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Growth and Ethylene Production in Eucalyptus Clones Sensitive to Shoot Blight, Submitted to Hypoxia and High Levels of Manganese

Fellip Janu ário Pinheiro Lacerda

Ivo Ribeiro da Silva & Fernanda Schultais

Vi çosa Federal University, Brasil.

Sarah Vieira Novais

Escola Superior de Agricultura Luiz de Queiroz (ESALQ),

S ão Paulo University, Brazil.

Fernando Palha Leite

Celulose Nipo Brasileira S/A, Belo Oriente, Brazil.

Roberto Ferreira Novais (Corresponding author)

Vi çosa Federal University, Campus Rio Parana íba, Brasil.

E-mail: rfnovais@ufv.br

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Abstract

Eucalyptus shoot blight in the Rio Doce Valley (ESBVRD) is a physiological anomaly that has been related to hypoxic environment and Mn excess. This study had the objective of understanding the mechanisms involved in the differential tolerance of eucalyptus clones to ESBVRD. Two experiments were carried out: (I) two clones, a sensitive and a ESBVRD tolerant, two O_2 concentrations (normal and hypoxic) and five concentrations of Mn (0, 5, to 90 mg L^{-1}); (II) same two eucalyptus clones, two O_2 concentrations (normal and mild hypoxic) and two Mn concentrations (30 and 300 mg L^{-1}). The hypoxic condition reduced the plant dry

weight and increased the production of ethylene in the two clones, both more strikingly in the sensitive clone. The O_2 deficiency was the first factor predisposing the clones to ESBVRD, in contrast to Mn excess, that appeared to act over a longer period for the expression of the symptoms.

Keywords: flooding, forestry nutrition, dieback of the Rio Doce Valley, ESBVRD.

1. Introduction

In the lowland regions of the Rio Doce Valley the occurrence of eucalyptus dieback (ESBVRD) has been observed for decades (Nascimento et al., 2006) and causes significant losses in productivity for the plantation. Although the symptoms are principally expressed in the apical region, they are visually different from those of the dieback caused by B deficiency and/or Ca observed in eucalyptus plantations in the region of Cerrado (Novais et al., 1990; Gonçalves et al., 2016).

In the initial stages brownish necroses are observed at the insertion of the secondary stems and petioles, especially in the region below the emission of new branches. The leaves become coriaceous and wrinkled and the lesions evolve rapidly leading to the death of the apical part of the branch, compromising plant growth. In the more severe case complete defoliation occurs, culminating in the death of the apex of the plant (Leite et al., 2014). According to these authors the intensity of visual symptoms is related to the age of the plants, as they are normally observed until the third or fourth year after planting. This is probably due to the increase in leaf area index when the plants become older (3-4 years), accommodating the higher transpirational demands of the plants. In this way the hypoxic condition can be minimized by the increase in the flux of water in the soil-plant-atmosphere system, reducing plant stress. In the lowland region of the Rio Doce Valley, ESBVRD has appeared sporadically and occurred with a higher intensity in years with very wet summers, such as 2002 and 2007. The visual symptoms reach their height between April and May (end of the wet season), but there is a recovery in growth at the end of the dry period (August-September) (Leite et al., 2014).

Under stress, such as frost, flooding and mechanical stress, the synthesis of ethylene by plants is increased (Taiz e Zeiger, 2006; Urano et al., 2017; Telewski, 2016; Zhang et al., 2016). That is because the expression of the gene that regulates ethylene synthesis is increased in hypoxic conditions (Alpuerto et al., 2016; Bailey-Serres and Voesenek, 2008; Dong et al., 2016; Keunen et al., 2016; Peng et al., 2005). Clemens and Pearson (1977), studying *Eucalyptus robusta*, observed that in roots and branches the rate of ethylene production was doubled after six days of hypoxia, but without the production of this compound in the leaves. Andrade et al. (2015) observed the excessive accumulation of ethylene in trees typical of a tropical forest (*Parkia gigantocarpa*) caused by the excess of Fe^{2+} and Mn^{2+} in flooded areas, causing a reduction in growth of even root death. Even in plants recognized as being tolerant to flood ethylene production was high in the secondary roots, allowing an increase in the absorption of oxygen and nutrients.

The hypoxic environment (reducing) caused the solubilization of Fe and Mn oxides and

hydroxides, making these elements available in toxic levels (Huang et al., 2015; Khabaz-Saberi et al., 2006). Thus, it is believed that hypoxia associated with elevated levels of Mn in the soil are the principal factors for the expression of the visual symptoms of ESBVRD.

