

EDUARDO FERREIRA MEDINA

PLANT RESPONSES TO SOIL ANOXIA AND HYPOXIA

Thesis presented to the Universidade Federal de Viçosa as part of the requirements of Pos-Graduate Program in Soil Science and Plant Nutrition for obtention of degree of *Doctor Scientiae*

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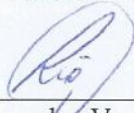
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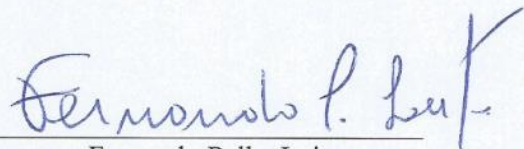
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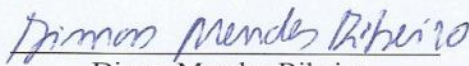
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ABSTRACT

MEDINA, Eduardo Ferreira, D.Sc., Universidade Federal de Viçosa, March, 2017. **Plant responses to soil anoxia and hypoxia.** Advisor: Ivo Ribeiro da Silva. Co-Advisor: Dimas Mendes Ribeiro.

The effects of soil hypoxia/anoxia in plants have been demonstrated in this work in different clones of Eucalyptus and in chickpea. In Brazil, the eucalyptus plantations have an important economic value to produce pulp, paper and charcoal. The problem is that in the last decades some physiological disturbances have been seen in eucalyptus plantations, and these problems are related to rainy periods and soil features, like poor drainage. Nevertheless, eucalyptus clones got differential tolerance to this disturb and this study has shown that there are metabolic and physiological differences between them. The concentration of aminoacids (specially alanine and GABA) have increased when plants are in hypoxic stress. However, changes in metabolites levels in different parts of the plant have shown differential behavior between clones. In another hand, the chickpea is an important legume used as source of protein in several parts of world, in special in Africa and Asia. In several areas, the chickpea plantations face strong rainy periods, leading to soil hypoxia and anoxia. The chickpea seed is full of starch reserves and it isn't clear if the root tips die during the hypoxic/anoxic stresses due to carbon starvation. In the experiment, we have seen less root tip mortality and better seedling root growth when the nutrient solution is supplied with glucose (50 mM) in comparison to the controls with no sugar. Even plants in anoxic system for 72 h had better root growth when the sugar was added than those which did not receive glucose in nutrient solution.

RESUMO

MEDINA, Eduardo Ferreira, D.Sc., Universidade Federal de Viçosa, março de 2017.
Resposta de plantas a anoxia e hipoxia no solo. Orientador: Ivo Ribeiro da Silva.
Coorientador: Dimas Mendes Ribeiro.

Os efeitos da hipoxia/anoxia no solo em plantas foram demonstrados neste trabalho em diferentes clones de eucalipto e em grão-de-bico. No Brasil, as plantações de eucalipto têm um importante valor econômico para produzir polpa, papel e carvão. O problema é que nas últimas décadas foram observados distúrbios fisiológicos em plantios de eucalipto e estes problemas estão relacionados aos períodos chuvosos e algumas características do solo, como o perfil sem boa drenagem. No entanto, os clones de eucalipto obtiveram tolerância diferencial a este distúrbio e este estudo mostrou que existem diferenças metabólicas e fisiológicas entre eles. A concentração dos aminoácidos (especialmente alanina e GABA) aumentou quando as plantas estão em estresse hipóxico. Entretanto, mudança em metabólitos e a forma como estes metabólitos aumentam ou diminuem seus níveis em diferentes partes da planta tem mostrado comportamento diferencial entre clones. Por outro lado, o grão-de-bico é uma importante planta leguminosa utilizada como fonte de proteína em várias partes do mundo, especialmente na África e na Ásia. Em várias áreas, as plantações de grão-de-bico enfrentam fortes períodos chuvosos, levando a hipoxia e anoxia do solo. A semente de grão-de-bico está cheia de reservas de amido e não está claro se o ápice da raiz morre durante o estresse hipóxico/anóxico devido à falta de carbono. No experimento, observamos menor mortalidade de ápices de raiz e melhor crescimento do sistema radial de plântulas quando a solução nutritiva foi suplementada com glicose (20 mM) em comparação com os controles sem açúcar. Mesmo plantas em sistema anóxico por 72h tiveram melhor crescimento da raiz quando o açúcar foi adicionado do que aquelas que não receberam glicose em solução nutritiva.

GENERAL INTRODUCTION

The lack of oxygen in soil is one of the most important stresses which plants have to deal in the nature. It is supposed that climatic changes might cause more rain in some regions probably causing more flooding in these places. The losses caused by waterlogging can be attenuated using tolerant species or clones to be cultivated in problematic areas (Dat *et al.*, 2006). But how these tolerant plants deal to survive in these areas, or maybe how these plants change their physiologic parameters and metabolism is still not clear.

Changes in the soil oxygen availability are quickly realized by roots as water fills the soil pores (Parent *et al.*, 2008). The effects of lack of oxygen display quick changes in the gene expression triggering changes in metabolic pathways. However, the hypoxic stress can last for days. It means that plants which have the ability to survive in soil hypoxic/anoxic conditions change not only the metabolism but also physiologic parameters and anatomic features (Jackson and Colmer, 2005).

The carbon net assimilation is very sensitive to the hypoxic stress, and the decrease in the photosynthesis occurs even with the maintenance of the normal stomatal conductance in rapid hypoxic events. However, when the hypoxia in soil extends for a long period of time root necrosis can drop down the hydraulic conductivity, leading to a decrease in the stomatal conductance decreasing the photosynthetic rates in flooded plants (Argus *et al.*, 2015).

Some responses to hypoxia are well known, like fermentation leading to lactate production first and then to ethanol (Kato-Noguchi, 2000b). However, Krebs alternative pathways have been shown in different plants (Rocha *et al.*, 2010). It is not yet clear whether the alternative pathways can play a crucial role in plants leading to a differential tolerance to waterlogging or it is just a common response that all plants share.

The accumulation of aminoacids, in special GABA and alanine, in the hypoxic stress is an important because this can explain how plants survive in anoxic and hypoxic environments changing the metabolism and linking the nitrogen to the carbon metabolic routes (Miyashita and Good, 2008). Plants might be different in the anatomical structure or they can have differential phenotypic plasticity to survive in flooded areas (Colmer, 2002), however the differential changes in the metabolism between them in order to fit better in these kind of environment is still a thrilling matter to be further investigated.

There are strong evidences that the soil hypoxia, or at least cycles of drought and hypoxia can trigger symptoms of the shoot dieback in eucalyptus (Lacerda *et al.*, 2010 and Leite *et al.*, 2014). But it remains unclear how the hypoxia causes these effects in eucalyptus plants in field, because the symptoms of the shoot dieback have not been recreated in controlled experiments yet.

Over the ages plants are dealing with a lot of different kind of situations changing quickly the genic expression, metabolic profile and then the growth rate to fit better during each event which may cause a stress. By studying the effects of soil hypoxia in plants we are preparing the agriculture for the future in a changing world.

This thesis is composed of 3 chapters, the first one analyses the shoot growth, metabolic profile and physiological parameters in *Eucalyptus* clones cultivated in field which have differential tolerance to the shoot dieback. The second discuss about the changes in metabolism and physiological parameters in *Eucalyptus* clones under hypoxic stress. The last studies the effects of anoxia and sugar addition in root growth and survival of chickpea seedlings. Chickpea grows quickly and it is a well-studied plant which gave us some advantages to evaluate how the anoxia plus sugar addition might change the root growth and increase the plant root survival rate.

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CHAPTER 1

METABOLIC AND PHYSIOLOGICAL DIFFERENCES BETWEEN *EUCALYPTUS* CLONES IN RESPONSE TO SHOOT DIEBACK

Abstract

The shoot dieback also known as physiologic disturb is an issue in many eucalyptus plantations in Brazil. Its occurrence is slightly correlated with rainy periods. Nevertheless, there are divergences between the clones planted, some are considered tolerant and others are sensitives to shoot dieback. This study was carried out to evaluate the physiological mechanisms that may be associated with differential tolerance of eucalyptus clones to shoot dieback under field conditions. The eucalyptus clones (tolerant and sensitive) grew in a farm plantation in the coastal area and it was evaluated the plant growth, gas exchanges (only in mature leaves), fluorescence and metabolomics in young and mature leaves as well as in shoot meristem. The tolerant clone grew more during the time analyzed (38 days). The tolerant clone exhibited higher carbon net assimilation (A) and lower values of the ratio of C_i with the atmosphere $CO_{2(g)}$ concentration (C_i / C_a) than the sensitive one. The reduction in the A in the sensitive clone is signal that shoot dieback is affecting all shoot causing a big reduction in the plant growing. The metabolic profile of shoot meristem, young leaves and mature leaves in each clone showed a distinct distribution of metabolites. The main differences between the metabolic profile of the clones were seen in the mature leaves. The behavior of metabolites in mature and young leaves shows that the shoot dieback affects first these parts and then the shoot meristem collapses last.

Keywords: adaptation, gas exchange, growth inhibition, metabolic plasticity, water regimes

INTRODUCTION

The eucalyptus plantations have a great role in Brazil's economy, and today there are around 6 million hectares planted (ABRAF, 2013). The wood is highly valued by the charcoal and pulp and paper industries (PÖYRY, 2013). The short rotation allows wood production for several other activities, helping to preserve the native forests from logging (IBA, 2014). During the last 30 years, some *Eucalyptus* clones have had lower timber production due to a physiological disturb in the shoot meristem, called shoot dieback. These losses often occur in areas with heavy raining periods and poorly drained soils.

The cause of shoot dieback is still unknown, but it is believed that edafoclimatic conditions play an essential role in triggering the disturb. The first cases of shoot dieback appeared during the 80's and were linked to a possible clonal productive decline. The main disturb symptoms are darkening of petioles and plagiotropic branches, leaf abscission and death of apical meristem. The plants that survive the shoot dieback lose the apical dominance and develop new buds in the trunk and become inappropriate to the wood industry (Ferreira, 1986). The shoot dieback has been reported in some studies as effect of lack of boron in eucalyptus plants. During dry periods this nutrient might become deficient in plant tissues even when it is provided by soil fertilization (Mattiello *et al.*, 2009). Others studies showed that the level of manganese seen in dieback sensitive plants is higher than in those tolerant ones, leading to a toxicity caused by manganese (Lacerda *et al.*, 2010). Plants in higher areas of valleys show less symptoms of shoot dieback than those in lower parts (Leite *et al.*, 2014). It has been shown the occurrence of shoot dieback in plants with age between 6 up 18 months (Ferreira, 1989). However, symptoms actually can occur in almost all ages. Eucalyptus plants with two months established in the field in drained areas showed symptoms of shoot dieback in coastal areas in Brazil with low concentration of manganese in the soils.

The shoot dieback causes severe decrease in timber productivity because of disruption on

the natural plant architecture (Maschio *et al.*, 1996) and decrease in the carbon net assimilation. The photosynthetic parameters are very sensitive to environmental stresses such as drought, salinity, temperature, plant disease and root hypoxia (Voesenek and Bailey-Serres, 2015). Besides of changes in plant growth, the stresses may cause strong differences in physiologic parameters. The carbon net assimilation and stomatal conductance are very sensitive to changes on root status (Chaves *et al.*, 2009). In this context, the plant metabolism is completely modified after different stresses (biotic or abiotic). The plant metabolic profile is important tool to understand how stresses may affect the whole plant by stopping the respiration or damaging the amino acidic pathways for example (Obata and Fernie, 2012).

Changes in specific metabolites can give us cues about what is happening in the molecular level of the shoot dieback. The main effects of this disturb are seen in the shoot meristem but what is happening in other parts of the plant are still unknown. The increase in content of aminoacids might be a signal of some stress is going on (Reggiani and Bertani, 2003). The accumulation of GABA and alanine is very sensitive to the hypoxic/anoxic stress in roots (Miyashita and Good, 2008). The effects caused by root hypoxia are easily noticed in root metabolism however root responses to hypoxia can affect the content of some metabolites in the shoot (Argus *et al.*, 2015). Probably the transpiration stream is carrying these metabolites from the roots to the shoot or the downregulation caused by the decrease in the root growth can change the concentration of sugars in leaves (Colmer and Greenway, 2005). Nevertheless, it is also unclear if these metabolites could act as a signal triggering adaptive responses in the shoot or if they are just been translocated from the roots to the shoot by the transpiration stream.

Here, we hypothesized that the differential tolerance of eucalyptus clones to shoot dieback is associated with changes in primary carbon metabolism. We demonstrated that the shoot dieback decreases the growth and net carbon assimilation and the sensitive clone had

increase in content of metabolites linked to the root hypoxic stress mainly in mature and young leaves.

MATERIALS AND METHODS

Plant Conditions

The experiment was conducted in Mucuri (18° 05' 11" S, 39° 33' 03" W), northeastern Brazil between April and June of 2015. The meteorological data were obtained from automatic weather stations of Instituto Nacional de Meteorologia (INMET) (Supplementary figure 1). Seedlings of two clones of *Eucalyptus urograndis* with differential tolerance to the shoot dieback (tolerant and sensitive) were cultivated in the field for three months. The clones are characterized as *Eucalyptus urograndis* obtained by controlled cross-pollination between *Eucalyptus grandis* and *Eucalyptus urophylla*.

Growth measurements

Plants cultivated for 3 months in the field of both clones had the stem elongation and stem thickening rate calculated in the same period, 38 days from the first to the last measurement. It was used a graduated tape to get the plant height from the soil up to the highest apical meristem. A digital caliper for measuring the stem thickness five centimeters from the soil surface. The same plants were used in both measurements, 6 plants of the tolerant and 6 plants of the sensitive clone.

Photosynthetic measurements

The net photosynthetic rates (A), stomatal conductances to water vapor (g_s) and internal-

to-ambient CO₂ concentration ratio (C_i/C_a) were determined using a Li-6400 open gas exchange system (Li-Cor, Lincoln, NE, USA) equipped with a blue/red light source (Li-6400-02B). The measurements were made in mature leaves (leaves located in the fourth or fifth pair of leaves from the shoot meristem in the middle third of the plant height) between 08:00 and 12:00 h at ambient temperature and air relative humidity (about 24 °C and 68 %, respectively) and under artificial light, air flow and CO₂ conditions (1400 μmol photons m⁻² s⁻¹ at the leaf level, air flow of 300 mol s⁻¹ m⁻² and 400 ppm respectively).

Metabolic profile

The metabolic profile was done in a gas chromatography-mass spectrometry (GC-MS) as described previously by (Liseč *et al.*, 2006). Briefly, 100 mg of mature leaves, young leaves (second or third pair of leaves from the meristem in the middle third of the plant height) and shoot meristems (until 2 cm down from the meristem tip in the middle third of the plant height) was homogenized with liquid nitrogen and extracted in 1.4 mL of methanol, and 60 μL of ribitol diluted in deionized water as internal standard (0.2 mg mL⁻¹) was subsequently added as quantification standard. The mixture was extracted for 15 min at 70 °C and mixed vigorously with 1.4 mL of water. To separate polar and nonpolar metabolites, 750 μL of chloroform was added to the mixture. After centrifugation at 2,200 g, the upper methanol/water phase was taken and dried in vacuum. Residues were redissolved and derivatized in 40 μL of 20 mg mL⁻¹ methoxyamine hydrochloride in pyridine at 37 °C for 120 min. Afterwards, the extract was treated with 60 μL of *N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide at 37 °C for 30 min. Eight microliters of a retention time standard mixture (0.029% [v/v] *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotracontane, and *n*-hexatriacontane dissolved in anhydrous pyridine) was added before trimethylsilylation. Sample volumes of 1 μL were then

injected in the GC-MS system comprised of an AOC-20i autosampler, and a QP2010 SE gas chromatograph-quadrupole mass spectrometer (Shimadzu, Tokyo, Japan), equipped with a column Rtx-5MS (Restek, Bellefonte, CA, USA). The derivatization, standard addition, and sample injection steps were carried out exactly as previously described (Lisec *et al.*, 2006). Both chromatograms and mass spectra were evaluated using TAGFINDER software (Luedemann *et al.*, 2008). Compounds were identified according to standardized guidelines (Dethloff F *et al.*, 2014) by mass spectral and retention time index matching to the mass spectral collection of the NIST11 database. Laboratory and reagent contaminations were identified by control treatments and removed from further analysis. Quantifications were based on the peak height values of the recorded mass feature. These values were corrected for the dry weight of each sample and by the response of the internal standard from each respective GC-MS chromatogram to obtain normalized responses.

Statistical Analysis

Experiments were designed with six replicates per treatment. In experiments to determine leaf gas exchange the sample unit was the mature leaf. The metabolic profile was made by using the shoot meristem, young leaves and mature leaves (described above). Analysis of variance ($P < 0.05$) was executed to determine the effects of treatments. Differences among means were examined by using the Student's *t*-test ($P < 0.05$). All the tests were performed using the SigmaPlot 11 (Systat Software Inc., Hounslow, London, UK)

RESULTS

Growth and photosynthesis

The two clones showed visual differences in growth (Figure 1A) and the shoot meristems

of the sensitive clone were dying (Figure 1B, C and D) but in the same plant of the sensitive clone was possible to see healthy and dead shoot meristems (Figure 1D). The tolerant plants exhibited normal growth with no kind of anomalies in the leaves or in the shoot meristems (Figure 1A, B and C).

