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CAN GENETIC VARIATIONS IN THE DEPLETION PROCESS OF STARCH STOCKS BE DRIVING CONTEMPORARY MICROEVOLUTION IN *Toona ciliata* VAR. *australis*?

Keywords:
Thermal performance curves
Neutral red dye
Thermal stress
Embolism
Cavitation

ABSTRACT: A simple method to establish a relationship between physiological responses of plants and thermal stresses is by quantifying the number of parenchyma cells with remaining starch stocks. The knowledge of the dynamic of starch depletion can be achieved by using statistical models such as thermal performance curves (TPC). The aim of this study was to quantify radial parenchyma cells with remaining starch stocks in order to evaluate changes in TPC regarding increases in temperature over seedlings of *Toona ciliata (*Australian Red cedar), in different heat induced treatments of matching both exposure time and temperature; besides of the assessment of variations in the TPC's and also to understand whether these changes are over genetic control. We used a protocol of heat induced treatment in the stems of the seedlings, anatomical cuts and staining with neutral red for the commercial clone BV1120, which was used as template to fit polynomial curves of TPC. After these mathematical fits and validation of these models with lignotubers of *Eucalyptus urophylla*, we defined a depletion time of 50% (TD50) from the starch stocks for each thermal treatment, so we could compare the performance for the others five commercial clones: BV1110, BV1121, BV1151, BV1210 and BV1321. The R^2 values were all above 85%. Results indicated that clone BV1110 had the highest value for remaining starch stocks at all heat induced treatments, in contrast to the clone BV1210, which had the lowest values for remaining starch stocks. The variation of the starch content indicates high values of broad-sense heritability, ranging from 97,43 to 98,48%, suggesting a possible contemporary microevolution process undergoing in Australian Red cedar. Thus, further selections can help improving the tolerance of Australian Red cedar to increasing temperatures on the environment.

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VARIAÇÕES GENÉTICAS DA DEPLEÇÃO DO AMIDO ESTÃO DIRECIONANDO UMA MICROEVOLUÇÃO CONTEMPORÂNEA EM *Toona ciliata* VAR. *australis*?

Palavras chave:
Normas de reação térmica
Corante vermelho neutro
Estresse térmico
Embolismo
Cavitação

RESUMO: Uma maneira de relacionar as respostas fisiológicas das plantas aos estresses térmicos é pela quantificação das células com amido remanescente, decorrente da sua despolimerização em açúcares simples que serão utilizados para reparar danos aos vasos embolizados do xilema. A compreensão da dinâmica dessa depleção poderá ser feita, por meio do uso de modelos estatísticos, que estabelecem curvas de performance térmica ou normas de reação térmicas (NRT). O presente estudo objetivou quantificar as células do parênquima radial que possuíam reservas de amido remanescentes, para avaliar as mudanças nas curvas das NRT, em resposta a aumentos de temperatura induzidos no caule de mudas de Toona ciliata (cedro australiano), sob diferentes escalas de tempo, além de avaliar as variações nas curvas das NRT de diferentes clones de Toona ciliata, bem como se essas variações são, predominantemente, genéticas e herdáveis. Foi utilizado um protocolo de indução de calor, no caule das mudas, cortes anatômicos e coloração com vermelho neutro, para o clone BV1120, que foi utilizado como modelo para ajustes polinomiais de terceiro grau das curvas das NRT. Após esses ajustes e validação dos modelos com lignotubers da espécie Eucalyptus urophylla, foi definido o tempo para depleção de 50% (td50) dos estoques de amido por tratamento térmico pela derivada de cada modelo ajustado, podendo, assim, comparar o comportamento para os outros cinco clones, o BV1110, BV1121, BV1151, BV1210 e BV1321. Os ajustes tiveram valores de R^2 acima de 85% e os resultados indicaram que o clone BV1110 apresentou maior estoque remanescente de amido, em todos os tratamentos térmicos, enquanto o clone BV1210 apresentou o menor estoque. Essa variação nos conteúdos remanescentes de amido indicaram altos valores de herdabilidade no sentido amplo, entre 97,43 a 98,4%, sugerindo um possível processo de microevolução contemporânea em andamento para cedro australino. Assim, seleções genéticas podem ajudar a aumentar a tolerância desses clones às alterações crescentes da temperatura ambiental.

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INTRODUCTION

The latest report on global climate change, published by the Intergovernmental Panel on Climate Change (2014), provides solid information to indicate that the Earth's surface temperature could significantly increase during the 21st century. In this report, the scientists project an increase of the Earth's surface temperature between 0.3 and 0.7°C, corresponding to the period of 2016 and 2035, which will strongly influence the frequency and duration of heat waves, with deep reflections in rainfall regimes over different regions on Earth. Predictions are that these climate changes will directly affect the dynamics of terrestrial ecosystems, increasing risks for food security and production of forest products, and also the maintenance of environmental services. The main studies, reviewed by Allen et al. (2010), emphasize the effects of these changes over plants, causing physiological stress, which can trigger a series of events that intensify trees' mortality.

Continuous rise in temperatures can lead plants to conditions of thermal stress that alter normal cellular homeostasis, constituting a significant threat to primary productivity in ecosystems. Plants under these conditions of stress may suffer changes in their metabolism by modifying mainly the enzymatic activities involved in carbon starch synthesis and its accumulation (MATHUR et al., 2014).

