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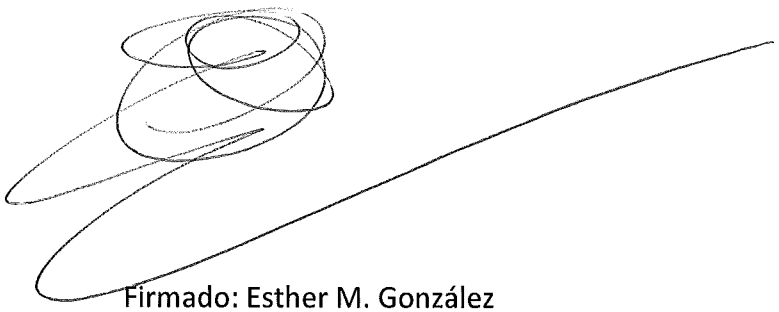

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Máster Universitario en Agrobiología Ambiental

A simple experimental approach to simulate drought stress conditions in agar Petri dishes: characterization of the response in *Medicago truncatula* plantlets.

Author: Nahikari López.

Tutors: Amaia Seminario and Esther M. González.

Natural Sciences Department. Public University of Navarra. 31006 Pamplona, Spain.

ABSTRACT

Drought stress is the major factor in the reduction of crop yields causing significant economic losses. However, this stress is difficult to standardize under controlled conditions for research purposes. For molecular biology studies, which usually need highly standardized protocols for screening of large populations, PEG, mannitol or NaCl are employed to apply water deficit conditions. However, these osmolites do not properly simulate drought stress effects. In the present study, a simple experimental approach employing different agar concentration to simulate drought stress conditions in Petri dishes is tested with *Medicago truncatula* seedlings. The response of root morphology, respiration and drought stress markers is characterized under mild (≈ -1.0 MPa) and moderate drought (≈ -1,5MPa). Mild drought provoked an increase of root length that was not observed under moderate drought. Root area, volume and diameter decrease progressively with the increase of drought severity. Respiration rate was only significantly affected under moderate drought conditions, without changes in cytochrome and alternative pathways capacity. According to this, sucrose, malate and α-ketoglutarate content decreased concomitantly with the increase of stress severity. Typical drought stress markers, as proline and amino acids, were also accumulated. Overall, these results validate this simple protocol to study drought stress response of seedlings under controlled conditions.

Keywords: drought, root, *Medicago truncatula*, respiration, root morphology.

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1. Introduction

Legumes are the second most economically relevant crop species due to their importance as a nitrogen source for animal feed and human consumption. In addition, they offer opportunities in studies of basic and applied plant biology due to plant-microbe, mycorrhiza and legume-pathogen interactions. Because of the difficulties involved in the study of crop legumes and limitations in the pace of the corresponding molecular analysis, it was necessary to find a model plant and the most promising advances have occurred in the legume species *Medicago truncatula* (Barker *et al*., 1990) which is a close relative of *Medicago sativa*, a forage crop. *M. truncatula* is considered as the model plant for temperate-climate legumes and it is widely used for genetic and molecular studies (Cook, 1999). Unlike most crop legumes, *M. truncatula* has a compact and small diploid genome (~5 x 10^8 bp), short lifecycle (3-4 months seed-to-seed), relatively high transformation, high levels of natural diversity, important collections of mutants and ecotypes, throughout its center of origin in the Mediterranean Basin. It is diploid and self-fertile with annual cycle and branching from the bottom. It is a creepy plant with trifoliate leaves and yellow flowers, which give a cylindrical and generally tight spiral fruit. It blooms in late winter and spring. This plant is studied in a large number of laboratories across the globe and the genetic and genomic tools are rapidly expanding. Moreover, its genome has been recently sequenced (Young *et al*., 2011).

Water stress suffered by plants during periods of water deficit is a determining factor of the final production obtained affecting almost all the plant development aspects causing significant economic losses. Drought is considered a major environmental factor limiting worldwide plant productivity (Boyer, 1982; 1996). This stress is unpredictable depending on many factors such as occurrence and distribution of rainfall, climatology, moisture reserve capacity of soils (Wery *et al*., 1994) and water demand of each particular crop.

Drought stress disturbs plant water relations and reduces leaf size, stems length and root proliferation, but cell growth, together with photosynthesis, is among the primary processes affected by this stress (Chaves, 1991). Hsiao (1973) described drought stress effects on plant physiology and showed that cell growth is the first process affected, being rapidly arrested even under mild water stress. Analyzing the plant response to drought stress, most research efforts have been traditionally focused on the aerial part of

the plant. However, the root system, essential for crop productivity (Paez-Garcia *et al*., 2015) and the first organ sensing the water deficit and, as such, with an essential signaling role, has received much less attention. The roots of dicotyledonous plants consist of a main root from which secondary roots emerge (Waisel *et al*., 1996). Different sections can be distinguished in the primary root (Figure 1), the apical meristematic section surrounded by a root cap, called root tip, the elongation section, the absorption area, containing the root hairs, and the upper root.

