results have been reported in plant species that normally cannot manage environmental temperatures as high as those tolerated by *P. euphratica* plants *in vivo*. This could occur due to the higher sensitivity of suspended cells in sensing temperature alterations when compared to whole plants growing in the field. In addition, a suspended cell alone or within small cell aggregates would not have the same ability to cope with a heat-stress situation as a cell integrated into a system of multiple interacting cells. It is only when integrated into a tissue that cells would probably achieve the capacity to serve as fundamental units of tissue homeostasis and repair during stressful situations. Therefore, it would be expected that the thermotolerance mechanisms activated by elevated temperatures in nature may be induced by lower temperature levels in cell suspensions.

Other morphological or physiological features not generally considered to be part of the temperature tolerance machinery might also be responsible for the natural heat tolerance of *P. euphratica* plants. It has already been demonstrated that this species' strong capability to tolerate drought is mostly due to the development of effective roots to access deep water tables [20]. Also, the adaptation to high-salinity environments appears to be related to the ability to adapt to higher osmotic stress by keeping cell integrity and effectively controlling ionic toxicity [11]. It should also be noted that *in vivo* plants encounter a combination of several abiotic stresses rather than one individually. In drought-stricken areas, like the natural habitat of *P. euphratica*, a combination of drought and other stresses,

such as heat or salinity, are encountered, and an integrated and unique response is employed by plants as the product of several interconnected responses [21, 22]. Therefore, we cannot exclude the possibility that the temperature tolerance presented by *P. euphratica* in the field, as demonstrated by the absence of related injuries, can be due to the capacity to achieve morphological or physiological modifications and/or overcome related stresses rather than specific thermotolerance mechanisms.

In the future, different approaches should be applied to take advantage of recently developed genomic and molecular tools for *Populus*. Also, the combination of genomic, proteomic and physiological approaches offers new possibilities for the elucidation of the function of the temperature stress machinery in *Populus*.

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