A way to correlate physiological responses of plants to thermal stresses is through quantification of the radial parenchyma cells with remaining starch stocks, in which the depletion of the starch is the result of depolymerization of simple sugars that are used to repair damage embolized xylem vessels (NARDINI et al., 2011). These authors suggested that sugars needed as osmoticum for refilling would be unloaded in the direction of embolized vessels via ray parenchyma. Those carbohydrates are then thought to be converted in to simple, low-molecular weight sugars, transported across the membrane on to the conduit wall, there by establishing a gradient to drive water movement away from the phloem.

The understanding of the dynamics of this depletion may be accomplished through the use of statistical models that establish thermal performance curves (TPC). These curves are adjusted to describe the effects of temperature on a given biological process that is desired to quantify. The development of TPC is being widely used to predict further scenarios on recent climate change by environmental and scientific organizations.

Studies which generate this knowledge are usually done under laboratory conditions, where temperatures are raised at different levels (KNIES, 2006). Schulte et al. (2011) suggest that for TPC studies, even under laboratory conditions, we should also consider exposure time scales in the heat induced treatments over plants. Thus, we can simulate closer conditions of climate change that happen on the natural environment. In addition, these authors also argue that the current processes involved in the mechanisms of responses of organisms to temperature changes are not fully understood, mainly due to the complex relationships between the temperature and other biotic and abiotic factors associated. Sears and Angilletta Jr. (2011) suggest that the scientific research related to climate change should identify specific characteristics of the selected individuals, document the genetic and phenotypic plasticity variations, and also consider how these characteristics will influence these processes within their own populations up to a higher level, such as the ecosystems.

In a more holistic view of TPC, when considering the cavitation/embolism of xylem vessels, it may be conjectured that the starch stocked in the radial parenchymal cells is the precursor to repairing the collapsed vessels and the depletion of this tissue may provide a better knowlegde of the changes in TPC (NARDINI et al., 2011).

This study aimed to address two major questions: 1 - Quantify radial parenchyma cells with remaining starch stocks to assess changes in the curves of TPC regarding heat induced temperatures in the stem of *Toona ciliata* seedlings (Australian Red cedar) under different exposure times; 2 - Evaluate the variations in TPC curves of different clones of this specie, and whether these variations are predominantly genetic and inheritable.

MATERIAL AND METHODS

Clonal seedlings of *Toona ciliata* (M. ROEMER) var. *australis* were grown for one year, during November 2014 to October 2015, in the nursery of the company Bela Vista Florestal Ltda., situated at Campo Belo city, Minas Gerais, latitude 20 ° 53 'S, longitude 45 ° 16' W and average altitude of 945 m. According to the Köppen climate classification, the climate is classified as Cwa: subtropical, humid and mesothermal. The region has an average annual temperature of 20.5°C and average annual rainfall is 1,406 mm.

The clones used in the experiment were: BV1110, BV1120, BV1121, BV1151, BV1210 and BV1321, registered in the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), under the numbers 31617, 31618, 31616, 31615, 31614 and 31613, respectively.

These cultivars have as maintainer the company Bela Vista Florestal Ltda.

The seedlings of the six clones of *T. ciliata* (M. ROEMER) var. *australis* were produced in 55 cm^3 polyethylene tubes, filled with substrate which was composed of vermiculite and carbonized rice husk with ratio of 3:1 (v/v). Seedlings grew in an experimental design spacing 10x10 cm from one another in growth trays during the first 12 months over sun light at nursery. After that period the experimental seedlings from each clone were selected, standardizing them by size, corresponding to about 25 cm of height and 1 cm of basal diameter.

The silvicultural treatments performed during the nursery growth period were: manual removal of weeds and microsprinkler fertigation by nozzles with a flow rate of 300 $L{\cdot}h^{-1}$, spaced 4x4 m. Seven shifts were used for daily irrigations, each shift had 6 minutes of microsprinkler duration.

After this period of 12 months, the seedlings were taken to the campus of the Federal University of Lavras - UFLA, situated in the city of Lavras, Minas Gerais, and taken to the forest nursery of the campus, where they could receive full sun light and regular daily irrigations, until the beginning of the laboratory experiment, conducted from November 2015 to January 2016, in order to quantify the cells of radial parenchyma, as well as the depletion levels in their starch stocks, due to the heat induced treatment of the stem of the selected clones. The study was then divided into three steps, which are described below.

First Step - Estimated time to 50% depletion of starch stocks (TD50)

In the first step, from the six clones of *T. ciliata* (M. ROEMER) var. *australis* available, one of them was chosen randomly (BV1120) to determine the estimated time for 50% depletion of stock starch (TD50), assessed by quantitation of the radial parenchyma cells with remaining starch stocks.

At the wood science and technology laboratory of UFLA, the seedlings were separated according to the heat treatment induction. The chosen thermal treatments were: 55°C, 52.5°C, 50°C and 47.5°C. The heat treatment of 55 °C was used as a threshold for comparison with wildfires studies in which they define this temperature as an initial value for the cambial tissue necrosis (BROWN; DEBYLE, 1987; GUTSELL; JOHNSON, 1996). Different timing periods for each of the four thermal treatments were assigned, as follows: 0, 10, 20, 30, 40 and 50 min for the temperature of 55 °C; 0, 20, 40, 60 and 80 min for the temperature 52.5°C; 0, 40, 60, 80, 100, 120 and 140 min for the temperature of 50°C; and 0, 30, 60, 90, 120 and 150 min for the temperature 47.5°C. The initial time "zero" represented the control for each treatment.

The variation of time on each chosen temperature was due to the pre-tests performed in the wood science and technology laboratory. It was found that for each temperature the depletion of starch behaved differently, e.g. for higher temperatures the break times for depletion of starch were smaller than that for the treatments performed at lower temperatures, where longer break times were used.

