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Selection of *Eucalyptus urophylla* cell lineages for tolerance to hydric and thermal stress

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Abstract
The present study was aimed at the evaluation of the possible relationship between high temperature sand increased tolerance to water deficiency of *Eucalyptus urophylla* cell lineages on their physiological and biochemical aspects. For this purpose, calluses cultures derived from hypocotyls *Eucalyptus urophylla* were maintained in tissue culture in an N7 culture medium with 5 mg L^{-1} Picloram and used to conduct tests. The calluses were treated at different temperatures (25, 30, 35, 40 and 45°C) for 2 h in BOD and then subjected to a selection pressure in N7 medium supplemented with 20% polyethylene glycol (PEG 6000) for 20 days under constant light exposure (42 µmol m^{-2} s^{-1} PAR) and temperature (26±2°C) conditions with a 12 h photoperiod. For evaluate the experiment, proline, total soluble proteins, glucose, polyose and glucuronic acid contents were determined as well as the relative growth rate by measuring dry weight and fresh weight. The results indicated that previous treatment with higher temperatures may increase tolerance to drought stress. Therefore tolerant cell lines to stress conditions can be selected using this methodology.
Key words: *In vitro* selection; Micropropagation; Cross-tolerance.

Introduction

Eucalyptus urophylla is a species which shows excellent productivity, high quality short fibers and high quantities of cellulose pulp production. However, the major limitation for the expansion of this species is the high sensitivity to stresses induced by variations in temperature and limited hydric availability causing productivity losses, occurring directly due to abiotic stress or diseases resulting from the environmental conditions.

The elevation of temperature is normally accompanied by the reduction in the available water content of the plants, which activates specific mechanisms at molecular, cellular and physiological levels for survival during alterations (Barnabás et al. 2008). Therefore, the selection of tolerant genotypes to both stress types resulting from abiotic factors is one of the main objectives within a genetic improvement program. The responses to tolerating effects combined with temperature and limited water supply stresses have barely been studied in comparison to works considering each isolated stress factor (Wahid et al. 2007).

Cross-tolerance is a phenomenon that is based on the existence of a common defense system in avoiding stress and postulates that a particular stress factor can induce tolerance in plants to a subsequent stressor that is different from the first (Rizhsky et al. 2002). Within this context, diverse genes encode various transcription factors which are induced or repressed under stressful conditions (Chen and Zhu 2004). The expression patterns of these transcription factors are highly complex and suggest that tolerance to stress and resistance is controlled to the level of transcription by an extremely intricate network of regulatory genes (Barnabás et al. 2008). Thus, the tolerance acquired for a known stress factor activates specific response patterns which are also involved in the tolerance to the other factor in question.

Tolerance to abiotic stress factors by vegetable cells is obtained through the accumulation of different osmolytes such as sugars (alcoholic sugars), proline and quaternary ammonium compounds (Sung et al. 2003; Sairam and Tyagi 2004; Kavi Kishore et al. 2005; Beck et al. 2007; Wahid et al. 2007). Proline is normally accumulated in large quantities in response to environmental stresses, being directly involved in the reduction of the redox potential of the cell (Kavi Kishore et al. 2005; Wahid and Close 2007). The carbohydrates such as glucose and other sugars such as maltose and sucrose occurs when the plants are subjected to a combination of hydric and thermal stresses, with sugars acting as osmoprotectors to the plants (Rizhsky et al. 2004). In addition, the presence of soluble sugars in the cells contributes to the maintenance of the osmotic potential (Regier et al. 2009).

The accumulation of specific proteins can also occur according to the type of environmental stress, including elevated temperatures, hydric deficiency, saline stresses and even pathogenic infections (Coelho et al. 2010). Among the proteins synthesized in response to thermal stress are those that are destined for the recuperation of damaged proteins, known as chaperones, whose activity is focused towards interacting with proteins to maintain correct conformation, prevent denaturation and conserve important functions in cells subjected to thermal stresses (Boston et al. 1996; Borges and Ramos 2005).

The selection of tolerant lineages can be made *in vivo* utilizing different explants to commence cultures (Mohamed et al. 2000; Haldrup et al. 2001; Taregyan et al. 2001). Calluses had been used in the majority of studies involving *in vitro* selection (Bhojwani and Razdan 1996). The use of this technology permitted the selection of millions of cells in a very small space with little effort and resources, as the characters selected at a cellular level can be re-checked in the regenerated plant (Bhojwani and Razdan 1996).