A large difference in the level of leaf Mn were observed between the sensitive (3070 mg kg^{-1}) and the tolerant (734 mg kg^{-1}) clone, indicating the involvement of this micronutrient in the appearance of ESBVRD (Leite et al., 2014). However, it is not known whether these levels of Mn in the sensitive clone are sufficiently elevated to cause damage to plant metabolism and growth, or the excess of Mn is the cause or only an effect of the inefficient mechanisms of the ESBVRD sensitive clones. Indeed, a lower tolerance to Mn could result from a lower restriction on its absorption and/or its translocation to the aerial part of the plant or its efficient inactivation inside the plant tissues. Lidon and Teixeira (2000) concluded that, depending on the species, the excess Mn could accumulate in vacuoles, the cell wall and the thylakoids, increasing tolerance to the high levels of Mn available in some species. It is known that plants subjected to high light intensity or elevated temperature are more likely to be predisposed to Mn toxicity (El-Jaoual and Cox, 1998). These factors are of great importance in lowland regions subjected to flooding, where ESBVRD occurs at a high frequency. These conditions of high temperature are observed practically throughout the whole year (Leite et al., 2014).

Muhammad et al. (2016) found a differential reduction in the chlorophyll content and photosynthetic attributes (rate of photosynthesis, transpiration and stomatal conductance), varying according to the genotype (variety), after the addition of toxic doses of Mn. The excess of this micronutrient is extremely damaging to photosynthesis, leading to a reduction in root growth and plant height. However, the most tolerant genotypes tend to have a lower level of Mn in their tissues, principally in the plasma membrane and the cell wall, resulting in higher plant biomass. (Xue et al., 2015) confirmed that tolerance to Mn varied according to chloroplast resistance. The accumulation of Mn in the chloroplast structure leads to the excess production of laminar granules, rupturing of the membrane and the entire structure of the chloroplast, causing one of the most common symptoms of Mn toxicity, dark spots on the leaves.

In general, Mn toxicity is characterized by necrotic spot on the leaves, petioles and stems, starting with the older leaves and spreading with time to the younger leaves. In some species, the symptoms of toxicity can start as leaf chlorosis that may cause the leaf to wrinkle and even tipping of the stem and petiole (Asati et al., 2016).

A common symptom of Mn toxicity is dark spots on the older leaves with chlorosis at the edges and he youngest become wilted with interneval chlorosis and along the edges (El-Jaoual and Cox, 1998). Wu (1994) suggested that brown spots are common in leaves, petioles and stems and are useful in the diagnosis of Mn toxicity in soybean. In apple trees, the symptoms of O_2 deficiency are characterized by the cracking of the bark of the trunk and secondary branches (Ernani, 2012), similar to the symptoms of ESBVRD. Winterhalder (1963), studying native Australian species, like *Eucalyptus gummifera*, verified that Mn

toxicity was indicated by the presence of small chlorotic leaves, with wrinkling in some cases and death of the apical tissue in extreme cases.

In view of the above, the objective of this work was to evaluate the tolerance of saplings eucalyptus clones, previously identified as having differential sensitivity to Rio Doce Valley dieback (ESBVRD), under hypoxic conditions and the excess of Mn.

2. Material and Methods

The eucalyptus seedlings utilized in the experiments were produced by min-cuttings from the nursery CENIBRA (Celulose Nipo-Brasileira S.A.), Belo Oriente - MG. The clones used 1213 (sensitive) and 2719 (tolerant to ESBVRD), were hybrids between the species *Eucalyptus urophylla x E. grandis* (*Eucalyptus urograndis*). The option to use these clones was due to the observations that in the field 1213 is more sensitive to dieback (Leite et al., 2014).

At 25 to 30 days old, the seedlings were transferred to the greenhouse. After five days of acclimatization in the original tubes, they were removed from the substrate, washed with deionized water and transferred to 10 L trays containing half strength Clark´s nutrient solution without Mn. The solutions were constantly aerated and the pH maintained at 5.0 (0.01 mol L^{-1} HCl or NaOH). The plants remained in the trays for 5 days in the greenhouse and were then transferred to a growth chamber. The clones were maintained under controlled illumination (600 µmol m^{-2} s^{-1}), with a 12 hour light 12 hour dark photoperiod, the temperature controlled to 30 ±2 ℃ and a relative humidity of approximately 70 %.

In *Experiment I*, the eucalyptus seedlings (clones 1213 and 2719) already acclimatized and in the growth chamber, were arrange individually in 3.4 L plastic recipients containing full strength Clark´s nutrient solution without Mn. The experimental unit was consisted one plant per recipient.

