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Phenolics metabolism provides a tool for screening drought tolerant *Eucalyptus grandis* hybrids

Clarice Noleto Dias[1], Edgard Augusto de Toledo Picoli*[2], Genaina Aparecida de Souza[2], Mohamed Ali Farag[3], Marcus Tullius Scotti[1], José Maria Barbosa Filho[1], Marcelo Sobral da Silva[1], Josean Fechine Tavares[1]

[1]Natural and Synthetic Bioactive Products Graduate Program, Federal University of Paraíba, João Pessoa, Paraíba, 58051-900, Brazil
[2]Plant Biology Department, Federal University of Viçosa, Viçosa, Minas Gerais, 36570-900, Brazil
[3]Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, 11562, Egypt

***Corresponding author: eatpicoli@gmail.com**

Abstract

Eucalyptus L'Hér. (Myrtaceae) is one of the most economically important plant genera worldwide. Breeding programs aim to adjust productivity and tolerance to abiotic stresses such as water deficit. Plants under stress can overcome the excessive production of reactive oxygen species *via* the production of phenolics. Therefore, the aim of the present study is to investigate the variation of leaf phenolics content among 4 commercial clones of *E. grandis* hybrids subjected to simulated water deficit (moderate and severe treatment). Seedlings of *E. grandis* hybrids were exposed to adequate water supply (control) and two concentrations of polyethylene glycol solutions (moderate and severe stress) for 60 days. Then, leaves were harvested and their phenolic contents were measured by high-performance liquid chromatography and colorimetric assays. Each clone showed a different tolerance level and reacted in a specific manner against this stress. Principal component analysis of controls of each clone revealed that caffeic acid and quercitrin contents were found at very low levels in the sensitive and in the tolerant clone leaves, respectively. The tolerant (*E. grandis* × *urophylla*- EGU–EGU1) and EGU3 clones had higher phenolic contents in the stressed seedlings than in the control ones. In contrast, this variation was not detected in the sensitive clone leaves (*E. grandis* hybrid–EGR). Chemical analysis of these phenolics can be used as a reliable screening tool for drought tolerant hybrids in *Eucalyptus*. Our results also support the selection of more efficacious *Eucalyptus* hybrids for field cultivation, thereby enhancing its environmental competitiveness.

Keywords: Abiotic stress; eucalypt dieback; flavonoids; HPLC; water stress.
Abbreviations: ANOVA_ analysis of variance; DAD_diode array detector; DPPH_2,2-diphenyl-1-picrylhydrazyl; EGU_*Eucalyptus urograndis* hybrid; EGR_*E. grandis* hybrid; GAE_gallic acid equivalents; HPLC_high performance liquid chromatography; IC_{50}_half-maximal inhibitory concentration; LOD_limit of detection; LOQ_limit of quantification; PCA_principal component analysis; PEG_polyethylene glycol; QE_quercetin equivalents; ROS_reactive oxygen species; TFC_total flavonoids content; TPC_total phenolics content; UV_ultraviolet.

Introduction

Eucalyptus L'Hér. (Myrtaceae), a native genus from Australia, is one of the most economically important and widely cultivated trees for wood crop purposes worldwide owing to its high-productivity characteristics. These plantations contribute to decreasing the exploitation of tropical flora and associated biodiversity (Domingues et al., 2011; Santos et al., 2013). Extensive plantations of *Eucalyptus* species can be found in Brazil and the dominant species are *E. grandis*, *E. urophylla* and their hybrids, named urograndis eucalypt (IBA, 2016).

The forest sector demands improvement in productivity and a considerable amount of raw material supply. From such perspective, it is deemed of interest to ensure the production of wood in a very efficient way, even for *Eucalyptus* plantations extended into marginal areas, without optimal growth conditions under environmental stresses (Moura et al., 2012; Bacha et al. 2016). Biotic and abiotic stresses inhibit the normal functioning of the plant and are some of the main problems affecting the agricultural industry, reducing crop yield and economic losses due to low productivity (Mahajan et al., 2005; Ayalew et al. 2016).

Among the stresses, water deficit is one of the most significant challenges that agriculture is facing. Drought and dehydration cause an imbalance in water supply of the soil water available to plants, which makes it insufficient to maintain growth, photosynthesis and transpiration demands (Tardieu et al., 2011; Zlatev and Lidon, 2012; Gall et al., 2015). A wide variety of growth-related processes in plants is affected by drought stress (Weemstra et al., 2013; He et al., 2014). Several studies have shown that water supply is critical to *Eucalyptus* development in plantations around the world (Costa e Silva et al., 2004; Stape et al., 2010).