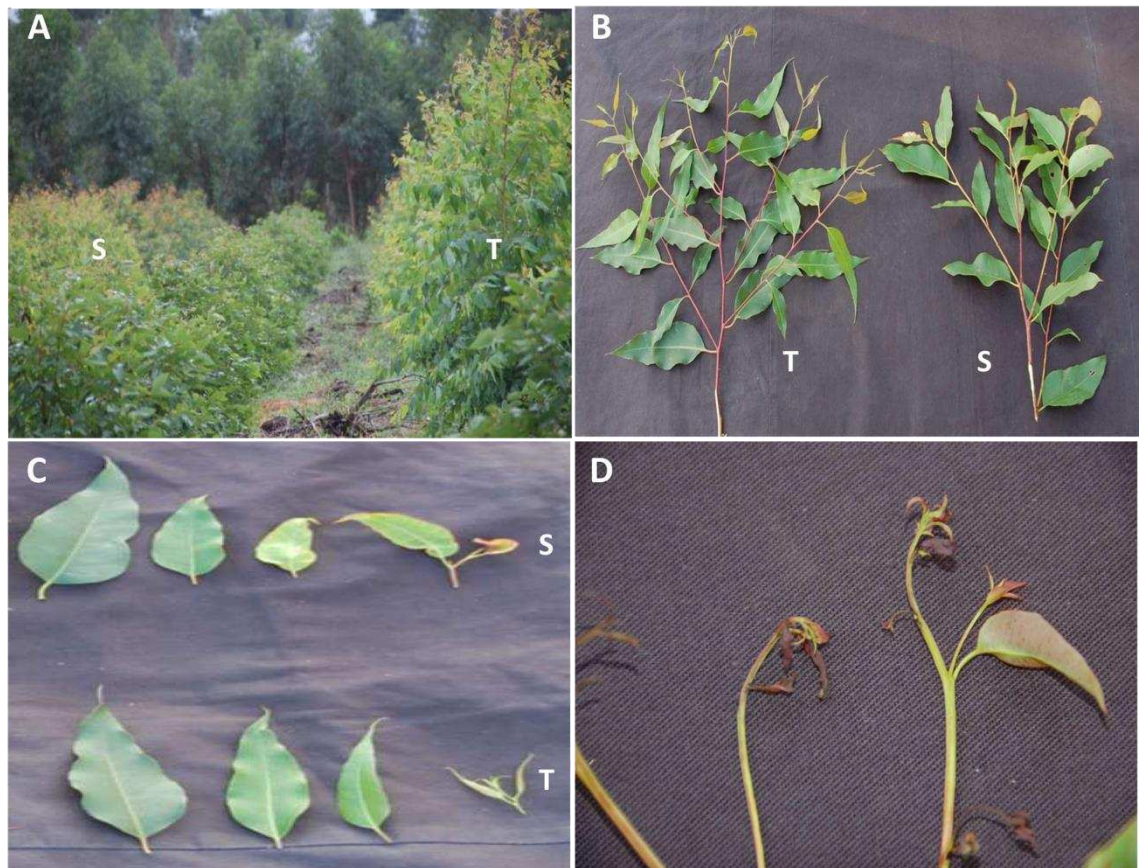


Figure 1. Detail of mature leaves, young leaves and shoot meristem (A), the shoot dieback in shoot meristems of the sensitive clone (B), picture of plants with the same age of both clones planted in the field (C), and apical stems and leaves of both clones (D). The letters T and S mean tolerant and sensitive clones, respectively.

The elongation rate in the main stem was approximately 3-fold higher in the tolerant clone than in the sensitive one (Figure 2A) during the evaluation period (38 days). The stem thickening did not show difference between the two clones (Figure 2B).

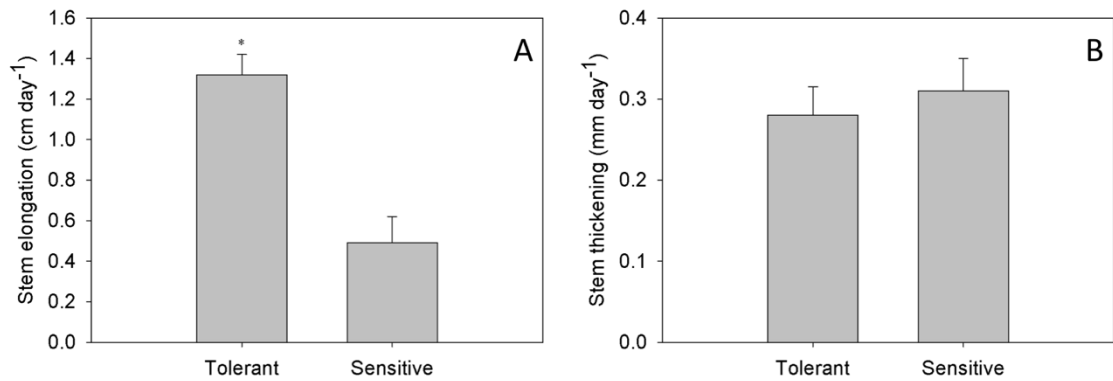


Figure 2. Grey bars represent the stem elongation (A) and stem thickening (B) calculated after a period of 38 days in two *Eucalyptus* clones. Asterisks indicate values determined by the Student's *t*-test to be significantly different from tolerant clone ($P < 0.05$). Values are presented as means of 6 replicates \pm SE.

The A measured only in completely expanded leaves (mature leaves) was about 10% higher in the tolerant clone than the sensitive one in the first measurement (Figure 3A). In the second measurement, the difference in the net carbon assimilation between the clones increased, the tolerant clone had a net carbon assimilation 39% higher than the sensitive clone (Figure 3D). The C_i/C_a was 7 and 13 % higher in the sensitive clone than in the tolerant one in the first and second measurements, respectively (Figure 3B and E). Furthermore, the g_s had the same value in both clones in both measurements (Figure 3C and F).

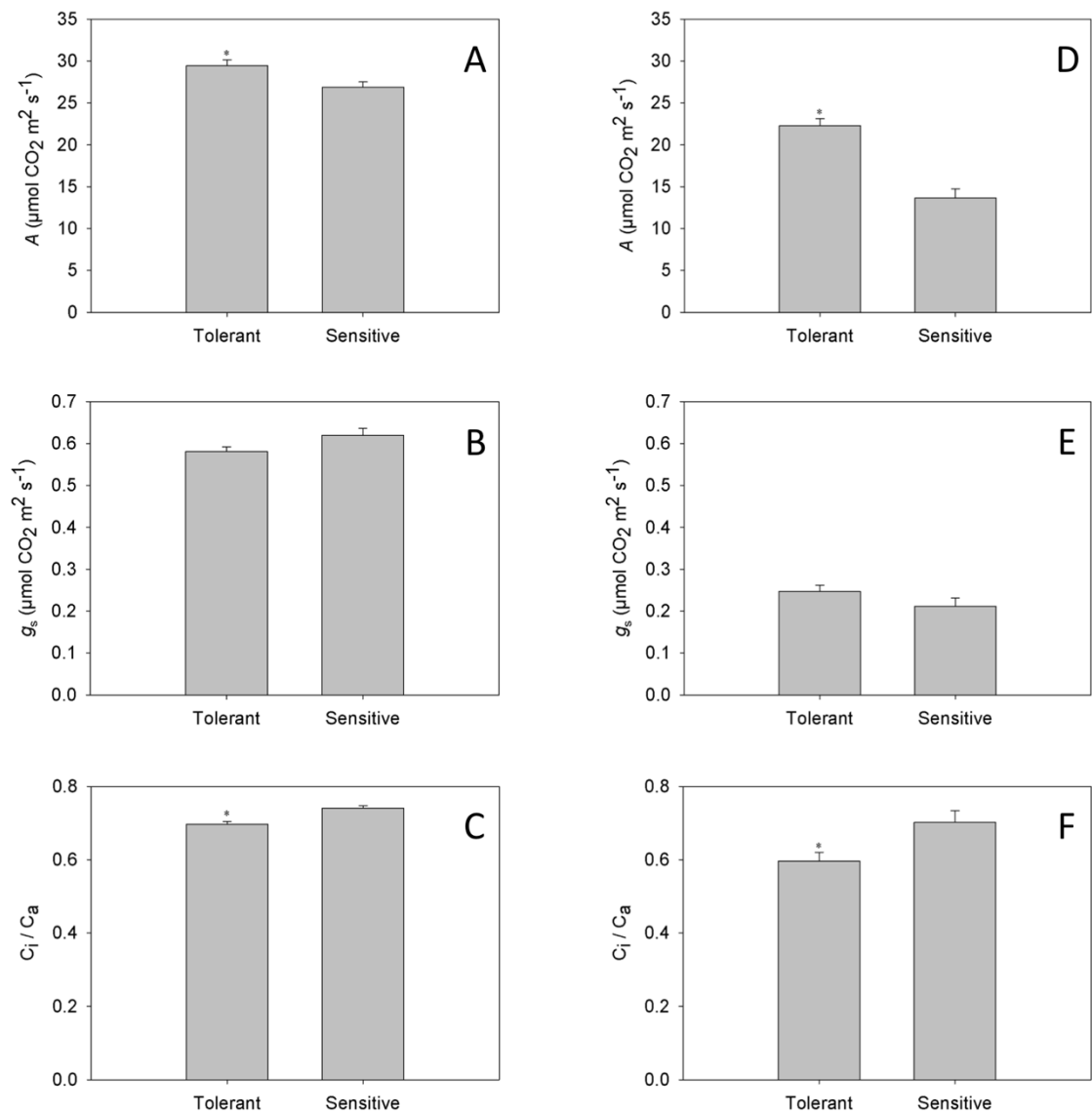


Figure 3. Grey bars represent the CO₂ net assimilation (A), C_i / C_a (B), g_s (C) on the first measurement. CO₂ net assimilation (D), C_i / C_a (E), g_s (F) 38 days after the first measurement in two *Eucalyptus* clones (tolerant and sensitive to the shoot dieback). Asterisks indicate values determined by the Student's *t*-test to be significantly different from tolerant clone ($P < 0.05$). Values are presented as means of 6 replicates \pm SE.

Metabolomics

The content of metabolites linked to the Krebs cycle is higher mainly in the mature leaves

of sensitive clones in comparison to the tolerant clone with the exception of fumarate (Figure 4 and Supplementary table 1). In young leaves the contents of isocitrate, malate, fumarate were the same in both clones (Figure 4). On the other hand, the contents of α -ketoglutarate, citrate and succinate increased 2.3, 2.5 and 1.8-fold respectively in the sensitive young leaves in relation to the young leaves of the tolerant clone. The shoot meristem of sensitive clone did not show the same variation to the tolerant clone as was seen in the young and mature leaves (Figure 4). In general, the values of tricarboxylic acids in shoot meristems of sensitive plants did not increase in relation to the tolerant ones with the exception of α -ketoglutarate which had a 1.3-fold increase (Figure 4 and Supplementary table 1). Actually, the contents of malate and isocitrate in shoot meristems decreased 10 and 20% respectively in the sensitive clone in comparison to the tolerant respectively.

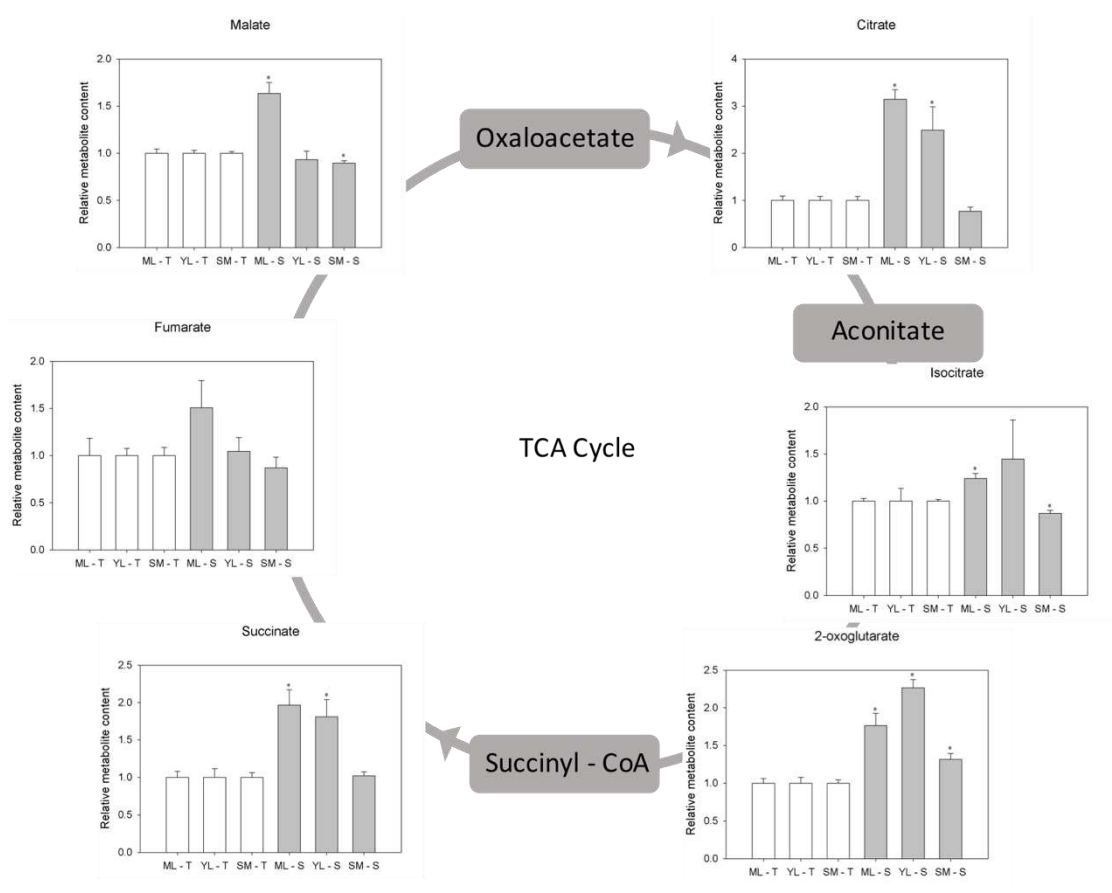


Figure 4. Relative content of tricarboxylic acids normalized to 1 (numeric value) for

the tolerant clone (white bars), grey bars represent the relative metabolite content of the sensitive clone. The plants parts analyzed were the shoot meristem (SM), young leaf (YL) and mature leaf (ML) of tolerant (T) and sensitive clone (S). Asterisks indicate values determined by the Student's *t*-test to be significantly different from tolerant clone ($P < 0.05$). Values are presented as means of 6 replicates \pm SE.

The content of glutamate, GABA, L-alanine, serine, L-valine and shikimate had a 2.1, 2.7, 4.8, 1.5 and 3.8-fold increase in the mature leaves of the sensitive clone in relation to the tolerant clone (Supplementary table 1). The shoot meristem of sensitive clone had a different behavior, now most of the aminoacids had a decrease in their concentrations in comparison to the shoot meristems of the tolerant clone (Figure 5). For example, the content of L-aspartate, glutamate, L-threonine and L-alanine in the sensitive were respectively 60, 60, 40 and 60% lower in shoot meristems of the sensitive clone in comparison to the tolerant one (Supplementary table 1). The content of L-aspartate, glutamate, L-proline, phenylalanine, L-threonine and serine was lower in the young leaves of the sensitive clone than in the tolerant one (Figure 5 and Supplementary table 1). GABA and shikimate contents were 2.7 and 1.4-fold higher in the shoot meristem of sensitive clone than in the tolerant one (Figure 5 and Supplementary table 1). Contents of sugars like sucrose, glucose and fructose was higher in the sensitive clone than in the tolerant clone (Supplementary table 1). For example in mature leaves the content of fructose, glucose and sucrose increased 1.3, 1.4 and 1.4-fold respectively. In the young leaves only the content of sucrose had a 1.8-fold increase. In the shoot meristem the contents of fructose and glucose had a 1.4 and 1.7-fold increase in relation to the tolerant clone.

Different aged leaves plus the different clones when evaluated might explain almost 97% of variation between the metabolites content between both clones (Supplementary figure 2). In general, each metabolite had a specific response depending on whether we are

evaluating mature leaves, young leaves or shoot meristem, the behavior of young and mature leaves of sensitive clone had some similarities (Supplementary table 1).