A total of 6x3 = 18, 5x3 = 15, 7x3 = 21 and 6x3 = 18 seedlings were used for heat treatments of 55°C, 52.5°C, 50°C and 47,5°C, respectively. Three replications for each time were used in each heat treatment.

Having the seedlings separated in the wood science and technology laboratory, they were then cut 1 cm down and 3 cm up the plant collar and then the edges of these cut stems were immediately sealed with fast curing acetic silica gel of fast curing, to minimize water loss during heat treatment. This sealing of the stems served to simulate a closer condition of the stomata closure when they are in real situations of high thermal stress.

Thermal treatments were applied inside a wood drying kiln with mechanical ventilation (Famem® model 320E) which allowed temperature maintenance during each of the time treatments, avoiding oscillations higher than 0.5 °C to keep the homogeneity of treatments. The cut stems were placed by their edges in a polystyrene support, which was chosen for not conducting heat to the stems by contact and ensured the uniformity of the heat treatment (convection) over the entire plant collar region. This support was loaded with three replicates of seedlings for each of the predetermined times of heat treatment.

At the end of each heat treatment inside the heater, the cut stems were immediately taken to the Wood anatomy laboratory of UFLA, and cut in the microtome slide (Leica® Model Jung SM 2000). The cuts were made with an approximate thickness of 8 μm at the plant collar region. The best transversal sections were separated and stained for 8 min in a neutral red solution 0.3 mM (0.01 wt%) dissolved in 7 mM KH_2PO/ Na_3PO_4 pH 7.4 as standardized by Stadelmann and Kinzel (1972) and, after being dyed, these cross-sections were washed in tap water to remove excess solution. Thus, microscope slides with these selected tranversal

sections were prepared and analyzed rapidly in the light microscope to avoid loss of the material.

The microscope slides focusing on phloem region were analyzed by WinCELL software (Regent Instruments Inc., Pro 2001a version), after being microphotographed by a camera (PixeLINK® PL-A662 model) attached to a light microscope (Olympus BX41 model) at 10x magnification. At least 10 images were captured from each repetition in order to have a representative mean of the phloem regions (external radial parenchyma), which contained stained cells. To quantify the stained cells with neutral red, we established an area of 1 mm^2, which was randomly selected for each transversal section, thus keeping a standard score of stained cells.

For each heat treatment the zero time (without treatment) was taken as a control and scored as 100%, to compare the data from each heat treatment and its time period in order to count the radial parenchyma cells with remaining starch stocks (stained cells), expressed in terms of percentage (%) and used as an indication of depletion of the starch stocks available in these cells in each respective time depending heat treatment.

To validate the use of neutral red for scoring radial parenchyma cells with remaining starch stocks as well as the adjustments of the starch depletion curves (TPC) for clone BV1120 model, we used 15 seedlings (three replicates and five times) of *Eucalyptus urophylla* which contained lignotubers at the same age and silvicultural treatments of *T. ciliata* seedlings, and we established a heat treatment of 55°C, at 0, 20, 40, 60 and 80 min time periods in the area of lignotubers (generally closer to plant collars), as these plant structures have a high content of starch stocks and they serve as protection of plants against biotic stresses, mainly due to high temperatures, for example, over forest fire regimes (KERR, 1925; MIBUS; SEDGLEY, 2000).

After collecting all the data percentage of cells with remaining starch stocks in each time depending on the heat treatment, we fitted polynomial models using GENES software (CRUZ, 2013). We then estimated for each heat treatment the time to deplete 50% (TD50) of starch stocks in the radial parenchyma cells, using derived math functions for each of the estimated polynomial models.

Second step - Test with glucometer

To validate the hypothesis that starch was converted to simple sugars (glucose) after each heat treatment, we repeated the same procedure used in the first step with the use of other 15 seedlings (3 replications and 5 times) of *Eucalyptus urophylla* which contained lignotubers with the same age and silvicultural treatment of *T. Ciliata* seedlings, but just using the heat treatment of 55°C, at 0, 20, 40, 60 and 80 min in the area of lignotubers.

After each time (depending on the heat treatment) the stems were immediately removed from the wood drying kiln, cut in half and the remaining sap was extracted utilizing pressure in the treated stem. A single drop of sap was necessary to measure glucose levels ($mg{\cdot}dL^{-1}$) in a glucometer (Accu-Chek Performa®), commonly used for verification of glucose levels in humans.

After data collection we fit an exponential model using the GENES (CRUZ, 2013) software, which served as a comparison to the polynomial model used to assess the remaining starch stocks in lignotubers for heat treatment of 55 °C carried out in the first stage of the experiment, in order to analyze the behavior of starch and glucose and verify that the stored starch (initial) was converted to simple sugar (glucose) after the same time, depending on the heat treatment.

Third step – clones' performance evaluation in their respective TD50

The TD50 obtained for each heat induced treatment over seedling collar area served as a common basis for the thermal treatments and comparison among the six *T. ciliata* clones regarding the maintenance of their starch stocks. Thus, four heat treatments were performed, each in their own TD50, previously calculated at the end of the first step, and applied over the five remaining *T. ciliata* clones (BV1110, BV1121, BV1151, BV1210, BV1321), in order to know each clone behavior and compare them with each other's performances and also with the clone used in the first step of the experiment (BV1120).

Fifteen seedlings were selected by heat treatment in their own TD50, which contained 3 replications for the five *T. ciliata* clones. The procedures for acquisition and conditioning of plants, seedlings' cuts, heat treatments, cuts in the microtome and scoring cells stained with remaining starch stocks (percentage) followed the same protocols that have been described in the first step of the experiment.