The treatments were arranged in a factorial scheme of 2 x 2 x 5, two clones (sensitive 1213 and tolerant 2719 to ESBVRD; two concentration of O_2 in the solution (normal 8 mg L^{-1} and severe hypoxic 1 mg L^{-1} of O_2), and five concentrations of Mn (as $MnCl_2.4H_2O$, 0, 5, 10, 30 and 90 mg L^{-1}). The treatments were arrange in a randomized block with four repetitions.

The nutrient solution was changed weekly and the pH adjusted daily to 5.0. The plants were grown under the defined experimental conditions for 25 days. To avoid darkening and to stimulate root growth 100 µmol L^{-1} of Al was added (Silva et al., 2004). The dissolved O_2 in each unit was measure with an oxymeter four time a day. In the hypoxic treatments, the low concentration of O_2 was maintained by the continuous injection of N_2 (Air Products) into the nutrient solution.

After 25 days in the growth chamber, each plant was divided into mature leaves, young leaves, stem and roots, washed three times in deionized water and oven dried at 72 ℃. The plant samples were then ground using a Willey mill and submitted to digestion in a nitric-perchloric acid mixture (4:1 v/v) according to the method of Malavolta et al. (1989). The digests were used to determine the level of Mn by atomic absorption spectroscopy.

In *Experiment II*, the same experimental conditions were used with a factorial scheme of 2 x 2 x 2, two eucalyptus clones; two concentrations of O_2 (normal 8 mg L^{-1} and mild hypoxic 6 mg L^{-1}), two concentrations of Mn (30 and 300 mg L^{-1}, defined by the results of Experiment I). During the experimental period the solution was constantly aerated, the pH adjusted to 5.0 daily and the solution changed weekly as described for Experiment I. The assay was conducted with eight replicates in a randomized block design.

The length of the root system was measured at 0, 2, 5, 10, 15, 18 and 27 days and the aerial part of the plant at 0 and 27 days. At the end of the experiment, each experimental unit was placed inside a glass bell chamber to isolate it from the external atmosphere. Each plant remained in the chamber for 20 min, the internal air being homogenized mini-fan. After this period, samples were taken, four repetitions per plant, of the internal atmosphere to determine the concentration of ethylene using a gas chromatograph equipped with a Porapak Q column and a flame ionization detector.

Again, at the end of the experiment the plants were divided into mature leaves, young leaves, stem and roots and oven dried at 72 ℃. After drying, the samples were ground using a Willey mill, submitted to digestion with nitric-perchloric acid (4:1 v/v, Malavolta et al., 1989) and the concentration of Mn determined by atomic absorption spectroscopy.

