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Physiological Responses to Hypoxia and Manganese in *Eucalyptus* Clones with Differential Tolerance to Vale do Rio Doce Shoot Dieback

Ignácio Harguindeguy[(1)], Gustavo Franco de Castro[(1)*], Sarah Vieira Novais[(2)], Leonardus Vergutz[(3)], Wagner Luiz Araujo[(4)] and Roberto Ferreira Novais[(5)]

[(1)] Universidade Federal de Viçosa, Departamento de Solos, Programa de Pós-Graduação em Solos e Nutrição de Plantas, *Campus* Viçosa, Viçosa, Minas Gerais, Brasil.
[(2)] Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Programa de Pós-Graduação em Solos e Nutrição de Plantas, Piracicaba, São Paulo, Brasil.
[(3)] Universidade Federal de Viçosa, Departamento de Solos, *Campus* Viçosa, Viçosa, Minas Gerais, Brasil.
[(4)] Universidade Federal de Viçosa, Departamento de Biologia Vegetal, *Campus* Viçosa, Viçosa, Minas Gerais, Brasil.
[(5)] Universidade Federal de Viçosa, Instituto de Ciências Agrárias, *Campus* Rio Paranaíba, Rio Paranaíba, Minas Gerais, Brasil.

ABSTRACT: Vale do Rio Doce shoot dieback (VRDSD) is an anomaly whose cause seems to be associated with hypoxic conditions and their consequences (excess Mn and Fe) triggered by elevation of the water table in areas with poor drainage. Different plants have distinct survival strategies under this form of stress. The objective of this study was to understand the physiological responses involved in the differential tolerance of eucalyptus clones to VRDSD and their relationship to hypoxia and excess Mn. A hydroponic experiment was carried out using a 2 × 2 × 2 factorial arrangement, two eucalyptus clones with different levels of tolerance to VRDSD (sensitive Urograndis hybrid - 1213; and the tolerant Rio Claro hybrid - *Eucalyptus grandis* x unknown - 2719), two concentrations of O_2 (8 and 4 mg L^{-1}), and two Mn concentrations (1.39 and 300 mg L^{-1}) in a randomized block design (RBD) with three replicates. Forty-day-old clones were maintained in Clark nutrient solution for 30 days. After this period, the treatments were applied for 11 days. Plant gaseous exchange shoot and root production, and the quantity of enzymes related to oxidative stress in leaves and roots were evaluated. In the tolerant clone, reactive oxygen species (ROS) were produced under hypoxic conditions, accompanied by reduction in production of dry matter, malondialdehyde (MDA), and in activity of the enzyme alcohol dehydrogenase (ADH). However, this clone had greater production of superoxide dismutase (SOD) under these conditions, an enzyme responsible for detoxification of ROS, which acts as part of the Low Oxygen Quiescence Syndrome (LOQS). In contrast, sensitive clones did not exhibit expressive reductions in growth or changes in the leaf/root ratio. These clones formed large quantities of adventitious roots and had high levels of MDA and ADH and low levels of SOD. Therefore, sensitive clones appear not to be prepared for detoxification of ROS and other toxic metabolites, but rather adopt morphological escape mechanisms, the Low Oxygen Escape Syndrome (LOES), in response to hypoxia. Thus, the period of soil waterlogging may cause the death of large numbers of roots in sensitive clones, limiting their ability to absorb water and nutrients and culminating in the death of these plants. Excess Mn seems to aggravate the damage caused by hypoxia, but it is not the causal agent of VRDSD.

Keywords: VRDSD, water table fluctuation, soil waterlogging, hydric stress, Mn toxicity.

*** Corresponding author:**
E-mail: gustavofcastro@ymail.com

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INTRODUCTION

Vale do Rio Doce shoot dieback in eucalyptus (VRDSD) occurs with higher intensity in soils located at the bottom of valleys (Inceptisols) and in soils of alluvial origin (Fluvisols) in locations with poor drainage (Leite et al., 2014). In rainy periods, eucalyptus from the lower third to the middle third of slopes may exhibit symptoms that appear, predominately, in the apical region of the plants, unlike symptoms induced by Ca and/or B deficiency that are commonly reported in Cerrado (Brazilian tropical savanna) regions with planted eucalyptus (Gonçalves et al., 2016). The symptoms of VRDSD, characterized by brown lesions, wilting, rapid leaf loss, and wrinkling and cracks on the lateral branches, evolve rapidly, leading to the death of the apical meristem and eventually the whole plant (Ferreira and Milani, 2002; Leite et al., 2014).

According to Leite et al. (2014), the hypoxic condition (low oxygen concentration) and its consequences (Mn and Fe toxicity) are the principle causes of VRDSD. Clones sensitive to VRDSD have high levels of Mn in the leaves. These authors assume that the highest intensity of symptoms observed at the age of establishment (third or fourth year after planting) are due to conditions favorable to disease development at that time. As the planted forest ages, developing greater leaf area, the transpiration rate increases, thus reducing the hypoxia condition by increasing the water flow through the system (soil/plant/atmosphere), such that VRDSD is not observed in plantations over four years old.

To overcome the problems caused by hypoxia, some plants adopt morphological escape mechanisms, known as Low Oxygen Escape Syndrome (LOES) (Voesenek and Bailey-Serres, 2015). Such mechanisms have high energetic costs, as they are activated by specific genes and are commonly found in plants adapted to long periods of waterlogging. Phenotypic traits, found in plants tolerant to VRDSD, include the formation of aerenchyma, the development of adventitious roots, anatomical leaf modifications, and gas pressurization by the porous tissues (Voesenek and Bailey-Serres, 2015). *Eucalyptus camphora* plants survived for 12 months in a waterlogged environment by forming adventitious roots and aerenchyma in the stem and roots (Greet, 2015).

Another response to hypoxia in plants is known as Low Oxygen Quiescence Syndrome (LOQS) (Voesenek and Bailey-Serres, 2015). The response of species with this characteristic is related to the mechanisms of quiescence, such as changes in metabolic pathways, increase in energetic efficiency, adjustment between the production and consumption of ATP, maintenance of cytosolic pH, and damage reduction by the elimination of reactive oxygen species (ROS) or other toxic compounds (Voesenek and Bailey-Serres, 2013). Fermentation is an alternative pathway for the production of pyruvate under hypoxia, normally produced in the Krebs cycle, when oxygen is available. However, fermentation has disadvantages in relation to the aerobic pathway, such as lower ATP production, accumulation of toxic metabolites, and reduction in cytosolic pH (Paul et al., 2016). The accumulation of metabolites, like lactate, alcohol, alanine, succinate, and even malate, are markers of active metabolic pathways (van Dongen and Licausi, 2015; Paul et al., 2016).

Although ROS are normally generated during abiotic stress as an escape mechanism, their accumulation causes oxidative damage to cells, such as chloroplasts and lipid membrane degradation. According to Ma et al. (2016), formation of ROS after three days of hypoxia caused a substantial loss in membrane selectivity, and these authors suggested that their formation is one of the first responses to hypoxia. Puyang et al. (2015) reported high degradation of the chloroplast after ROS generation, which was attributed to the chloroplast being the principal site of ROS production. Niu et al. (2017) reported the production of MDA, formed from lipid oxidation, as an indication of cell membrane damage due to ROS accumulation.

Finally, ROS production could also be associated with another consequence of hypoxia: excess Mn. Srivastava and Dubey (2011) reported high levels of ROS due to Mn toxicity in rice, which caused membrane damage. Consequently, enzymes of the antioxidant system, such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), and glutathione reductase (GR), increased their activities, mitigating oxidative stress caused by Mn phytotoxicity. Similar reports were made by Chen et al. (2016) and Moradi et al. (2017) in soybean.

Most studies that differentiate the responses in LOQS and LOES are carried out regarding herbaceous plants. Few papers working with woody plants evaluated how the differential responses of these plants influence root survival in deep soil and, even less, their responses to wetting and drying cycles, which appear to be associated with VRDSD. Additionally, it is speculated that VRDSD represents the symptoms of the damage caused by hypoxia, which may be intensified by Mn excess in waterlogged conditions.