Another factor off-putting *Eucalyptus* trees is the anomaly called eucalypt dieback, a disease of unknown etiology caused by interacting biotic and abiotic factors. Plants exhibiting this disease show deteriorated health, which in some severe cases can lead premature death (Leite et al., 2014; Ross and Brack, 2015). Some pests and pathogens

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attack plants, specifically, long-lived trees such as *Eucalyptus* species, which attempt to defend themselves over their whole lifetime against invaders (Naidoo et al., 2014; Jesus et al., 2015). One of the mechanism by which plants evade these threatens in nature is *via* the accumulation of phytoalexins, which are antimicrobial compounds synthesized mainly through the phenylpropanoid metabolic pathway. Phenolics have been reported as part of such defense mechanism that plays an important role in protecting the plant against pathogens (Rosa et al., 2010; Daayf et al., 2012).

In addition to phenolics antimicrobial potential, they also contribute to plants plasticity in response to abiotic stresses. Plants exposed to unfavorable environmental conditions can overcome the excessive production of reactive oxygen species (ROS) *via* the activation of different antioxidant mechanisms (Gill and Tuteja, 2010; Pereira et al., 2015). One of these mechanisms involves the biosynthesis of phenolics, by acting as ROS scavengers. Such flexibility on plant secondary metabolism provides a basis for the evolution of plant adaptation for changing environmental conditions (Farag et al., 2009), and the knowledge gained from its upregulation can be used for future metabolic engineering attempts in the development of drought tolerant plants. Understanding the qualitative and quantitative variations in the level of plant phenolics grown under water deficit is indeed a noteworthy strategy to manage indicators associated with stress tolerance in crop plants (Ma et al., 2014; Talhaoui et al., 2015).

Given the global commercial interest in the genus *Eucalyptus* and the lack of knowledge about the effects of water deficit on their phenolics, this work shows a chemical approach to assist the identification of *Eucalyptus* clones tolerant to drought. Previous studies showed no statistically significant effect on the concentration of formylated phloroglucinol compounds in *Eucalyptus* species grown under drought stress conditions (Wallis et al., 2010; McKiernan et al., 2014). Consequently, the main objective of the present study was to assess other types of phenolics in leaf samples from four different clones of *E. grandis* hybrid in response to artificial water deficit induced by polyethylene glycol (PEG).

Results and discussion

Methodology for phenolics detection and radical scavenging activity

Various analytical approaches have been adopted for the analysis of phenolics in plant extracts (Kala et al., 2016). Among the suitable technologies, HPLC-UV platform and colorimetric methods are reliable and low cost options to be directly applied by forest companies in the selection of tolerant plants. These methodologies may be used for relative quantification of this class of molecules due to their conjugated double and aromatic bonds that absorb UV radiation and, consequently, allow detection (Costa et al., 2015; Giusti et al., 2017).

Leaf methanol extracts of four different clones of *Eucalyptus* hybrids subjected to water stress treatments or not (control) were analyzed using colorimetric assays (TPC, TFC, and antioxidant activity) in parallel to HPLC-UV analysis. Fig 1. illustrates the HPLC-UV chromatograms of *E. grandis* hybrid leaves (EGU1) from control and severe drought stress plants, in which the chemical complexity of their extracts can be observed. Although visual inspection of the chromatograms shows differences among samples, five metabolites were identified and quantified in the samples to confirm the comparisons. The results of the linearity evaluation are presented in Table 1. Antioxidant activity, total and individual phenolic and flavonoid levels in dry leaves are presented in Table 2.

In this study, all analyzed methanol extracts showed significant DPPH radical scavenging activity, compared to ascorbic acid, which is the reference antioxidant compound. The control from the EGU2 clone exhibited the highest antioxidant activity among all samples, with an IC_{50} value of 9.70 μg mL^{-1}, likely to be mediated by the high level of total phenolics (54.12 mg GAE g^{-1} dry weight) as measured using Folin–Ciocalteu colorimetric assay.

Comparison between control samples of different Eucalyptus hybrids

Control samples from each clone were used to determine the differences in chemical composition (TPC, TFC, HPLC analysis) that are related to genetic factors, because they are samples from seedlings grown under the same conditions (Fig 2). Such comparative differences can contribute to determining the influence of leaf phenolic composition of these clones on resistance or non-resistance of anomaly called eucalypt dieback.

EGR clone was susceptible to this physiological disorder and its leaves exhibited the largest amount of total flavonoids. The lowest concentrations were found in leaf of the EGU1 clone (resistant to eucalypt dieback) and the EGU3 clone (in test phase). Prado et al. (2015) stated that the growth of *E. grandis* × *E. urophylla* cuttings treated with the flavonoid quercetin was not influenced by the presence of this compound. In contrast to the antioxidant activity expected for flavonoid supplementation, the authors observed that in this clone quercetin generated ROS. The highlighted observation may indicate that the high level of flavonoids in the clone susceptible to stresses (EGR) is related to an oxidative reaction of this compound class.

