ORIGINAL ARTICLE

Gaseous Exchanges and Formation of Aerenchyma in Eucalyptus Clones Submitted to Hypoxia and High Manganese Content

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Abstract: In lowlands of Vale do Rio Doce regions it is observed the occurrence of Eucalyptus shoot blight from Vale do Rio Doce (ESBVRD). This anomaly has been observed in regions with a high incidence of rainfall and soils conditions that predispose flooding (hypoxia) and, consequently, Mn available excess. The objective of this work was to evaluate nutritional, physiological and morphological changes possibly involved in the differential tolerance of eucalyptus clones to ESBVRD, under hypoxia conditions and Mn excess. To this end, an experiment was carried out in nutrient solution, in a growth chamber. Two clones of eucalyptus, a sensitive (1213) and a tolerant (2719), were submitted to four O_2 concentrations (1; 4; 6 and 8 mg L^{-1}), at the presence of 30 mg L^{-1} of Mn, concentration considered excessive or toxic. The lengths of the root system and shoots were measured, as well as photosynthesis, stomatal conductance, transpiration and CO_2 internal concentration. Also, roots samples were taken near the apex for anatomical analyzes. At the end of the experiment (21 days), the plants were harvested and separated into old and new leaves, stem and root. Afterwards, the levels of Mn in the tissues were determined. The hypoxia condition limited the total dry matter, similar to both clones, although the root growth was the most sensitive indicator of stress condition caused by hypoxia, with more marked effect in the sensitive clone. The tolerant clone replied more clearly to the increase of O_2 availability (lower hypoxia), with the increased metabolism due to the greater stomatal conductance, photosynthetic rate, transpiration and CO_2 internal concentration, comparing to the sensitive clone. Hypoxia condition caused the most intense formation of aerenchyma in the sensitive clone's root system, even though the arenchyma formation was also observed in the tolerant clone. Mn excess did not imply in great responses.

***Keywords*:** Flooding; Photosynthesis; Shoot Blight of Vale do Rio Doce

1. Introduction

Although the causes that lead to a predisposition of eucalyptus plants to Eucalyptus shoot blight from Vale do Rio Doce (ESBVRD) is not well explained, field observations have indicated that its occurrence coincides with years of higher rainfall, with the groundwater elevation in the lowland areas (Almeida *et al*., 2013). Thus, the long saturation period of water in the soil, combined with its high density - which makes gases diffusion even more difficult - can lead to hypoxia conditions in the root system region (Leite *et al*., 2014). This hypoxia condition in the soil causes the solubilization of Fe and Mn of the oxyhydroxides, making these nutrients available in toxic contents (Khabaz-Saberi *et al*.,

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2006; Huang *et al*., 2015). Thus, it is believed that the hypoxia associated with availability of high contents of Mn in the soil are the main contributors for the ESBVRD expression. Hypoxia can also lead to changes in the plants metabolism, causing physiological and anatomical changes in order to lead to survival in a deficit O2 condition (Medri *et al*., 2012; Carvalho *et al*., 2016; Oliveira *et al*., 2016). Additionally, there are evidences of differential tolerance to hypoxia of some eucalyptus clones (Leite *et al*., 2014).

Under hypoxia, the root system is not able to maintain the aerobic metabolism required for the nutrients absorption. Also under this condition, the plants produce metabolic signals in response to a decrease in the O2 endogenous concentration. Thus, there are changes in the architecture of roots, anatomy, metabolism and growth, as a survival strategy (Bailey-Serres and Voesenek, 2008; Medri *et al*., 2012; Oliveira *et al*., 2016; Luo *et al*., 2016). As a result of O2 concentration decrease, there is a negative regulation of Krebs cycle, main mechanism generator or C skeletons for plants synthesis and secondary metabolism compounds, such as the plant hormones (Visser and Voesenek, 2004; Voesenek *et al*., 2016). In O2 limited environment, there is a reduction of the citric acid cycle as well as the electron transporting chain. However, the plants and other organisms can overcome this limitation performing fermentation, that provides, alternatively, another route to pyruvate metabolism, different from the one that usually occurs in the presence of O2 through the Krebs cycle (Taiz and Zeiger, 2009; Paul *et al*., 2016). Nevertheless, this anaerobic metabolism results in severe stress to plants, such as (i) reduction in ATP formation, (ii) decline of the cytosol pH and (iii) accumulation of toxic metabolites (Marschner, 2012; Paul *et al*., 2016b).