After obtaining all the data we processed to the statistical analysis of variance for each heat treatment and we also estimated phenotypic, genotypic and environmental parameters, as well as means comparison test for genotypes (clone) for each heat treatment, according to the methodology described by Scott & Knott (1974).

In the analysis, the percentage of data cells with remaining starch stocks per clone, after the heat treatments in their own TD50, were subjected to analysis of variance organized in a factorial arrangement in an experimental design of randomized blocks with three repetitions for plant (ramete) per plot. In this arrangement the first factor consisted of six genotypes (C - clones): BV1110, BV1120, BV1121, BV1151, BV1210 and BV1321 and the second factor by the four heat treatments (T): 47.5 °C, 50°C, 55°C and 52.5 °C, where each T was conducted on a specific heat induction timing for TD50 defined for the template clone BV1120.

In this analysis we used the following statistical model [1], where: Yijk is the value observed in the i^{th} heat treatment (T) applied to the j^{th} clone (C) k^{th} block (B); μ is the observed overall mean; Ti is the i^{th} effect of heat treatment (fixed effect); i = 1, 2, ... t; Cj is the effect of the j^{th} clone (fixed effect); j = 1,2, ... c; TC (ij) is the effect of the interaction between the i^{th} heat treatment and the j^{th} clone (random effect); B/T (ki) is the effect of

$$Yijk = \mu + Ti + Cj + TC(ij) + B/T(ik) + Eijk \qquad [1]$$

k^{th} block within the i^{th} heat treatment (random effect); Eijk is the random error and independently associated with Yijk observations.

The individual analysis conducted to assess the effects of clones in each heat treatment was established for an experimental design of randomized blocks, with three replications and one plant (ramete) per plot.

In these analyzes, we used a statistical model [2], where: Yi_{jk} is observed at value in the j^{th} clone (C) k^{th} block; μ is the observed overall mean; Cj is the effect of the j^{th} clone (fixed effect); j = 1,2, ..., c; B_k is

$$Yi_{jk} = \mu + C_j + B_k + Ei_{jk} \qquad [2]$$

the effect of k^{th} block (random effect), and k = 1,2, ... b; Ei_{jk} is the random error associated with independent observations Yi_{jk}

The analysis of variance, individual analysis and comparisons between means were performed by the GENES software (CRUZ, 2013). Estimates of genetic and phenotypic parameters such as genetic gain - gain expected selection - selection intensity of 1 in 6 clones; CV_e - experimental variation coefficient; φ_F - phenotypic quadratic component σ^2_e - environmental variance; φ_c - genotypic quadratic component; h^2 - coefficient of genotypic determination (broad-sense heritability); CV_C - coefficient of genetic variation and CV_C/CV_e - variation index; were made by the expected mean squares, as Cruz (2013).

RESULTS AND DISCUSSION

In a preliminary test, neutral red was adequate to show the remaining starch stocks in the radial parenchyma cells of *T. ciliata* (Figure 1-C to F). This preliminary finding was validated by anatomical cuts of lignotuber of *E. urophylla* (Figure 1-A and 1-B), which have considerably large sources of starch stocks (BAMBER; MULLETTE, 1978).

The results presented in Figure 1 shows the quality of the images for anatomical features and for cell scoring with the remaining starch stocks. This allows a precise and quick score of these stained cells. These facts have enabled the assessment of a large numbers of samples, thus we could also have a great fit of the curves (TPC), as well as to select clones of *T. ciliata* with low starch depletion of stocks, when subjected to thermal stress.

In Figure 1 it is possible to visualise the positive results of the samples obtained in the cross-section coloring process from the stems subjected to different temperatures. Note in Figure 1E the fading effect of the red color within the radial biseriate parenchymal cells, suggesting that these cells are possibly in depolymerization process of their starch stocks. Thus, they were not used in the score process of cells stained by neutral red to avoid errors and standardize the quantification.

The results of this study and adjustments to the curves of TPC, as presented below, have been validated by data obtained from cross-sections of lignotubers of *E. urophylla* (Figure 1-A and 1-B), which exhibited a large expected amount of starch stocks. It is beneficial for plants to have this structure mainly when they deal with strong environmental stresses (KERR, 1925; MIBUS, SEDGLEY, 2000).

Most chemical processes for extraction and quantification of the total starch content in woody plant tissues are widely reported in the literature. Many of these processes are mentioned by Chow and Landhäusser (2004). However, when aiming to quantify radial parenchyma cells of woody plant stems with remaining starch stocks and over depolymerization process to form simple soluble sugars in order to repair embolized xylem vessels, as suggested by Bucci et al. (2013); Salleo et al. (2004), and Nardini et al (2011), many of the proposed methods are based on microscopic anatomical characterizations made on staining with specific dyes of cross-sections that allows the starch deposits scoring,