With regards to total phenolics, the clone with the highest levels was EGU2 (moderately resistant), followed by EGR. No differences were observed between the EGU1 and EGU3 clones. EGR leaves showed the lowest amount of caffeic acid and the highest of quercitrin, a flavonol. Considering all parameters, EGU2 leaves had the highest phenolic levels (TPC and phenolic acids), albeit EGR leaves were rich in flavonoids (TFC and quercitrin) (Fig 2; Table 2).

Leite et al. (2014) reported that susceptible clone plants with this anomaly have higher manganese (Mn) content in leaves than resistant clones and plants without the anomaly. In addition, the aforementioned authors reported an accumulation of phenolic compounds in affected tissues. These metabolites act as antioxidants and, consequently, protect the plant cells against the ROS generated by Mn toxic levels (Yan and Tam, 2011; Yao et al., 2012).

The total flavonoids and quercitrin content were higher in the leaves of the susceptible clone (EGR) than in the leaves of other clones. Nonetheless, for other monitored metabolites *viz*. gallic, chlorogenic, caffeic and ellagic acids no higher levels were observed in this clone versus others. It is noteworthy that caffeic acid level in the susceptible clone was found at very low levels (Fig 2). Pretreatment with exogenous caffeic acid was found to enhance dehydration tolerance of cucumber seedlings by stimulating antioxidant enzymes and increasing proline and soluble sugar contents that reduce growth inhibition (Wan et al., 2014). Accordingly, the low caffeic acid content in EGR clone can be correlated with its stress susceptibility.

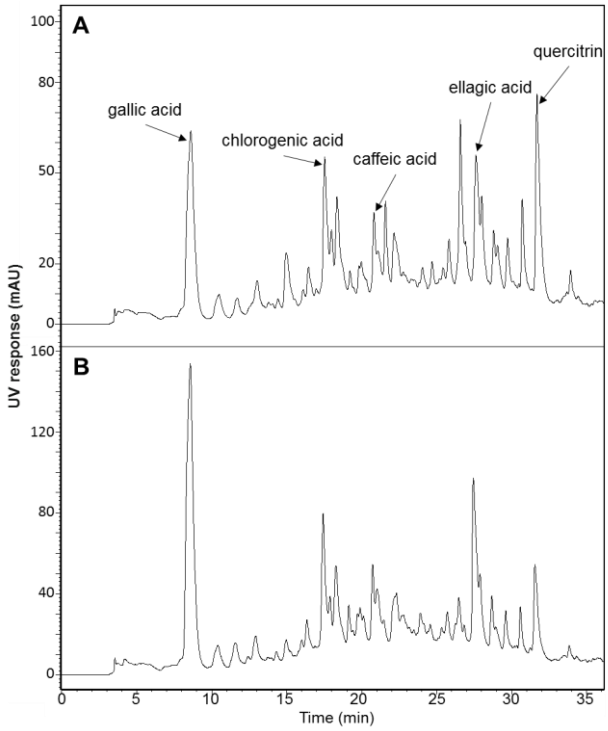

Fig 1. HPLC-UV chromatograms at 280 nm of the methanol extract of *Eucalyptus grandis* hybrid (EGU1) leaves. A: control sample; B: severe drought stress sample. Arrows indicate peaks corresponding to 5 phenolics quantified in *Eucalyptus*. Chromatographic conditions are described under Materials and Methods.

Table 1. Calibration data used for HPLC-UV quantification of phenolics in *Eucalyptus grandis* hybrids leaves.

Compounds	Calibration curve[a]	R^2	LOD[b]	LOQ[b]
Gallic acid	$y = 65047x - 73143$	0.9971	1.54	2.53
Chlorogenic acid	$y = 30103x + 6439.1$	0.9984	0.32	1.57
Caffeic acid	$y = 66470x + 1774$	0.9998	0.20	0.74
Ellagic acid	$y = 45774x - 120273$	0.9978	7.58	19.14
Quercitrin	$y = 16124x - 2398.8$	0.9983	1.21	3.96

[a]y = peak area, x = concentration in μg mL^{-1}. [b]LOD–limit of detection and LOQ–limit of quantification, both expressed in μg mL^{-1}.