The limitation to the expansion of commercial *Eucalyptus* sp. plants has often been linked to the high sensitivity to hydric and thermal stresses, causing productivity losses directly due to the abiotic stress suffered

or the diseases resulting from the environmental condition. Therefore, the early monitoring of these changes occurring in plant tissues can allow for the identification and understanding of the hydric and thermal stress control mechanism and can thus lead to the selection of tolerant species. Based on this, treatments utilizing high temperatures can increase the tolerance to hydric deficiency in *Eucalyptus urophylla* calluses.

This study therefore aimed to evaluate the possible relationship between increased temperature and increased tolerance to hydric deficiency as well as identifying and selecting *Eucalyptus urophylla* cell lines for their physiological and biochemical aspects.

Materials and methods

This study was conducted at the Tree Physiology Laboratory of the Forest Sciences Department of the Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (USP). *Eucalyptus urophylla* seeds were used, provided by the Forest Studies and Research Institute (IPEF) and identified as T10B71, lot AN578, collected from a base population at Anhembi Forest Garden, São Paulo State.

Disinfestation, inoculation and obtaining of explants

For the pre-cleaning disinfestation, the seeds were washed in running water and neutral detergent. Next, the seeds were disinfected through immersion and agitation in a manipulated sodium hypochlorite solution (3% active chlorine) containing two drops of Tween-20 for each 100 mL solution for 30 min. Next, within a laminar flow cabinet, the seeds were washed three times with autoclaved deionized water and then transferred to sterilized flasks containing 40 mL N7 medium (Simola 1985) for germination. Thirty seeds were inoculated per flask, with these remaining under consistent light (42 µmol m^{-2} s^{-1} PAR) and temperature (26±2°C) conditions, with a 12 h photoperiod. After 4 days the seeds germinated, and after 15 days the plant hypocotyls were excised and transferred to flasks containing N7 culture medium supplemented with 5 mg L^{-1} Picloram for callus induction.

Selection of lineages

At 120 days following the initiation of callogenesis (approximately 6 subcultures), the experimental base was formed and composed of 175 flasks with four calluses in each, resulting in a yield of 1,800 mg at the end of each subculture.

For this experiment, plant material was transferred to flasks containing 40 mL liquid N7 culture medium supplemented with 5 mg L^{-1} Picloram. For the hydric deficiency treatment, 200 mg L^{-1} polyethylene glycol (PEG 6000) was added to the medium as described above.

To favor the oxygenation of plants, all flasks utilized presented an acrylic blanket which supported the plant material and impeded the immersion of this in the culture medium. Following the transference to the liquid medium, 16 flasks per treatment were subjected to different temperatures (Table 1) in a B.O.D. type incubation chamber for 2 h and cultivated in a growth room for 20 days under consistent light (42 µmol m^{-2} s^{-1} PAR) and temperature (26±2°C) conditions, with a 12 h photoperiod.

Following the stipulated period, all treatments were evaluated for the relative growth rate and the levels of proline, total soluble proteins and non-structural soluble carbohydrates (glucose, polyose and glucuronic acid).

The experiment was conducted in a completely randomized design (2 x 2 factorial), with the factors consisting of two analytical steps (initial and final) and six different temperature treatments. For each treatment, four replications were evaluated with four calluses for each previously described variable (Table 1). The data obtained were evaluated comparing means by Analysis of Variance (ANOVA) and Least Significant Difference (LSD) using the Statistical Package for the Social Sciences (SPSS) program.

Table 1. Description of treatments made for the selection of *Eucalyptus urophylla* cell lineages for tolerance to thermal and hydric stresses.

Treatment	Description
T1	Calluses subjected to 25ºC for 2 h and transferred to N7 culture medium.
T2	Calluses subjected to 25ºC for 2 h and transferred to N7 culture medium with 20% PEG (6000).
T3	Calluses subjected to 30ºC for 2 h and transferred to N7 culture medium with 20% PEG (6000).
T4	Calluses subjected to 35ºC for 2 h and transferred to N7 culture medium with 20% PEG (6000).
T5	Calluses subjected to 40ºC for 2 h and transferred to N7 culture medium with 20% PEG (6000).
T6	Calluses subjected to 45ºC for 2 h and transferred to N7 culture medium with 20% PEG (6000).