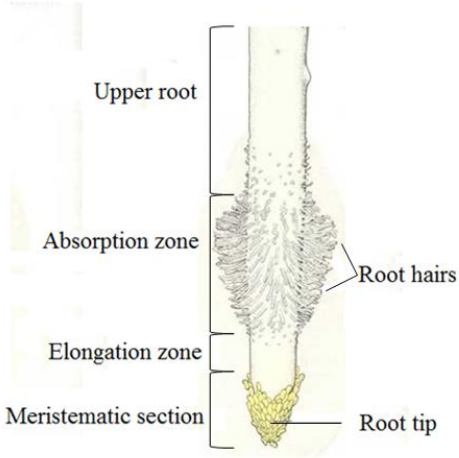

Figure 1: Sections of young *M. truncatula* root seedling. Adapted from www.riverpartners.com

Roots exposed to low water availability modify their growth and morphology (Blum, 2011) to explore the deeper areas with higher water availability, so deep rooting has been shown to be important under water deficiency (Gowda *et al*., 2011., Lynch, 2013., Wasson *et al*., 2012). Also, plants can be adapted to dry conditions reducing the diameter of roots, with greater root length per dry mass, increasing surface area in contact with soil water, increasing the volume of soil that can be explored and enhancing root hydraulic conductivity by reducing the volume of the apoplastic cells surrounding the xylem (Eissenstat & Achor, 1999; Rieger & Litvin, 1999; Huang & Eissenstat, 2000; Solari *et al*., 2006). Moreover, root hairs increase the contact surface with the soil improving water extraction (Bhat *et al*., 1979; Claassen & Jungk, 1982; Bates & Lynch, 2001; Mackay & Barber, 1985). On the other hand, an osmotic adjustment takes place in roots through the accumulation of compatible solutes, known as osmolytes, in order to maintain a positive flow of water into the root (Ruiz-Lozano, 2003). These osmolytes are ions, sugars and amino acids such as glycine, betaine, and

proline. These root adaptations serve to change their morphology attempting to access to areas with more water availability.

Plant carbon status is dependent on the balance between photosynthesis and respiration (Lambers *et al*., 1998; Flexas *et al*., 2006). Despite the close link between both processes, photosynthesis has been the most studied process because it is considered to have more importance in crop productivity (Flexas *et al*., 2005; Atkin & Macherel, 2009). Despite the vast knowledge of the effect of water stress on photosynthesis, there is much less known about its effect on respiration. Under conditions of water stress, photosynthesis is reduced but respiration is the process that provides energy and intermediates for cell growth and maintenance, and so cell respiration is directly involved on plant productivity (Flexas *et al*., 2005). Nevertheless, there is not a robust experimental evidence to describe the response of this physiological process to water stress. There are experiments that show either increased, unaffected or decreased rates of respiration in response to drought (Hsiao, 1973; Amthor, 1989). A better knowledge on the respiratory responses to drought stress may contribute to improve plant yield in semi-arid regions (Atkin & Macherel, 2009; Farooq *et al*., 2009).

Plant respiration comprises a set of reactions that are termed glycolysis, which is located both in the cytosol and in plastids, the oxidative pentose phosphate pathway, which is located in the plastids, the tricarboxylic acid (TCA) cycle or Krebs cycle, in the matrix of mitochondria, and the mitochondrial electron transport chain (ETC), which are in the inner mitochondrial membrane. Unlike animal mitochondria, there are two known pathways involved in plant respiration ETC: the cytochrome and the alternative pathways, which have their own regulation. The cytochrome pathway is cyanide-sensitive and is composed of several oxidoreduction reactions where electrons flow from several dehydrogenases to terminal oxidases via a redox-active ubiquinone (UQ) pool (Lambers *et al*., 2005) creating a proton gradient across the membrane that is used to generate ATP (Taiz & Zeiger, 2010). On the other hand, the alternative pathway, which is cyanide-resistant, is catalyzed by the enzyme alternative oxidase (AOX), a terminal oxidase that reduces oxygen to water (Vanlerberghe & McIntosh, 1997) and diverges from the cytochrome pathway at the level of the ubiquinone pool (Moore & Siedow, 1991) competing for electrons from the reduced ubiquinone pool (Hoefnagel *et al*., 1995; Ribas-Carbó *et al*, 1995) accepting electrons directly from ubiquinol. This alternative pathway is characterized by producing low amounts of ATP and is therefore

considered a wasteful pathway (Moore & Siedow, 1991). An increase in the alternative pathway under water stress has been shown in many cases, contributing to enhance photosynthetic electron transport due to the shift of electrons from the cytochrome to the alternative pathway (Ribas-Carbó *et al.*, 2005). There are several studies that showed an inducing AOX activity by H_20_2 (Wagner, 1995; Wagner & Krab, 1995; Neill *et al*., 2002). Other studies report that an increase in AOX activity could be due to non-specific disruption of cellular function (Maxwell *et al*., 2002) or due the inhibition of cytochrome pathway (Ribas-Carbó *et al*., 2005).

Under abiotic stress conditions, especially drought and salinity, production of ROS (Reactive Oxygen Species) has been shown to increase. ROS are formed as a natural by-product having important roles in cell signaling, but when their levels increase, they can become deleterious causing oxidative stress, which may result in significant damage to cell structures (Signorelli *et al*., 2012; Flexas *et al*., 2005; Hernández *et al*., 2012). The principal cause of ROS formation in mitochondria is the over-reduction of the ubiquinone pool due to the large difference in pH and electrical gradients. This is the alternative pathway, which diverts electrons from the cytochrome pathway to the AOX maintaining the ubiquinone pool in a more oxidized state even when the activity of the cytochrome pathway is blocked. Respiration via alternative pathway can help maintaining ubiquinone at a low level, probably through stabilizing the reduction state of mitochondrial ubiquinone pool (Purvis & Shewfelt, 1993). Therefore, AOX prevents over-reduction of respiratory chain components that might result in the production of ROS (Moller, 2001). Maxwell *et al*. (1999) confirmed the role of AOX in preventing oxidative stress due to transgenic manipulations although this idea had been suggested previously (Purvis & Shewfelt, 1993).