Therefore, the aim of the present study is to understand the physiological responses involved in differential tolerance of *Eucalyptus* clones to VRDSD and their relationship to hypoxia and excess Mn.

MATERIALS AND METHODS

A 2 × 2 × 2 factorial arrangement with two clones, two levels of oxygenation, and two concentrations of Mn was conducted in a greenhouse of the Soil Science Department of the Federal University of Viçosa from October to November 2012. The two *Eucalyptus* clones, recognized as having different sensitivities to VRDSD based on field observations, were produced by Nipo-Brasileira Cellulose S/A: Urograndis hybrid-1213 sensitive to VRDSD, and Rio Claro hybrid (*E. grandis* x unknown - 2719) tolerant to VRDSD. Oxygen was applied at two concentrations: normal (8 mg L^{-1}) and hypoxic (4 mg L^{-1}), adjusted through continuous injection of N_2. Manganese was applied at two concentrations: 1.39 and 300 mg L^{-1}, defined by previous experiments. The control treatment for both clones was defined as 8 mg L^{-1} O_2 and 1.39 mg L^{-1} Mn. The experiment was carried out in a randomized block design (RBD), with three replicates.

Initially, 40-day-old clones received half-strength Clark´s solution (Clark, 1975) at pH 5.5 with adequate oxygenation (8 mg L^{-1}) for 15 days. After this period of adaptation, the plants received full strength Clark´s solution for another 15 days. At the end of this 30-day period, the treatments were applied. The O_2 concentration was gradually reduced at 6-hour intervals until a concentration between 4 and 6 mg L^{-1} was reached. Eleven days after application of the treatments, the plants were harvested and divided into young leaves, old leaves, stem, and roots. Samples of fully expanded leaves and roots were frozen in liquid N_2 and stored at -80 °C until needed.

Nutrient levels in plants

The plant material (young leaves, old leaves, stem, and roots) was dried in a forced-air oven at 72 °C until constant weight. The weight was recorded and the material ground. The levels of Mg, Ca, Fe, Cu, K, Zn, and Mn were determined after nitric-perchloric acid digestion using atomic absorption spectroscopy; K was analyzed by flame photometry. The nitrogen contents were determined by the Kjeldahl method, as described by Bataglia et al. (1983).

Determination of gas exchange

Gas exchange was evaluated on the last day of the experiment, before material collection. The third or fourth completely expanded leaf from the apex of the orthotropic branch of the plants was used for this determination. The rates of net carbon assimilation (A), stomatal conductance (g_s), transpiration (E), and internal CO_2 concentration (C_i) were

measured between 7:00 and 11:00 a.m. under saturating artificial light (1,400 µmol m^{-2} s^{-1}) and environmental CO_2 concentration using an infrared gas analyzer (IRGA), LICOR model 6400XT (LI-COR, Lincoln, NE, USA). In all the analyses, the quantity of blue light applied was 10 % of the photosynthetically active radiation (*PAR*), in order to maximize stomatal opening. More details were described by Cavatte et al. (2012).

Determination of photosynthetic pigment levels

For evaluation of photosynthetic pigments, a 1 cm diameter leaf disc was placed in a glass tube with 7 mL of dimethylsulfoxide (DMSO) saturated with calcium carbonate and incubated in a water bath at 65 °C, in the dark, for 1.5 h. Absorbance was measured in a spectrophotometer at 665, 649, and 480 nm, as described by Wellburn (1994).

Enzymes and compounds of the antioxidant complex

To determine the enzymatic activity of SOD and catalase (CAT), an enzyme extract was prepared by macerating frozen plant tissue (0.060 g of leaf or 0.100 g of root) with 40 mg of PVPP (polyvinylpyrrolidone) in a chilled pestle and mortar. After that, 1 mL of pH 7.8 sodium phosphate buffer (100 mmol L^{-1} and 1 % Triton X-100) was added. The macerated material was centrifuged at 15,000 *g* for 20 min at 4 °C, and the supernatant was used for analysis of enzyme activity and quantification of total soluble proteins, as described by Bradford (1976).

Superoxide dismutase (EC 1.15.1.1) activity was measured by adding 30 µL of diluted (1:10) enzyme extract to 220 µL of reaction mixture containing 50 mmol L^{-1} sodium phosphate buffer (pH 7.8), 13 mmol L^{-1} methionine, 75 µL p-nitro blue tetrazolium (NBT), 0.1 mmol L^{-1} EDTA, and 2 µmol L^{-1} riboflavin. The reaction was carried out in an ELISA multi-well plate at 25 °C under fluorescent lighting (45 W). After 7.5 min exposure to light, the illumination was interrupted, and the formazan blue produced by the NBT photo-reduction was quantified in a spectrophotometer at 560 nm. The control reaction was kept in the dark for the same period (Giannopolitis and Ries, 1977). One unit of SOD is defined as the quantity of enzyme necessary to inhibit 50 % of the NBT photo-reduction (Beauchamp and Fridovich, 1971).

Catalase (EC 1.11.1.6) activity was determined by the consumption of H_2O_2 measured by the decrease in absorbance at 240 nm in 2 min, according to the method described by Aebi (1983). The reaction mixture is a 50 mmol L^{-1} potassium phosphate buffer (pH 7.0), with H_2O_2 at 40 mmol L^{-1} and 100 µL of the diluted enzyme extract (1:10), adjusted to a final volume of 1 mL. The control was the same reaction mixture without the enzyme extract. The calculations used the molar extinction coefficient of 36 mM^{-1} cm^{-1}, and a unit of catalase is defined as the quantity necessary to reduce the amount of H_2O_2 at a rate of 1 µmol min^{-1}.

The total antioxidant capacity was determined by the Ferric Reducing Ability of Plasma (FRAP) method, as described by Benzie and Strain (1996). Leaf and root tissues (0.050 and 0.100 g, respectively) were macerated with PVPP using a chilled pestle and mortar, and 1 mL of extraction sodium phosphate buffer [50 mmol L^{-1} (pH 6.8), 1 % Triton X-100, and 0.1 mmol L^{-1} EDTA] was added. The samples were centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatant was collected. The reaction mixture was prepared at a 10:1:1 ratio composed of 300 mmol L^{-1} acetate buffer (pH 3.6), 10 mmol L^{-1} 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mmol L^{-1} HCl, and 20 mmol L^{-1} $FeCl_3$. A volume of 2.5 µL of the enzyme extract for the roots or 5 µL of the diluted solution (1:100) were added, and the final reaction volume was completed to 300 µL in the ELISA plate wells. A standard curve using known $FeCl_2$ concentrations was prepared and incubated for 5 min at 37 °C, and absorbance was read at 593 nm. Total antioxidant capacity was expressed as µmol g^{-1} of dry matter.

Determination of oxidative damage

Leaf and root tissue (0.050 and 0.100 g, respectively) were macerated with 40 mg of PVPP using a chilled pestle and mortar. After the addition of 1 mL of extraction buffer containing 0.1 % (w/v) trichloroacetic acid (TCA), the samples were centrifuged at 12,000 *g* for 15 min at 4 °C. A volume of 500 µL of the supernatant was added to 1.5 mL of TCA 20 % (w/v) containing 0.65 % (w/v) of TBA (thiobarbituric acid) in a test tube with a screw top. The samples were vigorously mixed and incubated at 95 °C for 25 min in the dark and then cooled rapidly in an ice bath. After cooling the samples were centrifuged at 3,000 *g* for 10 min and the absorbance of the supernatants was read at 440, 532, and 600 nm in an ELISA plate reader. Calculations were made as suggested by Du and Bramlage (1992).

Hypoxia-responsive enzyme activity

The quantity of 0.110 g of frozen root tissue was macerated with 40 mg of PVPP in a chilled pestle and mortar. Then 1 mL of extraction buffer [50 mmol L^{-1} potassium phosphate (pH 7.0), 1 % Triton X-100, 10 mmol L^{-1} DTT (dithiothreitol), 1 mmol L^{-1} PMSF (phenylmethylsulfonyl fluoride), 10 mmol L^{-1} $MgCl_2$, and 1 mmol L^{-1} EDTA] was added. The material was centrifuged at 12,000 *g* for 15 min at 4 °C and the supernatant removed and aliquoted for enzyme analyses and quantification of total soluble proteins, as described by Bradford (1976).