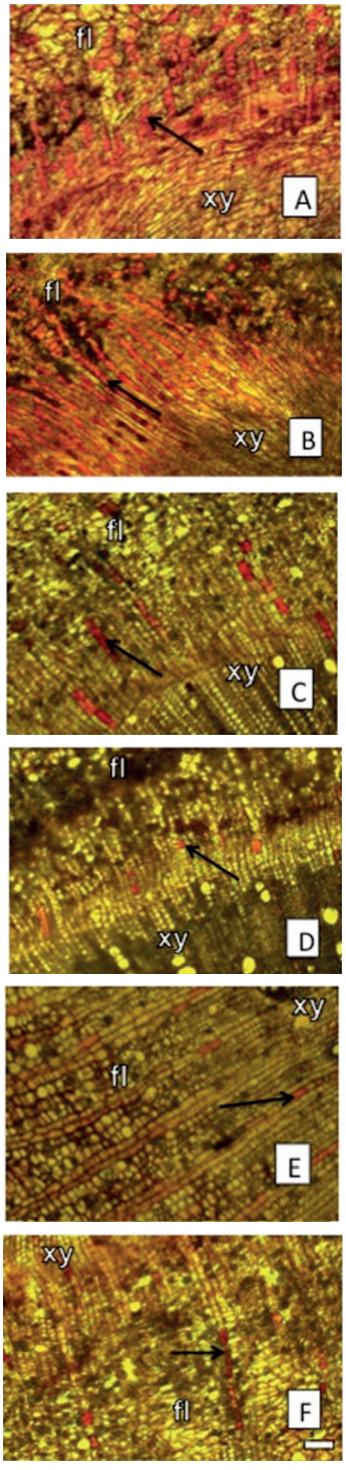

FIGURE 1 Starch stocks in the radial parenchyma cells stained in red by the neutral red dye (black arrows). A and B) cross-sections (8 μm thickness) from lignotuber and detection of large content of starch stocks in the radial parenchyma cells; C to F) detection of starch stocks in the radial parenchyma cells (stained red cells) of the BV1120 clone with the application of thermal treatments ranging in intensity and exposure time: 47.5°C/150min (C), 50°C/120 minutes (D) 52.5°C/72min (E), 55°C/30min (F).

such as iodine and safranin (BEGUM et al, 2010;. ISLAM; BEGUM, 2011; ORIBE; FUNADA; KUBO, 2003).

Neutral red dye, widely used for observation of live or dead cells, has the potential to discriminate the contents of vacuoles, such as starch, which is the main component of power reserve, as cells radial parenchyma are and structural carbon source (CHANTUMA et al., 2007, DIETZE et al, 2014.). For this reason it can be easily used to characterize necrosis of exchange of tissues when the plants are faced with forest fire situations (DICKINSON; JOHNSON, 2004), or heat stress caused by solar scalds or intense cold (MANION, 1991; TATTAR, 1989).

Estimated time for 50% depletion of starch stocks (TD50)

In the first step of this study, after all heat treatments were perfomed at different timing conditions for clone BV1120, used as a model to estimate for each temperature the specific time to cause a 50% depletion (TD50) in starch stocks in radial parenchyma cells, it was possible to obtain a quantification of the cells with remaining starch stocks by staining these cells with neutral red dye.

The performance curves presented in Figure 2 show how starch stocks change with increasing temperature. These different behaviors of BV1120 clone occurred when cut stems of seedlings were subjected to different temperatures and then fitted by

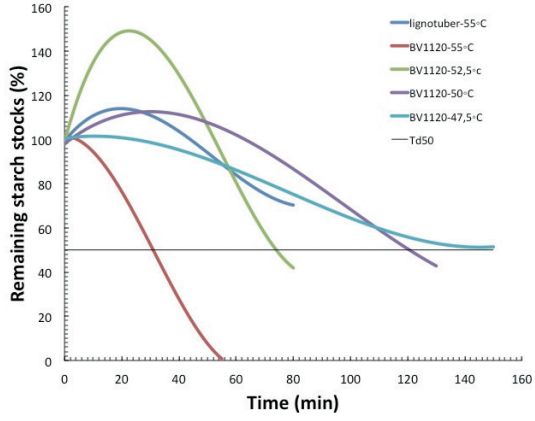

FIGURE 2 Starch depletion curves in the radial parenchyma cells of phloem region BV1120 adjusted to clone used as a template for assessing the exposure time that causes a 50% depletion starch (TD50). The TD50 for temperatures of 47.5; 50.0; 52.5 55.0°C and were, respectively, 150; 120, 72 and 30 minutes. DT50 for the starch stocks in the cells of radial parenchyma lignotubers of E. urophylla was not achieved in the experimented time.

third degree polynomials. All polynomial models were highly significant by Fisher's F test and they allowed fits of (R^2) 99.4; 85.6; 94.9 and 97.6% for the following heat treatments: 47.5; 50; 52; 55.5°C.

These fits of third degree polynomials are considered a common behavior for TPC (KNIES et al. 2006), which can allow the formation of physiological basis for interpretation of further evolutionary process. On that basis, if variations in the TPC are inherited, they provide evidence of evolutionary changes that make possible to conduct genetic selections aimed to improve plants' tolerance to environmental temperature changes (HUEY; KINGSOLVER, 1989).

The third degree polynomial was also fitted to remaining starch stocks from lignotubers of *E. urophylla*, used as a control for comparison purposes among different TD50 achieved in the induction heat treatments. This model was also highly significant and had an R^2 value of 87.8%.

All math derived functions matched with functions of the third degree polynomial models shown, allowing to estimate the respective times to reach the DT50, which assumed values of 30, 72, 120 and 150 min for the following heat induced temperatures: 55; 52.5; 47.5 and 50°C. Note that in the fit for the remaining starch stocks of lignotubers, DT50 in 55 °C temperature, has not been achieved to the experimented period of 80 min. Taking that temperature and just comparing the curves fitted for lignotuber and the clone BV1120, we can observe that this starch stock structure is tolerant to starch depletion compared to the clone template (BV1120).