It is possible to inhibit both cytochrome and alternative pathways adding cyanide (KCN) and salicylhydroxamic acid (SHAM) respectively (Bahr & Bonner, 1973; Theologis & Laties, 1978) to measure the fluxes of electrons in each pathway using an Oxygraph (oxygen electrode). This technique measures the capacities of each respiration pathway based on the fact that a pathway is saturated when the other is inhibited (Lambers *et al*., 2005). There is a third respiration pathway resistant to the inhibitors of the cytochrome and alternative respirations, which is called residual respiration. The nature of this residual respiration is unknown; the same as if this pathway occurs even in the absence of both inhibitors. This pathway may not be

associated with respiratory metabolism at all because it is not associated with mitochondria (Theologis & Laties, 1978) involving extramitochondrial metabolism.

Although the antioxidative enzymatic system removes a large amount of ROS (Wang *et al*., 2002), the enhancement of free radicals due to water deficiency causes damage in membranes of plant cells by lipid peroxidation. Damage in fatty acids of the biological membranes could produce malondialdehyde (MDA), the final product of plant cell membrane lipid peroxidation (Moussa & Aziz, 2008). Therefore, lipid peroxidation can be measured in terms of content of MDA (Smirnoff, 1995) and it is used as an indicator of oxidative damage.

Amino acids accumulation is one of the main responses of plants to environmental stress (Aspinall & Paleg, 1981; Mansour, 2000). This accumulation helps plants to overcome drought stress through osmotic adjustment (Greenway & Munns, 1980) which is one of the main mechanisms that alleviates some of the effects of water deficiency (Morgan, 1984). Proline is the amino acid that increases its concentration faster than others amino acids under stressed conditions as high temperature, starvation or water deficiency (Sanchez *et al*., 1998; Alexieva *et al*., 2001). Proline is used as a clear marker for environmental stress, especially for water stress (Routley, 1966). This amino acid contributes to the survival of the plant under stress (Stewart, 1981) due to its involvement in osmotic adjustment. Proline can be accumulated at high concentrations in the cell cytoplasm without interfering with cellular structure and metabolism (Yancey *et al*, 1982). Aside from its role as an osmoregulator, other functions of proline accumulation have also been proposed, including prevention of damage by contributing to radical oxygen scavenger and stabilization of membranes (Ashraf & Foolad, 2007), radical detoxification (Smirnoff & Cumbes, 1989), macromolecules stabilization and regulation of cellular redox status (Hare & Cress, 1997). On the other hand, accumulation of soluble carbohydrates in different parts of the plant is enhanced in response to environmental stresses and is also involved in osmoregulation helping plant to maintain turgor in water deficiency conditions. These carbohydrates are accumulated as storage substances that can be mobilized in periods of energy deficit. Irigoyen *et al*. (1992) (Irigoyen *et al*., 1992) observed an increase in total soluble carbohydrates in nodules of *Medicago sativa* causing changes in osmotic pressure. Starch plays important roles in the tolerance against various abiotic stresses such as drought. Studies

show that drought-tolerant varieties had higher starch content than susceptible varieties (Singh *et al.*, 2013).

There are different forms to study the effects of drought in plants. Field trials are employed to measure productivity, however, the variability of factors as soil mixture, climate, temperature variations, precipitations or biotic stresses limit the conclusions that can be obtained in this type of studies. Mature plants studies under controlled conditions are usually carried out using pots systems where drought stress is imposed by removing irrigation. Most studies on drought tolerance have focused on the late-stages development and reproductive periods, emphasizing the consequences of stress on plant performance. In molecular biology studies, other artificial growing system are usually employed as plant culture dishes, which usually apply mannitol or the polymer polyethylene glycol (PEG) of different molecular weights to modify the water potential of the culture medium (Kaufmann & Eckard, 1971). However, it has been shown that mannitol and PEG are absorbed by the plant, this latter causing a toxic effect (Mexal *et al*., 1975; Munns *et al*., 1979; Emmert, 1974). Finally, salinity is one of the most studied stress attending to the number of scientific references. Salinity stress is also a problem worldwide but its impact is much lower than that of drought stress and the balance between scientific studies does not match the impact of both stresses on crop production. Salinity stress is very simple to apply, easily to standardize and highly reproducible and it has an osmotic component in common with drought stress, but the ionic toxicity caused by sodium has a main impact on plant physiology. Therefore, salt effect on plant physiology cannot be extrapolate to that of drought stress. To our understanding, the simplicity to impose salinity lead to the high number of scientific studies focused on this stress. In this context, our laboratory (Perez, 2014) presented a very handling system to study drought response employing Petri dishes with different agar concentrations. This methodology simplifies the drought stress experiments, providing the molecular biology laboratories a standardized protocol for easy characterization of root response to drought in large populations, avoiding the interference of natural osmolites as mannitol or artificial polymers as PEG. **The objective of the present study is to characterize the drought response of *Medicago truncatula* plantlets attending mainly to root morphology and respiration and standard drought markers in order to validate the use of agar Petri dishes as a useful tool for drought stress studies.**

2. Materials and Methods

2.1. Experimental approach

Seeds of *Medicago truncatula* Gaertn. Cv. Jemalong A17 were surface decontaminated before transfer them in agar Petri dishes. Firstly, they were scarified by immersion in H_2SO_4 at 96 % for seven minutes, abundantly washed ten to fifteen times. Finally, they were soaked in NaClO at 3.5 % for five minutes and thoroughly washed with sterilized water. Seeds were stirring in water during four to six hours and then transferred to 0.7 % agar plates at 4 ºC for one day in the dark. Afterward, they were incubated 36 hours at 21 ºC in the dark.