Morphological adaptations, such as aerenchyma formation and adventitious roots, are also of great importance for aerobic metabolism maintenance (Magalhães *et al*., 2009; Phukan *et al*., 2016). A large part of these changes in plants subjected to hypoxic stress is mediated by ethylene production. One of these changes is aerenchyma formation (Loreti *et al*., 2016), tissue that contains interconnected channels allowing the gases diffusion, in order to maintain aerobic respiration and oxygenation of the rhizosphere, storing and distributing O2 in the tissues (Colmer, 2003; Takahashi *et al*., 2014). Van der Moezel *et al*. (1989), studying Eucalyptus camaldulensis after 11 weeks of flooding, observed aerenchyma formation in the plants root system. Greet (2015) reported seedlings ability of Eucalyptus camphora in surviving on waterlogged environment for 12 months and have attributed this ability to aerenchyma and adventitious roots rapid formation, both in the roots and the stem surface.

Mn acts mainly as an enzymatic cofactor, presenting, also, participation in photosynthesis photochemical phase, specifically in O_2 complex evolution (Malavolta *et al*., 1997; Santos *et al*., 2017). The Mn toxicity in rice is more severe in the aerial part, specifically in new branches than in the root system, due to its accumulation in high levels in leaves, thereby leaving this part of the plant prone to toxic levels of this nutrient (Zanão Júnior *et al*., 2010). This fact corroborates with field observations, where clones which are more sensitive to ESBVRD accumulate higher levels of Mn at the aerial part (Leite *et al*., 2014). High levels of Mn in the plant, in a eucalyptus plantation in areas of ESBVRD occurrence, may provide a better understanding of this micronutrient contribution so this disorder begins. The Mn foliar contents reach approximately four times higher in the sensitive clone (3,070 mg kg-1) than in the more tolerant clone (734 mg kg-1) to ESBVRD (Leite *et al*., 2014).

The objective of this work was to study nutritional, physiological and morphological changes at eucalyptus clones roots with differential tolerance to ESBVRD, when subjected to excessive Mn and to hypoxia in nutrient solution.

2. Material and methods

The experiment was conducted in a growth chamber with eucalyptus seedlings produced through mini-cuttings by CENIBRA’s greenhouses (Pulp Nipo-Brasileira S.A.), Belo Oriente - MG. The clones used were the 1213 and 2719, being these hybrids a crossing between the species of *Eucalyptus urophylla x E. grandis (Eucalyptus urograndis)*. The choice of these clones was due to field observations, which indicated that the former is more sensitive to the ESBVRD (Leite *et al*., 2014).

With 25 to 30 day old the seedlings were taken to a greenhouse. After five days of acclimatization in original plastic tubes, they were removed from the substrate, washed with deionized water and transferred to 10 L trays containing Clark nutrient solution (Clark, 1975), with half the concentration of the original solution and without the addition of Mn.

The solutions were continuously aerated and the pH maintained in 5.0 through the addition of acid or base (HCl, NaOH to 0.01 mol L^{-1}) The plants remained in the trays for five days, under greenhouse conditions and were then transferred to a growth chamber. In this new environment, they were subjected to controlled Lighting by halogen lamps (600 µmol m^{-2} s^{-1}), with photoperiod 12/12 h day/night. The temperature was controlled at 30 ± 2 ℃ and the relative humidity maintained around 70 %.

The seedlings already acclimatized to nutrient solution, were transferred to plastic pots of 3.4 L, and with two plants each, with complete Clark's nutritive solution but with the concentration of 30 mg L^{-1} of Mn (MnCl2.4H2O), instead of the 0.38 mg L^{-1} of Mn concentration in Clark's original solution. This concentration of Mn was defined based on previous experiment and taken as responsible for intermediary toxic effects to plants.

The treatments consisted of a 2 x 4 factorial design, being: two clones, a sensitive (1213) and a tolerant (2719) to ESBVRD and four concentrations of O_2 dissolved in nutritive solution– 1; 4; 6 and 8 mg L^{-1} of O_2. The O_2 concentrations were also defined according to previous studies, being considered normal, without hypoxia, the 8mg mL^{-1} of O_2 concentration. Randomized blocks was used as the experimental design, with four replicates. The treatments with O_2 were controlled by N_2 continuous bubbling in the nutritive solution, being the O_2 dissolved in each treatment measured by oximetry. At the implementation stage of the treatments, 100 µmol L^{-1} of Al^{3+} were added in all treatments to stimulate the seedlings rooting (Silva *et al*., 2004).