Growth rate determination

Following the selection period, four flasks were randomly selected for the determination of fresh and dry weights. For this, the calluses were placed in paper towel leaves for the culture medium remnants to be absorbed, and then weighed on an analytical balance. Following this, the material was subjected to drying in a greenhouse at a temperature of 60°C for a period of 48 h or until a constant mass was reached, then weighed on an analytical balance. After this data were obtained, the relative growth rate (RGR) was obtained through the following equation:

$$RGR=\frac{\ln M_{DM}(X_n)-M_{DV}(X_0)\ x\ 100}{T\ (Xn)}$$

Where: RGD = relative growth rate. M_{DM} = Mean mass of dry matter taken at the end of each sample. M_{DV} = Mean dry vegetable mass taken at the commencement of the experiment. T = Cultivation time (20 days).

Quantification of proline level

Proline was determined by the colorimetric method described by Bates et al. (1973), where the extraction of this amino acid resulted from the maceration of 300-500 mg fresh vegetable material in 10 mL of 3% sulfosalicylic acid. Following this, the extract was filtered to obtain a liquid which served as a raw material for the analysis. Next, 1 mL of the extract obtained was added to a test tube with 1 mL glacial acetic acid and 1 mL acid ninhydrin solution, maintained in a water bath for 1 h. After this time, the tubes were subjected to an ice bath. The solution was read on a spectrophotometer at an absorbance of 520 nm with four readings made per replication of each evaluated treatment.

Quantification of soluble proteins

Callus samples (300-500 g) were macerated in an extract solution containing 4 mL Tris-HCl buffer (pH 6.8), 1.6 mL 2-mercaptoethanol, 6.4 mL of sodium dodecyl sulphate (SDS) at 10%, 6.4 mL glycerol, 3.2 mL dimethyl sulfoxide (DMSO), 10.4 mL deionized water and one pinch of polyvinylpyrrolidone (PVP). Following this procedure, the vegetable matter remained for 1 h at room temperature in reaction and the mixture was heated to 100°C for 3 min. Following cooling, the samples were centrifuged at 12,000 rpm for 50 s to obtain the crude extract for utilization in quantifying the level of total soluble proteins. Following the reaction with Bradford's dye, the samples were quantified in a spectrophotometer at an absorbance of 595 nm with four

readings taken for each replication of each evaluated treatment (Bradford 1976).

Quantification of the level of soluble non-structural carbohydrates

Soluble sugars were extracted through callus maceration in 80% ethanol and subsequent filtration. Following the total evaporation of supernatant in an incubator at 50°C, the sugars were resuspended in 25 mL distilled, deionized water and filtered through a Millipore membrane (47 mm diameter, 0.45 µm porosity), then agitated in a Millipore membrane (25 mm diameter, 0.45 µm porosity). The separation of glucose from soluble carbohydrates occurred in a Shimatzu LC 10A high performance liquid chromatography (HPLC) system, which used an HPX87H column and was coupled with a RID-10[a] refraction detector. In this, the glucose, polyose and glucuronic acid levels were detected for each replication.

Results and discussion

The results showed that the developmental conditions in stress induction medium did not alter the recuperation capacity of the calluses studied, which did not cease its growth accumulating more dry matter post-stresses, except for the control (Fig. 1A). The same was verified by Lutts et al. (2004) in wheat calluses subjected to temporary hydric stress, which were able to recover in 2 days as the physiological effects of polyethylene glycol (PEG), did not impact on recovery abilities over 30 days of exposition, nor did the decreases in water levels or K^+ content. Souza et al. (2004) also observed this effect in *Eucalyptus grandis* shoots exposed to 41°C temperatures *in vitro*, which recovered from this thermal treatment due to the capacity of the species to adjust its metabolism in order to avoid damages from environmental alterations (homeostasis). The cells therefore resisted the disruptions caused by stress factors, avoiding damages in callus growth or visible morphological alterations.

The calluses in different treatments accumulated proline, total soluble proteins, glucose, polyose and glucuronic acid resulting from environmental variations that they were subjected to (Fig. 1B-F). According to Shvaleva et al. (2006), *Eucalyptus globulus* plants subjected to hydric stress also accumulated osmolytes (e.g., sugar and proline) in their organs.

The calluses accumulated proline in response to hydric deficiency (Fig. 1B) in T2 presenting higher proline levels. This result suggests that the absence of thermal pre-treatment with a temperature greater than 25°C induces the accumulation of this amino acid to allow for hydric deficiency tolerance, most probably due to the insufficiency of thermal shock proteins to aid in the osmotic regulation as had occurred clearly in T4 and T6 (Fig. 1B). Lechinoski et al. (2007) observed that the total soluble amino acid levels of *Tectona grandis* plants increased by 68% under hydric stress conditions and their total soluble protein levels decreased by 95%. This is most probably related to the increase of proteolytic enzyme activity, which exhausted the protein reserves of the plants resulting in an increase in the level of this amino acid and a decrease in protein synthesis as hydric stress affects all biochemical processes of the plant. However, proline accumulation was not observed in T5, demonstrating that the plant could have utilized another response mechanism to tolerate the stresses.