Three different agar solutions, 1.5 %, 3 % and 5 %, dissolved in Fahräeus medium (modified from Vincent, 1970) were used. The 1.5 % agar concentration simulates the control conditions (Barker, 2006) and plantlets growing in this condition exhibited a water potential around -0.77 MPa in shoots and -0.82 MPa in roots. The 3% and 5% agar concentrations were employed to simulate the two levels of drought, respectively. Plantlets growing in 3% agar, Drought 1 treatment (D1) exhibited a water potential around -0.82 MPa in shoots and -1.38 MPa in roots, whereas those growing in 5% agar presented a water potential around -1.38 MPa in shoots and -1.54 MPa in roots, according to (Pérez, 2014). The culture medium contains: 0.5 mM $MgSO_4$, $7H_2O$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, $2H_2O$, 50 µM Fe-EDTA, 1 mM $CaCl_2$ and 0.1 µL/L micronutrients $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, Na_2MoO_4. pH was adjusted at 6.5 before adding the agar. The culture mediums were sterilized by autoclaving (121ºC, 1 atm, 20 min) and finally $CaCl_2$ (1 mM) was added in.

Culture medium was poured in square Petri dishes (12 x 12 cm) under aseptic conditions within a laminar flow cabinet. Once it was solidified a filter paper was soaked onto the medium surface, in order to avoid direct root contact with the medium but allowing water and nutrient uptake by the seedlings (Sauviac *et al.*, 2005).

When rootlets were 1.5 cm length approximately, seedlings were placed on agar dishes of the different treatment under aseptic conditions in a laminar flow cabinet. Eight seedlings were placed equidistantly within each plate. A drop of water was applied to promote seed adhesion to the medium. The Petri dishes were sealed using a porous paper belt (Micropore 3M) allowing gas exchange and maintaining sterile conditions.

Petri dishes were placed at 30º slope to the vertical during four days in a growth chamber under controlled conditions (25/20 ºC day/night temperature, 70-80 % relative humidity, 200 µmol $m^{-2}s^{-1}$ (PPF), 16 h photoperiod). The root zone of the Petri dishes was covered with aluminum foil to keep roots in darkness simulating natural growing conditions.

2.2. Root morphology

Daily measurements of root growth were taken in the control and drought treatments. Root morphology was examined after four days exposure to the different agar concentrations. Total root length together with the position of each root section, upper root, root hair zone and root tip (considering elongation zone and tip), were manually determined using a ruler.

Root morphological parameters, area, volume and diameter, were determined by using the SmartRoot software. This is a semi-automated image analysis software, which analyzes digital images and streamlines the quantification of root growth and architecture for complex root systems (Lobet *et al*., 2011). SmartRoot is an operating system independent freeware, based on ImageJ, which uses cross-platform standards for communication with data analysis software.

2.3. Root respiration

Root respiration was measured using a Clark-type O_2 electrode (Hansatech Oxygraph, H. Sabur Laborbedarf, Reutlingen, Deutschland) connected to constant temperature circulating water baths (25 ºC). Primary roots (0.015 g) of each treatment were cut into small pieces and placed into Oxygraph chambers containing 1 mL of buffer (25 mM Imidazole-HCl, pH 6.5). The decrease of the oxygen in the chambers was measured under continuous stirring to promote oxygen diffusion within the chamber. This oxygen consumption rate was used to determine total respiration rate of the root. Once the decrease was constant, the inhibitors of the respiratory pathway, 10 µL 0.01 M KCN 20 mM HEPES pH 8 or 20 µL 1M SHAM were added. KCN inhibits the cytochrome pathway (CP) and SHAM inhibits the alternative respiration pathway (AP). Two different reactions were carried out for each sample; in a first one, KCN was added previously to SHAM, to measure the AP capacity, and in a second reaction to measure

the CP capacity, KCN was added after SHAM. Inhibitors were always added when the oxygen consumption in the cuvette reached a constant rate. Capacity is defined as the maximum rate that each pathway (cytochrome and alternative) can reach and it is estimated as the rate of consumption of oxygen remaining in the presence of the route inhibitor (Mcintosh, 1994). After the addition of both inhibitors, residual respiration (Rres) was determined. The cytochrome pathway capacity (CP) was estimated as the oxygen uptake, when alternative pathway is inhibited by SHAM divided by total oxygen uptake, and the alternative pathway (AP) capacity was estimated as the oxygen consumption whereas cytochrome pathway is inhibited by KCN. In both cases, Rres, obtained after the addition of both inhibitor was subtracted.

2.4. Malondialdehyde

Lipid peroxidation was measured as described Hodges *et al.* (1999) (Hodges *et al.*, 1999) with minor modifications, determining the amount of malondialdehyde (MDA) as the end product of lipid peroxidation process. Samples (0.05 g) were homogenized with 1 mL of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 13.200 g at 4 ºC for five minutes. Then, 750 µL of supernatant was collected and 750 µL of reagent solution (20 % (w/v) TCA + 0.01 % butylhidroxytoluene + 0.65 % TBA) was added and mixed with a vortex. The mix was heated in a water bath at 95 ºC and then the reaction was stopped by placing the samples on ice before centrifuged them again at 13.200 g 4 ºC for 5 minutes. Finally, 250 µL of supernatant was collected and absorbance at 440, 532 and 600 nm was determined using 0.1 % (w/v) TCA as blank. Equivalents of MDA were calculated by using the following formula:

MDA equivalents (mmol mL^{-1}) = [(A-B)/157 000] x 106

$$A = [(Abs532+TBA) – (Abs600+TBA)]$$

$$B = [(Abs440+TBA – Abs600+TBA) \times 0.0571]$$

2.5. Carbohydrates, proline and amino acids

Root and leaf aliquots (0.1 g), previously frozen with liquid nitrogen, were boiled with ethanol 80 % for 30 seconds and centrifuged at 5000 g, 4 ºC for five minutes to collect the supernatant. The action was repeated two times to perform an exhaustive extraction of soluble osmolites before the last extraction with cold ethanol was made. All

supernatants were collected together and evaporated in a Turbo Vap at 40 ºC. Dry residues were resuspended in 1 mL of deionized water, sonicated for 10 minutes and centrifuged at 2300 g, 4 ºC for 10 minutes. The supernatants were stored at -80 ºC to measure carbohydrates, amino acids and proline content.