The activity of Alanine Transaminase (AlaT; EC 2.6.1.2) was quantified as described by Good and Muench (1992). The reaction mixture was 100 mmol L^{-1} Tris-HCl (pH 8.0), 0.2 mmol L^{-1} NADH, 20 mmol L^{-1} L-Alanine, 1 unit of Lactate dehydrogenase, 30 µL of the enzyme extract, and 10 mmol L^{-1} 2-oxyglutarate (*Start*). To calculate the activity, the molar extinction coefficient of 6.22 mM^{-1} cm^{-1} was used, and one unit of AlaT was defined by the oxidation of 1 µmol min^{-1} of NADH.

The activity of Aspartate Transaminase (AspT; EC 2.6.1.1) was quantified using the method described by Griffith and Vance (1989). The reaction mixture was 100 mmol L^{-1} Tris-HCl (pH 8.0), 0.2 mmol L^{-1} NADH, 20 mmol L^{-1} L-Asparate, 30 µL of the enzyme extract, and 10 mmol L^{-1} 2-oxyglutarate (*Start*). To calculate the activity, the molar extinction coefficient of 6.22 mM^{-1} cm^{-1} was used, and a unit of AspT was defined by the oxidation of 1 µmol min^{-1} of NADH.

The activity of Alcohol dehydrogenase (ADH; EC 1.1.1.1) was quantified using the method of Kogawara et al. (2006). The reaction mixture contained 50 mmol L^{-1} Tris-HCl (pH 8.0), 2 mmol L^{-1} DTT, 0.25 mmol L^{-1} NAD^+, 30 µL of the enzyme extract, and 5 % ethanol. The activity was quantified by the direct oxidation of ethanol. To calculate the activity, the molar extinction coefficient of 6.22 mM^{-1} cm^{-1} was used, and a unit of ADH was defined by the oxidation of 1 µmol min^{-1} of NADH.

The data obtained were analyzed statistically according to an RBD, with the treatments arranged in a 2 × 2 × 2 factorial arrangement with two clones, two levels of oxygenation, and two concentrations of Mn, with three replicates. The data obtained were subjected to analysis of variance (Anova) and the means were compared using the *F* test ($p \leq 0.05$), with the help of Microsoft Excel® (Microsoft, Seattle, USA) software.

RESULTS

Dry matter production and root formation

The initial size of the plants used in the experiment was not the same, with the clone sensitive to VRDSD (1213) visibly bigger than the tolerant clone (2719). These differences were expected to reduce through the experiment, but this did not occur. Thus, at the

end of the experiment, in all treatments, the sensitive clone (1213) had a higher final dry matter weight. For that reason, these data are presented in a proportional value, comparing the effects of the treatments in relation to the control treatment (without hypoxia and with normal Mn concentration) for each clone.

The sensitive clone had a lower reduction in growth (approximately 9 %), whereas the tolerant clone exhibited a more pronounced reduction (approximately 44 %) under hypoxia. Manganese did not significantly influence any of the treatments (Figure 1). For the roots, the behavior between the clones was similar. Both had losses greater than 40 % in dry matter production under hypoxia (Figure 1). Again, no significant differences were observed in the presence of Mn.

This difference in response between the production of leaves and roots led to a difference in the relationship between leaves and roots under hypoxia. While the tolerant clone (2719) maintained the proportion, with a decline in the treatments under hypoxia (an average of 13.5 %), the sensitive clone exhibited an average increase of 67 % in this ratio.

In both clones, the formation of adventitious roots was observed; however, the intensity and frequency of this growth was greater in the sensitive clone (Figure 2).

Nutrient levels in plants

Hypoxia stimulated significant reductions in the levels of macro- and micronutrients, especially in the aboveground part, reaching contents considered low for Zn, Fe, and Mg (Table 1). In the roots, hypoxia elevated the levels of Fe in both clones, especially with the application of higher concentrations of Mn in the nutrient solution. In the tolerant clone (2719), the level of Fe in the roots increased 112 and 49 % for the

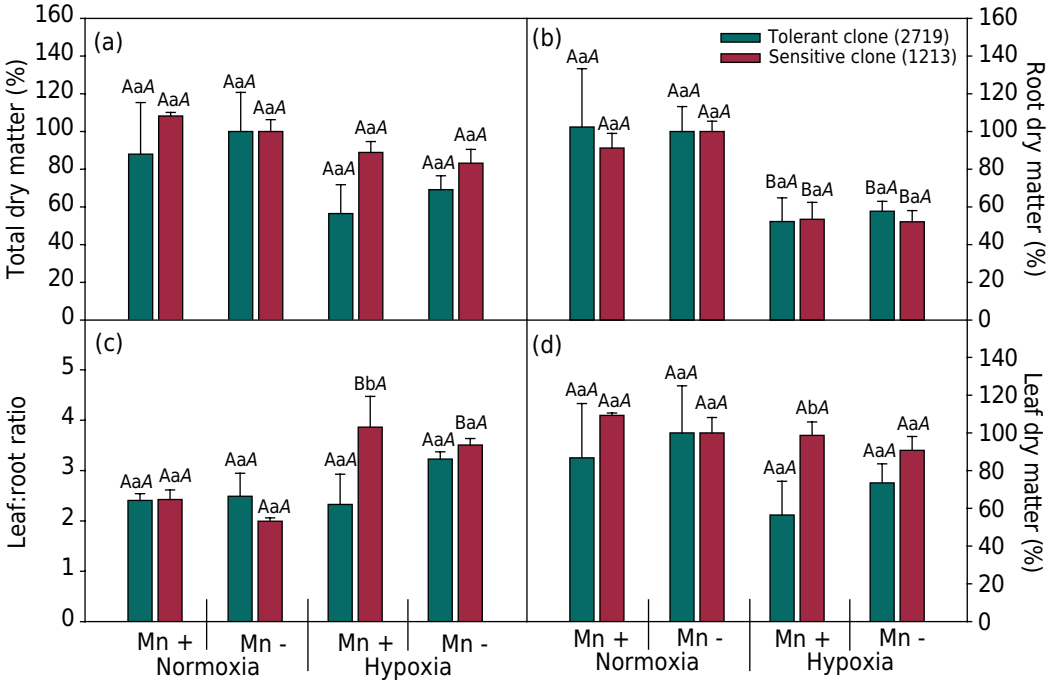

Figure 1. Dry matter production of the hydroponic experiment using two *Eucalyptus* clones. Total dry matter from the control treatment (a). Root dry matter from the control treatment (b). Relationship between leaf dry matter and root dry matter (c). Leaf dry matter relative to the control treatment (d). Uppercase letters compare the effect of O_2 (normoxia: 8 mg L^{-1}; and hypoxia: 4 mg L^{-1}) for the same clone and the same Mn concentration. Lowercase letters compare the effect of clones (tolerant and sensitive to VRDSD) for the same concentrations of Mn and O_2. Uppercase italic letters compare the effect of the Mn concentration of the nutrient solution (300 and 1.39 mg L^{-1}) for the same clone and the same O_2 concentration. Equal letters do not differ statistically at the level of 5 %.

Figure 2. Photographs of the experiment using two *Eucalyptus* clones with differential tolerance to VRDSD (a, b) subjected to different concentrations of O_2 (8 and 4 mg L^{-1}) and Mn (300 and 1.39 mg L^{-1}). Each experimental unit consists of two plants. The aboveground part (c) and root (d) of the tolerant clone under normoxia with 300 mg L^{-1} of Mn. Aboveground part (e) and root (f) of the sensitive clone under normoxia with 1.39 mg L^{-1} of Mn. Roots of the tolerant clone under hypoxia with 300 mg L^{-1} of Mn (g, h) and 1.39 mg L^{-1} of Mn (i) highlight the formation of less adventitious roots. Roots of the sensitive clone under hypoxia with 300 mg L^{-1} of Mn (j, k) and 1.39 mg L^{-1} of Mn (l, m) highlight the formation of more adventitious roots.