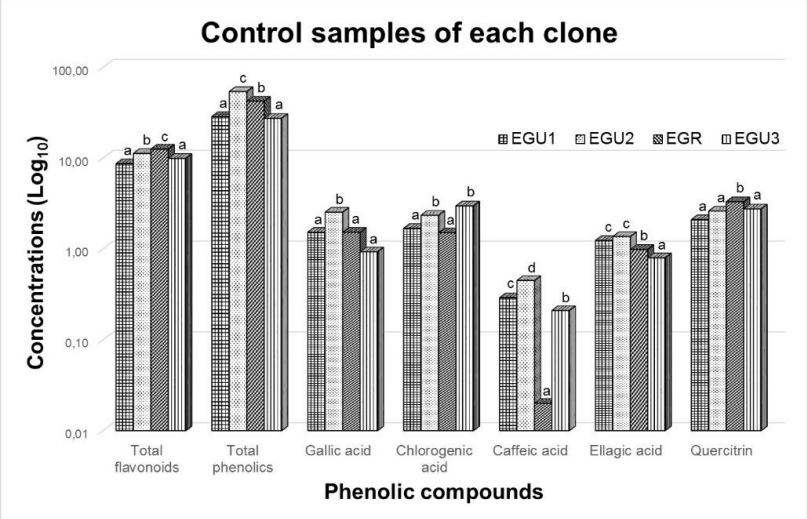

Fig 2. Concentrations of phenolics (mg g^{-1} dry weight) in leaves of four *Eucalyptus grandis* hybrids. EGU1–3 = different clones of *E. grandis* W. Hill × *E. urophylla* S. T. Brake; EGR = *E. grandis* hybrid measured by high-performance liquid chromatography and colorimetric assays. Results correspond to the mean values estimated from three independent determinations (standard deviation less than 5%). Different letters indicate statistically significant differences between treatments of the same clone ($P < 0.05$; Bonferroni's test).

Table 2. Antioxidant activity, total and individual phenolic and flavonoid levels in dry leaves of *Eucalyptus grandis* hybrids subjected to drought stress.

Assays	EGU1			EGU2			EGR			EGU3		
	Control	Moderate stress	Severe stress	Control	Moderate stress	Severe stress	Control	Moderate stress	Severe stress	Control	Moderate stress	Severe stress
DPPH radical scavenging assay[1]	19.84 (13.5–30.04)	22.26 (15.27–30.25)	18.07 (12.59–27.29)	9.70 (6.15–14.39)	21.23 (15.82–30.14)	16.46 (12.23–23.07)	18.48 (12.78–25.16)	21.89 (15.75–30.26)	29.74 (22.71–34.84)	31.21 (19.79–41.40)	18.55 (12.25–25.94)	15.5 (10.13-21.22)
Total flavonoids[2]	8.68 ± 0.28^{a}	14.53 ± 0.07^{c}	12.47 ± 0.12^{b}	11.25 ± 0.30^{a}	11.94 ± 0.61^{a}	14.62 ± 0.90^{b}	12.55 ± 0.55^{a}	12.57 ± 1.82^{a}	15.60 ± 0.58^{b}	9.94 ± 0.38^{a}	10.12 ± 0.024^{a}	19.74 ± 0.90^{b}
Total phenolics[3]	28.69 ± 1.09^{a}	52.95 ± 5.63^{c}	40.02 ± 1.08^{b}	54.12 ± 2.19^{a}	52.25 ± 0.44^{a}	60.22 ± 1.98^{b}	42.42 ± 0.60^{a}	49.89 ± 1.85^{b}	47.84 ± 2.72^{b}	27.39 ± 3.55^{a}	43.59 ± 2.92^{b}	87.00 ± 4.61^{c}
Gallic acid[4]	1.53 ± 0.09^{a}	3.30 ± 0.09^{b}	3.65 ± 0.15^{c}	2.54 ± 0.64^{a}	3.31 ± 0.02^{b}	3.70 ± 0.06^{b}	1.53 ± 0.04^{a}	2.78 ± 0.30^{b}	2.90 ± 0.21^{b}	0.93 ± 0.06^{a}	3.00 ± 0.02^{b}	4.79 ± 0.42^{c}
Chlorogenic acid[4]	1.69 ± 0.07^{a}	3.27 ± 0.28^{c}	2.77 ± 0.22^{b}	2.35 ± 0.21^{a}	2.38 ± 0.29^{a}	2.68 ± 0.07^{a}	1.51 ± 0.23^{a}	3.26 ± 0.35^{b}	1.86 ± 0.14^{a}	2.98 ± 0.16^{a}	3.33 ± 0.03^{a}	6.29 ± 0.52^{b}
Caffeic acid[4]	0.29 ± 0.01^{a}	0.97 ± 0.02^{c}	0.68 ± 0.06^{b}	0.45 ± 0.05^{b}	0.35 ± 0.06^{a}	0.51 ± 0.01^{b}	0.02 ± 0.00^{a}	0.04 ± 0.00^{b}	0.03 ± 0.00^{a}	0.21 ± 0.02^{a}	0.79 ± 0.01^{b}	1.72 ± 0.04^{c}
Ellagic acid[4]	1.24 ± 0.05^{a}	2.45 ± 0.03^{b}	2.27 ± 0.21^{b}	1.37 ± 0.18^{a}	1.39 ± 0.52^{a}	1.75 ± 0.25^{a}	0.99 ± 0.01^{a}	1.73 ± 0.02^{c}	1.61 ± 0.08^{b}	<LOQ^{a}	1.85 ± 0.03^{b}	3.57 ± 0.20^{c}
Quercitrin[4]	2.11 ± 0.07^{a}	2.27 ± 0.04^{a}	2.01 ± 0.21^{a}	2.62 ± 0.55^{a}	2.38 ± 0.03^{a}	3.39 ± 0.09^{b}	3.31 ± 0.08^{a}	3.85 ± 0.43^{a}	3.82 ± 0.26^{a}	2.76 ± 0.14^{a}	3.05 ± 0.05^{b}	4.51 ± 0.05^{c}