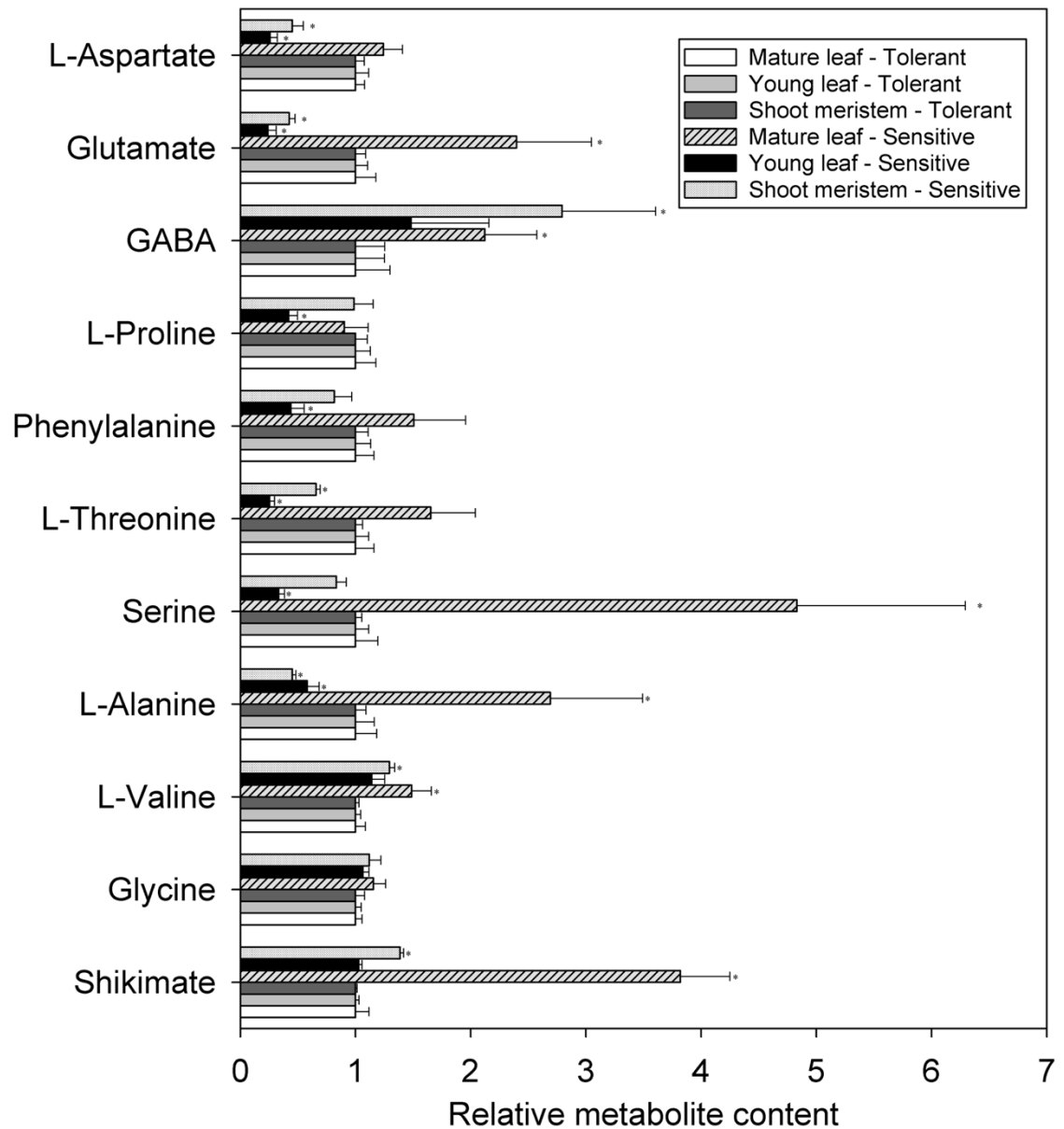


Figure 5. Values represented by bars of relative content of aminoacids normalized to 1 (numeric value) for the tolerante clone. Relative content of aminoacids in shoot meristem, young leaf and mature leaf of tolerant and sensitive clone. Asterisks indicate values determined by the Student's *t*-test to be significantly different from tolerant clone ($P < 0.05$). Values are presented as means of 6 replicates \pm SE.

DISCUSSION

Plant growth and photosynthetic parameters in plants with the shoot dieback

The beginning of 2015 was dry during the months of January and February with mean precipitation below of 50 mm per month. From March until June of 2015 the amount of rain increased substantially with mean precipitation values above 100 mm of rain per month (Supplementary figure 1). This situation with drought followed by intense rainfalls with the addition of high mean temperatures (Supplementary figure 1) might be the ideal scenario to the development of shoot dieback symptoms in eucalyptus. However, we still do not know how create the same stress in glasshouses, and how to predict when the shoot dieback will happen in the following seasons.

The shoot dieback influenced directly the stem elongation (plant height) by lack of apical dominance (Figure 1). It was observed that the shoot dieback caused a strong growth reduction in the plant height in the sensitive clone (Figure 2A), an effect observed also in eucalyptus productivity as shown by Maschio *et al* (1996). No difference was seen in the stem diameter growth rate during the 38 days of evaluation (Figure 2B). The carbon net assimilation was lower in the sensitive clone than in the tolerant at the beginning and at the end of the experiment (Figure 3A and D). The reduced A values of the sensitive clone caused a negative impact in plant height and future productivity as it is shown by Chaves *et al.*, (2002).

Both clones had the same stomatal conductivity (Figure 2B and E), showing that the sensitive clone did not have the decrease in the A due to the limitations on the stomatal aperture. The plants even suffering the disturbs of shoot dieback have shown to keep the root water uptake (Islam and Macdonald, 2004). So, the Rubisco (ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase) carboxylation sites did not consume the

carbon dioxide available in the internal leaf atmosphere increasing the values of C_i/C_a ratio (Figure 2C and F) (von Caemmerer *et al.*, 1981). But the decrease in the Rubisco activity can't be related to the lack of nitrogen in leaves of the sensitive clone. Because it was seen an increase in aminoacids content in the leaves of the sensitive clone (Reynolds *et al.*, 2012). The reduction in A can be induced by other non-stomatal factors as oxidative stress, negative regulation by the accumulation of carbohydrates and damage to the photosynthetic metabolism (Yordanova and Popova, 2007).

The systemic changes in the metabolism

The shoot dieback had an effect in all parts analyzed with more intensity in the mature leaves and young leaves than in the shoot meristems of the sensitive clone. The effects of intense rainfalls in the region in addition to a soil with a deficient drainage might have created a hypoxic environment to the roots causing the reduction in the carbon net assimilation and increasing the respiration by increasing the contents of the metabolites linked to the Krebs cycle in mature and young leaves (Figure 4) (Kreuzwieser and Rennenberg, 2014). Interestingly the amounts of sucrose were higher in mature and young leaves of sensitive clones in relation to the tolerant clone (Supplementary table 1). This fact might mean that the starch reserves have been mobilized to the respiration leading to the leaf senescence (Figure 1) when allied to the decrease in the A values (Figure 3A and D).

In general, aminoacids content tends to increase in response to a variety of different stresses in plants (Kinnersley and Turano, 2000). Plants under hypoxic stress exhibit some increase in the metabolite linked to the nitrogen metabolism or directly linked to the Krebs cycle. Here, we have seen the increase of aminoacids in mature and young leaves of sensitive plants (Figure 5). Metabolites like alanine and GABA had higher contents in

mature leaves of the sensitive clone in comparison to the tolerant one, interestingly these metabolites have its contents increased in plants that are suffering hypoxia stress (Rocha *et al.*, 2010). Besides these facts, shoot dieback sensitive eucalyptus clone under hypoxic condition increased more the amounts of alanine and GABA than the tolerant, nevertheless alanine, in special, can have its concentration changed in the shoot in response to root hypoxia (Medina *et al.*, 2017).

It was seen a slightly increase in sugars like fructose, glucose and sucrose in leaves and shoot meristems of the sensitive clone in relation to the tolerant one as it was seen by Argus *et al.*, 2014 (Supplementary table 1). This increase can be caused by the lack of growth in root leading to a metabolic depression of the consumption of these sugars (Colmer and Greenway, 2005).

The metabolomics is an excellent tool to understand how plants deal with environment stimuli, abiotic and biotic stresses (Hall, 2006). The Principal component analysis (PCA) showed a strong difference between the metabolic profile in all plant parts studied and same parts of different clones had difference is the PCA analysis as well (Supplementary figure 2).

It is possible to study some responses in specific groups of metabolites such as sugars, tricarboxylic acids and aminoacids in order to investigate the causes of the shoot dieback. However, there are evidences that the shoot dieback has the soil hypoxia as one of the main triggers of the disturb (Leite *et al.*, 2014) and studying the metabolic profile of sensitive and tolerant plants we have seen that the hypoxic stress might be playing an essential role to develop the symptoms of the shoot dieback.

The content of metabolites in the shoot meristem of both clones did not show a big difference like it was seen for the mature and young leaves. The shoot meristem is highest sensitive part in the shoot to any kind of stress. Probably during the evolution of shoot dieback symptoms some shoot meristems suffer the effects first than others (Figure 1).

So, in the same sensitive plant we have some healthy and dead shoot meristems. Then, small changes in the metabolites content make the shoot meristem collapse.

CONCLUSION

The reduction of A in the sensitive clone was important to cause the reduction in the plant growth. However, the causes of the reduction of Rubisco carboxylation which lead to increase the C_i/C_a ratio in mature leaves of the sensitive clone are still unclear.

The metabolites in general are diverging in the relative content in different parts of plant but it is important to say that the changes in the content of the metabolites in the sensitive clone were more evident in mature and young leaves than in the shoot meristem. As we said before is a matter for following studies to evaluate how the metabolites change immediately before the occurrence of shoot dieback, to understand better how the plants behave until the culmination of symptoms.

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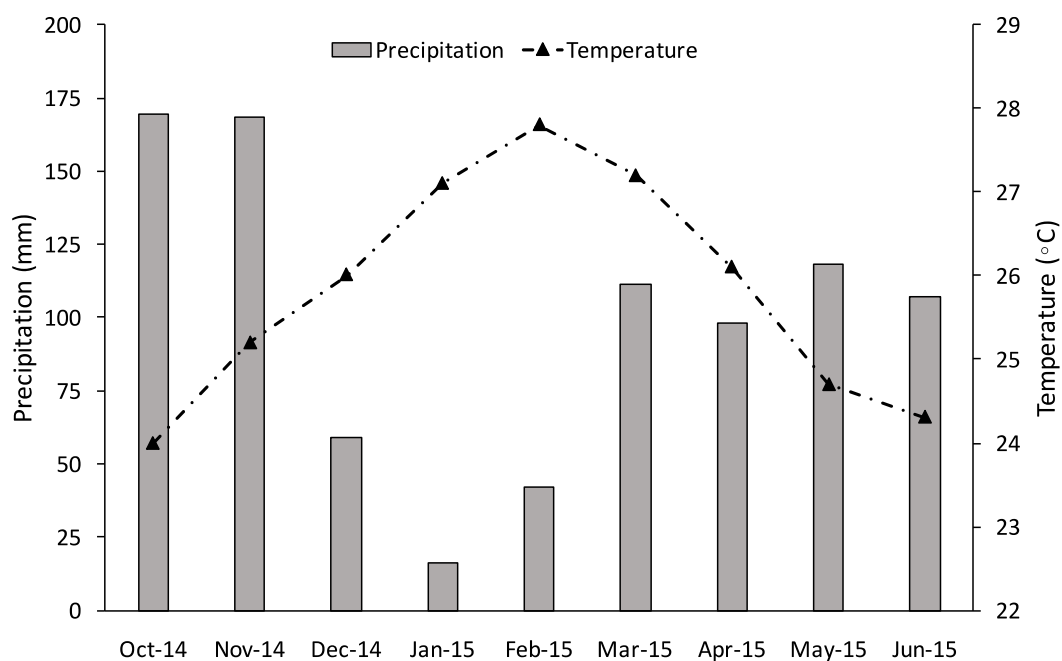
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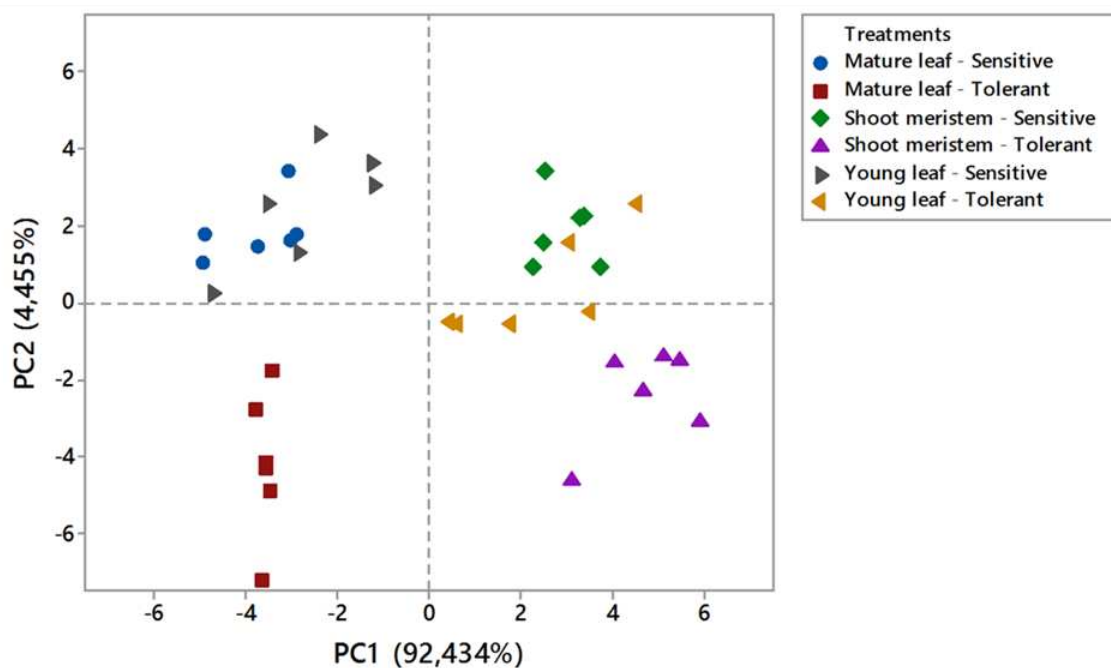
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SUPPLEMENTARY MATERIAL



Supplementary figure 1. Precipitation (columns) and mean temperature (line with triangles) from October 2014 to June 2015. The data were collected in the INMET weather station of Caravelas-BA. The experiment was evaluated from April 2015 to June 2015.



Supplementary figure 2. Principal component analysis of metabolic profile of mature leaf, young leaf and shoot meristem of 2 Eucalyptus clones (tolerant and sensitive to the shoot dieback). The total number of geometrical symbols represents the number of experimental units. Each symbol represents a different treatment.

	Tolerant			Sensitive		
	Mature leaf	Young leaf	Shoot meristem	Mature leaf	Young leaf	Shoot meristem
Lactate	1 ± 0.11	1 ± 0.08	1 ± 0.09	0.86 ± 0.18	1.1 ± 0.20	0.10 ± 0.19
Acetate	1 ± 0.11	1 ± 0.09	1 ± 0.09	0.92 ± 0.14	0.95 ± 0.08	0.80 ± 0.14
Oxalate	1 ± 0.31	1 ± 0.27	1 ± 0.14	4.21 ± 1.07	3.24 ± 1.33	2.48 ± 0.75
Succinate	1 ± 0.08	1 ± 0.12	1 ± 0.06	1.97 ± 0.20	1.81 ± 0.23	1.02 ± 0.05
Fumarate	1 ± 0.18	1 ± 0.08	1 ± 0.09	1.51 ± 0.29	1.04 ± 0.15	0.87 ± 0.11
Malate	1 ± 0.05	1 ± 0.03	1 ± 0.02	1.63 ± 0.12	0.93 ± 0.09	0.90 ± 0.03
Citrate	1 ± 0.09	1 ± 0.08	1 ± 0.09	3.14 ± 0.21	2.49 ± 0.50	0.77 ± 0.09
Isocitrate	1 ± 0.03	1 ± 0.14	1 ± 0.02	1.24 ± 0.06	1.45 ± 0.42	0.87 ± 0.03
α-Ketoglutarate	1 ± 0.06	1 ± 0.08	1 ± 0.05	1.76 ± 0.16	2.26 ± 0.11	1.32 ± 0.08
Phosphoric acid	1 ± 0.16	1 ± 0.20	1 ± 0.08	1.25 ± 0.32	0.30 ± 0.06	1.83 ± 0.19
Glycerate	1 ± 0.10	1 ± 0.04	1 ± 0.05	1.73 ± 0.19	0.33 ± 0.04	0.94 ± 0.09
L-Threonate	1 ± 0.13	1 ± 0.05	1 ± 0.06	1.47 ± 0.33	0.67 ± 0.08	0.25 ± 0.03
Ribonate	1 ± 0.06	1 ± 0.06	1 ± 0.08	1.10 ± 0.12	1.01 ± 0.07	1.02 ± 0.09
Galactarate	1 ± 0.04	1 ± 0.08	1 ± 0.06	1.23 ± 0.13	2.16 ± 0.24	2.28 ± 0.19
Benzoate	1 ± 0.08	1 ± 0.05	1 ± 0.04	2.89 ± 0.31	1.75 ± 0.08	1.38 ± 0.05
D-Gluconate	1 ± 0.07	1 ± 0.08	1 ± 0.35	1.08 ± 0.11	1.19 ± 0.22	0.83 ± 0.28
D-Glucuronate	1 ± 0.12	1 ± 0.05	1 ± 0.05	1.02 ± 0.25	0.60 ± 0.03	1.16 ± 0.10
Shikimate	1 ± 0.12	1 ± 0.03	1 ± 0.01	3.82 ± 0.43	1.03 ± 0.03	1.39 ± 0.03
Glycine	1 ± 0.06	1 ± 0.05	1 ± 0.08	1.15 ± 0.11	1.06 ± 0.05	1.12 ± 0.10
L-Valine	1 ± 0.09	1 ± 0.05	1 ± 0.03	1.49 ± 0.17	1.14 ± 0.11	1.30 ± 0.05
L-Alanine	1 ± 0.18	1 ± 0.16	1 ± 0.09	2.69 ± 0.80	0.58 ± 0.11	0.45 ± 0.03
Serine	1 ± 0.19	1 ± 0.12	1 ± 0.06	4.83 ± 1.46	0.33 ± 0.05	0.83 ± 0.09
L-Threonine	1 ± 0.16	1 ± 0.11	1 ± 0.06	1.65 ± 0.39	0.25 ± 0.04	0.66 ± 0.04
Phenylalanine	1 ± 0.16	1 ± 0.13	1 ± 0.11	1.51 ± 0.45	0.44 ± 0.12	0.82 ± 0.15
L-Proline	1 ± 0.18	1 ± 0.13	1 ± 0.10	0.90 ± 0.21	0.42 ± 0.08	0.99 ± 0.17
GABA	1 ± 0.30	1 ± 0.25	1 ± 0.25	2.12 ± 0.45	1.48 ± 0.68	2.79 ± 0.81
Glutamate	1 ± 0.18	1 ± 0.10	1 ± 0.09	2.40 ± 0.65	0.24 ± 0.07	0.43 ± 0.05
L-Aspartate	1 ± 0.08	1 ± 0.12	1 ± 0.08	1.24 ± 0.17	0.26 ± 0.06	0.45 ± 0.10
D-Fructose	1 ± 0.04	1 ± 0.04	1 ± 0.02	1.30 ± 0.06	1.05 ± 0.04	1.46 ± 0.03
D-Glucose	1 ± 0.06	1 ± 0.03	1 ± 0.04	1.37 ± 0.10	0.99 ± 0.04	1.77 ± 0.15
D-Erythrose	1 ± 0.10	1 ± 0.04	1 ± 0.11	1.83 ± 0.19	2.14 ± 0.26	1.76 ± 0.65
D-Mannose	1 ± 0.09	1 ± 0.03	1 ± 0.04	1.05 ± 0.14	1.76 ± 0.09	0.81 ± 0.06
D-Ribose	1 ± 0.05	1 ± 0.05	1 ± 0.08	1.19 ± 0.14	0.83 ± 0.06	1.54 ± 0.23
Glucose-6-P	1 ± 0.21	1 ± 0.05	1 ± 0.05	1.30 ± 0.56	0.35 ± 0.06	1.38 ± 0.08
Sucrose	1 ± 0.07	1 ± 0.05	1 ± 0.02	1.43 ± 0.15	1.81 ± 0.07	1.02 ± 0.04
Galactinol	1 ± 0.07	1 ± 0.08	-	1.46 ± 0.16	1.04 ± 0.16	-
Myo-Inositol	1 ± 0.19	1 ± 0.03	1 ± 0.02	4.65 ± 3.59	1.08 ± 0.02	1.26 ± 0.12
Hydroxylamine	1 ± 0.04	1 ± 0.03	1 ± 0.09	1.19 ± 0.05	1.08 ± 0.04	1.08 ± 0.09