The length of root system and shoots were measured at the beginning and at the end of the experiment, at 21 days after the transfer of the seedlings to the growth chamber. At 14 days, photosynthesis (*A*), stomatal conductance (*g*), transpiration (*E*) and internal concentration of CO_2, were measured, under saturating artificial light (1400 µmol m^{-2} s^{-1}) with the atmospheric concentration of CO_2 controlled (390 µmol mol^{-1}). The measurements were performed between 7:00 and 11:00 am, with the gaseous exchanges analyzer Li-Cor LI-6400XT, IRGA. Also at 14 Days, root samples were taken for the verification of possible anatomical modification in their tissues. For each selected root, a fragment from the apex, with 4±0.5 cm (Melo *et al*., 2007), was taken and washed with deionized water that then was fixed in FAA 50 for 48 h and in alcohol 70% for analysis (Johansen, 1940). In these roots fragments, transversal cuts were made in automatic advance rotary microtome (*Leica RM* 2125). The cuts were stained with toluidine blue (Feder and O'Brien, 1968) and mounted in synthetic resin *Permount*, being photographed in photomicroscope (Olympus AX 70), using the program *axion vision* 4.8. The quantitative analysis of the root anatomy was measured with the support of the program *Image Pro Plus*. Then, at 21 days, the plants were harvested, washed in deionized water and separated into old leaves and new, stem and root and oven-dried (65 ℃/72 h). Subsequently, the samples were ground and subjected to nitro-perchloric digestion in the proportion 4:1 v/v, according to Malavolta *et al*. (1997). The content of Mn was determined by atomic absorption spectrophotometry.

The dry matter production and the content of Mn in the plant components were subjected to analysis of variance. After the unfolding, the effect of O_2 concentrations was evaluated at production of plant dry matter, root growth, content of Mn, physiological and anatomical attributes, for both clones. For the evaluation of clones effect it was applied the models identity test by comparing the regressions. For the comparison between means the Tukey test at 5% was performed. The statistical program SAEG version 9.1 was used for the data processing and the software Sigma Plot for the graphs construction.

3. Results and discussion

3.1 Dry matter of aerial part and root growth

With the exception of dry matter of new leaves, all the other growth indicators were sensitive to the O_2 deficit condition, showing already a reduction in a condition of slight O_2 deficiency (6 mg L^{-1}) (**Figure 1**). A similar situation, observed for E. *camaldulensis* and E. *globulus,* in which hypoxia caused a reduction in dry matter production of all the plants compartments was reported by *Sena* Gomes and Kozlowski, 1980. Smith *et al*. (2017) monitored the growth rates and carbon sequestration in a region of Australia after the La Niña, which flooded a good part of the area. These authors observed a significant reduction in size and density of *Eucalyptus camaldulensis*, Acacia *salicina* and *Steno-*

phylla trees, causing the fall of carbon sequestration rates, since this attribute is directly correlated with the size of the tree.