Table 1. Nutrient contents in young and old leaves, roots, and stem of two *Eucalyptus* clones, tolerant (2719) and sensitive (1213) to VRDSD, subjected to different concentrations of Mn (300 mg L^{-1} and 1.39 mg L^{-1}) and O_2 (normoxia: 8 mg L^{-1} and hypoxia: 4 mg L^{-1})

O_2	Mn	N	K	Ca	Mg	Mn	Cu	Fe	Zn
— mg L^{-1} —		g kg^{-1}				mg kg^{-1}			
					Young leaves				
					Tolerant clone				
8	300	3.28 Aa*A*	1.10 Aa*A*	1.01 Aa*A*	0.44 Aa*A*	2993 Aa*A*	10.4 Aa*A*	51.2 Aa*A*	30.2 Aa*A*
	1.39	4.08 Aa*B*	1.51 Aa*A*	1.02 Aa*A*	0.44 Aa*A*	381 Aa*B*	12.3 Aa*A*	79.5 Aa*A*	32.4 Aa*A*
4	300	2.38 Ba*A*	0.70 Aa*A*	0.76 Aa*A*	0.29 Aa*A*	1977 Ba*A*	5.6 Ba*A*	26.3 Aa*A*	14.7 Ba*A*
	1.39	2.35 Ba*A*	0.98 Aa*A*	0.65 Aa*A*	0.31 Aa*A*	243 Aa*B*	6.6 Ba*A*	34.1 Ba*A*	17.2 Ba*A*
					Sensitive clone				
8	300	3.41 Aa*A*	1.27 Aa*A*	0.95 Aa*A*	0.41 Aa*A*	5083 Ab*A*	11.2 Aa*A*	69.7 Aa*A*	35.5 Aa*A*
	1.39	3.51 Aa*A*	2.32 Aa*B*	1.03 Aa*A*	0.61 Aa*B*	557 Aa*B*	10.3 Aa*A*	70.3 Aa*A*	30.8 Aa*A*
4	300	2.14 Ba*A*	0.97 Aa*A*	0.69 Aa*A*	0.28 Aa*A*	2811 Ba*A*	5.8 Ba*A*	29.4 Ba*A*	14.6 Ba*A*
	1.39	2.22 Ba*A*	0.93 Ba*A*	0.73 Aa*A*	0.35 Ba*A*	315 Aa*B*	6.0 Ba*A*	30.9 Ba*A*	15.9 Ba*A*
					Old leaves				
					Tolerant clone				
8	300	2.89 Aa*A*	1.11 Aa*A*	1.01 Aa*A*	0.29 Aa*A*	2407 Aa*A*	11.3 Aa*A*	63.5 Aa*A*	32.4 Aa*A*
	1.39	3.13 Aa*A*	1.60 Aa*A*	1.32 Aa*A*	0.36 Aa*A*	714 Aa*B*	14.9 Aa*A*	99.3 Aa*B*	35.8 Aa*A*
4	300	2.30 Aa*A*	0.73 Aa*A*	1.10 Aa*A*	0.29 Aa*A*	1748 Aa*A*	6.3 Ba*A*	74.1 Aa*A*	23.6 Ba*A*
	1.39	2.50 Aa*A*	1.02 Ba*A*	1.05 Aa*A*	0.31 Aa*A*	629 Aa*B*	7.3 Ba*A*	63.7 Ba*A*	23.0 Ba*A*
					Sensitive clone				
8	300	3.24 Aa*A*	1.13 Aa*A*	1.03 Aa*A*	0.33 Aa*A*	3899 Ab*A*	12.9 Aa*A*	88.2 Aa*A*	26.8 Aa*A*
	1.39	3.48 Aa*A*	1.38 Aa*A*	1.24 Aa*A*	0.45 Ab*B*	777 Aa*B*	13.2 Aa*A*	99.3 Aa*A*	26.6 Ab*A*
4	300	2.30 Aa*A*	1.00 Aa*A*	0.74 Aa*A*	0.27 Aa*A*	1301 Ba*A*	7.7 Ba*A*	56.9 Ba*A*	17.4 Ba*A*
	1.39	2.14 Ba*A*	0.75 Ba*A*	0.74 Ba*A*	0.25 Ba*A*	608 Aa*A*	8.5 Ba*A*	57.0 Ba*A*	19.3 Aa*A*
					Roots				
					Tolerant clone				
8	300	2.01 Aa*A*	1.12 Aa*A*	0.99 Aa*A*	0.36 Aa*A*	3055 Aa*A*	60.5 Aa*A*	1691 Aa*A*	252 Aa*A*
	1.39	1.97 Aa*A*	0.88 Aa*A*	1.33 Aa*A*	0.37 Aa*A*	328 Aa*B*	57.9 Aa*A*	1863 Aa*A*	230 Aa*A*
4	300	1.65 Aa*A*	0.59 Aa*A*	1.91 Aa*A*	0.36 Aa*A*	4169 Aa*A*	83.1 Aa*A*	3588 Ba*A*	172 Aa*A*
	1.39	1.58 Aa*A*	0.84 Aa*A*	2.47 Ba*A*	0.37 Aa*A*	380 Aa*B*	54.9 Aa*B*	2780 Aa*A*	146 Aa*A*
					Sensitive clone				
8	300	2.26 Aa*A*	0.90 Aa*A*	1.12 Aa*A*	0.38 Aa*A*	2535 Aa*A*	28.6 Ab*A*	1453 Aa*A*	117 Ab*A*
	1.39	1.63 Aa*B*	1.42 Aa*A*	1.21 Aa*A*	0.45 Aa*A*	143 Aa*B*	27.9 Ab*A*	1662 Aa*A*	181 Aa*A*
4	300	2.35 Ab*A*	1.21 Ab*A*	1.43 Aa*A*	0.43 Aa*A*	6014 Bb*A*	63.7 Ba*A*	4258 Ba*A*	183 Aa*A*
	1.39	1.89 Aa*A*	0.94 Aa*A*	2.05 Aa*A*	0.44 Aa*A*	261 Aa*B*	47.3 Aa*A*	2989 Ba*B*	140 Aa*A*
					Stem				
					Tolerant clone				
8	300	1.16 Aa*A*	1.52 Aa*A*	1.05 Aa*A*	0.29 Aa*A*	1981 Aa*A*	12.0 Aa*A*	51.2 Aa*A*	33.6 Aa*A*
	1.39	1.10 Aa*A*	1.60 Ba*A*	1.63 Aa*A*	0.27 Aa*A*	280 Aa*B*	12.6 Aa*A*	56.7 Aa*A*	30.1 Aa*A*
4	300	1.08 Aa*A*	1.14 Aa*A*	1.21 Aa*A*	0.19 Ba*A*	1801 Aa*A*	8.1 Ba*A*	51.9 Aa*A*	22.6 Ba*A*
	1.39	1.04 Aa*A*	0.94 Aa*A*	0.88 Ba*A*	0.16 Ba*A*	156 Aa*B*	8.6 Ba*A*	40.7 Aa*A*	19.5 Ba*A*
					Sensitive clone				
8	300	1.08 Aa*A*	1.22 Aa*A*	1.31 Aa*A*	0.25 Aa*A*	2749 Ab*A*	13.4 Aa*A*	46.3 Aa*A*	25.5 Aa*A*
	1.39	1.06 Aa*A*	1.35 Aa*A*	2.18 Aa*B*	0.34 Aa*B*	237 Aa*B*	15.0 Aa*A*	53.2 Aa*A*	21.1 Ab*A*
4	300	0.88 Aa*A*	1.11 Aa*A*	1.29 Aa*A*	0.13 Ba*A*	1704 Ba*A*	7.5 Ba*A*	23.4 Bb*A*	12.8 Bb*A*
	1.39	0.92 Aa*A*	1.23 Aa*A*	1.59 Aa*A*	0.16 Ba*A*	110 Aa*B*	7.6 Ba*A*	37.9 Aa*A*	12.5 Ba*A*

Uppercase letters compare the effect of the O_2 concentration for the same clone and the same Mn concentration. Lowercase letters compare the effect of clones for the same concentrations of Mn and O_2. Uppercase italic letters compare the effect of the Mn concentration of the nutrient solution for the same clone and the same concentration of O_2. The determination of nutrient contents was performed according to Malavolta et al. (1997). Equal letters do not differ statistically at the level of 5 %.

concentrations of 300 mg L^{-1} and 1.39 mg L^{-1} Mn, respectively. However, in the sensitive clone, the level of Fe in the roots increased 193 and 79 % for the same concentrations of Mn, respectively.