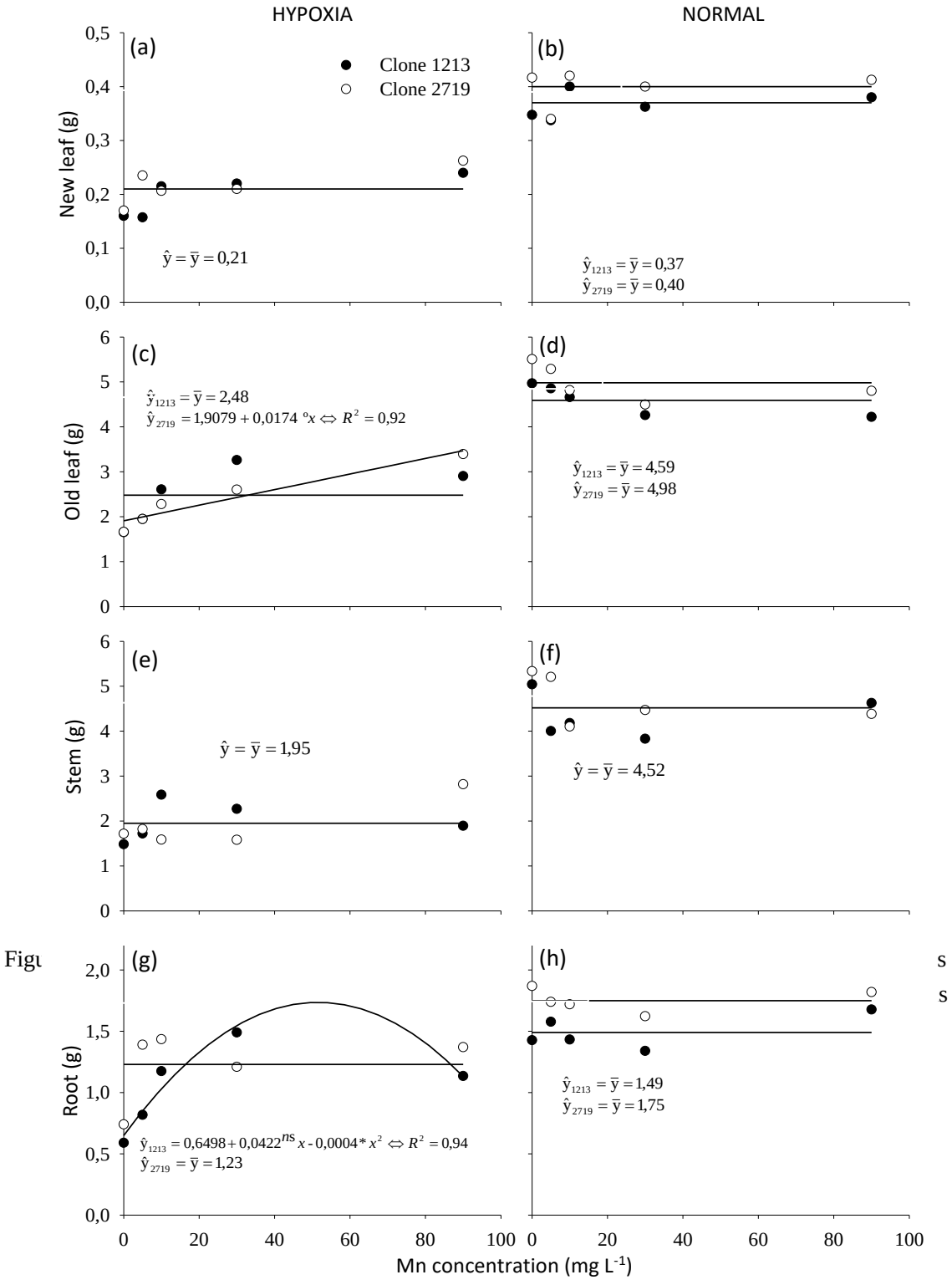
The results were submitted to an analysis of variance. Subsequently, the degrees of freedom of treatments were defined and using regression analysis, in Experiment I, the effect of Mn concentrations on the production of dry vegetable matter, the concentration of Mn on the growth of the aerial part of the plant in the two clones was investigated. Similarly, in Experiment II, the effect of hypoxia and the concentration of Mn on daily root growth in both clones was investigated and using a test of means (Tukey a 5 %), the effect of Mn on the growth of the aerial part of the plant, its height, diameter, concentration of ethylene and root growth. To investigate the effect of the clones the model identity test was applied comparing the regression analyses. The data was analyzed using the statistical package SAEG version 9.1 and the software Sigmaplot to produce the graphs.

3. Result and Discussion

Experiment I

3.1 Analyses of the Dry Matter from the Plant Components

In general, both the clones presented a reduced production of dry matter under hypoxia (Figures 1 e 2), certainly, the most important factor is the reduced offer of energy for plant growth under this condition. The plants have reduced metabolism and absorb and transport of nutrients to the aerial part (George et al., 2012), reductions also occur in the transport of water, hormones and other solutes via mass flow (Wang et al., 2016).



Figu s
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Figure 1. Dry matter yield of new leaf (a, b) and old (c, d), stem (e, f) and root (g, h) in eucalyptus seedlings (clones 1213®- sensitive- and 2719 To the ESBVRD) influenced by concentrations of Mn and O2 (Hypoxia and Normal) at 25 days.

Figure 2. Detail of the aerial part and root system of the sensitive clone (1213®) to SPEVRD submitted to the normal oxygenation condition (+ O2) (A and C) and severe hypoxia (- O2) (B and D).

Level of Mn

Despite the absence of visual symptoms of deficiency, Mn leaf levels as low as 59 mg kg^{-1} were observed in young leaves when Mn was not supplied in the nutrient solution (Figure 3). This level of Mn was probably the residue from the initial production phase of the seedlings. Although there was a difference in the absorption of Mn by the clones, the level of Mn considered deficient was similar varying between 10 and 20 mg kg^{-1}, as observed by Shao et al. (2016) in fully expanded leaves and independent of the species, variety and environment (Broadley et al., 2012). On the other hand, levels higher than 8000 mg kg^{-1} were observed in mature leaves under hypoxia and the higher concentrations of Mn (Figure 3c), for both clones as well as the sensitive clone under normal oxygen levels (Figure 3d). These levels are well above those considered critical for eucalyptus leaves about 400-500 mg kg^{-1} of Mn (Gonçalves et al., 1996). Differences in leaf Mn levels were observed between the clones under normal oxygenation, with the sensitive clone showing higher levels (Figure 3b e 3d). Under hypoxia, it was evident that there were higher levels of Mn in the roots (Figure 3g) and the stem (Figure 3e) of the tolerant clone. Thus, lower leaf levels of Mn were expected in this clone, probably due to the lower translocation of this micronutrient, which could be considered an adaptive mechanism in response to high Mn levels. However this was not