Fructose, glucose and sucrose were measured by capillary electrophoresis (Beckman PACE System 5500) (Warren & Adams, 2000). A fused-silica capillary, 30/40.2 cm long and 50 μm internal diameter (Beckman Coulter Inc., USA) was employed.

Amino acid content was determined spectrophotometrically (Yemm & Cocking, 1955). 500 μL citrate buffer (16.8 g anhydrous citric acid and 6.4 g NaOH in 100 mL H_2O) and 430 μL ninhydrin reagent (0.05 g ascorbic acid in 5 mL H_2O and 1.25 g ninhydrin in 125 mL methoxyethanol) were added to each sample. The mixture was boiled at 100 ºC for 20 min and cooled in ice. Then, 1 mL ethanol 60 % was added to each sample and stirred. Absorbance at 570 nm was measured using glycine for the standard curve.

Free proline was measured spectrophotometricaly (Bates *et al.*, 1973). A 0.2 mL extract aliquot, 0.3 mL phosphoric acid 6M and 0.6 mL ninhydrin solution (12.5 mL glacial acetic acid and 0.5 g ninhydrin acid) were mixed and boiled for 60 min. When the reaction mixtures reached room temperature, 3 mL toluene was added. It was centrifuged at 5000 g for 1 minute to separate the phase with the chromophore and its absorbance was measured at 515 nm using a standard curve of L-proline.

2.6. Organic acids

Organic acid content was measured according to the method of Wilson & Harris (1966) (Wilson & Harris, 1966). Root and leaf aliquots (0.1 g), previously frozen in liquid nitrogen, were homogenized with 1.5 mL TCA 5 % (w/v) and centrifuged at 1750 g for 4 min. The mixture was washed with diethyl ether saturated with water two times. The aqueous phase was purged with helium to evaporate the ether residues and then filtered through a 0.45 μm syringe filter. Succinate, malate, α-ketoglutarate and citrate levels were determined by ion chromatography in a DX-500 system (Dionex) by gradient separation with a Dionex IonPac AS11 column according to the application method suggested by the supplier (2.5 mol m_3 NaOH/ 18% methanol to 45 mol m_3 NaOH/18 % methanol in 13 min).

2.7. Statistical analysis

For all the variables determined in this study, we calculated the average as a central statistic and standard error as statistical of dispersion.

For the length of different root sections, we use the average values of 36 biological replicates. For estimations of area, volume and diameter, average values of 16 biological replicates were used. For root respiration measurements, average values of 20 biological replicates were used. For measurements of soluble carbohydrates, organic acids and drought stress markers a pool of roots was used due to the material shortage and three technical replicates were measured for each parameter. Significant differences among treatments were determined with Student's t-test ($P \leq 0.05$).

3. Results and discussion

3.1. Root morphology

Seedlings having a 1.5 cm rootlet, previously germinated under standard conditions, were transferred to Petri dishes containing different agar concentration, 1.5 %, 3 % and 5 % during four days. These conditions lead to three treatments with different water availability for the seedlings. The 1.5 % agar concentration simulates the control conditions (Barker, 2006) and plantlets growing in this condition exhibited a water potential around -0.77 MPa in shoots and -0.82 MPa in roots. The 3 % and 5 % agar concentrations were employed to simulate the two levels of drought, respectively. Plantlets growing in 3 % agar, Drought 1 treatment (D1) exhibited a water potential around -0.82 MPa in shoots and -1.38 MPa in roots, whereas those growing in 5 % agar presented a water potential around -1.38 MPa in shoots and -1.54 MPa in roots, according to (Pérez, 2014).

Root length was measured after four-day exposure to the different water availability treatments. Figure 2 presents a comparative image of different agar dishes treatments. Root length of D1 treatments was significantly higher than that in control whilst D2 treatment did not show this growth induction. This response under mild drought stress could be associated to an attempt to reach other soil areas with more water availability (Sharp *et al*., 1988). Conversely, the lack of growth in D2 could be due to loss of cell turgor (Larcher, 2006) and oxidative stress.

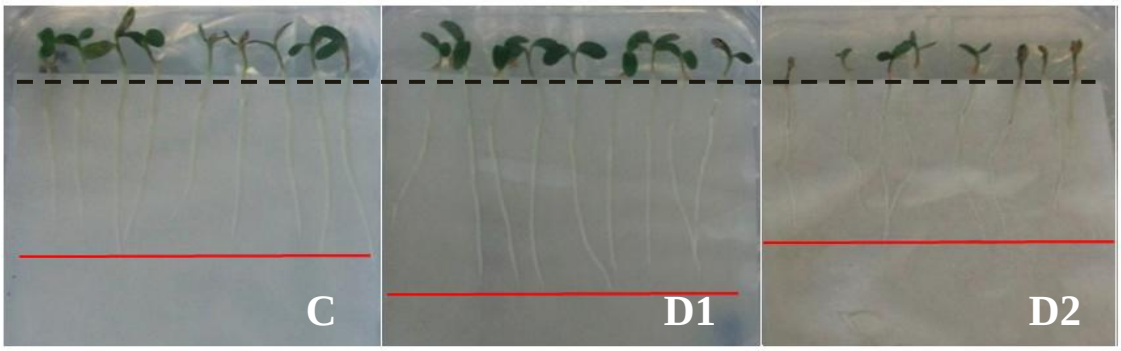

Figure 2: Plant growth after four-day exposure to different water availability treatments. C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54MPa in root).