In both clones, hypoxia reduced Mn levels in young leaves by 34.1 % for the tolerant clone and 44.1 % for the sensitive one. Under hypoxia, in the presence of excess Mn, the Mn levels in the roots of the tolerant clone increased from 3,055 to 4,169 mg kg^{-1} and, in the sensitive one (1213), from 2,535 to 6,014 mg kg^{-1}.

Photosynthetic rate, gas exchange, and pigments

The photosynthetic rate in both clones behaved similarly, with significant reductions (37 %) caused by hypoxia, with no apparent effects from Mn (Figure 3). The same happened with stomatal conductance, which was 70 % lower in relation to normoxia (normal oxygen levels). However, internal CO_2 did not exhibit the same changes in these treatments. The chlorophyll level behaved similarly in both clones, with reductions up to 12.92 % caused by hypoxia, with no significant effects from Mn. However, these reductions did not change the chlorophyll *a*/chlorophyll *b* ratio (Figure 3).

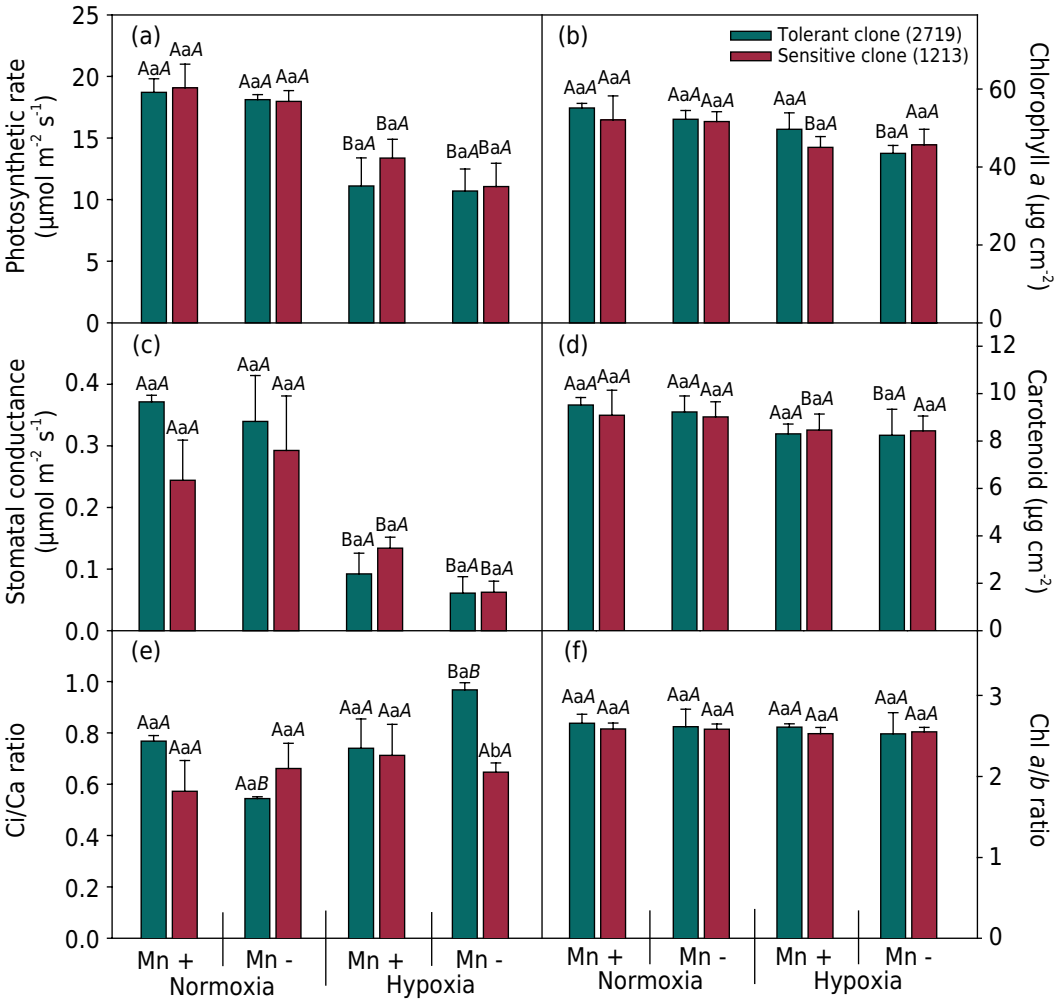

Figure 3. Gas exchange and pigment levels in leaves from two *Eucalyptus* clones in hydroponics in response to the treatments. Photosynthetic rate (a); Level of chlorophyll *a* (b). Stomatal conductance (c). Carotenoid level (d). Internal carbon concentration (e). Chlorophyll *a* to chlorophyll *b* ratio (f). Uppercase letters compare the effect of O_2 (normoxia: 8 mg L^{-1} and hypoxia: 4 mg L^{-1}) for the same clone and the same Mn concentration. Lowercase letters compare the effect of clones (tolerant and sensitive to VRDSD) for the same concentrations of Mn and O_2. Uppercase italic letters compare the effect of the Mn concentration of the nutrient solution (300 and 1.39 mg L^{-1}) for the same clone and the same O_2 concentration. Equal letters do not differ statistically at the level of 5 %.

Cell damage and antioxidant complex in leaves

The level of MDA in the leaves of the tolerant clone (2719) increased in the treatments with the highest Mn concentration (300 mg L^{-1}) from 17 to 25 nmol g^{-1} per dry weight (Figure 4).

Although normoxia did not induce an increase in this metabolite in the tolerant clone (2719), in the sensitive one (1213), under conditions of low concentration of Mn, there was an increase, with an average value of 45 nmol g^{-1} (Figure 4).

The tolerant clone (2719) under normoxia and both Mn concentrations showed higher SOD activity in leaves (29.6 % higher) compared to the sensitive clone (1213) under the same conditions (Figure 4). However, levels of catalase in the leaves did not differ between clones, with small variations among treatments. Hypoxia caused increases of around 50 % in total antioxidant capacity, while the treatment with excess Mn (300 mg L^{-1}) had values that were, on average, 20 % lower in both clones.

Cell damage, antioxidant complex, and fermentative enzymes in roots

The level of MDA in the roots of the tolerant clone (2719) was stable in all the treatments (Figure 5). In contrast, in the sensitive clone (1213), MDA levels were influenced by hypoxia and the higher Mn application rate (300 mg L^{-1}). In the treatments under hypoxia with excess Mn, the sensitive clones had MDA levels that were, on average, 100 % higher than in the tolerant clone. Excess Mn in the roots of the sensitive clone under hypoxia increased MDA levels by 50 %.