Results correspond to the mean values estimated from three independent determinations ±SD. Values followed by different superscript letters indicate statistically significant differences between treatments of the same clone ($P < 0.05$; Bonferroni's test).
EGU1–3 = different clones of *E. grandis* W. Hill × *E. urophylla* S. T. Brake; EGR = *E. grandis* hybrid. [1] IC_{50} in µg mL^{-1}. The antioxidant activity of the reference compound ascorbic acid was IC_{50} = 10.04 µg mL^{-1} [2] mg of quercetin equivalents per g of dry weight [3] mg of gallic acid equivalents per g of dry weight [4] mg of the individual compound per g of dry weight.

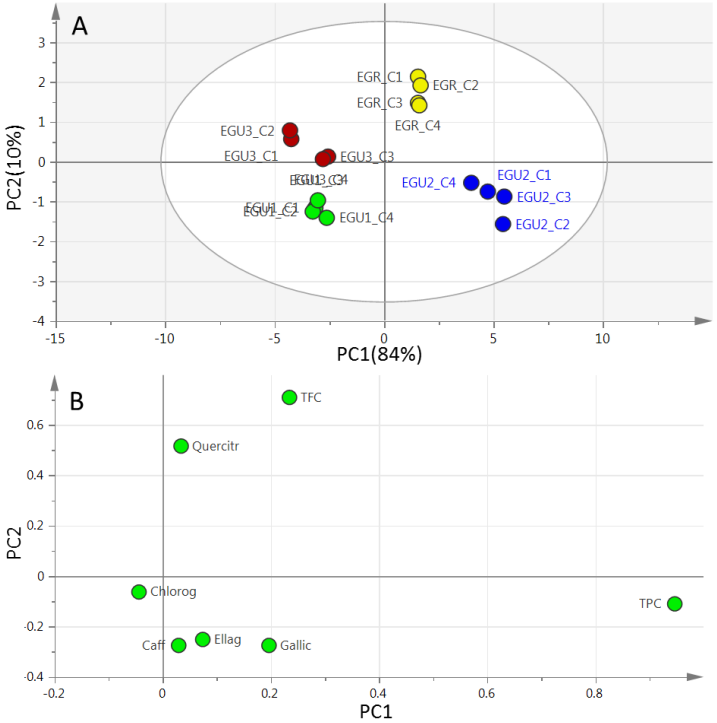

Fig 3. PCA score plot of PC1 and PC2 scores from the leaf phenolics of the controls (c – adequately watered plants) of four *Eucalyptus grandis* hybrids and their replicates (A). Loading plot for PC1 and PC2 with contributing variables (B). EGU1–3_different clones of *E. grandis* W. Hill × *E. urophylla* S. T. Brake; EGR_*E. grandis* hybrid. Variables: TPC and TFC_total phenolics and flavonoids content; Gallic, Chlororog, Caff, Ellag and Quercitr_compounds quantified *via* HPLC.

In the present study, principal component analysis (PCA) of the leaf phenolic profiles viz. (TPC, TFC, HPLC analysis) was further performed to compare clones in order to classify samples and to better define the correlation between sensitivity to eucalypt dieback and drought stress to the high levels of these key metabolites in leaves. In the score plot of PC1 *vs* PC2 scores (Fig 3A), replicates from the same sample clustered together, confirming the repeatability of the methods used in this study. PC1 accounted alone for 84% of the variation between samples and clear discrimination between the tolerant (EGU1 – green dots) and the sensitive (EGR – yellow dots) clones could be observed along this PC. Moreover, on the left side of the plot, EGU1 and EGU3 clones were positioned with negative PC1 score values, whereas EGU2 and EGR clones were placed in the right side of PC1 (positive score values).

The loading plot (Fig 3B) revealed the variables that contributed significantly to the group segregation. Total flavonoids and quercitrin are more enriched in EGR versus phenolic acids (caffeic and chlorogenic acids) abundance in EGU1. Considering these two genotypes are diametrically on opposite sides as they are negatively correlated. In other words, caffeic acid and quercitrin contents were found at very low levels in EGR and in EGU1 clone leaves, respectively.