CHAPTER 2

IDENTIFYING QUANTITATIVE CHEMICAL AND PHYSIOLOGICAL INDICATORS OF HYPOXIA DURING SEEDLING GROWTH OF *EUCALYPTUS* CLONES

Abstract

Floods in areas with eucalyptus cultivation in south-eastern Brazil have caused large losses in timber production. The aim of this work was to study physiological responses between two *Eucalyptus* clones in order to explain the differential tolerance to soil hypoxia. Photosynthetic quantification of ethylene, abscisic acid (ABA) and indol-3-acetic acid (IAA) levels were used to study the root and shoot responses to soil hypoxia. ABA levels and stomatal conductance (g_s) were higher and lower respectively in clone B under hypoxic condition than in clone A. Ethylene emanation by roots of both clones was 4-fold higher in the hypoxic condition than in the normal condition. However, hypoxic treatment caused the same root ethylene production in both clones. Analysis of 1-aminocyclopropane-1-carboxylic acid (ACC) showed clone B produced more ACC in hypoxic condition than clone A. In contrast, 3-indole acetic acid (IAA) showed an opposite result with reductions under hypoxic conditions. The metabolic profile of roots from both clones showed increases in almost all amino acid concentrations under hypoxic conditions. In particular, the γ -aminobutyric acid (GABA) had an 8-fold increase for clone A and a 12-fold increase for clone B, and alanine increased 14-fold for clone A and 73-fold for clone B. These last two metabolites are directly linked with carbon metabolism by the Krebs Cycle and may play a crucial role in root survival during hypoxic periods acting as non-cycle alternative pathway to regenerate NAD^+ . Together, these results are indicative of a variety of means by which central metabolism and stress signaling could be integrated.

Keywords: aminoacids, ethylene, growth inhibition, hormones, metabolites, waterlogging.

INTRODUCTION

Eucalyptus plantations contribute significantly to the global economy, with around 6 million hectares planted in Brazil alone (ABRAF 2013). *Euclayptus* wood is the basis for several industries such as charcoal, engineered products and pulp and paper (PÖYRY 2013). Furthermore, the forest rotation allows to keep wood production for several activities, helping to preserve the native forests from logging (IBA 2014). Recent losses in timber production due to a range of environmental factors, such as flooding, drought and disease, have been extensive and with many incidences impacting particular clonal susceptibilities to site conditions. For flooding, losses often occur in areas which face periods with heavy rains (1700 mm per year) and in soils with bad drainage.

Plant tolerance to flooding is associated with both morphological and physiological adaptations. The morphological changes are usually preceded by changes in stomatal aperture, photosynthesis and transpiration (Voesenek and Bailey-Serres, 2015). As consequence of waterlogging, roots are exposed to internal variations in O₂ (hypoxia or anoxia in severe situations) and CO₂ concentrations, as well as increase in ethylene and reactive oxygen species. Ethylene biosynthesis and systems modified by ethylene have been shown to be involved closely with the flooding stress responses (Cao *et al.*, 2007; Pierik *et al.*, 2007). For example, the ability to produce adventitious roots is commonly associated with enhanced tolerance to flooding and their development has commonly been associated with ethylene production and perception (Voesenek *et al.*, 1993; Mergemann and Sauter, 2000; Steffens *et al.*, 2006). However, the ethylene-regulated processes interact with events controlled by other hormones, including abscisic acid, gibberellins and auxin to control growth under flooding conditions (Steffen and Sauter,

2009). Despite many reports in the literature on the roles of hormones in acclimation to flooding stress, little information is available concerning their effects on the coordination of primary metabolism and growth of *Eucalyptus* in response to O₂ deprivation.

Growth and development of plants are influenced by carbon availability via tightly controlled reactions in close proximity to the primary photosynthetic reactions (Sulpice *et al.*, 2014). This enables rapid feedback mechanisms to prevent damage to highly sensitive photosynthetic apparatus. Plants in flooded soil often show a decreased net photosynthetic rate (Kreuzwieser and Rennenberg, 2014), which can significantly affect root carbohydrate metabolism (Colmer and Voesenek, 2009). A decrease in stomatal conductance can be caused by reductions in root hydraulic conductance or from ‘signals’ from the O₂-deficient roots (Vartapetian and Jackson, 1997; Argus *et al.*, 2015). However, low-O₂ stress responses vary widely depending on the plant species, genotype and stage of development (Voesenek and Bailey-Serres, 2015). To establish appropriate *Eucalyptus* plantations on soils likely to experience flooding, a better understanding on the impacts of O₂ on primary metabolism is useful and if such efforts differ between clones then selection of more tolerant materials should help to mitigate the risks associated with soil flooding on plantation establishment. It is known that seedlings are particularly vulnerable to carbohydrate starvation and stress (Graf *et al.*, 2010). A key question is therefore whether *Eucalyptus* seedlings acclimate to decreased O₂ concentration. Here, physiological analysis of *Eucalyptus* seedlings growing under a low O₂ regime in the root-zone is combined with shoot and root metabolite profiling to evaluate how low O₂ concentration in roots affects metabolism. It is anticipated that low anoxic conditions will impart a change in metabolites abundance (both quantitatively and qualitatively) that may be used in early detection of stress conditions.

MATERIALS AND METHODS

Plant Growth Conditions

The experiment was conducted in Viçosa (20° 45' S, 42° 54' W), south-eastern Brazil. One month old seedlings of two clones (clone A and clone B) were used. The plants were obtained from Veracel company plant nursery. The seedlings were cultivated in a greenhouse during the months of March and April of 2014. The average temperature was 21.3 °C. The clones are characterized as *Eucalyptus urograndis* obtained by controlled cross-pollination between *Eucalyptus grandis* and *Eucalyptus urophylla*. The clones A and B are two of the most widespread planted in Brazil and selected based on wood volume, density and cellulose production (Bison *et al.*, 2006). Clone A is considered tolerant and clone B is sensitive to the shoot dieback that is a physiological disturb that appears in eucalyptus plants after rainy periods. The seedlings were cultivated in small tubes with top soil and were acclimated to the nutrient solution before the beginning of the experiment. During the acclimation period the plants were kept for 7 days in Clark's solution with 12.5 % of its normal concentration (Clark, 1975). During the second week, the concentration used was 25.0 % of the normal concentration of Clark's solution. After the third week, the plants were kept in Clark's solution with 50 % of its normal concentration until the end of the experiment. Roots with the nutrient solution were placed in plastic pots of 8 liters. After the acclimation period (3 weeks), a group of plants was maintained in nutrient solution in equilibrium with air, control treatment with air bubbling keeps the O₂ concentration above 8 mg/L, and another group was subjected to hypoxic condition, nutrient solution was bubbled with a mix of nitrogen and air (O₂ concentration of 4 mg/L), during 14 days.

Determination of damage to shoot and root

Shoot and root were sampled 14 days after the imposition of hypoxia. The shoot and root weight were obtained using a precision scale and root volume was measured by the water volume displaced in a 250 mL cylinder flask. Also, photographs of the root tips of all treatments were taken on the stereoscopic microscope Zeiss Stemi 2000-C. Images were obtained 5 days after imposition of hypoxia. Excised roots were placed in a forced draft oven for 3 days at 70 °C and then weighed to quantify dry mass.

Determination of ethylene levels

Whole roots of *Eucalyptus* seedlings with approximately 2 g of fresh mass (FM) were placed in sealed plastic bottles of 750 mL, containing at the bottom, 4 mL of deionized water. The inside atmosphere of the flask was mixed occasionally with a syringe with long needle. Following 18 h incubation time, sample of 1 mL was taken from the flask headspace with syringes. Ethylene produced by roots was measured by gas chromatography according to the technique described by Siva *et al.* (2014).

Measurements of photosynthetic parameters

Chlorophyll fluorescence in mature leaves (fourth pair of leaves from the stem tip) was measured using a fluorometer with modulated amplitude pulse (Li-Cor LI-6400XT). After adaptation to the dark for 30 min, leaf tissues were initially exposed to a weak pulse of far-red light ($0.03 \text{ mmol m}^{-2} \text{ s}^{-1}$) for the quantification of the initial fluorescence (F_0) followed by a saturating light pulse with irradiance of $6000 \text{ mmol photons m}^{-2} \text{ s}^{-1}$ and lasting 0.8 s to estimate the maximum emitted fluorescence (F_m). Furthermore, it was

estimated the maximum photochemical efficiency of PSII (F_v / F_m), from the simple ratio of the variable fluorescence (F_v), which is subtracting F_0 from F_m and (F_m).

Net photosynthetic rate (A), stomatal conductances to water vapor (g_s) and tissue intercellular to ambient CO_2 concentration ratios (C_i/C_a) were determined in the same leaves, using a Li-6400 open gas exchange system (Li-Cor, Lincoln, NE, USA) equipped with a blue/red light source (Li-6400-02B). Measurements were taken between 08:00 h and 12:00 h at ambient temperature, air relative humidity and CO_2 conditions (about 23 °C, 81 % and 390 ppm respectively) under artificial light with $1400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the leaf level and air flow of $300 \text{ mol s}^{-1} \text{ m}^{-2}$.

Quantification of phytohormones

Hormones were extracted from *Eucalyptus* leaves and roots following the methodology described previously (Müller and Munné-Bosch, 2011) with modifications. Briefly, a 110 mg of fresh tissue was powdered in liquid nitrogen and 400 μl of extraction solvents has been added (methanol: isopropyl alcohol: acetic acid 20: 79: 1). The samples were mixed in vortex (4 times for 20 s), sonicated (5 min) and kept on ice (30 min). After centrifugation (13000 g, 10 min in 4 °C), 350 μl of supernatant was removed and put into a new tube. The process was repeated with the pellet resultant and then the supernatants were pooled. A last centrifugation (20000 g, 10 min in 4 °C) was make to remove the rest of tissue in suspension. The sample was automatically injected (5 μl) in the system LC - MS/MS using an Agilent 1200 Infinity Series coupled to a Mass Spectrometry type triple Quadrupole (QqQ), model 6430 Agilent Technologies. Chromatographic separation was carried out on a column Zorbax Eclipse Plus C18 (1.8 μm , 2.1 x 50mm) (Agilent) in series with a guard column Zorbax SB-C18, 1.8 μm (Agilent). The solvent used was: (A) acetic

acid 0, 02 % in water and (B) acetic acid 0, 02% in acetonitrile in a gradient of time /%B: 0/5; 11/60; 13/95; 17/95; 19/5; 20/5. The solvent flow rate was 0.3 ml/min in a column temperature of 30 °C. Ionization used was an ESI (Electrospray Ionization) with the following conditions: gas temperature of 300 °C, nitrogen flow rate of 10 L/min, nebulizer pressure of 35 psi and capillary voltage of 4000 V. The equipment was operated in MRM mode (multiple reaction monitoring). The mass of the precursor ion/fragment established was monitored by fragmentation tests of each molecule: cytokinins (zeatin) (220/136), ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (102, 1/56,2), ABA (263/153), IAA (176/130), SA (137/93), GA₃ (345/142.9), JA (209/59), GA₄ (331/21). Cytokinins, AIA and ACC were scanned in the positive mode, while ABA, AS, GA₃, GA₄ e JA in the negative mode. A calibration curve (0.1 ng to 200 ng) using the respective standards of each hormone was generated to determine the absolute quantification. The generated data were analyzed in the software “MassHunter Workstation” to obtain the peak areas for each hormone in the sample and the results were expressed in ng/g of fresh tissue.

GC-MS-based metabolite analysis

Metabolite extraction was performed following a gas chromatography-mass spectrometry (GC-MS)-based metabolite profile as described previously (Lisec *et al.*, 2006). Briefly, leaf and root tissue (100 mg) were homogenized with liquid nitrogen and extracted in 1.4 mL of methanol, and 60 µL of internal standard (0.2 mg ribitol mL⁻¹ water) was subsequently added as a quantification standard. The mixture was extracted for 15 min at 70°C and mixed vigorously with 1.4 mL of water. To separate polar and nonpolar metabolites, 750 µL of chloroform was then added to the mixtures. After centrifugation at 2,200 g, the upper methanol/water phase was taken and reduced to dryness in vacuum.

Residues were redissolved and derivatized in 40 μl of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine at 37 °C for 120 min. Afterwards the extract was treated with 60 μl of *N*-methyl-*N*-[trimethylsilyl]trifluoroacetamide at 37°C, for 30 min. Eight microliters of a retention time standard mixture (0.029% [v/v] *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotracontane, and *n*-hexatriacontane dissolved in anhydrous pyridine) was added before trimethylsilylation. Sample volumes of 1 μL were then injected in the GC-MS system comprised an AOC-20i autosampler, and a QP2010 SE gas chromatograph-quadrupole mass spectrometer (Shimadzu, Tokyo, Japan), equipped with a column Rtx-5MS (Restek, Bellefonte, CA, USA). The derivatization, standard addition, and sample injection steps were carried out exactly as previously described (Lisec *et al.*, 2006). Both chromatograms and mass spectra were evaluated using TAGFINDER software (Luedemann *et al.*, 2008). Compounds were identified according to standardized guidelines (Dethloff *et al.*, 2014) by mass spectral and retention time index matching to the mass spectral collection of the NIST11 database. Laboratory and reagent contaminations were identified by non-sample control experiments and removed from further analysis. Numerical analyses were based on the peak height values of the recoded mass feature, i.e., the response values. These values were corrected for the dry weight of each sample and by the response of the internal standard from each respective GC-MS chromatogram to obtain normalized responses.

Statistical Analysis

Experiments were designed in randomized order, with six replicates per treatment. To determine the concentration of ethylene, the sampling unit was a plastic bottle of 750 mL closed, containing whole excised roots. In experiments to determine leaf gas exchange and parameters of chlorophyll a fluorescence, the sample unit was a in general the third

pair of leaves. Differences between means were detected by Tukey test, at 5% probability. The metabolite profile statistical analysis was made by using the *t*-test 5%.

RESULTS

Growth and photosynthesis

Shoot dry mass did not suffer from the hypoxic condition in both clones (Figure 1A). However, hypoxia treatment reduced the rate of net photosynthesis (A) of clone A and B by 29 % and 32 % as compared with their respective controls (Figure 1B). Furthermore, the sub-stomatal CO_2 concentration (C_i) and the ratio of internal to atmospheric CO_2 concentration (C_i/C_a) were unaltered in shoot of clone A, but hypoxia led to a significant decrease of C_i and C_i/C_a in shoot of clone B (Figures 1C and D). The maximum photochemical efficiency of PSII [maximum variable fluorescence/maximum yield fluorescence (F_v/F_m)] was not affected by the hypoxia treatment both in clone A and B (Figure 1D). The results also showed a decrease in stomatal conductance (g_s) of 34% and 53%, as compared with control, in plants of clone A and B under hypoxia condition, respectively (Figure 1E).