Daily measurements of root length were taken in the different treatments in order to ascertain how the root growth induction occurs in the D1 treatment. These determinations were done manually and results are not conclusive enough to be presented in this study. However, it was observed that root growth induction started rapidly, just at day 2 after starting the 3 % agar exposure.

Table 1: Length (in cm) of the different root sections in C: Control (-1.18 MPa), D1: Drought 1 (-1.38 MPa) and D2: Drought 2 (-1.54 MPa) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n=36).

Root Sections	**C**	**D1**	**D2**
Upper root	2.07 ± 0.12	2.36 ± 0.08*	2.35 ± 0.10
Root hair	2.33 ± 0.19	2.84 ± 0.15*	1.93 ± 0.12#
Root tip	0.79 ± 0.11	0.93 ± 0.08	0.79 ± 0.07
Total root length	5.19 ± 0.22	6.13 ± 0.21*	5.07 ± 0.17#

Total length was manually measured using a ruler. At the same time, the length of the different root sections were recorded: upper root (UR), absorption section with root hairs (RH) and elongation and tip zone (RT) (Figure 3). As it is exposed in table 1, upper root and absorption zone are significantly longer in D1 than in others as well as

total length. The elongation zone (RT), shows no significant differences among all treatments, although a slight increase was observed in D1.

Figure 3: Plant growth after four-day exposure to different water availability treatments C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54 MPa in root). Upper root, RH: roots hair zone, RT: Elongation and tip zone.

This increase in total length in D1 can be possible because plants can modulate their root system architecture changing the proportion of root cell division and differentiation in the root tip modifying their growth (Silva-Navas *et al.*, 2015). This higher growth under mild stress is also observed in hypocotyl under abiotic stress conditions (Youssef *et al.*, 2015) where they also observed that this increase in length is mainly due to maximal cell length rather than a higher cell division. In roots, it is not known if this increase is due to a higher cell division or an increased cell growth, which could be subject of further studies.

Measurements of root area, volume and diameter were obtained using a computer software called SmartRoot. A significant decrease in area, volume and diameter is observed in D1 compared to control and a greater difference is observed in D2 (Figure 3). Root hair zone diameter was also analyzed to verify that the difference in diameter observed in all treatments remained in the root hairs area, which was demonstrated as shown in figure 3D. Differences in the thickness of the root can be observed in figure 3, and it is corroborated in figure 4 where root diameter is shown. This thickness is greater in root of control treatment and it decreases as the degree of drought increases. Previous studies (Sharp *et al.*, 1988), shown this behaviour in maize plants roots, which were thinner when they were grown at low water potential.

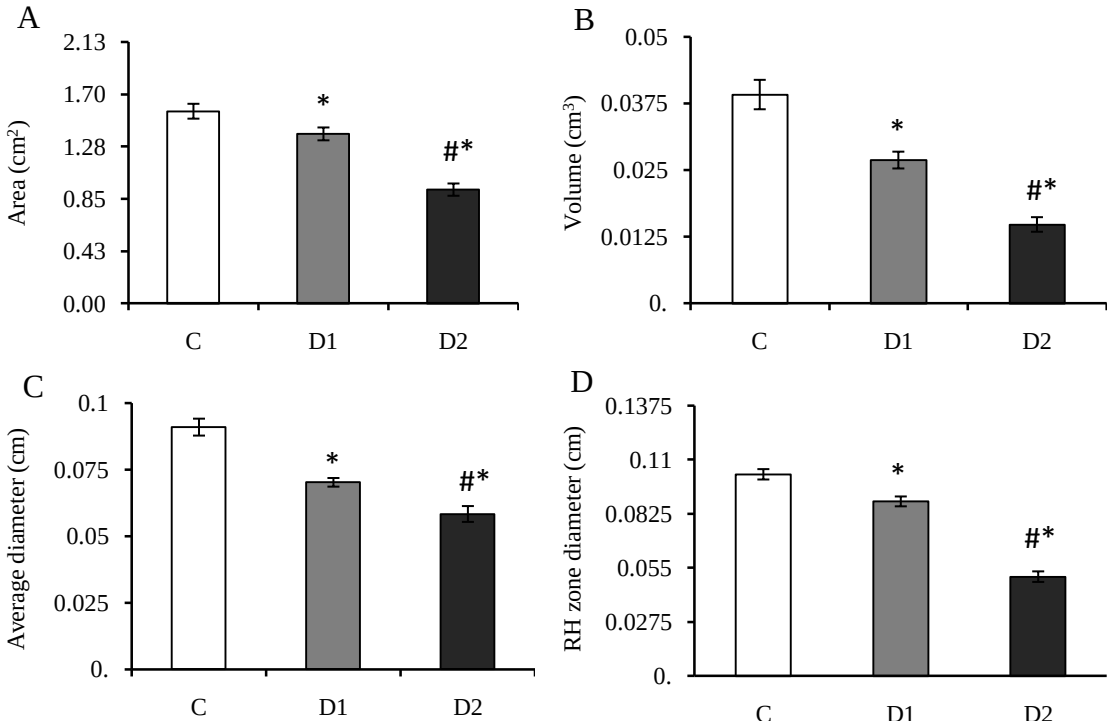

Figure 4: Area (A), volume (B), root average diameter (C) and root hair zone diameter (D) in C: Control (-0.82 MPa), D1: Drought 1 (-1.38 MPa) and D2: Drought 2 (-1.54 MPa) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n=16).