Some fungus species threaten *Eucalyptus* crops; *Puccinia psidii* Winter and *Calonectria* spp. commonly cause diseases in trees cultivated in tropical and subtropical climates (Varshney et al., 2012; Santos et al., 2014; Alfenas et al., 2015). The Suzano Pulp and Paper Company provided the phenotypic data of the clones studied in relation to their tolerance to these pathogens. The EGU1, EGU2, and EGU3 clones are considered tolerant to *P. psidii* and *Calonectria* spp., with EGU3 being moderately tolerant to *Calonectria* spp.; the EGR clone was found to be tolerant to *P. psidii*. The high phenolic levels in the samples as analyzed *via* HPLC (Fig 2) provide chemical based evidence for these fungal tolerances (Cheynier et al., 2013; Mikulic-Petkovsek et al., 2013).

Comparison between treatments (control, moderate and severe water stress) of the same clone

Each clone was examined separately to overcome genetic difference among them and to better assess the effect of water stress on *Eucalyptus* clones with different tolerance levels. The tolerant clone (EGU1) was able to increase its leaf phenolic contents (TPC, TFC, gallic, chlorogenic, caffeic, and ellagic acids) when subjected to moderate and severe water stress compared to adequately watered plants (control samples). Only quercitrin content showed no change in the stressed compared to the control plants (Table 2).

The leaves of the moderate stress and control samples of the moderately tolerant clone (EGU2) present the same level of phenolic compounds. However, a slight increase in these compound contents could be observed when this clone was subjected to severe drought stress. The clone sensitive to drought (EGR) showed no quantitative difference in quercitrin levels among stressed and no-stressed plants. The levels of chlorogenic, caffeic, and ellagic acids in leaves were found lower with severe stressed compared to moderate stressed plants. The clone in test phase (EGU3) exhibited higher amount of leaf phenolics and flavonoids in stressed plants when compared to adequately watered plants, with the highest levels revealed from the severe stressed samples (Table 2). A plant that has the ability to protect itself against ROS generation by environmental stress can be considered tolerant and reduce the oxidative damage *via* the activation of antioxidant metabolites, *i.e.*, accumulation of phenolic compounds (Hura et al., 2009; Boscaiu et al., 2010; Yadav et al., 2014). The increase of phenolic compounds in *Triticum aestivum* L. genotypes is suggested to be closely related to water deficit tolerance (Ma et al., 2014). The effect of prolonged water stress treatment in *Artemisia annua* L. led to an increase in phenolic content, *ca*. 300 and 960% higher due to mild and moderate water stress, respectively (Yadav et al., 2014). Regarding with the clone's chemical responses to water stress, it was found that each clone reacted in a specific manner to such stress. In other words, stress can affect the performance and survival of these plants differently. The effects of abiotic stress in plants are dependent on numerous factors linked to the plant's performance (Gall et al., 2015). Warren et al. (2012) investigated the response of polar metabolites in *E. pauciflora* and *E. dumosa* leaves to long and severe water stress and revealed that even closely related species responded differently to this stress. In the EGU1 and EGU3 clones, phenolic content increased with both water stress treatments when compared with the control samples. Additionally, these two control clones clustered together in the PCA score plot suggesting their relatedness and or similar secondary metabolic pathways regulatory network (Fig 3). Although, there is no previous phenotypic information for EGU3 with regard to its drought tolerance, our results indicate EGU3 is similar to EGU1 clone. This data suggests that as well as EGU1, EGU3 is tolerant to drought. Another response pattern was detected in plants (Król et al., 2014). Young *E. globulus* and *E. viminalis* trees subjected to limited water availability for 3 months have shown a decrease in phenolic concentrations (McKiernan et al., 2014). Leaves from both species in the moderate- and low-water treatments contained respectively 17 and 19% less phenolic content than the control samples. Similarly, the caffeic acid content in the moderate stressed plants of EGU2 clone was lower than in the adequately watered plants of this clone. EGU2 is moderately tolerant to water deficit and the decrease of caffeic acid content in response to drought can be associated to a relatively susceptibility to this stress. For the drought sensitive clone (EGR), HPLC analyses revealed that the levels of some foliar phenolic acids were lower in the severe stress than moderate stress treatment. Such specific response pattern can be related with the plant failure to mitigate against water stress. The biosynthesis of phenolics for protection against abiotic stress can be controlled by several regulatory signals or networks at the genetic and epigenetic levels, where they are defined from how a specific plant lineage reacts in the course of its evolution to the environmental challenges for survival. Some plants are able to adapt to these ecological changes and reprogram their metabolism to reach a status that ensures fitness and endurance (Sanchez et al., 2012; Cheynier et al., 2013).