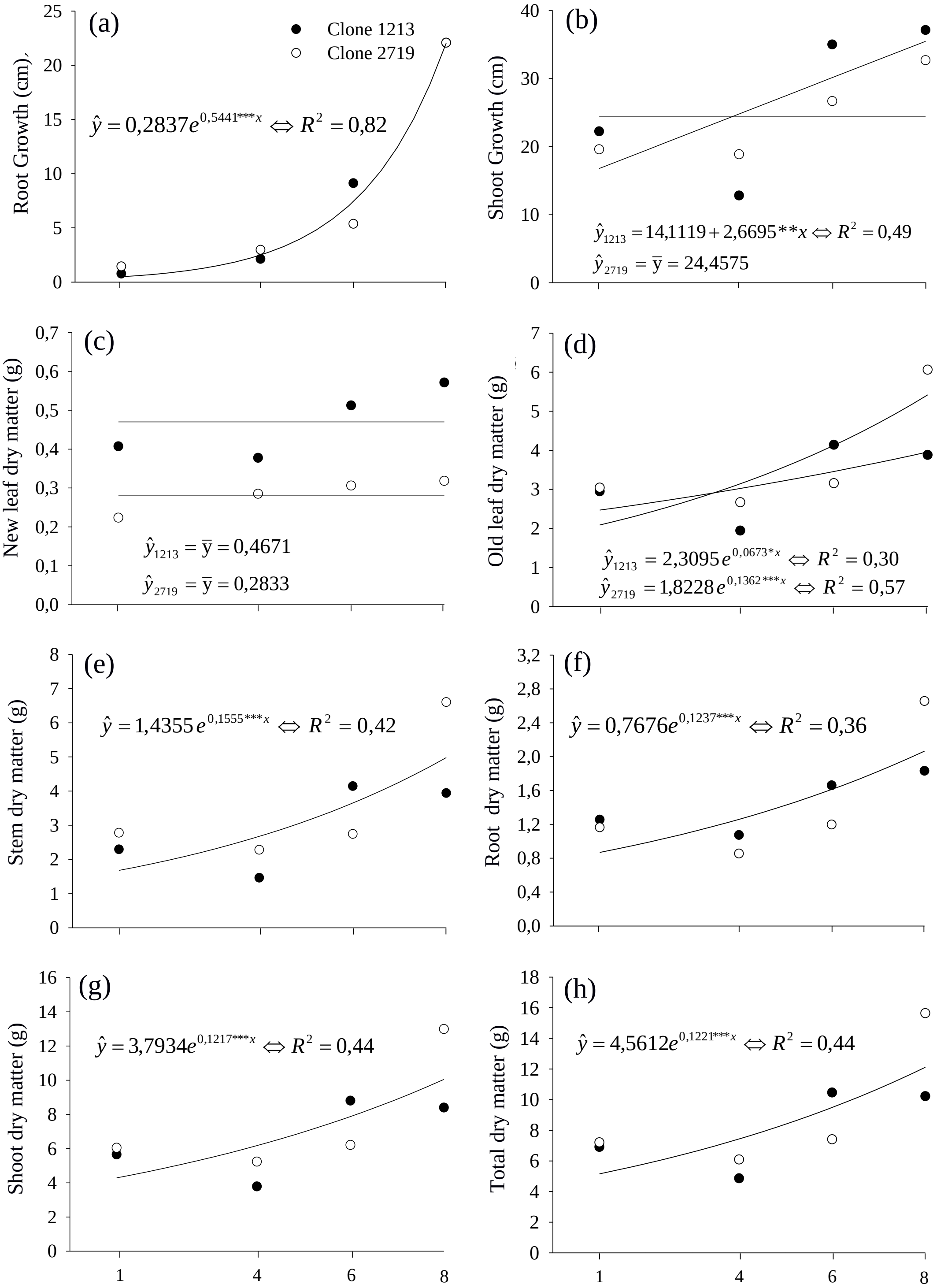

Figure 1; Root growth (a) and aerial part (b), dry matter of new leaf (c), old leaf (d), stem (e), root (f), shoot (g) and total (h) in eucalyptus seedlings (clones 1213®-sensitive- and 2719®-tolerant- Cenibra SA) influenced by O_2 concentrations at 21 days. *, **, ***: significant at 5, 1 and 0.1%, respectively, by the test F. Test of identity of 10% models exemplified in (a), (e), (f), (g) and (h) estimated similar growth pattern for both clones**.**

According to Phukan *et al*. (2016), the limited aeration (availability of O_2)) with the establishment of conditions that lead to hypoxia caused by excess of water in the soil (or in a condition of lack of O_2 in nutritive solution) causes initially drastic changes in the plants metabolism, such as reducing the water absorption and the stomatal conductance, with a consequent decrease in photosynthesis and plant growth. Then, with the lowest roots permeability, there is less absorption of nutrients, alteration in the hormonal balance such as ethylene production, leaves epistasis, followed by chlorosis and leaf abscission. With the permanence of the hypoxia condition there is a formation of aerenchyma and adventitious roots, with the consequent death of plant with the extension of the period of limited O_2 availability.

The root growth was the most affected by this stress condition (Figures 1a and 2), such as a small reduction in the O_2 availability leading to a large reduction in the root system, growth by up to 70 % for both clones.

Figure 2; Growth of the aerial part and root system of the clones 1213®-sensitive- and 2719®-tolerant- Cenibra S.A., being the first, from the left to the right, the clone 1213, in O_2 decreasing concentration. The numerical values represent the concentrations of O_2 (mg L^{-1}) in Clark's nutrient solution**.**

Rocha *et al*. (2010), studying *Lotus japonicus*, *Kozlowski* (1984), in woody plants, observed that the root growth of these plants was negatively influenced by the O_2 deficit condition. In more extreme cases, for example, in the temporary absence of O_2 (anoxia), there might be at the death of the root system.

In conditions of high hypoxia (1 mg L^{-1} of O_2), both clones presented intense darkening of the root system, suggesting a low activity or even their death (**Figure 2**). On the other hand, the aerial part seems to tolerate more this condition. In this situation, the accumulation of toxic metabolites such as lactate and, or, ethanol, the decline in cytosolic pH (George *et al*., 2012; Voesenek *et al*., 2016) and the highest production of reactive species of O_2 (Bai *et al*., 2010) are intense, culminating with the loss of the cells integrity, as evidenced by the darkening of the entire root tissue.

The aerial part from both clones was changed by the O_2 concentration reduction (**Figure 2**). However, there was no equation adjustment for the tolerant clone (**Figure 1**b), for which it is suggested that the period of hypoxia was not sufficient to transfer the stress caused on the root system for the aerial part. The opposite was observed for the most sensitive clone. George *et al*. (2012) argue that the root system of some species ceases its growth immediately after the start of the hypoxia, which may reach to death after a few days; on the other hand, the aerial part keeps its growth, reducing it only the long-term. This is possible due to the stock of nutrient that the aerial part accumulated prior to the stress period.

In a hypoxia condition, the genes that regulate the expression of protein carriers of nutrients have their expression reduced (Kreuzwieser and Gessler, 2010; Loreti *et al*., 2016), leading to a reduction in the absorption of nutrients by roots and its transport to the aerial part.