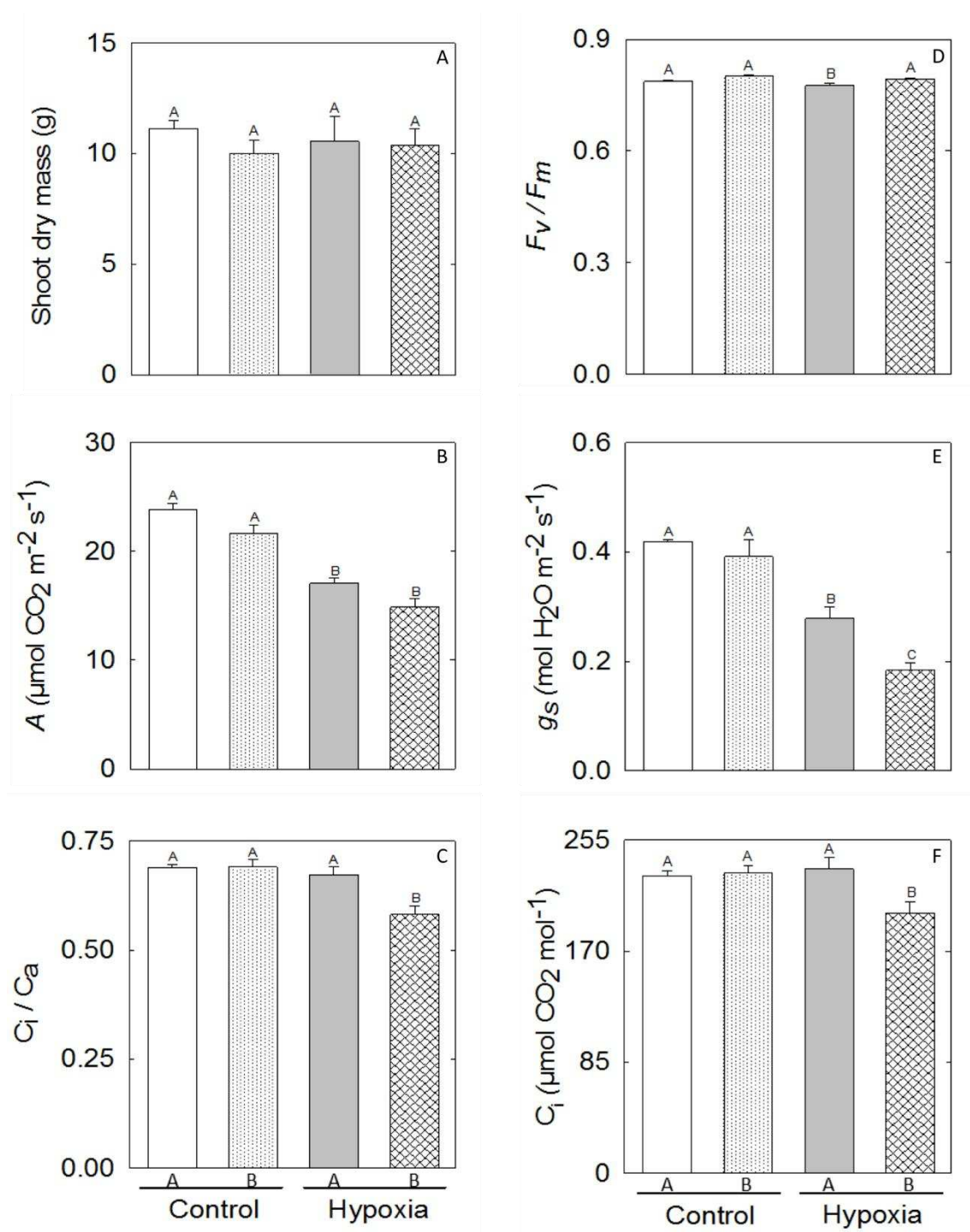


Figure 1. Physiological impacts observed in plants under hypoxic condition. (A) Shoot dry mass. (B) A , Net photosynthesis. (C) C_i/C_a , sub-stomatal CO_2 concentration/ambient CO_2 ratio. (D) F_v/F_m , maximal photochemical efficiency of photosystem II. (E) g_s , stomatal conductance. (F) C_i , sub-stomatal CO_2 concentration. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Values are means \pm SE of 6 replicates.

Change in hormones

The results showed an increase in ABA levels of 175 % and 150 %, as compared with control, in leaves of clone A and B in hypoxic condition, respectively (Figure 2A). The levels of 1-aminocyclopropane-1-carboxylic acid (ACC) were increased in shoot of clone B, with no changes in shoot of clone A under hypoxia condition (Figure 2B). In addition, the levels of indol-3-acetic acid (IAA) in both shoots of clone A and B under hypoxia treatment were similar to those observed in control plants (Figure 2C).

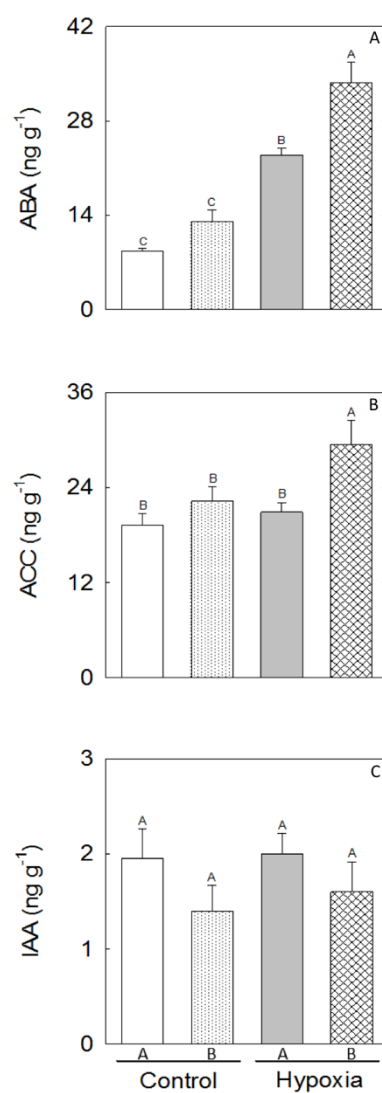


Figure 2. Levels of abscisic acid, 1-aminocyclopropane-1-carboxylic acid and indol-3-

acetic acid in shoot of plants under hypoxic condition. (A) ABA, abscisic acid. (B) ACC, 1-aminocyclopropane-1-carboxylic acid. (C), IAA, indol-3-acetic acid. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Values are means \pm SE of 6 replicates.

To further investigate the functional link between hypoxia and growth impairment we next performed a physiological characterization in root tissues of clones A and B. Five days after the hypoxia treatment, root tip turned black over a distance of 1-2 cm (Figure 3A). Furthermore, hypoxia treatment reduced root dry mass and root volume of clone A and B (14 days) in comparison with control plants (Figures 3B and C).

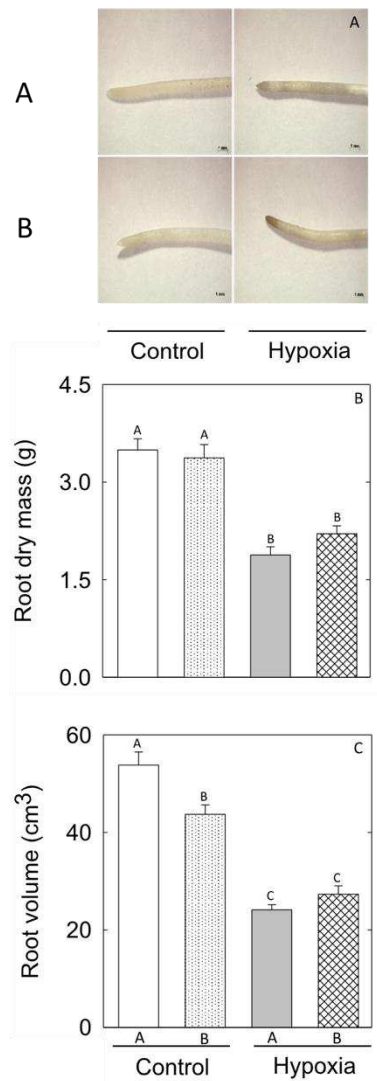


Figure 3. Phenotypic changes of root caused by hypoxic condition. (A) Photography of

root sections. (B) Root dry mass. (C) Root volume. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Values are means \pm SE of 6 replicates.

The levels of IAA in root decreased approximately 5-fold for clone A and 2-fold for clone B in the hypoxic condition (Figure 4A). By contrast to the situation observed for IAA, the levels of ACC and ethylene were strongly increased in roots of clone A and B under hypoxia treatment, as compared with controls (Figures 4B and C).

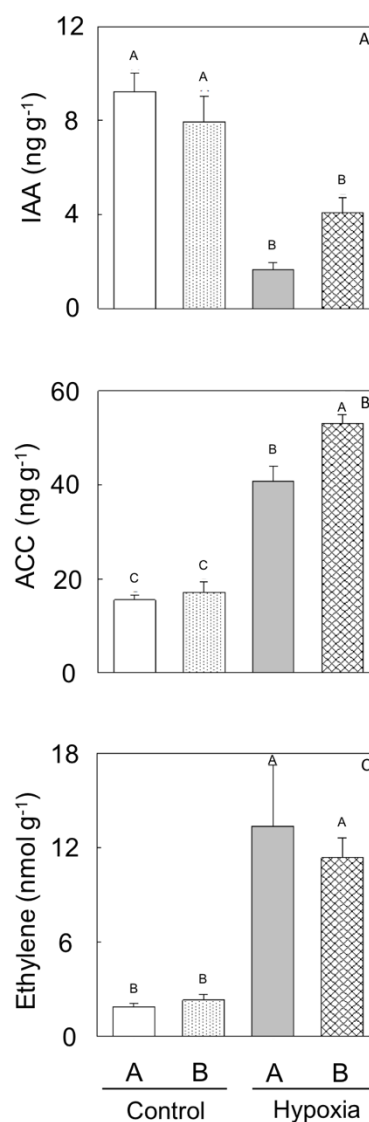


Figure 4. Levels of indol-3-acetic acid, 1-aminocyclopropane-1-carboxylic acid and ethylene in root of plants under hypoxic condition. (A) IAA, indol-3-acetic acid. (B) ACC, 1-aminocyclopropane-1-carboxylic acid. (C) ethylene. Bars followed by the same

letters do not differ statistically at the 5 % level by Tukey test. Values are means \pm SE of 6 replicates.

Metabolite profiles

To enhance our understanding of the changes in growth in plants exposed to hypoxia condition, we performed an extensive metabolic characterization in both shoot and root tissues of clone A and B. The analysis revealed that the hypoxia regime did not lead to significant changes in the levels of sugars and sugar derivatives such as glucose, fructose, sucrose, mannose, myo-inositol and maltose in shoots of clone A and B (Supplementary table 1). However, levels of galactinol was increased by hypoxia treatment in shoots of both clones A and B. A significant increase in myo-inositol and sucrose levels was only observed in root of clone B under hypoxia condition (Supplementary table 1). There were no significant differences in fructose in roots of clone A and B under hypoxia condition. Analysis of amino acids levels revealed that the alanine was the only amino acid that increased in the shoots, approximately 2.2-fold, (Clone B) in hypoxic condition (Figure 5A). Shikimate levels were also increased by hypoxia treatment in shoots of clone A and B in comparison to control plants (Figure 5A). On the other hand, shikimate levels were decreased by hypoxia treatment in roots of clone A with no changes in roots of clone B in comparison to control plants (Figure 5B). Furthermore, roots of clone A and B under hypoxia condition were characterized by increased levels of alanine, proline, GABA, serine, threonine and glutamate (Figure 5B). GABA and alanine showed the highest accumulation among the amino acids. GABA had an increase of 8-fold for clone A and 12-fold for clone B; alanine increased 14-fold for clone A and 73-fold for clone B. Hypoxia treatment caused an increase in aspartate and phenylalanine in roots of clone B but not in roots of clone A (Figure 5B). Significant decreases in tyrosine levels were only observed in roots of clone A under hypoxia condition. Glycine and valine levels were

unaltered by hypoxia treatment in roots of clone A and B.

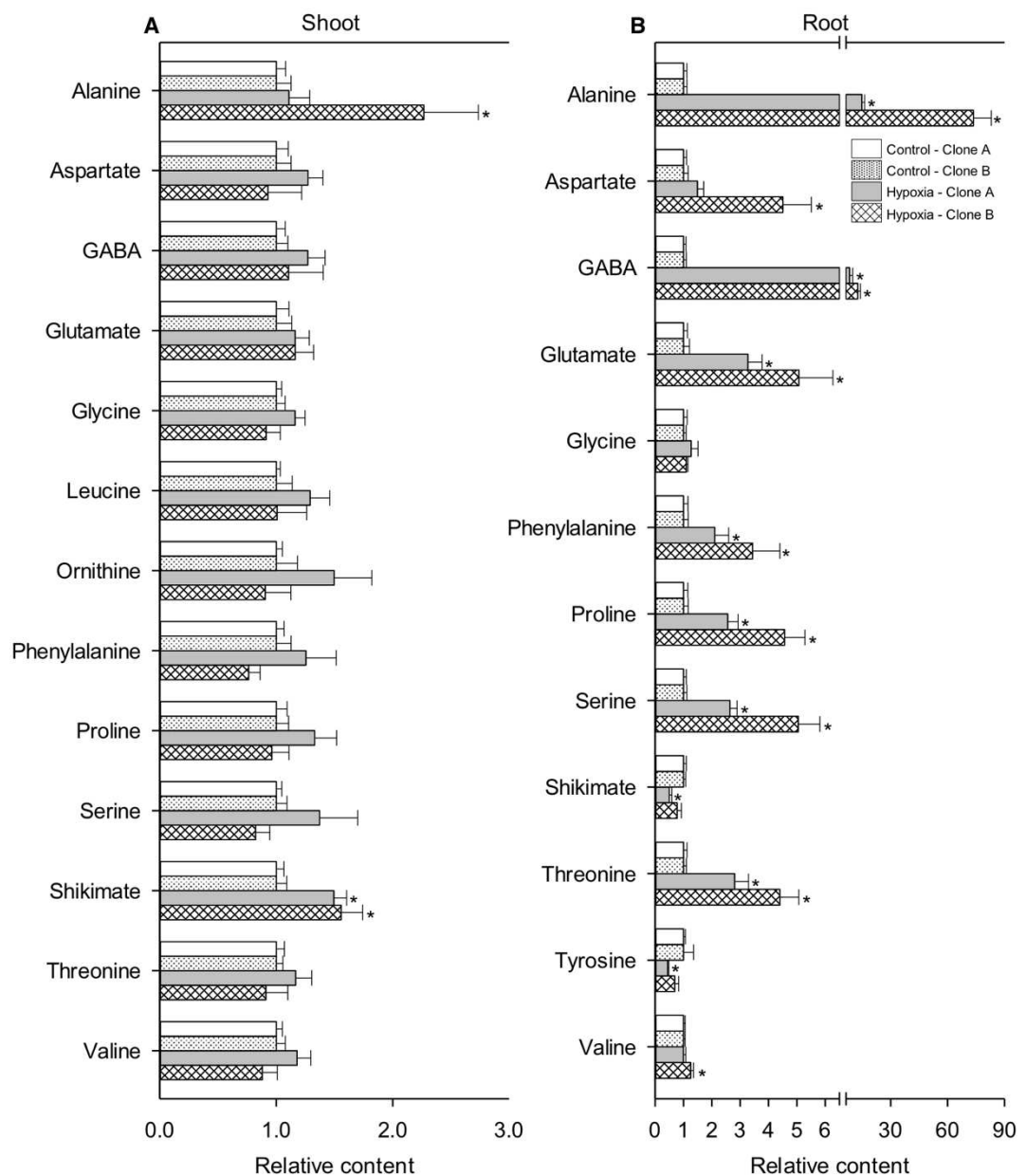


Figure 5. Changes in metabolite profiles in shoot (A) and root (B) of plants under hypoxic condition. Data are normalized with respect to mean response calculated for the control treatment in each organ. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$). Values are presented as means of 6 replicates \pm SE. The full dataset from the metabolite profiling study is available as Supplementary Tables S1 and S2.

Interestingly, the levels of tricarboxylic acid (TCA) cycle intermediates such as citrate, succinate, fumarate, malate, isocitrate, and α -ketoglutarate in shoots of clone A and B

under hypoxia condition were similar to those observed in shoot of control plants (Figure 6A). On the other hand, levels of α -ketoglutarate were consistently reduced in root of clone A, with no changes in roots of clone B under hypoxia condition (Figure 6B). Furthermore, succinate had an increase of 2.5-fold in roots of both clones in hypoxic condition. There were no significant differences in the levels of citrate, fumarate, isocitrate and malate both in root of clone A and B under hypoxia condition, as compared with control plants (Figure 6B).

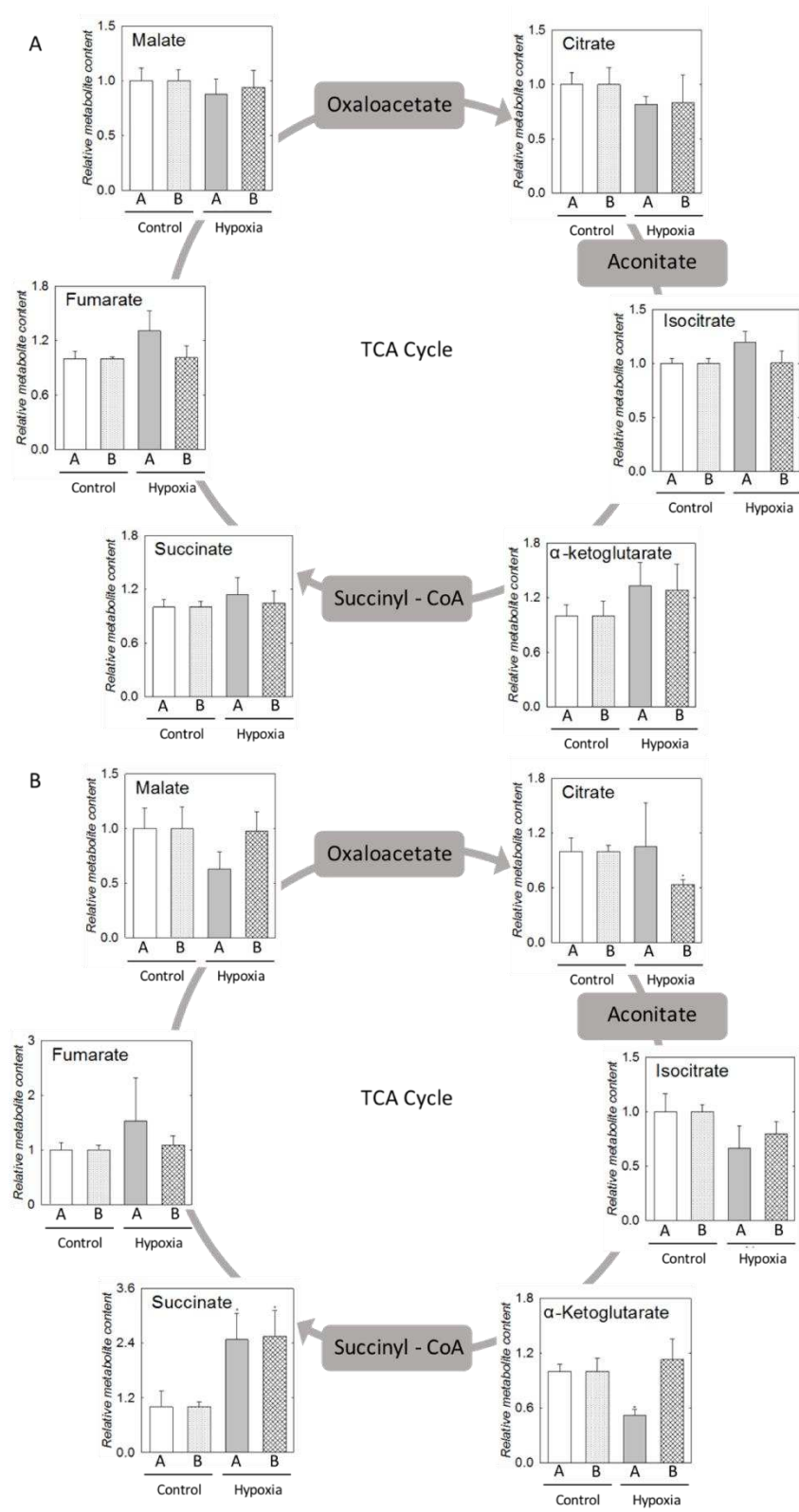


Figure 6. Changes in tricarboxylic acid cycle intermediates in shoot (A) and root (B) of plants (Clone A and clone B) under hypoxic condition. Data are normalized with respect to mean response calculated for the control treatment in each organ. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$).