The increase of temperature in the pre-treatments caused an elevation in the level of total soluble proteins in all treatments, with all being significantly different to the control (Fig. 1C). This result was also observed by Boston et al. (1996) and Borges and Ramos (2005), where heat shock proteins were synthesized in response to temperature stresses. In addition, thermal stress can alter the fluidity of the cell membrane with denaturation of the proteins probable. However, a superproduction of chaperones, which are involved in the cellular antioxidant system, could have assisted in the stabilization of membranes and in the maintenance of the cell metabolism (Abril et al. 2011). This is an indicator of synthesis of certain proteins during thermal stress conditions, having been reduced or inhibited in production when the stress factor is absent.

Calluses from different treatments accumulated glucose after exposition to hydric deficiency (Fig. 1D), with greater accumulation in T4 and T5. In addition, when temperature increased during pre-treatments, there was a gradual elevation in the glucose levels of T2 and T5 during selection. The T6 calluses however presented a reduction in their glucose level, which did not differ from treatments T3 and T4 (Fig. 1D). The accumulation of glucose observed in the selection can be related to the utilization of this sugar as a precursor of diverse cell components such as hemicellulose and proteins. The increase in hemicellulose content of the cell wall under hydric stress was observed by Iraki et al. (1989) and Rose et al. (1998) and can prevent cell wall collapse, as was observed by Hossain et al. (2006), where sugars were found to be incorporated in the cell wall.

Relating to the sugars evaluated, there was also a considerable increase in the levels of glucuronic acid and polyose during stressful conditions in the majority of the treatments assigned. The highest accumulation of polyose occurred in T2, T3 and T5 treatments (Fig. 1E), with glucuronic acid occurring in a larger quantity in T2, T4, T5 and T6 (Fig. 1F). All of these carbohydrates influenced the movement properties of the cell wall matrix, which is important for the protection of cells. Lutts et al. (2004) affirmed that the synthesis of fibrous compounds from the cell wall can induce the formation of a polymetric gel in the apoplast capable of fixing ions and retaining consistent quantities of water. This process could have contributed efficiently to adjusting the symplastic osmotic compartment beyond supersynthesis of compatible specific organic solutes. The results therefore suggest that the levels of glucuronic acid and polyose can be used as response parameters to hydric and thermal stress.

According to the cross-tolerance theory (Rizhsky et al. 2002), it was verified that when the temperature was elevated prior to the hydric deficiency treatment (T3 and T6), the cellular energetic cost could have been reduced with the catabolization of proline, as the treatments subjected to elevated temperature presented lower proline levels than those from T2, which were not subjected to this stress factor. The reduction in the accumulation of this amino acid can be related to the increase in the production of total soluble protein observed in this experiment (Fig. 1C).This indicates that this could have caused an increase in the tolerance of calluses to hydric deficiency when subjected to elevated temperatures during pre-treatment (Rizhsky et al. 2002).

The results demonstrated that the treatments were efficient at simulating stressful conditions, as the calluses altered their entire metabolism, accumulating solutes to survive the stresses. It is emphasized that other solutes could have been accumulated, which were not contemplated in this study, although the results inferred that the levels of glucuronic acid and polyose can be used as parameters indicating the response to hydric and thermal stresses in plants, as was reported in the literature regarding proline and glucose.

In relation to the forms of response, it was observed that the cells utilized different metabolic pathways to respond to the environmental perturbations, as could be observed in

treatment T4 with the elevation not altering proline levels in cell lineages but being sufficient to buffer the effects of hydric and thermal stress. Corroborating with the results of this experiment, Grigorova et al. (2011) suggested that effects separate from hydric and thermal stress in wheat plants could not be extrapolated for the combination of the two stresses in conjunction. New studies should thus be conducted with the objective of observing the effects of thermal and hydric stresses in conjunction, as well as the influence of thermal stress resistance under hydric stress.