Treatments with an evident peak behavior were only observed at temperatures of 50 and 52.5 °C of clone BV1120 and for 55 °C treatment of lignotuber. These increased values in starch stocks indicate that the maximum starch peak concentrations occurred within an exposure time ranging from 20 to 30 minutes.

For *T. ciliata* these increase in starch stocks indicate the existence of plant strategies to tolerate thermal stresses. However, it should be noted that at the temperature of 55 °C this strategy may occur, but it was not possible to be detected in the curve performance. This might be because of the minimum exposure time used of 10 minutes in the first break time of this specific heat induced treatment. In 47.5 °C treatment the peak expression did not occur as well, even despite the fact that the polynomial fit had a R^2 value of 99%.

Test with glucometer

The initial peak of TPC is developed with the starch accumulation over an abiotic stress before the following depletion of these starch stocks and they are controlled by specific signals. Nardini et al. (2011) suggest that the increase in intracellular [Ca^{2+}] after the embolism of the vessels and vibration of the cell walls activates signal translations which lead to changes in the starch metabolism in simple sugars.

These changes are suggested by the results presented in Figure 3, in which we can observe the changes in remaining starch stocks (% of red stained cells) and the glucose concentration (mg·dL^{-1}) with increasing application of heat dependent time treatment. In this figure it can be noticed that the initial stored starch was converted to simple sugar (glucose) after the time

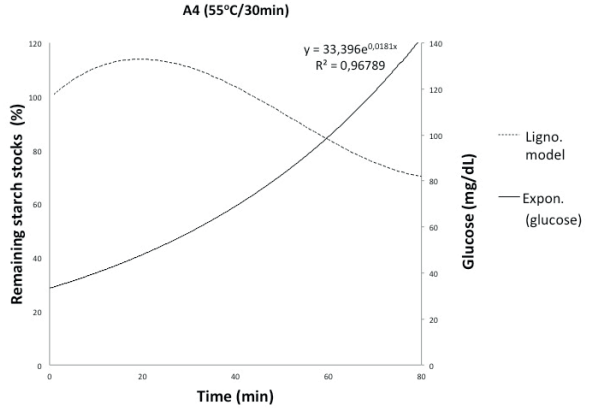

FIGURE 3 Performance of parenchymal cells with remaining starch and glucose in E. urophylla lignotubers for heat treatment of 55°C.

of heat induced treatment in lignotubers of *E. urophylla*. After the initial increasing peak in starch stocks a parallel exponential rise in glucose levels was also noticed, which can be explained by a model with R^2 value of 96%.

Clones' performance evaluation in their respective TD50

Variance analysis of a specific factor in the experiment showed that the remaining starch stocks suffer highly significant effects of clones and thermal treatments, depending on the magnitude of the temperature and exposure time. The interactions between these two factors were also significant, indicating the existence of phenotypic plasticity for the depletion of starch stocks, provide by the heat induced treatments.

The existence of such plasticity reveals the importance of this study, once it shows the possibility of establishment, through plant breeding, of adjustments of environmental conditions where thermal stress are quite pronounced.

After finding these interactions, we proceeded to the analysis of variance to evaluate the significance of the effects of clones within each heat induced treatment. Table 1 presents these analysis, showing that the

TABLE 1 Summary of variance analysis for the six T. ciliata clones and estimates of genetic and phenotypic parameters per heat time treatments: 47.5°C/150min (T1), 50°C/120min (T2), 52.5°C/72min (A3) and 55°C/30min (T4).

Variation Source	DF	Mean Squares			
		T1	T2	T3	T4
Blocks	2	14.39	3.50	4.06	6.06
Thermal Treatments	5	586.22*	410.37*	1,846.59*	1,171.42*
Residual	10	14.86	10.57	22.52	15.46
Mean		40.56	36.50	49.61	61.11
CVe (%)		9.50	8.91	9.57	6.43
φF		195.41	136.79	615.53	390.47
σ^2e		4.95	3.52	7.51	5.15
φc		190.46	133.27	608.02	385.32
h^2 (%)		97.47	97.43	98.78	98.68
CVc (%)		34.03	31.63	49.70	32.12
CVc/CVe		3.58	3.55	5.20	4.99
Gain (%)		50.49**	46.92**	74.24**	47.95**

* Significant at 5% probability by the F test. ** genetic gain expected for selection (selection intensity 1 in 6 clones). Subtitle: CV_e: coefficient of experimental variation, φF: phenotypic quadratic component, σ^2e: environmental variance, φc: genotypic quadratic component, h^2: coefficient of genotypic determination (broad-sense heritability), CV_C: coefficient of genetic variation, CV_C/CV_e: variation index.

coefficient CV_e experimental variation (%) ranged from 6.43 to 9.57%, indicating that the design and evaluation technique of remaining starch stocks with neutral red dye had low experimental errors magnitude, providing reliability over the data collection process.

In Table 1 we can also observe that the effect of clones was highly significant in all heat induced treatments. This indicates the existence of high levels of genetic variation, independent of thermal condition assessed. High values of genotypic coefficient of variation in relation to the experimental coefficient (CV_C/CV_e) indicate the possibility of pronounced genetic gains by clones' selection. The estimates of these genetic gains in the maintenance of starch stocks exceeded the levels of 46.9% and can be extended to 74.2% when the temperature was induced to 52.5°C for a period of 72 minutes, which was the highest estimated genetic gain.

Another interesting fact was the high values of genotypic determination coefficients, results for clones were all above 97%. This reveals that the gene of resistance to temperature increments is already fixed in the studied population of clones. This indicates that the depletion of starch in order to produce simple sugars and subsequent repairing xylem embolism is under strong genetic control.