Materials and Methods

Chemicals

Quercitrin was previously isolated by our group. Milli-Q water was used for high performance liquid chromatography (HPLC) analysis; HPLC-grade methanol and acetonitrile were supplied from Tedia Brazil (Rio de Janeiro, Brazil). All other chemicals and standards were purchased from Sigma Aldrich (St. Louis, MO, USA) and were used for phenolics detection and quantification.

Plant material

Plants with the same age (110 days) of four commercial hybrids of *E. grandis* (EGU1-3 and EGR) were obtained from Suzano Pulp and Paper Company (São Paulo, Brazil). According to a confidentiality contract, the identification of the genotypes that is adopted by the Suzano Pulp and Paper Company were suppressed. Arbitrary identification was given to identify the clones as depicted in Table S1.

The seedlings were transferred into pots (2 L) filled with commercial substrate (Santa Carolina, Brazil) supplemented with osmocot formulation and grown for 30 days under optimal conditions (mean temperature: 25°C, natural light, and daily irrigation) in a greenhouse at the CLONAR Company (Cajuri, Brazil). The conduction of plants was performed according to standard protocols of CLONAR Company.

Water stress induction

After the acclimatizing period, the plants were randomly divided into three groups: control (C), moderate and severe water simulated stress treatments, with five plants of each clone line in each treatment. The experimental conditions were as follows:

- Control samples (C): Plants watered daily at least two times per day (adequately watered);
- Moderate and severe water stress samples: Artificial water stress induced with polyethylene glycol PEG-6000 solutions. The use of high molecular weight PEG is a common approach in physiological experiments to induce osmosis and simulate drought stress (Hamayun et al., 2010; Yu and Li, 2014). The plants exposed daily to 100 mL 100 g L^{-1} PEG-6000 (moderate water stress samples) and 100 mL of 300 g L^{-1} PEG-6000 (severe water stress samples). In addition, all plants received standard irrigation.

The level of stress provided to the plants was defined in previous experiments, for the same genotypes and treatments, where the methodology for water deficit simulation was established. The substrate water potential was measured for every seedling pot on the last day of the experiment and was determined using a crioscope (ITR model MK540). The sample freezing point in Horvet degrees (°H) were converted to MegaPascal (MPa) according to Szijarto and van de Voort (1983). Higher values mean more water availability conditions and were found for the control samples, whereas medium and higher values denoted for drier conditions and were found for the moderate and severe water stressed samples, respectively. The water potential in control, 100 PEG and 300 PEG were equivalent to -0.08, -0.35 and -0.48 MPa, respectively.

Treatments and controls included five replicates, producing 60 experimental units. These treatments were carried out for 60 days. Thereafter, twenty fully expanded leaves were collected dried at room temperature and stored prior to analysis.

Phenolics extraction procedure

All leaves collected from the same seedling were pooled in a single sample. These samples were ground with a pestle in a mortar into small pieces. One gram was extracted with 100% methanol (10 mL) using ultrasound bath for 30 min. The extracts were then filtered through Whatman filter paper No. 1 and concentrated to dryness.

Total phenolics content (TPC) assay

Total phenolics were determined using the Folin–Ciocalteu colorimetric method (Hosu et al., 2014). This assay measures the total content of phenolic hydroxyl groups and is an auxiliary technique in determining the antioxidant capacity (Karabourniotis et al., 2014). The method principle is the electrons transference from phenolic molecules to phosphomolybdic/phosphotungstic acids in basic medium forming blue complexes (Costa et al., 2015). Briefly, 0.5 mL of Folin–Ciocalteu reagent (10%) was added to 0.120 mL of the appropriately diluted crude extract. The reaction mixture was allowed to react for 5 min, and 0.4 mL of 7.5% sodium carbonate was then used to neutralize the mixture. Samples were incubated at room temperature, in a dark place for 120 min, and absorbance at 765 nm was measured with a spectrophotometer (BioTek, Winooski, USA). The content of total phenolics was calculated using a calibration curve of gallic acid (the linearity range: 0–300 µg mL^{-1}, R^2 = 0.9988) and expressed as mg gallic acid equivalents (GAE) per g dry weight.

Total flavonoids content (TFC)

Total flavonoids were determined using the aluminum chloride colorimetric method described previously (Hosu et al., 2014), with slight modifications. Briefly, 0.1 mL of the crude extracts was treated with 0.1 mL of 2.5 µg mL^{-1} $AlCl_3$. After 30 min, the absorbance of the mixture at 410 nm was measured with a spectrophotometer. The content of total flavonoids was calculated using a calibration curve of quercetin (linearity range: 0–250 µg mL^{-1}, R^2 = 0.9956) and expressed as mg quercetin equivalents (QE) per g dry weight.