0.05). Values are presented as means of 6 replicates \pm SE. The full dataset from the metabolite profiling study is available as Supplementary Tables S1 and S2.

DISCUSSION

Hypoxia causes severe damages in the shoot in a long-term or in severe situations (Argus *et al.*, 2015). In the present experiments, no difference was found in shoot dry mass in hypoxic condition for both clones (Figure 1A). However, the root dry mass and root volume were decreased by hypoxia equally for both clones (Figure 3B), indicating that the hypoxic treatment imposed in this study resulted in stress imposition on the roots.

Increased production of ACC by clone B roots can explain the higher concentration of ACC in leaves of the same clone compared to that of clone A. O₂ deficiency stimulates the ACC synthase in roots (Peng *et al.*, 2005) and by the transpiration stream the ACC might reach the leaves.

The roots of both clones had the same increase in ethylene production under the hypoxic conditions (Figure 4C). Ethylene biosynthesis genes are up regulated during hypoxic stress (Peng *et al.*, 2005), where ethylene acts as a regulator of root morphology. It is likely that the most important responses of the root system to tolerate the prolonged hypoxia are to develop aerenchyma (and this increased gas-filled porosity) and the formation of adventitious roots (Drew *et al.*, 2000; Sauter, 2000; Voesenek *et al.*, 2006; Perata and Voesenek, 2007; McDonald and Visser, 2003). In addition to these anatomical and morphological changes, plant hormones also play a crucial role to root survival when the soil environment changes from aerated to hypoxic for short periods of time (Armstrong and Drew, 2002). The increase in ethylene biosynthesis might enhance the root sensitivity to endogenous auxin promoting the formation of adventitious roots (Visser *et al.*, 1996). In this context, the hypoxic root environment reduced the concentration of IAA in roots (Figure 4A). Auxins are well known to affect root growth

by inducing cell division and/ or expansion (Tanimoto, 2005). Here we observed that hypoxia negatively affect the accumulation of IAA in root of clone A and B, while remained unchanged in shoot of both clones (Figures 2C and 4A). This suggests that there is lower flexibility in auxins metabolism in autotrophic organs. The plant hormone ABA is associated with regulating many developmental and physiological processes in plants including responses in stomatal regulation (Zou *et al.*, 2015). Our results demonstrated that levels of ABA were higher in clone B leaves clone in hypoxic condition than clone A leaves (Figure 2A). In keeping with this observation, g_s decreased more slowly in clone A than in clone B under hypoxia condition (Figure 1E), suggesting the involvement of ABA in stomatal regulation under hypoxia condition leading to a decrease in the A in both clones. In addition, both clones suffered a decrease in A in hypoxic condition (Figure 1B). The C_i/C_a ratio and g_s were lower in clone B in hypoxic condition, and the A was the same as clone A. Even in hypoxic condition clone A kept same C_i of leaves of plants with roots in the aerated treatment. Under hypoxic conditions, even with a lower g_s in the hypoxic condition the clone B kept the same A as clone A causing a decrease in the C_i/C_a ratio (Figure 1C). Decreases suffered in A perhaps are reflected by decreases in C_i/C_a values of clone B and as such may influence long term leaf function and longevity compared to clone A. The effects of hypoxia in roots and how they change the stomatal closure, in each clone, may define the long-term tolerance to the stresses caused by flooding but is not the only factor responsible for the decrease in A (Liao and Lin, 2001).

In roots, levels of succinate increased upon exposure to hypoxic conditions in both clones (Figure 6B). Additionally, clone B had higher concentrations of sucrose in the root under hypoxic conditions compared to clone A (Supplementary table 2) that might result from decreased consumption of this metabolite by respiration and fermentation. Most amino acids under hypoxic conditions are higher in roots for both clones. For example, GABA and alanine showed the highest accumulation. GABA and alanine can act as a carbon

storage in stress periods, and can be a way to maintain the TCA cycle (Rocha *et al.*, 2010). There is an alternative non-cycle pathway from alanine and GABA to the succinic acid, that can regenerate the succinate using the reducing power (NADH) in hypoxic and anoxic stresses (Rocha *et al.*, 2010; Gibbs and Greenway, 2003). In the shoots, alanine was the only amino acid that increased (Clone B) for plants with roots in hypoxic condition (Figure 5A). Probably this increase was related to the high accumulation of alanine in roots of clone B (Figure 5B).

In hypoxic stress the pyruvate is headed off to fermentation process leading to a decrease in the TCA cycle activity (Bailey-Serres *et al.*, 2012). However, the increase in succinate levels has been seen in response to hypoxia in roots of both clones (Figure 6B). Succinate is accumulated due the reduced activity of mitochondrial succinate dehydrogenase when the lack of oxygen inhibits electron transport at cytochrome *c* oxidase (Branco-Price *et al.*, 2008). Nevertheless, succinate levels might play an important role to ameliorate the effects of lack of oxygen (Branco-Price *et al.*, 2008). The conversion of 2-oxyglutarate produces ATP at substrate level, depending on NAD⁺ presence. Besides reverse TCA cycle from oxaloacetate to succinate would regenerate NAD⁺ that could be used in glycolysis to produce ATP at substrate level (Sweetlove *et al.*, 2010).

The change in the relative content of root metabolites shows how each clone have a different metabolic response to hypoxia (Figures 5B and 6B; Supplementary Figures 1 and 2). However, these changes were not in the same intensity in the shoot (Figures 5A and 6A). The hypoxic treatment in shoot caused smaller alterations in the relative content of metabolites than in root (Supplementary tables 1 and 2).

CONCLUSION

Characterizing early responses to root hypoxia is important for early detection protocols

that enable rapid mitigation via altered management prescriptions. Identifying early (root) responses with the potential for above ground chemical and/or physiological markers presents a major challenge for developing rapid, practical detection methods. Here we have illustrated several chemical entities (ACC, succinate) capable of indicating anoxic conditions that are known to participate in maintaining energetic balance throughout the TCA cycle. Importantly, we illustrate several chemical components that may indicate differential tolerance of some *Eucalyptus* clones to flooding. Confirmation of these results across a broader range of eucalypt species with contrasting environmental origin will assist in the appropriate selection of tree species/genotypes for flooding prone areas.

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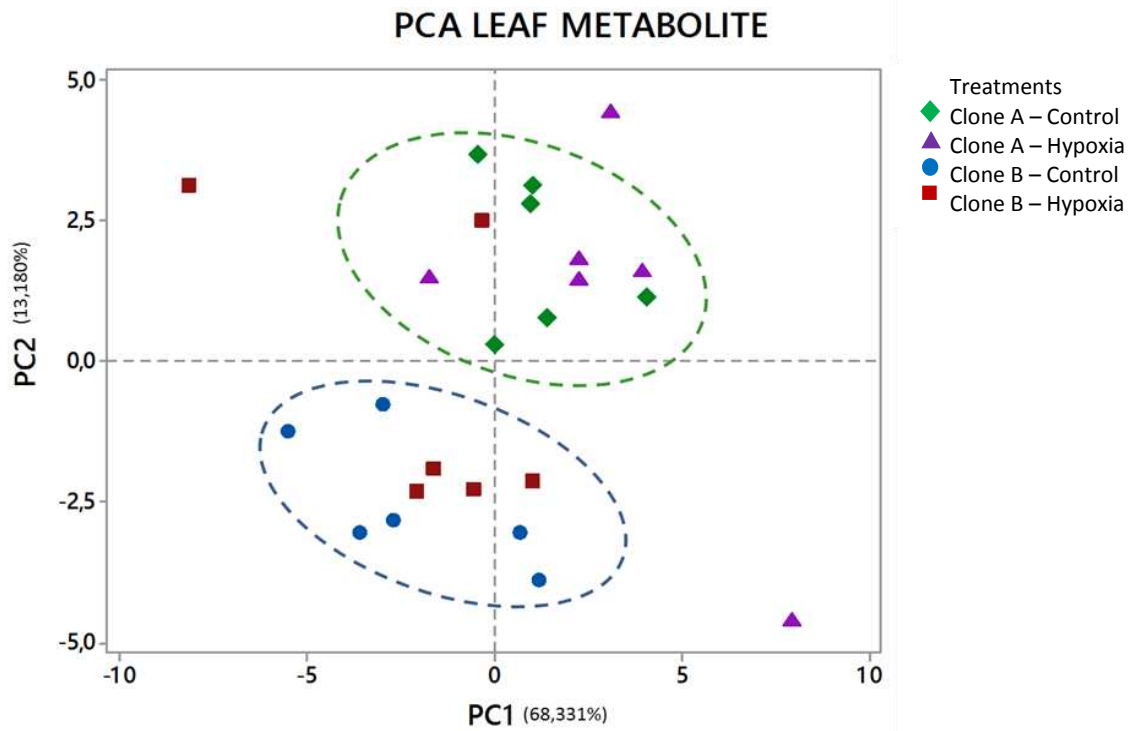
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Shoot metabolic profile table, black letters means indicate values determined by the student's *t*-test to be significantly different from the control ($P < 0.05$).

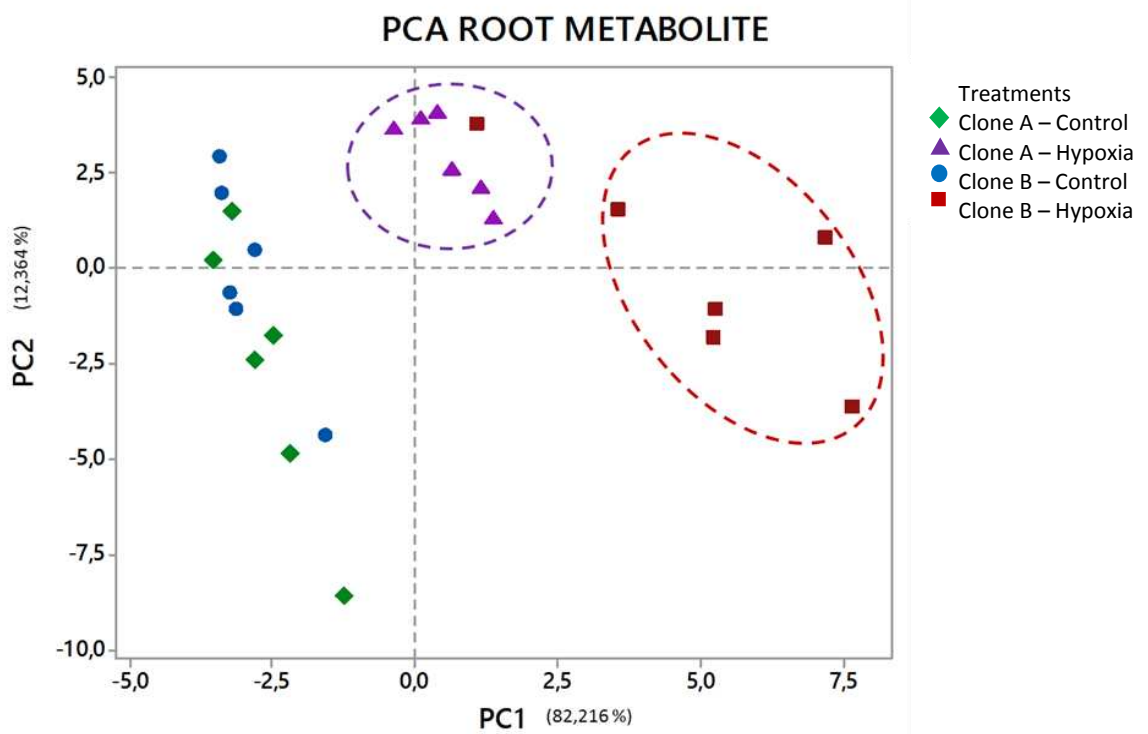
	Control		Hypoxia	
	Clone A	Clone B	Clone A	Clone B
Lactate	1 ± 0.093	1 ± 0.111	0.757 ± 0.109	0.860 ± 0.186
Oxalate	1 ± 0.105	1 ± 0.112	1.283 ± 0.200	0.715 ± 0.177
Succinate	1 ± 0.081	1 ± 0.064	1.139 ± 0.192	1.043 ± 0.139
Fumarate	1 ± 0.083	1 ± 0.020	1.308 ± 0.218	1.010 ± 0.130
Malate	1 ± 0.120	1 ± 0.102	0.876 ± 0.138	0.941 ± 0.156
Citrate	1 ± 0.108	1 ± 0.157	0.816 ± 0.074	0.834 ± 0.253
Isocitrate	1 ± 0.047	1 ± 0.047	1.195 ± 0.105	1.006 ± 0.107
α -Ketoglutarate	1 ± 0.118	1 ± 0.160	1.333 ± 0.257	1.284 ± 0.284
Phosphoric acid	1 ± 0.078	1 ± 0.065	1.290 ± 0.242	0.990 ± 0.118
L-Threonate	1 ± 0.100	1 ± 0.116	0.910 ± 0.129	0.603 ± 0.149
Ribonate	1 ± 0.127	1 ± 0.103	1.392 ± 0.298	1.720 ± 0.445
Galactarate	1 ± 0.160	1 ± 0.221	0.696 ± 0.191	3.028 ± 2.089
Benzoate	1 ± 0.084	1 ± 0.101	1.633 ± 0.214	1.151 ± 0.188
D-Gluconate	1 ± 0.084	1 ± 0.204	0.761 ± 0.066	1.438 ± 0.572
D-Glucuronate	1 ± 0.109	1 ± 0.118	0.877 ± 0.091	1.257 ± 0.227
Shikimate	1 ± 0.066	1 ± 0.090	1.494 ± 0.110	1.557 ± 0.185
Glycerate	1 ± 0.107	1 ± 0.118	1.348 ± 0.388	1.046 ± 0.195
Glycine	1 ± 0.046	1 ± 0.076	1.161 ± 0.086	0.913 ± 0.122
L-Valine	1 ± 0.052	1 ± 0.078	1.179 ± 0.117	0.880 ± 0.129
L-Alanine	1 ± 0.080	1 ± 0.127	1.109 ± 0.178	2.267 ± 0.469
Serine	1 ± 0.047	1 ± 0.092	1.372 ± 0.327	0.822 ± 0.123
L-Threonine	1 ± 0.069	1 ± 0.057	1.167 ± 0.137	0.910 ± 0.189
Phenylalanine	1 ± 0.067	1 ± 0.127	1.255 ± 0.260	0.763 ± 0.098
L-Proline	1 ± 0.093	1 ± 0.106	1.329 ± 0.189	0.962 ± 0.147
Pyroglutamate	1 ± 0.075	1 ± 0.083	1.499 ± 0.097	0.844 ± 0.132
GABA	1 ± 0.077	1 ± 0.100	1.271 ± 0.147	1.105 ± 0.299
Glutamate	1 ± 0.109	1 ± 0.133	1.162 ± 0.121	1.162 ± 0.159
L-Aspartate	1 ± 0.103	1 ± 0.127	1.272 ± 0.129	0.929 ± 0.289
L-Leucine	1 ± 0.035	1 ± 0.137	1.291 ± 0.169	1.008 ± 0.253
β -Alanine	1 ± 0.088	1 ± 0.200	1.382 ± 0.397	0.880 ± 0.169
DL-Ornithine	1 ± 0.052	1 ± 0.182	1.497 ± 0.323	0.905 ± 0.220
D-Fructose	1 ± 0.051	1 ± 0.045	1.255 ± 0.237	1.037 ± 0.071
D-Glucose	1 ± 0.026	1 ± 0.059	1.143 ± 0.107	1.064 ± 0.125
D-Mannose	1 ± 0.032	1 ± 0.090	1.294 ± 0.170	1.102 ± 0.061
Maltose	1 ± 0.181	1 ± 0.093	1.957 ± 0.618	0.859 ± 0.133
Sucrose	1 ± 0.027	1 ± 0.134	1.048 ± 0.056	1.241 ± 0.203
Glucose-6-P	1 ± 0.144	1 ± 0.230	1.594 ± 0.343	2.444 ± 0.933
D-Ribose	1 ± 0.059	1 ± 0.120	1.002 ± 0.058	1.132 ± 0.203
Myo-Inositol	1 ± 0.053	1 ± 0.049	1.035 ± 0.061	0.994 ± 0.075
Galactinol	1 ± 0.131	1 ± 0.101	1.530 ± 0.184	1.731 ± 0.142
Hydroxylamine	1 ± 0.117	1 ± 0.057	1.428 ± 0.238	0.860 ± 0.147

Supplementary Table S2. Root metabolic profile table, black letters means indicate values determined by the student's *t*-test to be significantly different from the control ($P < 0.05$).