High phenotypic plasticity values for the studied clones, genetic variability and the extremely high estimates of genotypic determination coefficients are strong indications that in the life story of *T. ciliata*, microevolutionary processes for storage and depletion of starch stocks to protect xylem cavitation and embolism repair has occurred in past ages (HENDRY; KINNISON, 2011 STOCKWELL et al., 2003) .

Phenotypic plasticity is a very important factor, it addresses phenotypic changes in natural environments and should be implemented in the maintenance of template populations (CHEVIN; LAND; MACE, 2010). These authors state that in studies involving changes in TPC, plant individuals must have focused on ecological importance of phenotypic aspects such as, morphology, physiology, behavior or phenology, affecting the population growth through its influence on the plant life cycle.

Furthermore, this study suggests that the TPC curves allows for the prediction of evolutionary dynamics of tolerance curves for the analyzed species. With the genetic variation demonstrated by the difference in TPC behavior, the extent of tolerance in different environments can be developed as a result of the analyzed feature for phenotypic plasticity. Thus, this approach can be used to test predictions in specific cases, which are based on variability of estimates, heritability and phenotypic plasticity of the feature in question (CHEVIN; LAND; MACE, 2010).

Table 2 shows the mean of all clones in their thermal induced treatments. It is observed that the best performance was for the BV1110 clone, as the remaining starch stocks were higher than the other clones in 47.5; 50; 52 and 55°C heat treatments. In this most extreme temperature and over 30 min exposure time, this clone

TABLE 2 Summary of mean test for the six clones in the following heat induced treatments: 47.5°C/150min (T1), 50°C/120min (T2), 52.5°C/72min (T3), 55°C/30min (T4).

Clone	T1	T2	T3	T4
1110	62.7 a	47.7 a	76.3 a	88.3 a
1120	51.0 b	44.7 a	56.0 b	47.7 b
1121	33.3 c	36.3 a	51.7 b	61.0 b
1151	39.3 c	45.0 a	42.3 b	81.0 a
1210	24.3 c	18.3 b	21.7 c	38.3 b
1321	32.7 c	27.0 b	32.7 c	50.3 b

Means followed by the same letter in the column do not differ by 1% probability by Scott Knott test.

kept the starch stocks well above the other clones, and the levels of these stocks were close to those in the cells of radial parenchyma from lignotuber of *E. urophylla* (Figure 4-D), although the number of these cells with starch stocks are higher, as shown in Figure 1-A and 1-B.

Figure 4 shows the mean results for *T. ciliata* clones in the different heat induced treatments and their respective TD50 along the third degree polynomial models, which were fitted for the template clone BV1120 and for the lignotuber of *E. urophylla*. In Figure 4 we can observe the mean results for each heat induced treatments, as showed in Table 2, evidencing changes of ranking of clones concerning their remaining starch stocks. In Figure 4-D, we can also note the TPC for lignotubers of *E. urophylla* compared to the other clones of *T. ciliata*, which was the plant structure and species used to validate these curves.

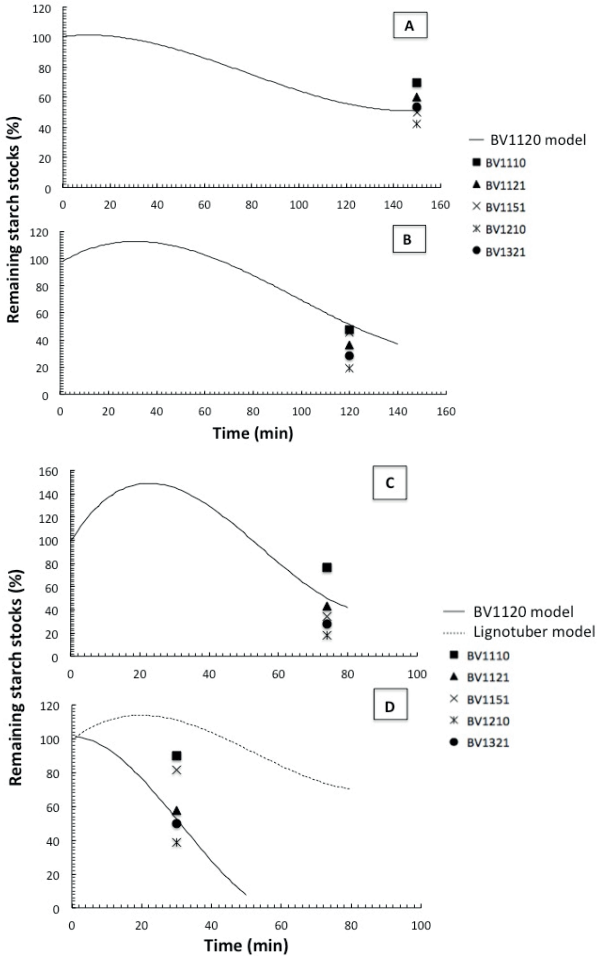

FIGURE 4 TPC performance for radial parenchymal cells after depletion of starch stocks for clone BV1120 (template) of *T. ciliata* when assessed over the temperatures of 47.5°C (A), 50°C (B), 52.5°C (C) and 55°C (D). Depletion of starch stocks for the five clones of *T. ciliata* when assessed over the temperatures in their respective DT50 are highlighted in the charts. In D is shown the starch depletion curve of lignotubers of *E. urophylla*.