The SOD activity in the roots was 38 % higher in the tolerant clone (2719) than in the sensitive clone (1213) under hypoxia at the higher Mn application rate (Figure 5). While the tolerant clone had slightly higher total antioxidant capacity in roots, under normoxia,

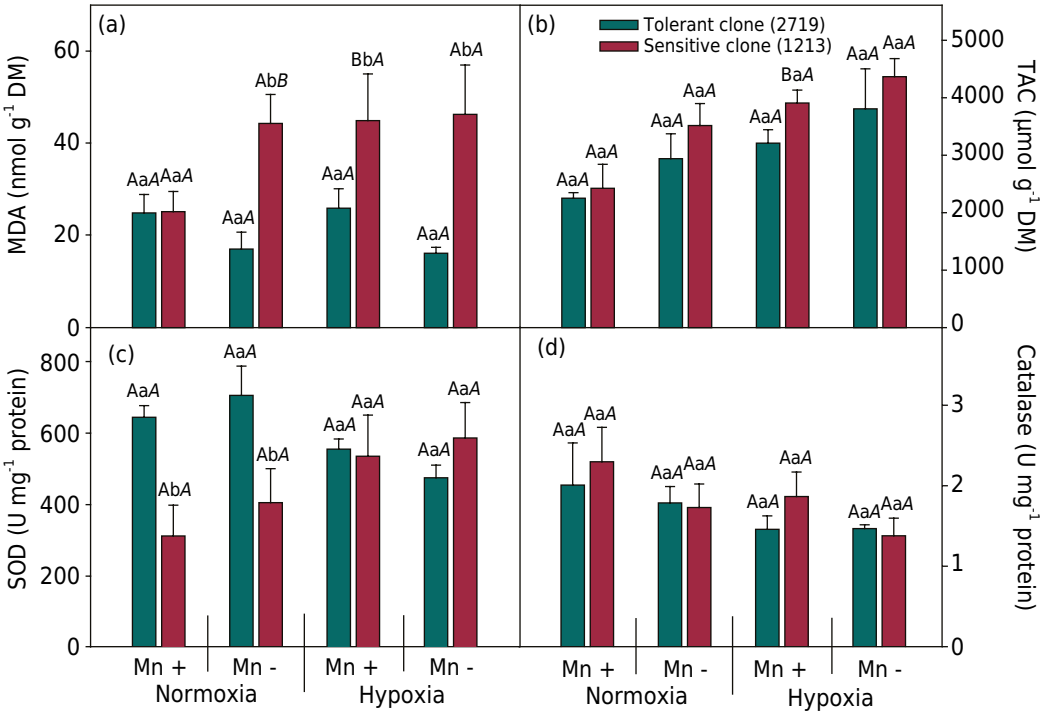

Figure 4. Enzymatic activity and metabolites in leaves from two *Eucalyptus* clones in hydroponics in response to the treatments. Level of malondialdehyde (a); total antioxidant capacity (b); superoxide dismutase activity (c); and catalase activity (d). Uppercase letters compare the effect of O_2 (normoxia: 8 mg L^{-1} and hypoxia: 4 mg L^{-1}) for the same clone and the same Mn concentration. Lowercase letters compare the effect of clones (tolerant and sensitive to VRDSD) for the same concentrations of Mn and O_2. Uppercase italic letters compare the effect of the Mn concentration of the nutrient solution (300 and 1.39 mg L^{-1}) for the same clone and the same O_2 concentration. Equal letters do not differ statistically at the level of 5 %.

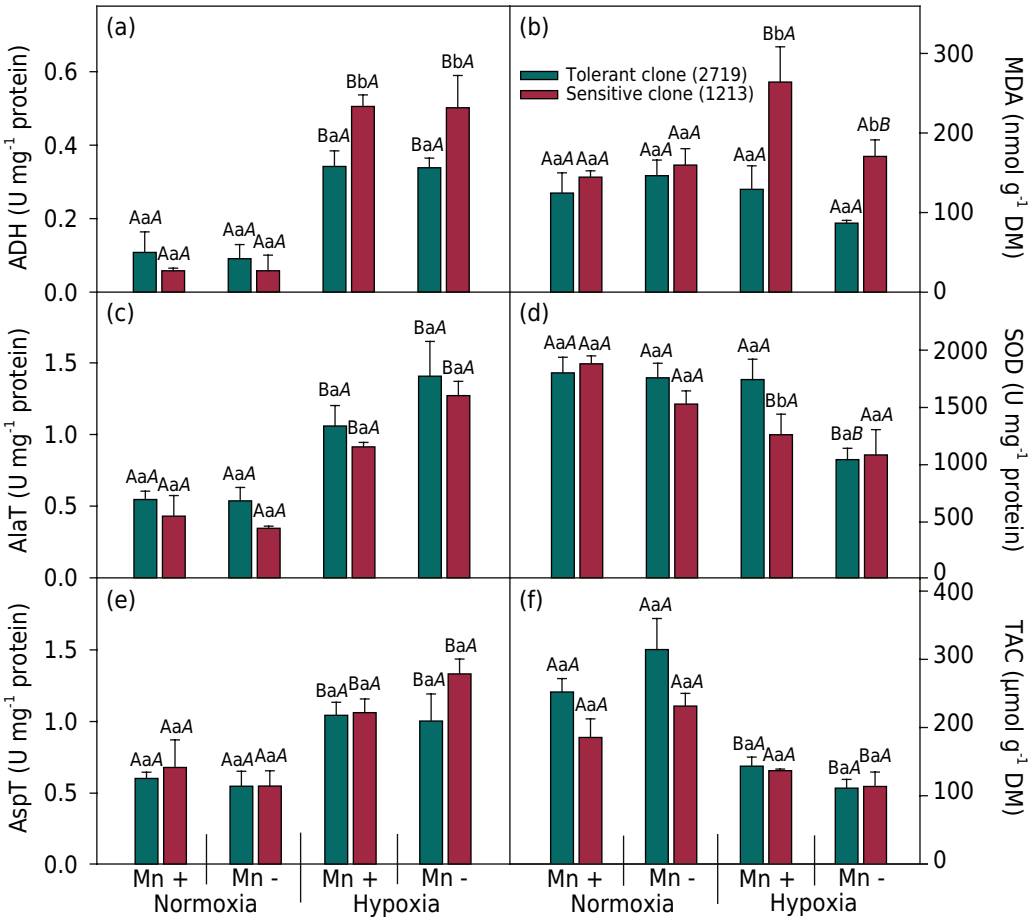

Figure 5. Enzymatic activity and metabolites in roots from two *Eucalyptus* clones in hydroponics in response to the treatments. Alcohol dehydrogenase activity (a). Level of malondialdehyde (b). Alanine transaminase activity (c). Superoxide dismutase activity (d). Aspartate transaminase activity (e). Total antioxidant capacity (f). Uppercase letters compare the effect of O_2 (normoxia: 8 mg L^{-1} and hypoxia: 4 mg L^{-1}) for the same clone and the same Mn concentration. Lowercase letters compare the effect of clones (tolerant and sensitive to VRDSD) for the same concentrations of Mn and O_2. Uppercase italic letters compare the effect of the Mn concentration of the nutrient solution (300 and 1.39 mg L^{-1}) for the same clone and the same O_2 concentration. Equal letters do not differ statistically at the level of 5 %.

it showed lower SOD activity and a lower total antioxidant capacity, similar to roots in the sensitive clone under hypoxia. The sensitive clone showed higher ADH activity in roots under hypoxia compared to the tolerant clone (Figure 5). However, AlaT and AspT had similar responses in both clones.

DISCUSSION

Higher production of dry matter in the sensitive clone compared to the tolerant one (Figure 1) was explained by the escape mechanism adopted by these plants. Whereas the sensitive clone used LOES, as evidenced by the greater formation of adventitious roots in this plant (Figure 2), the tolerant clone used LOQS, using differentiated metabolic routes. Even though alternative metabolic routes ensure survival under stress, they have disadvantages, including lower production of ATP and accumulation of toxic metabolites, resulting in less growth for these plants (Voesenek and Bailey-Serres, 2015). The higher leaf/root ratio of the susceptible clone under hypoxia is contrasted by leaf production cessation in the tolerant clone, which does not occur in the sensitive clone (Figure 2), a common escape strategy for this plant (Voesenek and Bailey-Serres, 2015).

Lower values of MDA and higher values of SOD in leaves and roots are in agreement with the lower dry matter production of the tolerant clones and the escape mechanism used

(LOQS). Malondialdehyde is a compound produced by lipid oxidation, characteristic of ROS formation, while SOD is the first ROS detoxification enzyme to be induced (Voesenek and Bailey-Serres, 2013). Thus, the higher production of SOD in the tolerant clone corroborates the hypothesis that such plants have a greater ability to avoid the formation of ROS, as well as to detoxify them. In addition to higher production of ROS in tolerant clones, there is also greater accumulation of these metabolites in certain parts of the plant (Wang et al., 2016). These authors found concentrations up to three times higher in the elongation zone (roots) compared to the concentrations found in the mature part (shoot). Bailey-Serres et al. (2012) observed that the plant with a quiescent response had higher antioxidant activity, as well as lower oxidative damage in the submerged tissues.