Regarding the difference between clones, the tolerant was superior in dry matter production of old leaves in a condition of mild hypoxia (**Figure 1**d). However, in conditions of severe hypoxia the opposite was observed. The dry matter of new leaves was not changed by the O_2 concentration (**Figure 1**c). Moreover, the sensitive clone showed greater growth of new leaves. Regarding the growth characteristics, no differences were obtained between clones ($p>0.10$).

3.2 Manganese Content

The levels of Mn in old leaves were influenced by the O_2, concentration, presenting an increase with the increase in oxygenation for both clones (**Figure 3**a). In addition, the tolerant clone showed higher levels regarding the sensitive clone, but with a greater reduction in Mn content when approaching the condition of intense hypoxia (1 mg L^{-1} of O_2). Although the Mn contents in new leaf not showing influence of O_2 concentration, once more, the Mn contents were higher in the tolerant clone (**Figure 3**b). This observation contradicts what was observed in the field, in which the sensitive clone accumulates higher contents of Mn in the aerial part of the plant (Leite *et al*., 2014). However, it should be emphasized that the Mn leaf contents are somewhat higher and, on the other hand, the values observed in the field are up to five times higher for the clone sensitive to ESBVRD. Perhaps, as intermediate concentrations of Mn were used (30 mg L-1), it was not possible to verify the same effect occurred in the field.

As it was expected, the opposite was observed in roots and stems (**Figure 3**c and 3d), for which the sensitive clone accumulated higher Mn contents. The tolerance to excess of Mn, as well as of other metallic micronutrients, in several plant species has been attributed to greater retention of the excess in the roots. This capacity depends, to some extent, on the formation of complexes in the root system (Han *et al*., 2006; Muhammad *et al*. 2016). However, the increased tolerance to Mn can also be attributed to the accumulation in the thylakoid (Lidon and Teixeira, 2000) or in the chloroplast (Xue *et al*., 2016), suggesting that the aerial part, has also an important role to control the excess of Mn.

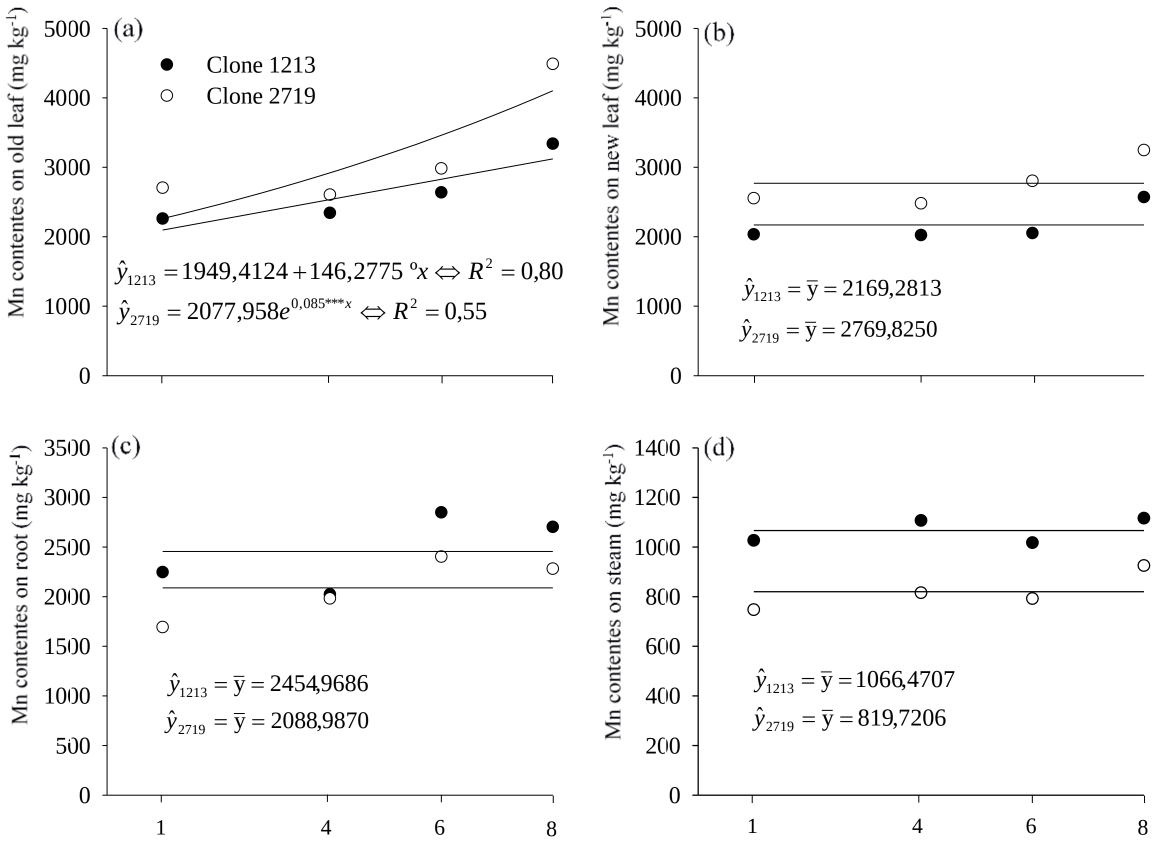

Figure 3; Mn contents on old (a) and new leaf (b), root (c) and stem (d) eucalyptus seedlings (clones 1213®-sensitive and 2719®-tolerant- Cenibra SA) influenced by concentrations of O_2 at 21 days. *, **, ***: significant at 5, 1 and 0.1%, respectively, by the test F**.**