observed. A possible cause could be related to a reduction in the metabolic activity in the roots of the sensitive clone, as this tissue was the first to be negatively altered under hypoxia (Figure 2D). Besides, the intensity of the hypoxia applied may have been too severe.

EXPERIMENT II

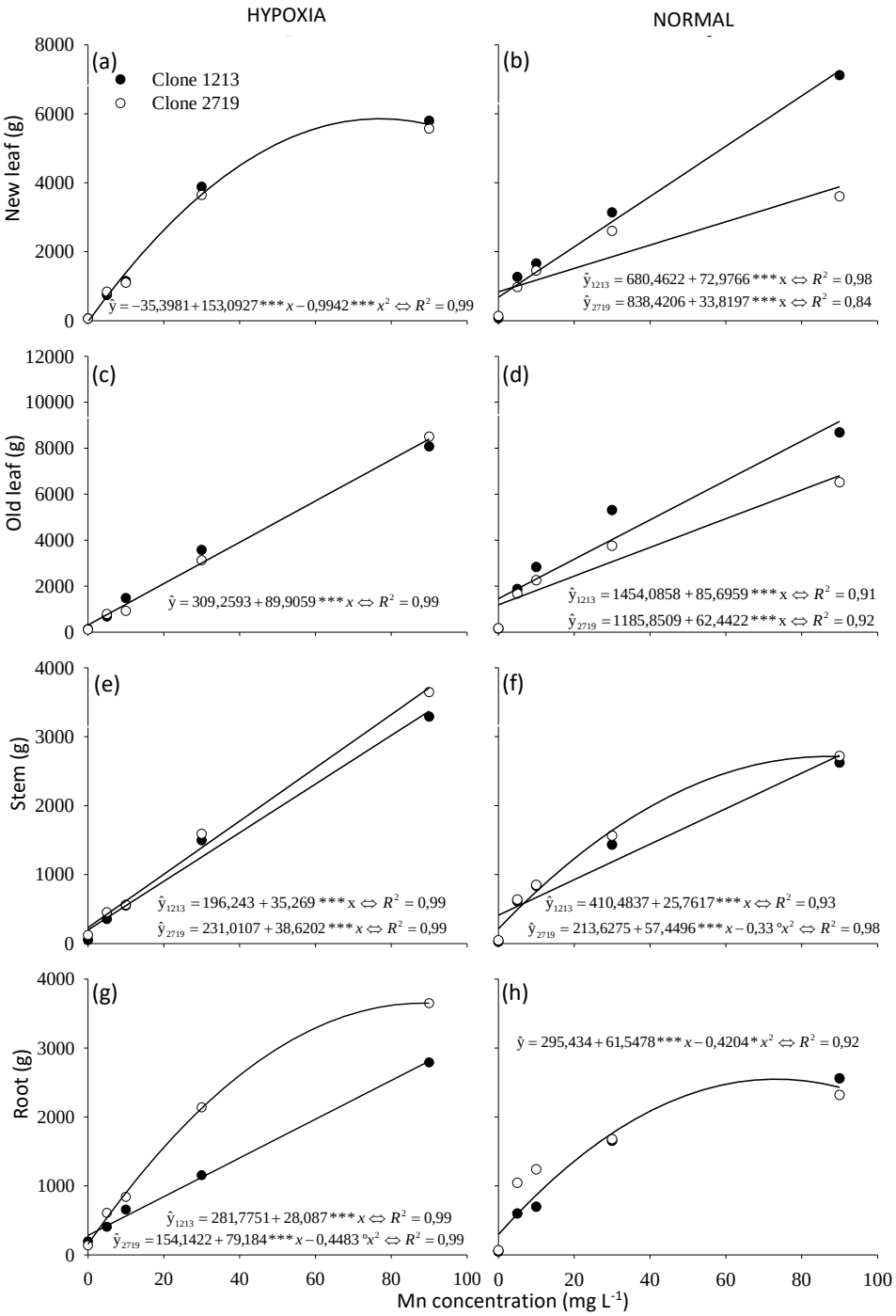

Figure 3. Mn contents in new leaves (a, b) and old (c, d), stem (e, f) and root (g, h) in eucalyptus seedlings (clones 1213® and 2719® CENIBRA SA) influenced by concentrations of Mn and O2, at 25 days. °; *; ***: significant at 10, 5 and 0.1%, respectively, by the F test.