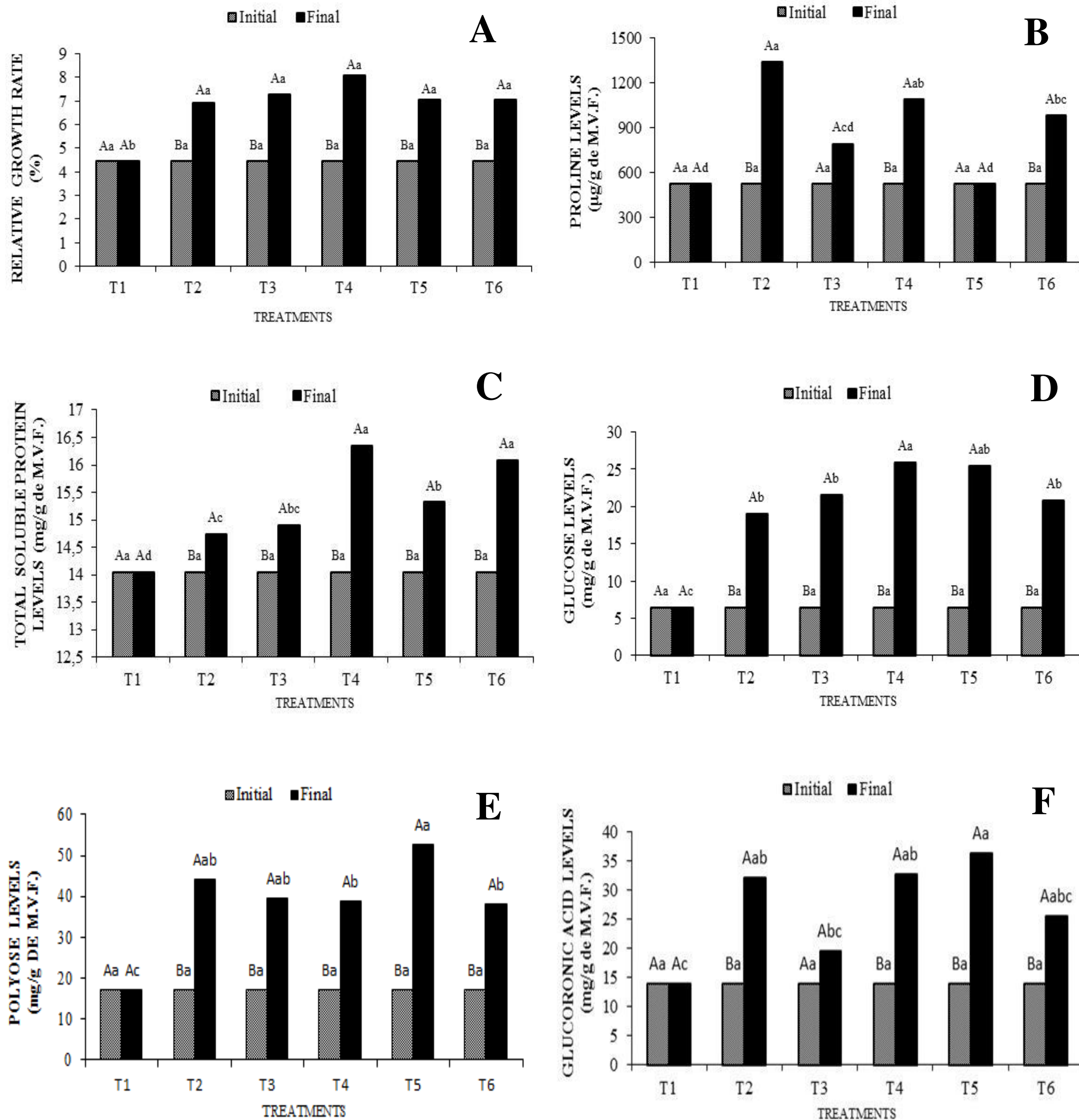

Figure 1. Measurements taken for *Eucalyptus urophylla* calluses subjected to hydric and thermal stresses. (A) Relative growth rate of calluses. (B) Proline levels. (C) Total soluble protein levels. (D) Glucose levels. (E) Polyose levels. (F) Glucuronic acid levels. Capital letters refer to each treatment and lower case letters to a determined experimental phase, with different letters representing a significant difference by the LSD test ($p < 0.05$).

Conclusions

The pre-treatments with temperature elevation increased the tolerance to hydric deficiency of *Eucalyptus urophylla* calluses.

The results permitted the inference that glucuronic acid and polyose levels could be used as parameters indicating responses to hydric and thermal stress in plants, just as proline and glucose.

The cell lineages selected presented variations in response mechanisms; although all were tolerant to hydric deficiency, those subjected to 35°C for 2 h and maintained in an N7 culture medium with 20% PEG 6000 presented the highest tolerance.

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