	Control		Hypoxia	
	Clone A	Clone B	Clone A	Clone B
Lactate	1 ± 0.135	1 ± 0.220	0.847 ± 0.118	1.269 ± 0.278
Oxalate	1 ± 0.196	1 ± 0.069	3.510 ± 2.607	0.933 ± 0.067
Succinate	1 ± 0.348	1 ± 0.116	2.481 ± 0.569	2.558 ± 0.555
Fumarate	1 ± 0.129	1 ± 0.085	1.529 ± 0.785	1.092 ± 0.164
Malate	1 ± 0.183	1 ± 0.195	0.629 ± 0.156	0.978 ± 0.172
Citrate	1 ± 0.146	1 ± 0.062	1.050 ± 0.483	0.635 ± 0.053
Isocitrate	1 ± 0.162	1 ± 0.063	0.664 ± 0.204	0.794 ± 0.113
α -Ketoglutarate	1 ± 0.079	1 ± 0.148	0.517 ± 0.067	1.131 ± 0.226
Phosphoric acid	1 ± 0.210	1 ± 0.284	2.565 ± 1.025	5.489 ± 2.231
L-Threonate	1 ± 0.207	1 ± 0.292	0.122 ± 0.030	0.290 ± 0.068
Ribonate	1 ± 0.206	1 ± 0.194	0.504 ± 0.111	0.933 ± 0.175
Galactarate	1 ± 0.102	1 ± 0.103	0.760 ± 0.190	0.798 ± 0.095
Benzoate	1 ± 0.144	1 ± 0.065	0.959 ± 0.236	1.190 ± 0.117
D-Gluconate	1 ± 0.208	1 ± 0.285	0.515 ± 0.201	0.460 ± 0.094
D-Glucuronate	1 ± 0.072	1 ± 0.154	0.577 ± 0.085	1.358 ± 0.267
Shikimate	1 ± 0.098	1 ± 0.076	0.501 ± 0.091	0.775 ± 0.149
Glycine	1 ± 0.131	1 ± 0.092	1.270 ± 0.241	1.094 ± 0.061
L-Valine	1 ± 0.060	1 ± 0.059	0.997 ± 0.083	1.255 ± 0.097
L-Alanine	1 ± 0.116	1 ± 0.120	14.798 ± 1.534	73.630 ± 9.374
Serine	1 ± 0.099	1 ± 0.116	2.636 ± 0.253	5.048 ± 0.767
L-Threonine	1 ± 0.125	1 ± 0.101	2.805 ± 0.490	4.399 ± 0.669
Phenylalanine	1 ± 0.160	1 ± 0.167	2.101 ± 0.493	3.438 ± 0.962
L-Proline	1 ± 0.152	1 ± 0.168	2.561 ± 0.365	4.570 ± 0.716
Pyroglutamate	1 ± 0.076	1 ± 0.182	0.936 ± 0.141	2.634 ± 0.531
GABA	1 ± 0.085	1 ± 0.099	8.350 ± 1.610	12.710 ± 1.394
Glutamate	1 ± 0.139	1 ± 0.212	3.275 ± 0.497	5.069 ± 1.201
L-Aspartate	1 ± 0.108	1 ± 0.165	1.494 ± 0.219	4.509 ± 0.999
L-Tyrosine	1 ± 0.066	1 ± 0.354	0.443 ± 0.041	0.693 ± 0.139
D-Fructose	1 ± 0.073	1 ± 0.164	0.747 ± 0.097	1.321 ± 0.188
D-Glucose	1 ± 0.125	1 ± 0.260	0.592 ± 0.107	1.856 ± 0.439
Sucrose	1 ± 0.108	1 ± 0.132	1.458 ± 0.207	2.571 ± 0.321
Myo-Inositol	1 ± 0.192	1 ± 0.203	0.999 ± 0.261	2.889 ± 0.707
Hydroxylamine	1 ± 0.207	1 ± 0.205	1.047 ± 0.345	2.006 ± 0.623



Supplementary figure 1. Principal component analysis of leaf metabolic profile.



Supplementary figure 2. Principal component analysis of root metabolic profile.

Supplementary table 1. Metabolic profile, bold numbers means indicate values determined by the student's *t*-test to be significantly different from the tolerant ($P < 0.05$).

CHAPTER 3

CHICKPEA SEEDLINGS TOLERANCE TO ANOXIA: IS ROOT TIP DEATH DUE TO CARBON STARVATION?

Abstract

The effects of sugar addition in plants under anoxic stress still remain under investigation. It is unclear whether the plants die in anoxia because of lack of energy or because of ethanol toxicity. In this study chickpea (*Cicer arietinum*) seedlings were subjected to anoxia and how the sugar addition might influence their response. Seeds were germinated, hypoxically pretreated and then they were kept in the anoxic system with sugar addition or not (treatments) for 0, 12, 24, 36, 48, 60 and 72 h. After that, the plants were moved to an aerated system and stayed on it for 3 days. Plants kept in anoxia for 12 h showed a similar root growth pattern in both treatments (with sugar addition or not). From 24 to 72 h without oxygen the plants grew slower without sugar. The sugar supply increased the number of lateral roots up to 72 h in anoxia in comparison to those with no sugar. It resulted in an increase in seedling fresh weight when sugar was added (24, 60 and 72 h). There was no seedling root tip mortality up to 12 h in anoxia in both treatments. However, anoxia increased mortality to 56, 78, 100, 89 and 89 % in the 24, 36, 48, 60 and 72 h, respectively, when no sugar was added. On the other hand, root tip mortality decreased to 11, 11, 22, 33 and 44 % in the 24, 36, 48, 60 and 72h in anoxic systems with glucose added respectively.

Keywords: Flooding tolerance, respiration, metabolomics.

INTRODUCTION

The oxygen is the essential element for the mitochondrial activity to generate energy in plants (Zabalza *et al.*, 2009). However, sometimes the oxygen availability can be diminished to certain organs, for instance, in the roots after some flooding events. The flooding is one of the most important abiotic factors that cause stress in plants (Bailey-Serres and Voesenek 2008). Different species present differential tolerance to the hypoxia depending on their age, genotype and duration of the flooding (Jaeger *et al.*, 2009).

Chickpea (*Cicer arietinum*) is an important legume cultivated in Mediterranean-climatic regions like several countries in Africa, Asia and southern-west Australia, where its production has increased recently (Jettner *et al.*, 1999). The seeds are rich in carbohydrate and protein and it is an important part of human nutrition in several places (Iqbal *et al.*, 2006). Some important chickpea producer's regions face problems with rains leading to waterlogging and hypoxia in soil (Solaiman *et al.*, 2007).

Tolerance to waterlogging is an important and useful characteristic among different plant species, allowing us to understand how plants change their metabolism or anatomy in order to survive in this kind of situation (Dennis *et al.*, 2000). Carbohydrate starvation is one of the detrimental effects of hypoxic stress in plants, the ability of plants to keep distributing sugars from the shoot to the root system is important to flood tolerance in plants (Colmer and Greenway 2005). One of the effects of the hypoxic/anoxic stress is the decrease in the net carbon assimilation, this fact plus the lack of oxygen causes an imbalance in the root energy acquisition (Colmer and Voesenek 2009).

The effects of hypoxia in plants are well known, including plants death and decreasing root growth rate, number of lateral roots, and fresh weight. What is still unknown is whether root tissues under anoxia die because of ethanol toxicity, cell acidification or sugar starvation (Loreti *et al.*, 2005). However, providing sugar for the roots helps increasing its survival under anoxia, particularly the root tips, which are more vulnerable to carbohydrate starvation (Niinemets, 2010).

In general, plants survive better under anoxic situation when some energy source is supplied than with no sugar added to the cultivation system, for example sucrose or glucose or pyruvate (Colmer *et al.*, 2001). Nevertheless, plants under hypoxic stress regulate the respiration rates avoiding to create an anoxic environment in the root system (Geigenberger *et al.*, 2000). There are studies showing that when some sugars like glucose are supplied to plants in hypoxic system the respirations rates does not change significantly (Zabalza *et al.*, 2009).

Pyruvate is a molecule that links the glycolysis in the cytosol to the Krebs cycle under the mitochondrion and it affects the plant respiration rates more than the glucose and sucrose when they are supplied in the nutrient solution (Vanlerberghe and McIntosh, 1997). Another interesting fact is the accumulation of aminoacids in plants under the hypoxic/anoxic stress (Reggiani and Bertani, 2003). The main two aminoacids in this case are GABA and alanine (Rocha *et al.*, 2010; Miyashita and Good, 2008). Some authors say that these aminoacids could act as a carbon stock during the stress period and they would help the plants to survive after the hypoxia or anoxia providing carbon to the respiration (Miyashita *et al.*, 2007; Rocha *et al.*, 2010). Actually, the alanine aminotransferase uses the pyruvate as substrate producing alanine in a convertible way (Miyashita *et al.*, 2007).

In the following experiments, our main aims were therefore first to investigate whether the response of root growth to anoxia depends on the carbon supply; and second to analyze the effect of anoxia on seedling survival.

MATERIAL AND METHODS

General experiment design

The experiment was divided in 2 main parts, the first one we assessed the root tip survival in nutrient solution under anoxic stress. The second one we studied the effects of anoxia with glucose addition or not in some growth parameters. Each part of experiment has a specific sampling time. The controls plants were maintained always aerated (no hypoxic pretreatment) and they were sampled according to the anoxic times (18 h of hypoxic treatment + anoxic times)

Plant Material

The seeds of Australian chickpea (*Cicer arietinum*) variety Genesis836 were surface sterilized in sodium hypochlorite (0.04 %) and imbibed in aerated 0.5 mM CaSO₄ solution for 3 h. A small needle prick was done in the seed-coat after 1 h to help the water go into the seed and make all the seeds germinate at the same time. All procedures occurred at 20 °C. Imbibed seeds were placed on floating mesh which kept them in contact with the nutrient solution without drowning the seedlings. The nutrient solution was continuously sprayed with air. The seedlings grew for 5 days when tap roots were ~ 5-6 cm and before any lateral roots were visible. The nutrient solution concentrations were: 0.5 mM Ca²⁺, 0.4 mM K⁺, 0.438 mM NO₃⁻, 0.541 mM SO₄⁻², 0.0625 mM NH₄⁺, 0.04 mM Mg²⁺, 0.02

mM H_2PO_4^- , 0.01 mM Na^+ , 0.01 mM H_4SiO_4 5 μM Cl^- , 0.01 mM Fe-sequestrene, 2.5 μM H_3BO_3 , 0.2 μM Mn^{2+} , 0.2 μM Zn^{2+} , 0.1 μM Ni^{2+} , 0.005 μM Cu^{2+} , 0.05 μM MoO_4^{2-} . 2-(N-morpholino)ethanesulfonic acid (MES) buffer was added to the nutrient solution at 0.5 mM and the pH adjusted to 6.5 using KOH, bringing the final K^+ concentration in the solution to 0.475 mM. The 5 d old seedlings were exposed to a hypoxic pretreatment by spraying a gas mixture (10 % of $\text{O}_{2(\text{g})}$ the level in air; i.e. 2.1 % of $\text{O}_{2(\text{g})}$ in $\text{N}_{2(\text{g})}$ ultrapure, or ~ 0.021 mM O_2 in solution at 20 °C; for 18 h: Waters *et al.*, 1991b) in the nutrient solution. Aerated control seedlings were kept in continuously aerated solution. Hypoxically pretreated seedlings were exposed to anoxia by transferring the plants on the mesh floats to an opaque, sealed 5 L pots with a lid and small gas outlet, so that roots were submerged in nutrient solution (high-purity $\text{N}_{2(\text{g})}$) and shoots were in the gas phase. Seedlings that were reaerated following anoxia were placed into fresh aerated solution at the appropriate time. Glucose was supplied at 50 mM to evaluate the effect of sugar addition in the seedling survival in anoxic condition. This system was used to evaluate post-anoxic recovery of seedlings in terms of root elongation, lateral root growth, seedling survival and biomass production.

Assessing seedlings survival

Anoxia was imposed for different periods (0, 3, 6, 9, 12, 24, 36 and 48 h) and seedling root growth rate was assessed when sugar was added to the solution or not. After anoxia, the seedlings were kept for 3 days in an aerated solution in order to study whether the main roots were still able to grow or not.

Seedling growth

The seedling growth parameters evaluated were root elongation, lateral root growth, main root tip mortality and biomass production. Plants after the hypoxic pretreatment were kept in the anoxia for 0, 12, 24, 36, 48, 60 and 72 h. After that, plants of each anoxic time were moved to the aerated system (recovery system) and stayed on it for 3 days. The root elongation was measured using a graduated ruler every day during the recovery in the aerated system. The lateral root growth was analyzed every day by counting the number of lateral roots. The main root tip mortality was evaluated by the ability of main roots to grow after the end of the third day in the recovery system. And the biomass production was measured in seedlings 5 days after the germination, and in the end of the third day in the recovery system, using a precision balance.

Statistical analysis

Experiments were designed in randomized blocks order, with three blocks with three replicates. Analysis of variance ($P < 0.05$) was executed to determine the effects of treatments. Differences among means were examined by using the Student's t -test ($P < 0.05$). The statistical analysis for the assessing seedling survival topic was made by using the Tukey at 5 % of probability. All the tests were performed using the SigmaPlot 11 (Systat Software Inc., Hounslow, London, UK).

RESULTS

Assessing main root tip survival and plant growth

The root growth rate increased with the days in recovery system (aerated nutrient solution) reaching the maximum level in the third and last day in the recovery system (Figure 1). The root growth rate in the end of the experiment was similar in chickpea

plants from 0 h until 24 h in the anoxic treatment (Figure 1). After 36 h in hypoxia the root growth did not recover as in the others treatments. After 48 h in anoxia all root tips were virtually dead (Figure 1).

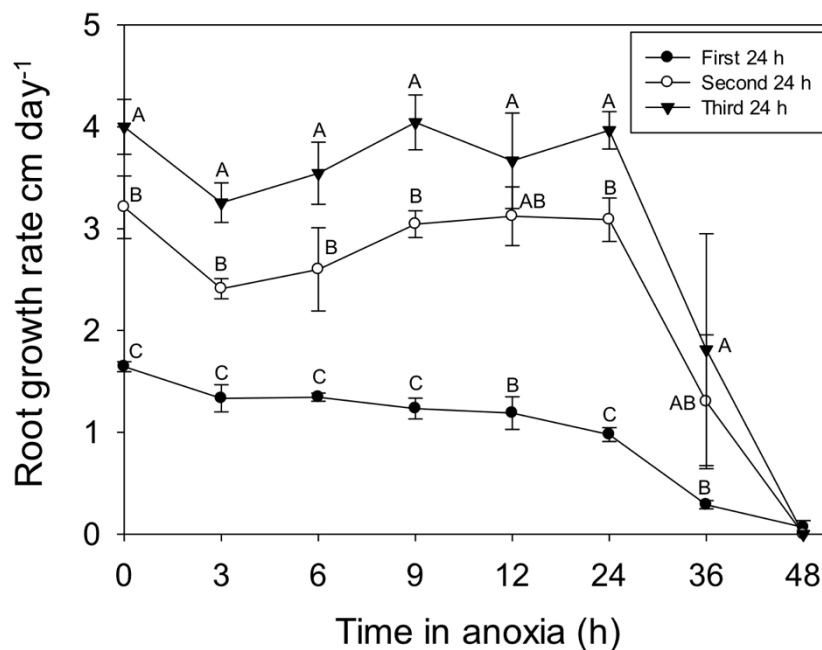


Figure 1. Root growth rate in chickpea plants (5 days old) which were kept in anoxia for 0, 3, 6, 9, 12, 24, 36 and 48 h. The plants had 3 days of reaeration in the recovery system fully oxygenated. The plants that were kept aerated (control) had a root growth rate of 3.8, 2.6 and 3.1 cm day⁻¹ on the first, second and third days (recovery days) of evaluation, respectively. Different letters indicate significantly different values determined by the Tukey at 5 % of probability. Values and standard error represented by symbols and error bars in the graph are means of 3 replicates.

The treatments with sugar added in nutrient solution increased the seedling fresh weight in comparison to those without sugar in the nutrient solution. The seedling weight increased 24, 30 and 50 % higher following 24, 60 and 72 h in anoxia with sugar, respectively, than those without sugar (Figure 2). The fresh weight increase was the same after 72 h in anoxia as 0 h in anoxia when the sugar was added to the nutrient solution.

On the other hand, the fresh weight increase was after 72 h in anoxia when no sugar was supplied.