Figure 4. Growth of aerial part (A) and root system (B) of the sensitive clone (1213®) submitted to the normal condition (8 mg L-1 of O2) (+ O2) and hypoxia (6 mg L-1 of O2) (- O2), combined with two Mn concentrations (30 and 300 mg L-1); Initial symptom of Mn toxicity in new leaf (C) and, in a more advanced phase (D), in clone 1213® (6 mg L-1 of O2 and 300 mg L-1 of Mn).

Table 1. Mean values of shoot height (MSH), shoot height (SH), ethylene concentration (EC), stem diameter (D) and root growth (RG) of eucalyptus seedlings 1213®-sensitive and 2719®-SPEVRD-tolerant), as a function of Mn and O2 concentrations

O_2	Mn	MSH	SH	EC	D	RG
mg L^{-1}		cm	cm	µg kg^{-1}	mm	cm
			Clone 1213			
6	30	21,4 Ab*B*	65,5 Ab*B*	144,4 Aa*A*	5,2 Aa*A*	7,7 Ab*B*
6	300	22,5 Ab*A*	69,0 Aa*A*	126,9 Aa*A*	4,9 Aa*A*	7,6 Ab*B*
8	30	26,1 Ab*A*	71,6 Ab*A*	94,9 Aa*B*	5,8 Aa*A*	13,3 Ab*A*
8	300	22,7 Bb*A*	63,2 Bb*B*	77,4 Ba*B*	5,2 Aa*A*	13,0 Aa*A*
			Clone 2719			
6	30	32,5 Aa*A*	75,3 Aa*A*	110,8 Ab*A*	5,5 Aa*A*	16,1 Aa*A*
6	300	32,1 Aa*A*	55,8 Bb*B*	111,7 Aa*A*	5,4 Aa*A*	12,2 Aa*A*
8	30	35,4 Aa*A*	77,2 Aa*A*	77,6 Ab*B*	5,4 Aa*A*	19,5 Aa*A*
8	300	30,0 Aa*A*	74,4 Aa*A*	58,8 Bb*B*	5,3 Aa*A*	14,1 Ba*A*

Means followed by upper case vertical letters for the same clone and O_2 do not differ in concentrations of Mn by the Tukey test at 5%. Means followed by lower case vertical letters for the same Mn and O_2 concentration do not differ for clones by the Tukey test at 5%. Means followed by equal vertical and vertical capital letters for the same clone and concentration of Mn do not differ as to O_2 concentrations by the 5% Tukey test.

Table 2. Mean dry matter of root (R), stem (S), new leaf (NL), old leaf (OL), shoot (S) and total (T) seedling (1213®-sensitive clones and 2719 ® -resolerant to SPEVRD), as a function of Mn and O_2 concentration

O_2	Mn	R	S	NL	OL	S	T
mg L^{-1}				g $plant^{-1}$			
				Clone 1213			
6	30	4,7 Ab*A*	6,6 Aa*B*	0,35 Ab*B*	5,3 Aa*B*	12,2 Aa*B*	17,0 Aa*B*
6	300	4,6 Aa*A*	7,0 Aa*A*	0,31 Aa*A*	5,2 Aa*A*	12,5 Aa*A*	17,2 Aa*A*
8	30	4,9 Aa*A*	9,1 Aa*A*	0,48 Aa*A*	6,1 Aa*A*	15,7 Aa*A*	20,6 Aa*A*
8	300	4,6 Ab*A*	6,8 Bb*A*	0,31 Bb*A*	5,8 Aa*A*	12,9 Ba*A*	17,6 Bb*A*
				Clone 2719			
6	30	5,4 Aa*A*	7,5 Aa*B*	0,53 Aa*A*	5,0 Aa*A*	13,1 Aa*A*	18,5 Aa*A*
6	300	4,7 Ba*B*	5,7 Bb*B*	0,36 Ba*B*	2,8 Bb*B*	8,9 Bb*B*	13,7 Bb*B*
8	30	5,2 Aa*A*	8,9 Aa*A*	0,55 Aa*A*	5,4 Aa*A*	14,9 Aa*A*	20,2 Aa*A*
8	300	5,5 Aa*A*	8,5 Aa*A*	0,55 Aa*A*	6,1 Aa*A*	12,2 Aa*A*	20,8 Aa*A*

Means followed by upper case vertical letters for the same clone and O_2 do not differ in concentrations of Mn by the Tukey test at 5%. Means followed by lower case vertical letters for the same Mn and O_2 concentration do not differ for clones by the Tukey test at 5%. Means followed by equal vertical and vertical capital letters for the same clone and concentration of Mn do not differ as to O_2 concentrations by the 5% Tukey test.