Higher ADH in the tolerant clone also corroborates the hypothesis of the predominant quiescent response of these plants. Plants with quiescent responses temporarily intensify the activity of this enzyme, which decreases in the following days (Ismail, 2013). Similarly, ADH activity that intensified in the first few days in the submerged tissue of açaí palm was observed by Gonçalves et al. (2010).

However, differential fermentation rates do not directly confer greater tolerance to hypoxia. Thus, there is also a relationship between an increase in cell damage and increased ADH activity, since the inverse reaction may occur, producing acetaldehyde (Wu et al., 2015). These escape responses may offer a palliative solution for the sensitive clone (1213), which allows survival during the period of hypoxia, but this makes the plant more susceptible to subsequent water deficit (Voesenek and Bailey-Serres, 2015). During waterlogging, sensitive plants loose a large part of their deep root system, but maintain the aboveground part. After waterlogging, comes a period of intense evapo-transpiration, in which the reduced root system is not able to meet this demand for water, culminating in symptoms of VRDSD and plant death.

Significant reductions in macro- and micronutrients in the leaves were influenced by defense mechanism commonly observed in several species subjected to high concentrations of nutrients and characterized by the formation of binding sites in the root cell walls (Reichman, 2002; Gandini et al., 2017). Thus, for toxic levels of Zn and Cu, for example, higher contents of these nutrients are observed in the roots. Therefore, it is possible that the clones subjected to an excessive application rate of Mn, produced a greater number of binding sites in response. The accumulation of Fe in the roots was observed in a similar experiment using the same clones as in this study (Lacerda, 2013).

Reduction in nutrient uptake capacity causes reductions in the aboveground part. This is partially explained by a decrease in water absorption, which reduces transport. Another contributing factor is a decrease in active uptake by the roots, caused by the limitation of available energy after glycolysis reduction (Parent et al., 2008; Marschner, 2011; Tóth, 2016). A quiescent root tissue should reduce energy expenditure to a minimum, as well as the active uptake of nutrients.

Manganese seems to play a secondary role in these phenomena as a consequence of hypoxia, and its damage intensifies during water deficit. Along with reduction in uptake of other nutrients by hypoxia, the high leaf/root ratio, and the lower uptake of water and nutrients in the dry period due to lack of deep roots comes an increase in the Mn/Mg ratio, which may intensify leaf damage (Le Bot et al., 1990; Davis, 1996), causing early senescence.

Reductions in photosynthetic rate and stomatal conductance were not attributed to CO_2 deficiency for either clone, since the internal CO_2 did not undergo such changes. Trees in tropical areas with seasonal floods have a similar response (Rengifo et al., 2005), with a reduction in photosynthetic rate and stomatal conductance, but an accumulation of starch in leaves. The accumulation of starch and sugars in leaves compromises photosynthetic efficiency due to feedback-inhibition (Kreuzwieser et al., 2004; Dalberto et al., 2017).

The normal chlorophyll *a*/chlorophyll *b* ratio corroborates the stable internal CO_2 values (Figure 3). Since light energy was not reduced, it can be concluded that the reduction in photosynthetic rates is caused by other limiting factors. A limitation in the activation or action of Rubisco, imbalances in the Calvin cycle, or even feedback-inhibition due to sugar accumulation may have occurred, resulting in this decrease. Feedback-inhibition by the accumulation of photoassimilates is commonly observed in plants with roots under hypoxia (Kreuzwieser et al., 2004; Rengifo et al., 2005). Regardless of the form of photosynthetic inhibition, excess light energy must be adequately dissipated or it may cause cell damage (Carvalho and Amancio, 2002; AbdElgawad et al., 2016).

The high antioxidant capacity of the leaves, which was higher in the treatments with hypoxia than those treated with Mn, demonstrates that both clones respond to stress and the damage caused by Mn is minimal in comparison to that caused by hypoxia. *Prunus cerasifera* L. clones (Pistelli et al., 2012) tolerant and sensitive to hypoxia had high total antioxidant capacity in the leaves when the roots were under hypoxia. Lacerda (2013) demonstrated that the damage caused by hypoxia in the roots of these same clones was faster and more intense than the damage under high Mn concentrations.

CONCLUSIONS

Mechanisms of VRDSD tolerance appear to be related to and activated by hypoxia, since the morphological and quiescence responses were triggered only after waterlogging. Furthermore, although excess Mn appears to intensify some responses, its role in this anomaly seems to be secondary. The differential behavior of these clones after the treatments confirms the difference between genotypes. The tolerant clone (2719) responded in a manner characteristic of LOQS, suspending growth, especially in the leaves, and redirecting its energies for defense against and prevention of leaf and root damage. However, the sensitive clone (1213) responded in a manner characteristic of LOES, promoting the formation of roots near the surface to maintain the growth of the above ground part of the plant. If, on the one hand, this clone manages to maintain its high growth rate without changing the leaf/root ratio, on the other, it is less efficient in damage prevention, which compromises the survival of its deep roots during the period of hypoxia (which eventually comes to an end), resulting in difficulty in taking up water and nutrients.

REFERENCES

AbdElgawad H, Zinta G, Beemster GTS, Janssens IA, Asard H. Future climate CO_2 levels mitigate stress impact on plants: increased defense or decreased challenge? Front Plant Sci. 2016;7:1-7. https://doi.org/10.3389/fpls.2016.00556

Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. 3nd ed. Weinheim: Verlag Chemie; 1983. p.273-7.

Bailey-Serres J, Lee SC, Brinton E. Waterproofing crops: effective flooding survival strategies. Plant Physiol. 2012;160:1698-709. https://doi.org/10.1104/pp.112.208173

Bataglia OC, Furlani AMC, Teixeira JPF, Furlani PR, Gallo JR. Métodos de análise química de plantas. Campinas: Instituto Agronômico de Campinas; 1983. (Boletim Técnico, 78).

Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem. 1971;44:276-87.https://doi.org/10.1016/0003-2697(71)90370-8

Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay. Anal Biochem. 1996;239:70-6. https://doi.org/10.1006/abio.1996.0292

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54. https://doi.org/10.1016/0003-2697(76)90527-3

Carvalho LC, Amancio S. Antioxidant defence system in plantlets transferred from in vitro to ex vitro: effects of increasing light intensity and CO_2 concentration. Plant Sci. 2002;162:33-40. https://doi.org/10.1016/S0168-9452(01)00524-6

Cavatte PC, Oliveira AAG, Morais LE, Martins SCV, Sanglard LMVP, Damatta FM. Could shading reduce the negative impacts of drought on coffee? A morphophysiological analysis. Physiol Plantarum. 2012;144:111-22. https://doi.org/10.1111/j.1399-3054.2011.01525.x

Chen Z, Yan W, Sun L, Tian J, Liao H. Proteomic analysis reveals growth inhibition of soybean roots by manganese toxicity is associated with alteration of cell wall structure and lignification. J Proteomics. 2016;143:151-60. https://doi.org/10.1016/j.jprot.2016.03.037

Clark RB. Characterization of phosphatase of intact maize root. J Agr Food Chem. 1975;23:458-60. https://doi.org/10.1021/jf60199a002

Dalberto DS, Martinazzo EG, Hüther CM, Posso DA, Bacarin MA. Photosynthetic activity of young *Ricinus communis* L. plants under conditions of flooded soil. Semin-Cienc Agrar. 2017;38:73-84. https://doi.org/10.5433/1679-0359.2017v38n1p73

Davis JG. Soil pH and magnesium effects on manganese toxicity in peanuts. J Plant Nutr. 1996;19:535-50. https://doi.org/10.1080/01904169609365141

Du Z, Bramlage WJ. Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. J Agr Food Chem. 1992;40:1566-70.

Ferreira FA, Milani D. Diagnose visual e controle das doenças abióticas e bióticas do eucalipto no Brasil. 2nd ed. Viçosa: Editora UFV; 2002.