Low water potentials studies in vermiculite have shown that root diameter is decreased to explore efficiently soil water at minimum cost (Sharp *et al.*, 1988). It is difficult to find previous references where root diameter is decreased, in fact there are reports of thicker roots when grown in dry soils (Vartanian *et al.*, 1981), possibly due to a mechanical impedance to root penetration in compact soils thus grows in thickness rather than length (Wilson *et al.*, 1977). Length increase that occurs in D1 does not

compensate the reduction of diameter because the roots showed a reduced area and volume. Biomass of shoot and root was also measured. Although significant differences were not observed among the treatments, a downward trend was observed (Perez, 2014).

3.2. Root respiration

Figure 5 presents the maximum total respiration rate of roots after four-day exposure to the different water availability treatment. As it is shown, total respiration rate decreased significantly in D2 but it was not affected in D1. In the mild drought treatment, total respiration remains constant in order to fuel the root growth induction observed under these conditions. The significant decrease in total respiration rate observed in D2 may be associated to the reduced root growth occurring in these conditions. It could be also a consequence of ROS damages in mitochondria or a decrease in the rate of ion uptake and associated energy demand (Atkin & Macharel, 2009). In this context, it has been described that a reduced respiration would promote root survival during extended drought (Wit, 1978; Sisson, 1989).

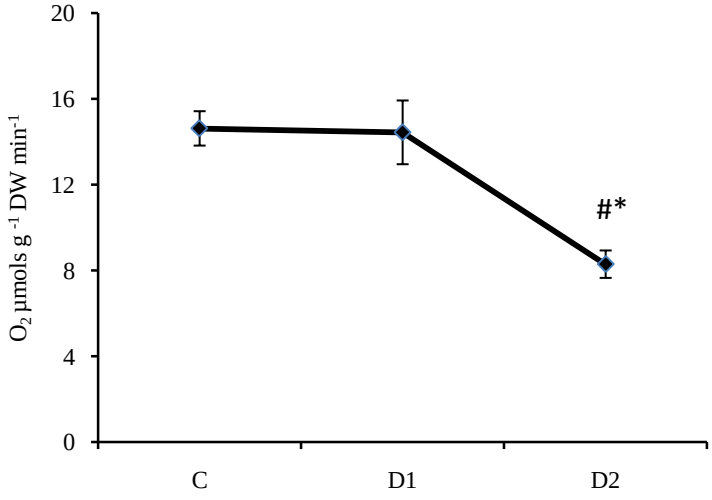

Figure 5: Total respiration rates in C: Control (-0.82 MPa), D1: Drought 1 (-1.38 MPa) and D2: Drought 2 (-1.54 MPa) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n=20).

Respiration capacities are presented in figure 6. There are no significant differences between treatments in cytochrome respiration capacity although a decreasing trend is observed. Alternative respiration capacity decreases significantly in D1 but not in D2

suggesting a different energy rearrangement occurring depending on the drought severity. Residual respiration capacity significantly increase in both D1 and D2 treatment, what could be due to the activation of certain oxidases involved in peroxisome/glyoxysome fatty acid peroxidation (Moller *et al.*, 1988), mechanism in which this respiration pathway participates. Although both cytochrome and alternative are two well-known pathways, the importance and mechanism of residual respiration is still unknown.

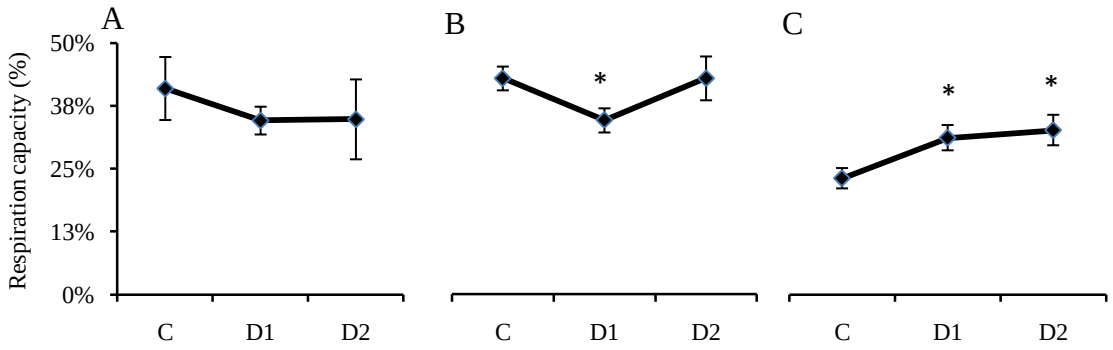

Figure 6: Respiration capacities: Cytochrome respiration capacity (A), alternative respiration capacity (B), residual respiration capacity (C) in C: Control (-0.82 MPa), D1: Drought 1 (-1.38 MPa) and D2: Drought 2 (-1.54 MPa) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n=20).

A controversial response of root respiration to water stress can be observed by revising the literature. Some authors (Dhopte & Ramteke, 1991; Dhopte *et al.*, 1992) reported an increased root respiration in drought conditions, especially for drought-sensitive genotypes, but other studies (Nicolas *et al.*, 1985) reported decreased rates, in both drought-tolerant and drought-sensitive wheat genotypes, in water stress. More recently studies, using PEG as drought simulator, showed a decrease in root respiration as drought stress increased (Mohammadkhani & Heidari, 2007) which is supported by previous findings (Haupt-Herting *et al.*, 2001; Ghashghaire *et al.*, 2001). This decline in respiration could be part of a systemic metabolic response due to oxidative stress and mitochondria damage.