Figure 5 shows individual and group behavior for the five *T. ciliata* clones in different heat induced treatments. The BV1120 clone behavior is not shown in Figure 5, once its behavior was previously set to be randomly chosen to establish the time of DT50 in all simulated heat induced treatments. Thus, in all these treatments, its behavior does not change because in all of them, it keeps 50% of depletion or maintenance of starch stocks.

In Figure 5, it is observed that the clone BV1110 showed a higher percentage of cells with remaining starch stocks and it always showed higher results then compared to the other assessed clones; on the other hand, clone BV1210 had the lowest values in all heat treatments. Note that for the BV1121 and BV1321 clones' behavior, considering different heat induced treatments, they demonstrated intermediate levels for starch stocks maintenance.

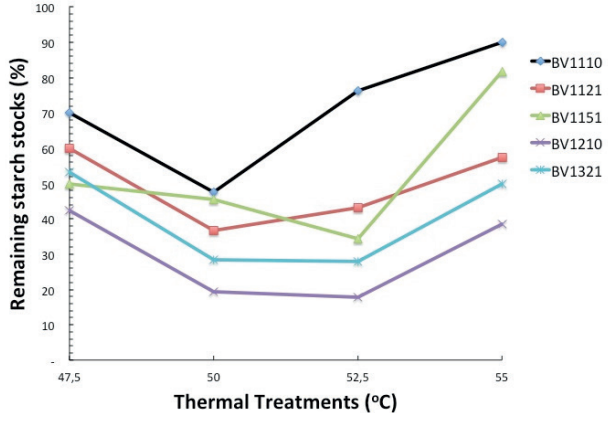

FIGURE 5 *T. ciliata* clones' behavior under the heating effect assessed at their DT50 in the respective heat induced treatments: 47.5°C/TD50=150min, 50°C/TD50=120min, 52.5°C/TD50=72min and 55°C/TD50=30min.

Considering these six clones assessed in respect to the remaining starch stocks, four of them were also studied by Rodrigues et al. (2013) concerning their physiological characteristics. The authors stated that the clone BV1110, which in our study showed the largest remaining starch stocks after all heat treatments, also showed higher photosynthetic rate, while clone BV1321, which presented one of the lowest remaining starch stocks in our study, had the lowest photosynthetic rate. These relationships, for these two clones, imply that the largest remaining starch stocks are related to the initial starch stocks, i.e. before we cut the stem seedling for the experiment, when they were still under photosynthetic activity.

The behavior of BV1121, BV1210 and BV1321 clones were similar to each other and also divergent when compared to clones BV1110 and BV1151. The behavior of clone BV1110, which had a strong drop in starch stocks in the heat induced treatment of 50°C/ DT50 = 120min, changed its behavior in relation to the three clones previously mentioned, but did not change at a level to promote change in the clones' ranking, indicating that for these four *T. ciliata* clones, the interaction was simple. On the other hand, BV1151 clone behavior in the heat induced treatment of 52.5°C/ DT50 = 72min showed a strong decline in starch stocks, which caused a change in the ranking of the five clones for this specific temperature and exposure time, revealing a possible complex interaction. Probably, the main cause of significant interaction between clones and heat induced treatments, as shown in Table 1, was due to the behavior of clones BV1110 and BV1151.

These facts reveal a great influence of temperature and exposure time levels of remaining starch stocks in the radial parenchyma cells. For the higher simulated temperature and considering a shorter exposure time, as the heat induced treatment 55°C/TD50 = 30min, clones showed the highest stocks values for remaining starch stocks, while for the lower temperature and considering longer exposure time, as the heat induced treatment at 50°C/TD50 = 120min, clones showed lower values for remaining starch stocks; i.e. in this condition the depletion of starch stocks were more pronounced.

Under these combined conditions of temperature and exposure time, and considering the theory of Nardini et al. (2011), in which the starch is depolymerized in simple and soluble sugars which migrate through the radial parenchyma towards the cavitatied xylem vessels in order to repair them, it can be assumed that in the condition of low stressful temperatures and longer exposure times, there will be greater depletion of starch stored to repair the embolism in the xylem vessels, while at higher temperatures, but with low exposure time, the need for depolymerization of starch can be inconspicuous, as in this last condition, the starch stocks remained at higher levels. Therefore, as shown in Figure 5 and Table 2, it can be observed that in the case of clones BV1110 and BV1151 that have kept starch stocks at 88.3 and 81% respectively, the depletion of starch stocks were just 11.7 and 19%.

These considerations on the variations in temperature and exposure time are according to Schulte at al. (2011) who mention that for laboratory experiments of TPC we can not only consider the temperature as stress factor, but we also should consider the exposure time at which the temperatures remain to cause stress in plants. Thus, we can infer that the interaction between these environmental conditions constitutes strong abiotic stresses that restrict ontogenetic processes in plants.

CONCLUSION

Neutral red dye was found to be suitable for the coloration of radial parenchyma cells with starch stocks, making it easier to score process.

The heat treatment combined with a rise in temperature and exposure time reduced starch stocks and allowed the adjustment of thermal response curves with high precision. These fits were made with a third degree polynomial model with all R^2 values above 85%.

There is a significant variation between *T. ciliata* clones, assessed as the remaining starch stocks after heat induced treatments. This variation indicates that changes in thermal performance curves standards with respect to depletion of starch in the radial parenchyma cells follow the principles of natural selection.

This variation in the remaining starch content is highly heritable, above 97%, and suggests that the genes that control this feature are fixed in the population, but further molecular studies need to be addressed to confirm this hypothesis as well field experiments. In addition, selections can probably help improve the tolerance of plant species to temperature changes on the environment.

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