Gandini C, Schmidt SB, Husted S, Schneider A, Leister D. The transporter SynPAM71 is located in the plasma membrane and thylakoids, and mediates manganese tolerance in *Synechocystis* PCC6803. New Phytol. 2017;215:256-68. https://doi.org/10.1111/nph.14526

Giannopolitis CN, Ries SK. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 1977;59:309-14. https://doi.org/10.1104/pp.59.2.309

Gonçalves JFC, Lima RBS, Fernandes AV, Borges EEL, Buckeridge MS. Physiological and biochemical characterization of the assai palm (*Euterpe oleracea* Mart.) during seed germination and seedling growth under aerobic and anaerobic conditions. Rev Árvore. 2010;34:1045-53. https://doi.org/10.1590/S0100-67622010000600010

Gonçalves JLM, Alvares CA, Souza AHBN, Arthur Junior JC. Caracterização edafoclimática e manejo de solos das áreas com plantações de eucalipto. In: Schumacher MV, Viera M, organizadores. Silvicultura do Eucalipto no Brasil. Santa Maria: Editora UFSM; 2016. p.111-54.

Good AG, Muench DG. Purification and characterization of an anaerobically induced alanine aminotransferase from barley roots. Plant Physiol. 1992;99:1520-5. https://doi.org/10.1104/pp.99.4.1520

Greet J. The marked flooding tolerance of seedlings of a threatened swamp gum: implications for the restoration of critical wetland forests. Aust J Bot. 2015;63:669-78. https://doi.org/10.1071/BT15128

Griffith SM, Vance CP. Aspartate aminotransferase in alfalfa root nodules: I. Purification and partial characterization. Plant Physiol. 1989;90:1622-9. https://doi.org/10.1104/pp.90.4.1622

Ismail AM. Flooding and submergence tolerance. In: Kole C, editor. Genomics and breeding for climate-resilient crops. Vol. 2 Target traits. Berlin: Springer;2013. p.269-90. https://doi.org/10.1007/978-3-642-37048-9_7

Kogawara S, Yamanoshita T, Norisada M, Masumori M, Kojima K. Photosynthesis and photoassimilate transport during root hypoxia in *Melaleuca cajuputi*, a flood-tolerant species, and in *Eucalyptus camaldulensis*, a moderately flood-tolerant species. Tree Physiol. 2006;26:1413-23. https://doi.org/10.1093/treephys/26.11.1413

Kreuzwieser J, Papadopoulou E, Rennenberg H. Interaction of flooding with carbon metabolism of forest trees. Plant Biol. 2004;6:299-306. https://doi.org/10.1055/s-2004-817882

Lacerda FJP. Papel da hipóxia e do manganês na tolerância diferencial de clones de eucalipto à Seca de Ponteiro no Vale do Rio Doce [dissertação]. Viçosa: Universidade Federal de Viçosa; 2013.

Le Bot J, Goss MJ, Carvalho MJGPR, Beusichem MLV, Kirkby EA. The significance of the magnesium to manganese ratio in plant tissues for growth and alleviation of manganese toxicity in tomato (*Lycopersicon esculentum*) and wheat (*Triticum aestivum*) plants. In: van Beusichem ML, editor. Plant nutrition - Physiology and applications. Dordrecht: Kluwer Academic Publishers; 1990. p.223-8.

Leite FP, Novais RF, Silva IR, Barros NF, Neves JCL, Medeiros AGB, Ventrella MC, Villani EMA. Manganese accumulation and its relation to "eucalyptus shoot blight in the Vale do Rio Doce". Rev Bras Cienc Solo. 2014;38:193-204. https://doi.org/10.1590/S0100-06832014000100019

Ma Y, Zhu M, Shabala L, Zhou M, Shabala S. Conditioning of roots with hypoxia increases aluminum and acid stress tolerance by mitigating activation of K^+ efflux channels by ROS in barley: insights into cross-tolerance mechanisms. Plant Cell Physiol. 2016;57:160-73. https://doi.org/10.1093/pcp/pcv175

Malavolta E, Vitti GC, Oliveira SA. Avaliação do estado nutricional das plantas: princípios e aplicações. 2. ed. Piracicaba: Associação Brasileira para Pesquisa da Potafos; 1997.

Marschner P. Marschner's mineral nutrition of higher plants. 3rd ed. Amsterdam: Academic Press; 2011.

Moradi P, Ford-Lloyd B, Pritchard J. Metabolomic approach reveals the biochemical mechanisms underlying drought stress tolerance in thyme. Anal Biochem. 2017;527:49-62. https://doi.org/10.1016/j.ab.2017.02.006

Niu K, Ma X, Liang G, Ma H, Jia Z, Liu W, Yu Q. 5-Aminolevulinic acid modulates antioxidant defense systems and mitigates drought-induced damage in Kentucky bluegrass seedlings. Protoplasma. 2017;254:2083-94 https://doi.org/10.1007/s00709-017-1101-4

Parent C, Capelli N, Berger A, Crèvecoeur M, Dat JF. An overview of plant responses to soil waterlogging. Plant Stress. 2008;2:20-7.

Paul MV, Iyer S, Amerhauser C, Lehmann M, van Dongen JT, Geigenberger P. Oxygen sensing via the ethylene response transcription factor RAP2.12 affects plant metabolism and performance under both normoxia and hypoxia. Plant Physiol. 2016;172:141-53. https://doi.org/10.1104/pp.16.00460

Pistelli L, Iacona C, Miano D, Cirilli M, Colao MC, Mensuali-Sodi A, Muleo R. Novel *Prunus* rootstock somaclonal variants with divergent ability to tolerate waterlogging. Tree Physiol. 2012;32:355-68. https://doi.org/10.1093/treephys/tpr135

Puyang X, An M, Xu L, Han L, Zhang X. Antioxidant responses to waterlogging stress and subsequent recovery in two Kentucky bluegrass (*Poapratensis* L.) cultivars. Acta Physiol Plant. 2015;37:197. https://doi.org/10.1007/s11738-015-1955-z

Reichman SM. The responses of plant to metal toxicity: a review focusing on copper, manganese and zinc. Melbourne: Australian Minerals & Energy Environment Foundation; 2002.

Rengifo E, Tezara W, Herrera A. Water relations, chlorophyll *a* fluorescence, and contents of saccharides in tree species of a tropical forest in response to flood. Photosynthetica. 2005;43:203-10. https://doi.org/10.1007/s11099-005-0034-x

Srivastava S, Dubey RS. Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings. Plant Growth Regul. 2011;64:1-16. https://doi.org/10.1007/s10725-010-9526-1

Tóth VR. Reed stands during different water level periods: physico-chemical properties of the sediment and growth of *Phragmites australis* of Lake Balaton. Hydrobiologia. 2016;778:193-207. https://doi.org/10.1007/s10750-016-2684-z

van Dongen JT, Licausi F. Oxygen sensing and signaling. Annu Rev Plant Biol. 2015;66:345-67. https://doi.org/10.1146/annurev-arplant-043014-114813

Voesenek LACJ, Bailey-Serres J. Flood adaptive traits and processes: an overview. New Phytol. 2015;206:57-73. https://doi.org/10.1111/nph.13209

Voesenek LACJ, Bailey-Serres J. Flooding tolerance: O_2sensing and survival strategies. Curr Opin Plant Biol. 2013;16:647-53. https://doi.org/10.1016/J.PBI.2013.06.008

Wang F, Chen ZH, Liu X, Colmer TD, Shabala L, Salih A, Zhou M, Shabala S. Revealing the roles of GORK channels and NADPH oxidase in acclimation to hypoxia in Arabidopsis. J Exp Bot. 2016;12:3191-204. https://doi.org/10.1093/jxb/erw378

Wellburn AR. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol. 1994;144:307-13. https://doi.org/10.1016/S0176-1617(11)81192-2

Wu J, Dai Y, Rui S, Cui N, Zhong F, Cheng S. Acclimation of Hydrilla verticillata to sediment anoxia in vegetation restoration in eutrophic waters. Ecotoxicology. 2015;24:2181-9. https://doi.org/10.1007/s10646-015-1549-y