3.2 Analyses of the Dry Matter from the Plant Components

At the highest concentration of Mn (300 mg L^{-1}) under normal oxygenation (8 mg L^{-1}) provoked reduced growth on the aerial part of the plant in the sensitive clone (Tables 1 and 2).

Under mild hypoxia (6 mg L^{-1}), only the height of the tolerant clone was affected negatively by the highest concentration of Mn, not causing reduced growth of roots, stem and stem diameter in both clones (Table 1). However, there was a lower production of dry matter for all the plant components at the highest dose of Mn under 6 mg L^{-1} of O_2 for the tolerant clone (Table 2). The same effects were not observed for the sensitive clone. These observations suggest that excess Mn was more damaging to the growth of the sensitive clone under normal O_2 conditions. On the other hand, under hypoxia the highest dose of Mn was the most limiting for the growth of the tolerant clone. This was confirmed by the data from the total production of dry matter of the plants, in which the sensitive clone produced the highest accumulation of biomass under hypoxia. In contrast, under normal O_2 level and the highest dose of Mn, the tolerant clone performed the best (Table 2).

Table 3. Mean levels of manganese in eucalyptus seedlings (clones 1213®-sensitive and 2719®-SPEVRD-tolerant), as a function of Mn (treatment) and O2 concentration

O_2	Treatment(1)	Mn	O_2	Treatment(1)	Mn
	mg L^{-1}	mg kg^{-1}		mg L^{-1}	mg kg^{-1}
	Root			New leaf	
		Clone 1213			
6	30	4704 Bb*A*	6	30	7267 Ba*A*
6	300	17298 Aa*A*	6	300	19152 Aa*A*
8	30	4788 Bb*A*	8	30	9647 Ba*A*
8	300	16625 Aa*A*	8	300	12461 Aa*B*
		Clone 2719			
6	30	6524 Ba*A*	6	30	4560 Bb*B*
6	300	15310 Aa*B*	6	300	8983 Ab*A*
8	30	6134 Ba*A*	8	30	6061 Bb*A*
8	300	19006 Aa*A*	8	300	9326 Ab*A*
	Stem			Old leaf	
		Clone 1213			
6	30	2779 Bb*B*	6	30	5412 Ba*A*
6	300	5471 Aa*A*	6	300	11030 Aa*A*
8	30	3326 Ba*A*	8	30	5921 Ba*A*
8	300	5398 Ab*A*	8	300	9484 Aa*A*
		Clone 2719			
6	30	3004 Ba*B*	6	30	4235 Ba*B*
6	300	5294 Aa*A*	6	300	7173 Ab*B*
8	30	3664 Ba*A*	8	30	5856 Ba*A*
8	300	5868 Aa*A*	8	300	10123 Aa*A*

(1)Treatment: Mn concentration added for each treatment; means followed by upper case vertical letters for the same clone and O2 do not differ in Mn concentrations by Tukey test at 5%. Means followed by lower case vertical letters for the same concentration of Mn in the treatment and O2 did not differ for clones by Tukey test at 5%. Means followed by equal vertical and vertical capital letters for the same clone and concentration of Mn do not differ as to O2 concentrations by the 5% Tukey test.

Root growth

Excess Mn had no effect on root growth in both the clones, except of the tolerant clone under normal O_2 (Table 1). The root growth of the sensitive clone was extremely sensitive to hypoxia, presenting a 41 % reduction in growth, independent of the Mn concentration. Thus hypoxia is extremely damaging to the growth of the root system, as observed by other authors (George et al., 2012; Júnior et al., 2015; Muhammad et al., 2016; Shao et al., 2016; Xue et al., 2015). The same effect was not observed in the tolerant clone, supporting the idea that its root system is superior under mild hypoxia. Additionally, the reduced root growth could be attributed to the elevated concentration of ethylene (Muhammad et al., 2016). In flooded soils,

the ethylene produced by the roots stimulates the biosynthesis of auxin (Lynch et al., 2012), when transported to the zone of root growth could inhibit cellular elongation, reducing root growth (Shimamura et al., 2016).