3.3 Gaseous exchange

The reduction of the O_2 concentration led to fall in the values of most attributes, being more expressive in tolerant clone (**Figure 4**). There was a decrease in the net photosynthetic rate (A) for both clones, which may reach 70 % for the tolerant clone in intense condition of hypoxia (1 mg L^{-1} of O_2) (**Figure 4**a). Martinazzo *et al.* (2013), studying the plum three (*Prunus salicina Lindl.*), observed a reduction of A when these plants were subjected to flooding. This reduction may be associated with the stomata closure, which triggers several processes, such as the reduction of CO_2 internal concentration CO_2, transpiration and changes the relation between internal CO_2 (Ci) and ambient (C_a). In plants of carnaúba palm (*Copernicia prunifera* (Mill.) H.E. Moore) was observed that the condition of flooding causes stomatal conductance reduction and photosynthesis (Arruda and Calbo, 2004). Silveira *et al.* (2015) observed that when raising the CO_2 concentration, there was a reduction in the stomatal conductance than in photosynthetic rates, leading to an increase in the efficient use of water, as a strategy for the coffee plant survival. As expected, the relation between the intercellular CO_2 and the atmospheric was reduced.

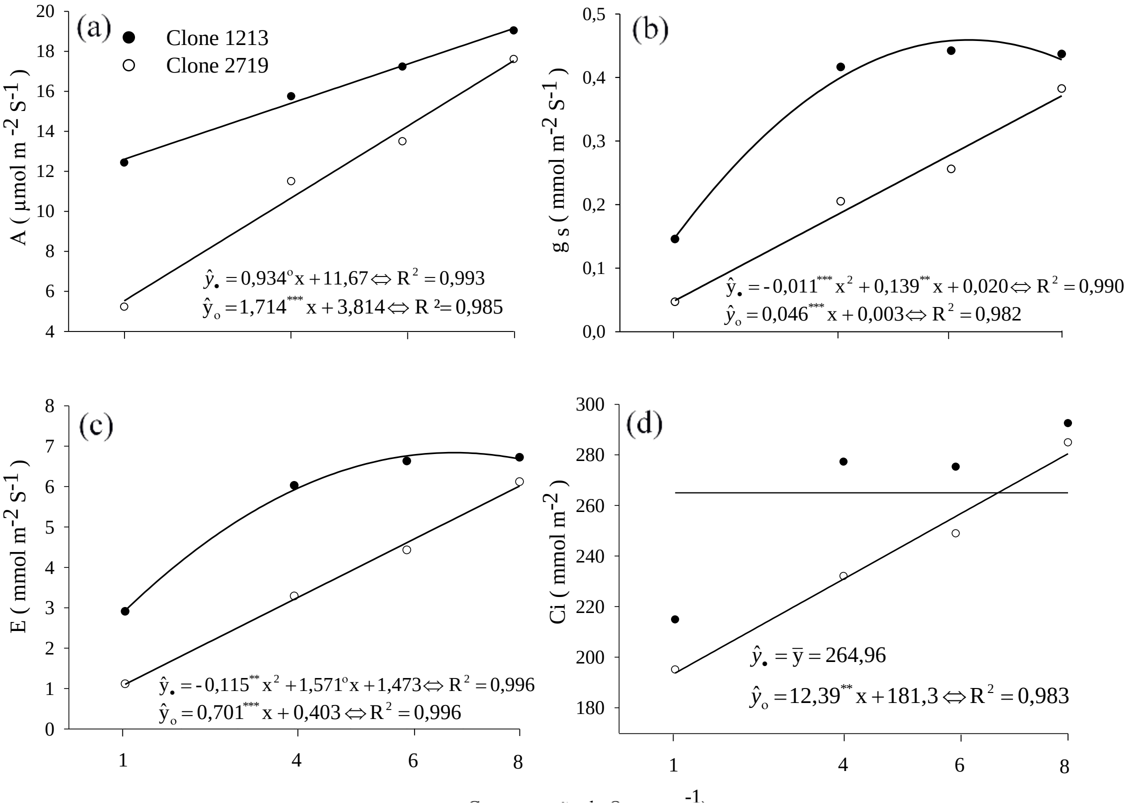

Figure 4; Photosynthetic rate (a), stomatal conductance (b), transpiration (c) and internal CO2 concentration (d) in eucalyptus seedlings (clones 1213® - sensitive - and 2719®- tolerant- CENIBRA SA) in 14 days Of experiment. Regression equations were adjusted for each attribute above, with levels of significance (p <0.1), ** (p <0.05), *** (p <0.01) and ns (nonsignificant) by t-test**.**