DPPH radical scavenging assay

The free radical scavenging activity of the samples was measured *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brem et al., 2004; Borrás-Linares et al., 2015). Briefly, 0.1 mL of 0.03 mM DPPH was added to 0.1 mL extract at different concentrations (0–200 µg mL^{-1}). After 30 min of incubation in the dark at room temperature, the absorbance at 518 nm was measured with a spectrophotometer. Ascorbic acid was used as the reference. Percentage inhibition of DPPH free radical was calculated on basis of the control reading by the following equation:

$$\text{DPPH scavenged (\%)} = \frac{(Acont - Asample)}{Acont} \times 100$$

Where, A_{cont} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the extract/standard.

The antioxidant activity of the extract was expressed as IC_{50}, which is the concentration (in µg mL^{-1}) of extract that inhibits the formation of DPPH radicals by 50%.

HPLC-UV analysis

The HPLC system consisted of an Autosampler SIL-20A (105 vial capacity), LC-20AD pumps, and an SPD-M10A diode array detector (DAD) (Shimadzu, Kyoto, Japan). The separation of the compounds was carried out with a gradient elution program at a flow rate of 1 mL min^{-1}, using an Ace C18 (250 mm × 4.6 mm × 5 µm) column supplied by Advanced Chromatography Technologies (Aberdeen, Scotland).

The injection volume in the HPLC system was 20 μL, and the mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). The following linear gradient was used: 0–3 min: 5% B, 3–15 min: 5–15% B, 15–50 min: 15–45% B, 50–70 min: 45–95% B, 70–80 min: 95% B, followed by re-equilibration of the column for 10 min before the next run. Double online detection was carried out in the DAD, at 280 and 340 nm, and ultraviolet (UV) spectra in the range 200–400 nm were also recorded. Before the injection, each extract was dissolved in $CH_3OH/CH_3CN/H_2O$ (1:1:1 v/v) HPLC grade to obtain a final extract concentration of 5 mg mL^{-1}, and then filtered through a 0.45 μm-PVDF syringe filter. Several phenolic compounds known as antioxidants and available in-house were injected under the same conditions as the extracts. The identified phenolics were confirmed by comparing their retention time, spectra as well as by adding the standard solution to the extract.

Phenolics quantification using HPLC-UV

Calibration curves were obtained by injecting caffeic, chlorogenic, ellagic, gallic acids and quercitrin standard solutions, each at five different concentrations between 1.25 and 100 μg mL^{-1} on the HPLC system using same gradient described above. In addition to the linearity, the limits of detection (LOD) and quantification (LOQ) were also estimated using the signal-to-noise ratio (S/N) approach (n = 5). The LOD and LOQ were determined as an S/N of 3:1 and 10:1, respectively. The calibration curves and additional relevant analytical data are shown in Table 1. The concentrations were calculated from the peak areas at 280 nm, where the mean value was determined from triplicate injections.

Statistical analyses

All determinations were carried out in triplicate and repeated on at least three different days. The half-maximal inhibitory concentration (IC_{50}) was calculated using the probit regression model (SPSS program, version 13.0), assuming a confidence level of 95% ($P < 0.05$). Differences between groups were analyzed by one-way analysis of variance (ANOVA) with the Bonferroni post-hoc test using GraphPad Prism® version 5.0, considering $P < 0.05$ as statistically significant. Multivariate data analysis was performed from quantified metabolites and total phenolics assay as variables using the program SIMCA-P Version 13.0 (Umetrics, Umeå, Sweden). All variables were mean centered and scaled to Pareto variance. Principal component analysis (PCA) was run for obtaining a general overview of the variance of metabolites among specimens.

Conclusion

Understanding the defense mechanisms against biotic (pests and pathogens) and abiotic (drought) stresses in forest trees is an interesting strategy to enhance crop production. The results presented here showed that there are chemical composition differences between *Eucalyptus* hybrids according to their level of tolerance to stress conditions and that each of them reacted in a particular manner against water deficit. Furthermore, the leaf content analysis of phenolic acids, *viz*. caffeic acid and flavonoids is a reliable screening tool for drought tolerant hybrids in *Eucalyptus*. Therefore, phenolic measurements could be used as screening tool for assessment of tolerant plants. Such data can help researchers in defining indicators that correlate secondary metabolites *i.e.* phenolics and crop tolerance with some stress conditions and for future breeding efforts. Further studies including a large number of genotypes (inter- and intra-species/hybrids), morphophysiological and nutritional approach, and analysis of more classes of metabolites (untargeted metabolomics) should be performed to enable the identification of a comprehensive *Eucalyptus* response patterns. This knowledge can be used for the development of trees that are able to tolerate drought conditions but not to the detriment of yield by minimizing the stress impact on wood productivity.

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