Throughout the experimental period (27 days), the daily root growth was reduced in both clones in response to O_2 and Mn concentrations (Figure 4). Again, the effect of hypoxia was much more pronounced than that of the high concentration of Mn. Under hypoxia, independent of the concentration of Mn or the clone used, the daily root growth was always lower than that under normal oxygenation.

Synthesis of ethylene

Anaerobic stress triggered significant ethylene production in both clones (Table 1), with the sensitive one showing a larger increase. However, Mn did not influence this increase under hypoxia. Similar results occurred in *E. camaldulensis*, *E. globulus*, *E. obliqua* (Blake and Reid, 1981) and *E. robusta* (Clemens and Pearson, 1977) under flooding, suggesting that the production of this hormone is one of the principal physiological modifications that occur under hypoxia in eucalyptus. Under hypoxic conditions expression of the gens that regulate the production of ethylene is intensified (Alpuerto et al., 2016; Dong et al., 2016), altering the growth and morphology of the root system. This higher production of ethylene corroborates the results presented in experiments I and II, since reduction in the growth of the root system (Phukan et al., 2015) and the formation of aerenchyma (Drew et al., 1989; He et al. 1996, Shimamura et al., 2016), are closely correlated with the production of this hormone in hypoxic conditions.

Mn toxicity

The increase in the concentration of Mn from 30 to 300 mg L^{-1}, under hypoxia or not, elevated the level of this micronutrient in all the plant compartments in both clones (Table 3). The sensitive clone accumulated more and in general the higher levels were observe in the leaves. This result together with those observed in the field, where the clones are more sensitive to ESBVRD, presented higher levels of Mn in the leaves (Leite et al., 2014). In this study, the levels of Mn in the sensitive clone reached 11000 and 19000 mg kg^{-1} in mature and young leaves, respectively. For the tolerant clone, close to 6000 and 9000 mg kg^{-1} were observed in mature and young leaves, respectively. However, this did not result in higher levels of Mn in the root system of the sensitive clone, indicating that the higher tolerance of the tolerant clone to Mn excess, although the levels of Mn in the roots were similar to those found in the roots of the sensitive clone, could be linked to a limitation in the rate of absorption or translocation to the aerial part of the plant.

Under hypoxia and Mn excess, the sensitive clone showed loss of apical dominance and a brownish necrosis in young leaves, and with time they became wrinkled and necrotic (Figures 4C and D). The level of leaf Mn in this condition was 19000 mg kg^{-1}, suggesting that this level is toxic for the sensitive clone (Table 3). Similar symptoms have been observed in eucalyptus by Winterhalder (1963); however, in this study, besides these symptoms, the leaves were small and chlorotic. This can be attributed to the limitation that the excess of Mn

causes on the absorption of Fe (Eroglu et al., 2015). In the same study by Winterhalder (1963), *E. gummifera* was the most sensitive to Mn in the soil, while *E. saligna* was able to absorb large quantities of this micronutrient without showing symptoms of toxicity. The levels of Mn observed in the leaves of *E. gummifera* and *E. saligna* were 2040 and 4250 mg kg^{-1}, respectively, with an adequate level of Mn being 510 mg kg^{-1}. These observations suggest that there are different levels of tolerance to Mn among the species of eucalyptus, as reported by El-Jaoual and Cox (1998), with *E. urograndis* being generally classified as one of the more tolerant species to an excess Mn.

Among the symptoms characteristic of Mn toxicity are brownish lesions, loss of apical dominance, leaf wrinkling (Asati et al., 2016), cracks in the lateral branches also occur with high frequency in ESBVRD. As previously described, some of these symptoms resemble those presented in the field, such as brownish lesions and leaf wrinkling. However, lesions in stems/stalks, petioles and branches were not observed under greenhouse conditions. Such information indicates that the time of exposure to Mn could have been too short for the full expression of these symptoms. However, indicating Mn as the cause of the ESBVRD is still not reliable, mainly because the plants that are exposed to excess Mn are subject to several other complications arising from the hypoxic condition.

4. Conclusions

Pronounced hypoxia causes reduced growth of both clones, sensitive and ESBVRD-tolerant, although the effect is more accentuated in the sensitive clone, being root growth the best indicator of the stress caused by hypoxia; Mn did not influence this evaluation. Mild hypoxia triggered the greatest increase in ethylene production in the sensitive clone and Mn did not influence this increase as well. Nevertheless, the visual symptoms of Mn toxicity occurred only in young leaves of the sensitive clone, characterized by the formation of brownish necrosis on the leaf edges, followed by wrinkling, with hypoxia being the first factor that predisposes the clones to ESBVRD which Mn excess acts in the long term for the complete expression of the characteristic symptoms.

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