The stomatal conductance (g_s) is one of the first physiological changes that plants, in general, are in a flooding condition. Bradford and Hsiao (1982), studying a variety of tomato (*Lycopersicon esculentum*), observed a reduction of 30% in g_s when the plant remained for 24 h in a flooding condition. For the two species of eucalyptus *(E. camaldulensis and E. lesouefii*), it was also observed that the hypoxia causes alteration of stomatal conductance, and to *E. lesouefii* the reduction was more intense (Van der Moezel *et al.*, 1989). For the eucalyptus clones in this study, the reduction in g_s was more intense for the tolerant clone, being that a small variation in the concentration of O_2 from 8 to 6 mg L^{-1} caused a reduction in g_s of 33 %, unlike the sensitive clone that showed no variation (**Figure 4**b).

The transpiration (*E*) was also changed by hypoxia, being reduced with the increase of the O_2 restriction to the root system (**Figure 4**c). This fact is explained primarily by the g_s reduction. This statement corroborates with the study by Martinazzo *et al.* (2013), where the imposition of hypoxia caused a reduction in the plum tree transpiration.

The reduction of CO_2 (C_i) internal concentration was similar to other photosynthetic attributes, but it was not significant for the sensitive clone (**Figure 4**d). In the tolerant clone this reduction suggests that in hypoxia conditions, the net photosynthetic rate can be controlled by g_s.

The tolerant clone responds more quickly to hypoxia than the sensitive, because a small decrease in O_2 concentration causes a reduction in g_s and, consequently, in photosynthesis by reducing the concentration of C absorbed. However, it maintains C in the carboxylation site and this allows to keep the photosynthesis at rates sufficient to survive and avoid degradation by the O_2 reactive species. This mechanism seems to be an advantage for the plant recovery after the stress.

3.4 Aerenchyma formation

Differences were observed in the formation of intercellular spaces in the roots of both clones submitted to hypoxia, with aerenchyma formation in both clones (**Figure 5**). Apparently, the origin of the aerenchyma is exogenous, evidenced by the cells removal from the cortical parenchyma. Dantas *et al.* (2001) observed that hypoxia also induced the formation of large intercellular spaces, forming lysigenous aerenchyma in roots of corn cultivar Saracura BRS- 4154, due to the increase in the cellulase activity. In eucalyptus species there are also reports of aerenchyma formation. Van der Moezel *et al*. (1989), studying E. *camaldulensis*, observed formation of aerenchyma in the root system, supporting the idea that the eucalyptus, as well as other species, has a hypoxia escape mechanism, suggesting tolerance to flooding conditions.