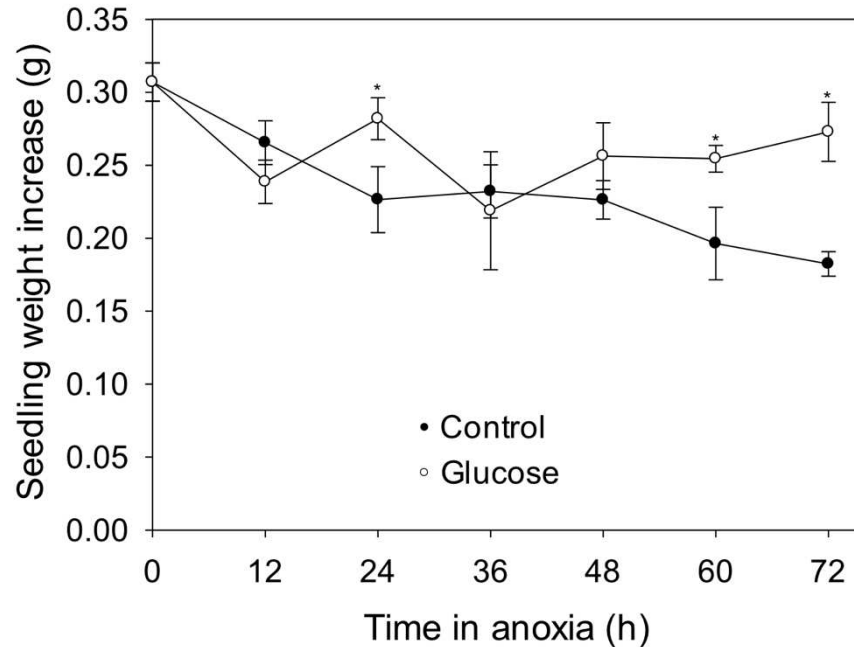


Figure 2. Seedlings fresh weight increase of chickpea plants under anoxia, with and without sugar. Initial fresh weight was obtained 5 days after germination (experiment start time, before the hypoxic pretreatment) and the final weight was obtained in end of experiment, after the anoxic and recovery time. The seedlings were kept in anoxia for 0, 12, 24, 36, 48, 60 and 72 h with or without sugar addition (50 mM glucose), after that they stayed for 3 days in the recovery system. Plants that did not receive the hypoxic pretreatment and were kept aerated had 0.35 g of fresh matter increase. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$). Values and standard error represented by symbols and error bars in the graph are means of 3 replicates.

The number of lateral roots was evaluated during recovery for 3 days. On the first day, none of the plants had lateral roots. On second day, the mean number of lateral roots per treatment was bigger in plants treated with sugar in the treatments of 36 and 72 h in anoxia. On the third day, the number of lateral roots when sugar was added was 45, 68

and 61% higher than when no sugars was supplied in 24, 60 and 72 h anoxic treatments respectively (Table 1). In the end of the experiment the number of lateral roots in chickpea plants under anoxia for 72 h supplied with sugar was the same of them which stayed for 0 h in anoxia. The plants that did not receive the sugar after 72 h in anoxia had the lowest number of lateral roots.

Table 1. Main root tip mortality (%) in chickpea plants which were kept in anoxia for 0, 12, 24, 36, 48, 60 and 72 h with sugar addition (50 mM glucose) or not. Plants always maintained in the aerated system with no hypoxic pretreatment had 0 % of root tip mortality. The root tip was considered dead when it did not grow anymore. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$). Values and standard error represented in the table are means of 3 replicates.

Time in anoxia (h)	Root tip mortality (%)	
	Control	Glucose
0	0 ± 0	0 ± 0
12	0 ± 0	0 ± 0
24	55 ± 29	11 ± 11
36	78 ± 11	11 ± 11*
48	100 ± 0	22 ± 11*
60	89 ± 11	33 ± 19*
72	89 ± 11	44 ± 29

The root tip mortality was higher in plants with no sugar supplied than sugar was added to the system. Following 0 and 12 h of anoxic conditions no root tip mortality was seen in both systems (with sugar added to the nutrient solution or not). Nevertheless, the root tip mortality reached the maximum value (100% of root tip mortality) after 48 h in anoxia, when no sugar was added. On the other hand, root tip reached the maximum mortality value (44%) in 72 h with the plants in the anoxic systems with glucose added (Figure 3).

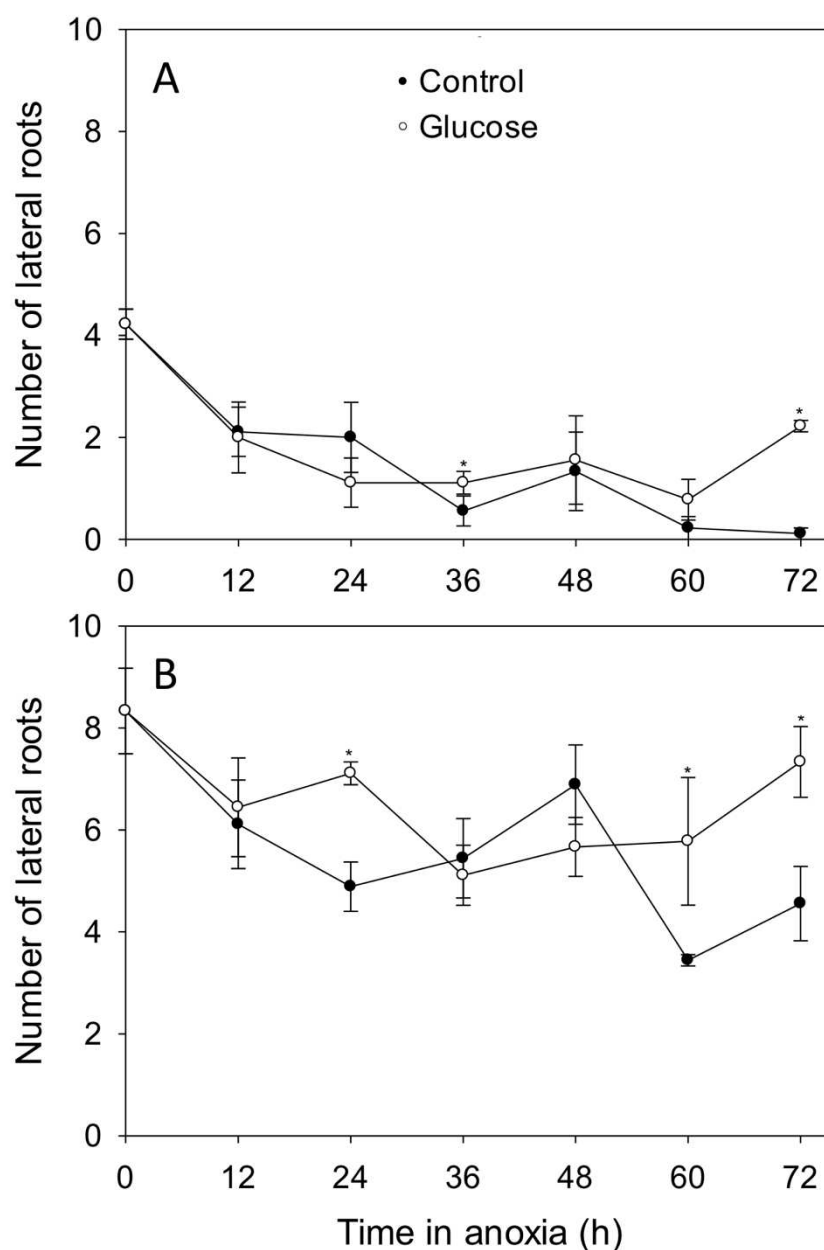


Figure 3. Number of lateral roots in chickpea plants (5 days old) which were kept in anoxia for 0, 12, 24, 36, 48, 60 and 72 h during the recovery day 2 and 3 (A and B) with sugar addition (50 mM glucose) or not. Plants of each treatment (anoxic times) were kept for 3 days in the aerated system. The number of lateral roots of plants which were kept always aerated was 7.6 and 11.6 on days 2 and 3 of evaluation. On the first day in the aerated system no lateral roots were seen. Asterisks indicate values determined by the Student's *t*-test to be significantly different from control ($P < 0.05$). Values and standard error represented by symbols and error bars in the graph are means of 3 replicates.

The root growth rate in plants decreased after 12 h in anoxic system with no sugar added. In general, the root growth rate was bigger, on the third day of recovery. This difference hasn't been seen in plants where the sugar was added however from 48 to 72 h in anoxic system a variation in root growth rate has been noticed (Figure 4).

Plants in the recovery system with sugar added in the nutrient solution had root growth rate 2, 4 and 11-fold bigger than those with no sugar added in 24, 36 and 72 h anoxic systems respectively. After the second day, all treatments from 24 to 72 h in anoxia exhibited a bigger root growth rate in system where the sugar was provided than the other one (Figure 4).

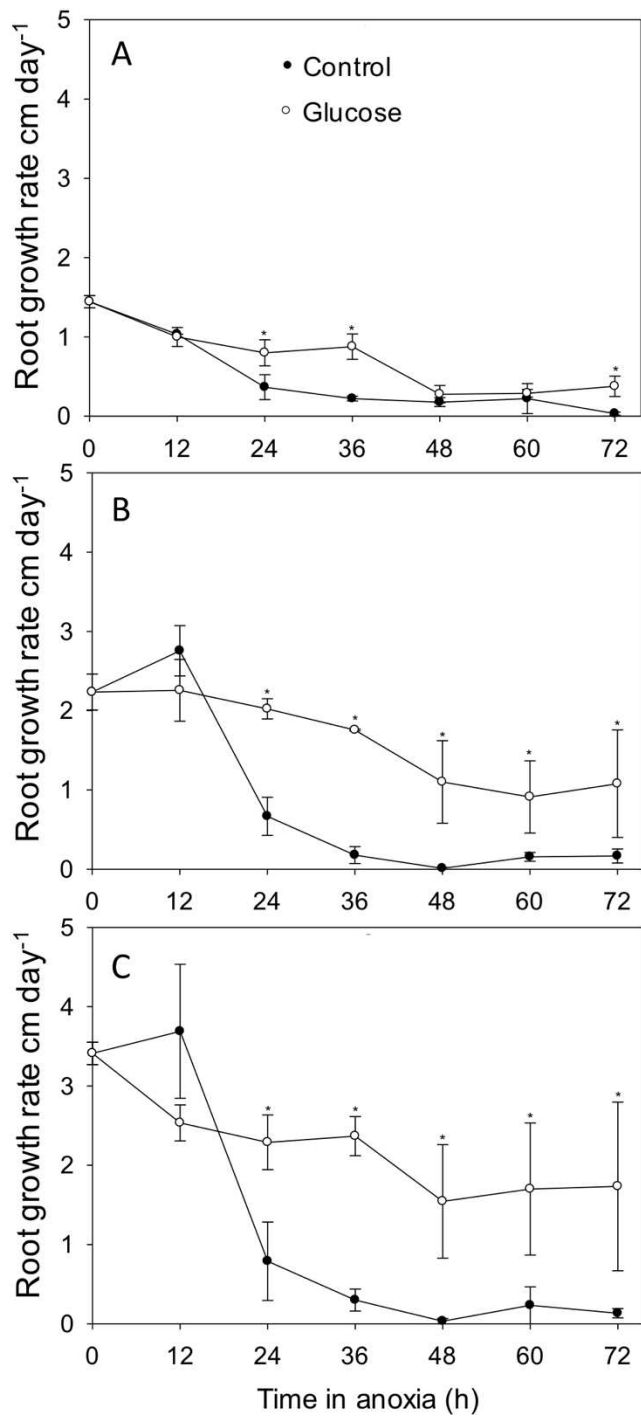


Figure 4. Main root growth rate in chickpea plants (5 days old) which were kept in anoxia for 0, 12, 24, 36, 48, 60 and 72 h. After the anoxic time the plants were kept in the aerated system for 3 days. The same plants were evaluated during the 3 days. The letters A, B and C represents the first, second and third day in the aerated system respectively. Plants in the system always aerated (control plants) had mean root growth rate of 2.5 cm / day during the 3 days of evaluation. The nutrient solution had sugar addition (50 mM glucose) or not. Asterisks indicate values determined by the Student's *t*-test to be significantly

different from control ($P < 0.05$). Values and standard error represented by symbols and error bars in the graph are means of 3 replicates.

DISCUSSION

Once the plants were moved to the aerated system the roots started to grow again (Figure 1). After 3 days in the aerated system plants exhibited the greatest root growth rates getting over the effects of anoxia. It takes few days from the changes in metabolism, after the restoration of the oxygen supply, until any response in plant growth can be seen (Thomas *et al.*, 2005). Plants that stayed for more than 24 h in the anoxic system had a breakdown in root growth rate. It seems that after 24 h the anoxia caused a permanent damage in the root tips causing this reduction on the root growth rate in chickpea plants. The effects of sugar addition on respiration might cause a strong difference in plant survival in anoxic situations as it was shown by Bouny and Saglio (1996). Even the increase on fresh weight was greater in seedlings treated with sugar (Figure 2). The increase in the fresh weight in chickpea plants treated with sugar was the same in plants which stayed for 0 h until 72 h in anoxia. The sugar added to the solution was used by plants to get energy saving sugar their own reserves in the seeds. On the other hand, plants that did not receive sugar in solution had to spend the own sugar from the seed reserve to keep the glycolysis and fermentation (Huang *et al.*, 2005).

The root tip is a sensible zone to stresses, most likely because it is a region with high metabolic activity with a high rate of cell division and respiration (Taiz and Zeiger, 2010). The root tips are supposed to be the first root region to recognize the anoxic stress and suffer the consequences like cell death. During the first 12 hours in anoxic stress none of plants showed root tip death however the mortality increase strongly in 24 h when no sugar was added to solution (Table 1). The plants treated with sugar had root tip mortality lower than those with no sugar in nutrient solution. The effects of sugar on the

maintenance of energy to keep the cells alive are evident even in this high sensitive root region (Saglio *et al.*, 1980). The sugar requirements to keep the tissues alive are higher in anoxic situations because the energy production yield is lower than in aerated environments, then tissues with high metabolic activity are quite sensitive the lack of oxygen (Igamberdiev and Hill, 2004). We here observed that on the first day following the recovery that the roots had a small growth rate, for example in plants that stayed for 48 h in anoxia with no sugar added to the nutrient solution, and then after the second day in the recovery system the root growth stopped. This fact might have been caused by the stresses of returning this tissue to the aerated system leading to death the already damaged root tip (Blokhina and Fagerstedt 2010).

The number of lateral roots was another example how the sugar supplement can change some plant features (Figure 3). With more energy to spend, plants can invest quickly in growing characters by using the energy and carbon available in solution. The carbon provided by sugar to roots was essential to develop lateral roots even in treatments with a long time in anoxic system (Armstrong *et al.*, 1994). Interestingly chickpea plants even with no sugar added in the nutrient solution had an increase in the amount of lateral root in all the anoxic times analyzed. As it was seen after 24 h in the anoxic system plants exhibited root tip death at different percentages depending on the sugar addition but the number of the lateral roots was the same in chickpea seedlings in both systems (with sugar added in nutrient solution or with no sugar added) until 48 h in anoxia.

During the recovery time the plants increased they ability to grow as soon they were out of anoxic stress (Figure 4). After the anoxic stress when the plants were returned to the aerated system, the oxidative stress caused by the contact with the oxygen. The amounts of proteins linked to the oxidative stress increase significantly after long periods in the anoxia (VanToai and Bolles, 1991). The growth started to stabilize in the standard rates as the control treatments (aerated system) day after day in the aerated system (Figure 4).

Plants that were kept in hypoxic system until 12 h had the same growth rate in both systems (with sugar or not). After 12 h in anoxia, the glucose played an important role to keep the root elongating and growing as a source energy. The sugar addition allowed the plants even in extreme conditions of 72 h in anoxia to keep growing when they were returned to the aerated system, the enhance in root survival in plants treated with sugar was showed by Colmer *et al.*, 2001. The main effect of the anoxia was in the root tip survival, because plants with the root tip still alive in both systems, after 3 days in the recovery system they exhibit normal root growth rate. The glycolysis plus the fermentation are the two main metabolic ways to increase the root tips survival time in anoxia when exogenous sugar is supplied in the nutrient solution (Drew, 1997). With no oxygen in the system the seed reserves were efficient to keep the root tips alive until 24 h in the anoxic system with a sudden increase in the root tip mortality after 36 h in the anoxia. The seed reserves still had carbohydrates to feed the root tip because it was seen the growth of new lateral roots after the plants were moved to the aerated system. Some impedance occurred in the translocation of these sugars from the seed reserve to the root tip (Colmer and Greenway 2005).

CONCLUSION

The survival of root tips of chickpea seedlings and root growth rate decreased significantly after 36 h in the anoxic system when no sugar was supplied to the nutrient solution. The difference between the number of lateral roots in both systems (with sugar added or not) was less dependent on the sugar addition.

The sugar addition alleviates the effects of anoxia by providing energy to keep the plants growing. The time in anoxia was determinant to cause damage in plants, and the sugar

addition had a strong importance to keep the root tips alive keeping higher root growth rates in plants treated with sugar.

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GENERAL CONCLUSION

The study of hypoxia/anoxia effects in plants is an important matter still. Plants have to deal every day with a large number of stresses and the soil hypoxia/anoxia is one of the most important in the nature.

It was seen in the first chapter that eucalyptus clones had differential responses to the hypoxic stress, and even the metabolism changes of this plants under the hypoxic stress were different. Some metabolites could be used in the future as biomarkers to study the tolerance to the hypoxia in eucalyptus plants.

In the second chapter, we concluded that the shoot dieback in eucalyptus plants change the whole metabolism of the sensitive plants causing differences not only in the shoot meristem but in young and mature leaves.

In the third chapter, supplying nutrient solution with glucose increased the root tip survival and root growth in the chickpea plants in anoxia.

FUTURE WORKS

The metabolic profile of the chickpea roots and shoots has been performed in the University of Sydney in Australia to add more information to the effects of sugar addition in chickpea plants under the anoxic stress.

Metabolic profile has been performed in the University of Sydney to study the effects of the anoxia in metabolism in different eucalyptus species (*E. globuls*, *E. diversicolor*, *E. camaldulensis* and *E. grandis*). This species inhabits different environments in the Australia and they have differential tolerance to the flooding. The future results of this study will help to understand the differential metabolic behavior of these species in order to tolerate or not the root hypoxia/anoxia.

The shoot dieback still needs more studies to be well understood. The meristem disturbs caused by the shoot dieback can't be produced in the controlled situations yet. It is also needed to study the plant physiology, weather parameters and soil *in situ* before, during and after the shoot dieback.