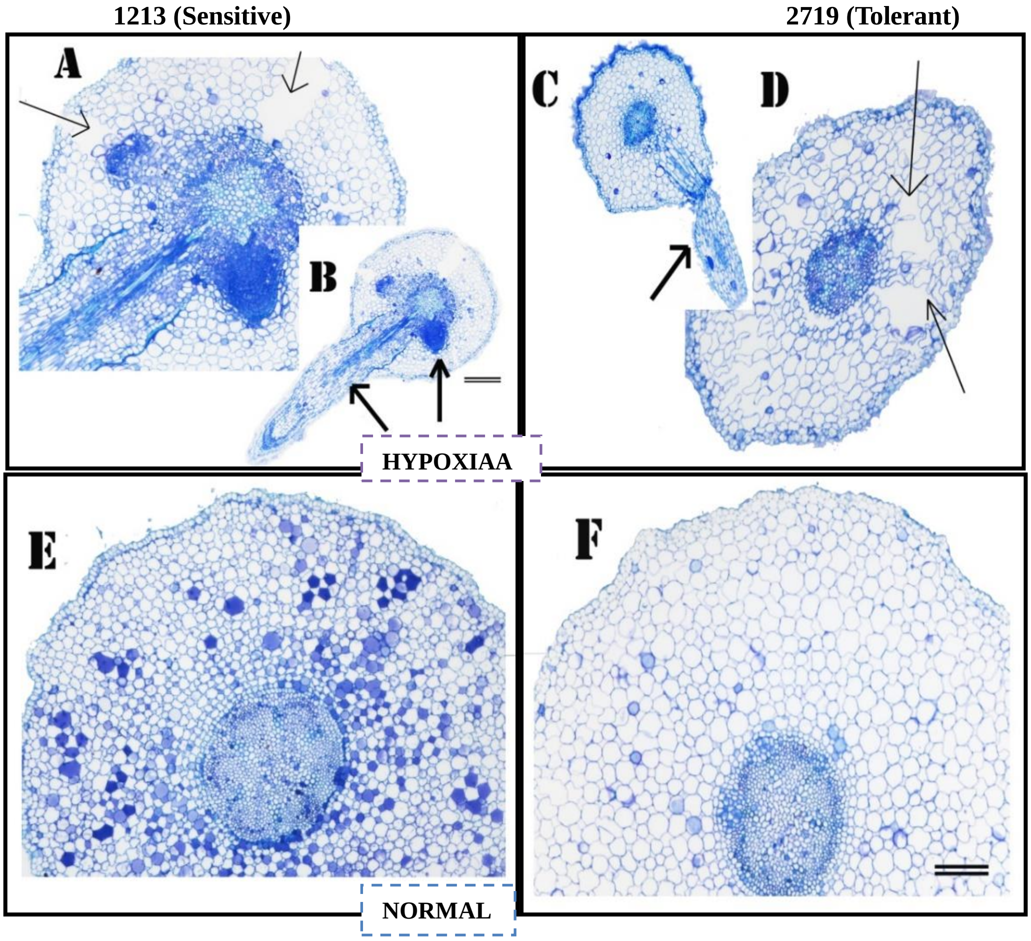

Figure 5; Root cross section of eucalyptus seedlings (clones 1213® and 2719® Cenibra S.A.), under growth in nutrient solution in hypoxia (4 mg L-1) and in normal O_2 (8 mg L-1) conditions. The figures (A, D, E, F) and (B, C) are represented in the scales 200 and 100 µm respectively indicated in figure A and F by two parallel bars. The full arrows indicate the launching of lateral roots and the thinner arrows indicate intercellular spaces**.**

In aerobic conditions, there was no aerenchyma formation in the root system of the clones in this study. In a hypoxia situation, the two clones, exhibit increased size and reduction at the number of cells in the root cortex (Table 1) and disorganization (**Figure 5**). When the plants grew in normal conditions of O_2, both, sensitive and tolerant clone, had roots with smaller cells, more organized and less intercellular spaces. Moreover, the two clones exposed to hypoxia showed a higher number of lateral roots, as a possible response to this condition (**Figure 5**). Regardless the O_2 concentration, the sensitive clone showed no significant difference for the variables evaluated, except for the number of cells in the cortex. This suggests the lower differentiation of cells of the same area of cortex for the genotype. The sensitive clone also presented lower area of cortex in comparison to the tolerant clone, regardless O_2 concentration. On the other hand, except for the root area and the mean number of cells, all of the variables evaluated, in normal condition of oxygenation (8 mg L^{-1} de O_2), were significantly superior to the hypoxia condition for the tolerantclone. These results suggest the tendency to maintain the same number of cells to a smaller cortex area.

Clone	O_2	RA	AE	CA	COA	RD	DCM	MNC
mg L^{-1}		------------------------- µm² ----------------------				------- µm ------		
1213	4	576601 Aa	84830 Aa	67041 Aa	426823 Aa	858 Aa	263 Aa	8,8 Ba
1213	8	765638 Aa	91856 Aa	62710 Aa	609248 Aa	983 Aa	271 Aa	14,6 Aa
2719	4	417847 Aa	87642 Ba	28797 Bb	357945 Ba	728 Ba	184 Ba	8,7 Aa
2719	8	874518 Aa	107910 Aa	48370 Ab	680095 Aa	1028 Aa	254 Aa	13,5 Aa

Vertical mean values followed by the same capital letter do not differ to O_2 effect, for the same clone, by the Tukey test ($p < 0.05$). Vertical mean values followed by the same lowercase letter did not differ for clones to the same O_2 dose by the Tukey test ($p < 0.05$).

Table 1. Root area averages (RA); Area of the epidermis (AE); Cylinder area (CA); Cortex area (COA); Mean root diameter (RD); Mean cylinder diameter (DCM); Mean number of cells (MNC) in two eucalyptus clones, 1213 (sensitive) and 2719 (tolerant), before O_2 concentrations in nutrient solution

Despite the maintenance of the root area, the possibility of transport reduction in the vascular system, due to reduction in the area of central cylinder and the relevance of changes in the epidermis area in relation to the reduction of O_2 concentration, has to be considered.

4. Conclusions

The hypoxia causes reduction in the growth of both clones, the sensitive and tolerant to ESBVRD, although the effect is more marked in the sensitive clone, being the root growth the best indicator of this fact; Mn does not change it. This condition (hypoxia) is more limiting to the stomatal opening of the tolerant clone than for the sensitive clone, consequently reducing the photosynthetic rate, respiration and the internal CO_2 concentration. The mild hypoxia allows the aerenchyma formation in both clones.

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