3.4. Soluble carbohydrates content

Carbohydrate content was determined in shoot and root for the better understanding of carbon reserve mobilization occurring in the different water availability treatments. Fructose, glucose and sucrose content are shown in figure 7.

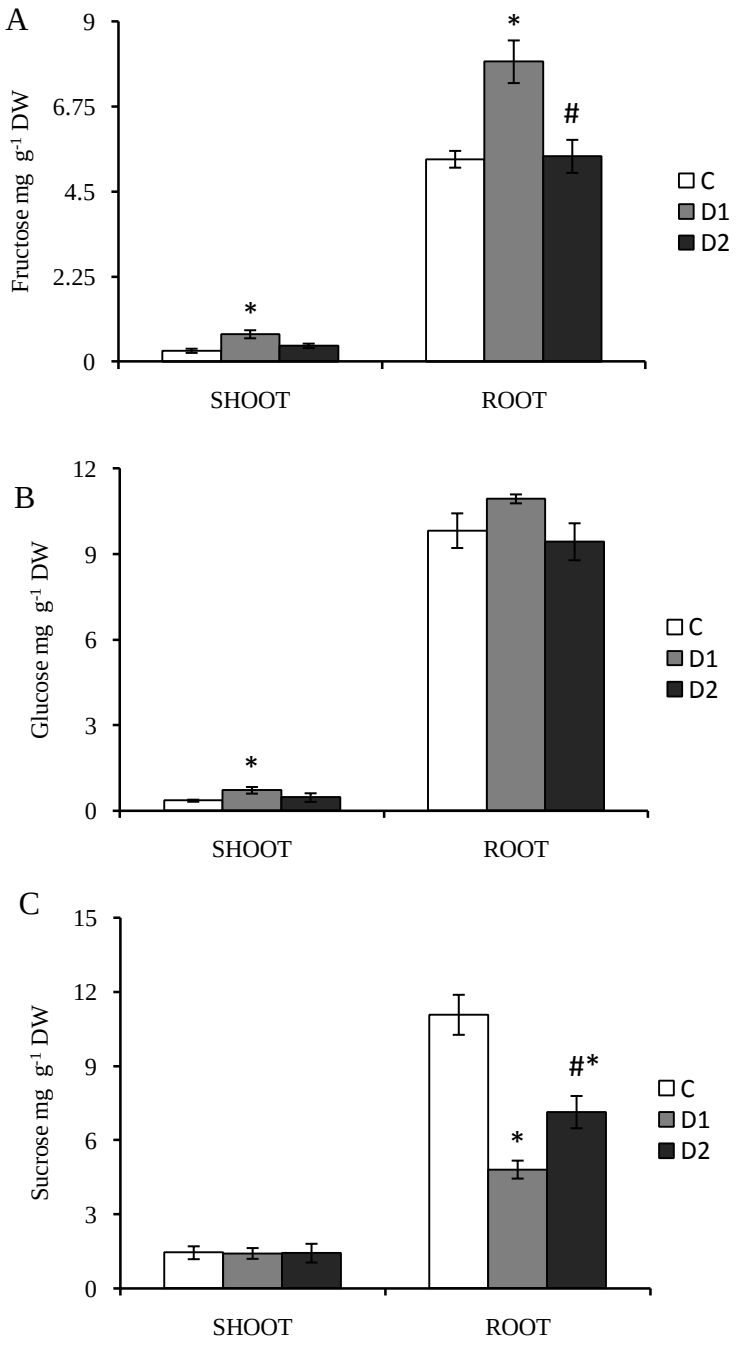

Figure 7: Fructose (A), glucose (B) and sucrose (C) content in shoot and root of *M. truncatula* plantlets exposed to C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54 MPa in root) treatments.. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatment; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n= 3).

Glucose, fructose and sucrose contents were much higher in roots than in shoots suggesting a more active role of this organ for the future implementation of the plant. Mild drought stress (D1) provokes an accumulation of fructose in shoot and root which did not occur under higher water limitation conditions (D2). A similar pattern was observed for glucose, being the effect only significant in the shoot part.

Sucrose, the main carbon source transported through the plant, was not affected at shoot level, suggesting that drought stress imposed was not limiting the carbon availability in the aerial part. However, the sucrose content decrease significantly in root under D1 conditions to values 50 % lower than that of control treatment. This may be due to the fact that D1 roots have an immediate energy requirement to fuel growth induction which was observed to occur only two days after starting the treatment. Sucrose content was also significantly lower in D2 compared to control treatment but increase significantly with respect to D1, reflecting a reduced respiration activity and a lack of growth induction.

3.5. Organic acids

Malate, α-ketoglutarate and succinate contents are shown in figure 8. The content of all of them was higher in roots than in shoots indicating the high activity of the root organ in this plant stage.

The content of malate, the more abundant organic acid, showed a significant reduction in both organs when compared to controls, although the difference was not significant in shoot of plantlets exposed to D1. Succinate and α-ketoglutarate, important carbon skeleton involved in primary nitrogen assimilation, showed a similar response in shoots, increasing significantly in D1 with respect to controls and decreasing back to control values in D2 treatment. However, these organic acids showed a decreasing trend in roots, more marked in the case of α-ketoglutarate, which showed significant differences in D2 compared to C and D1.

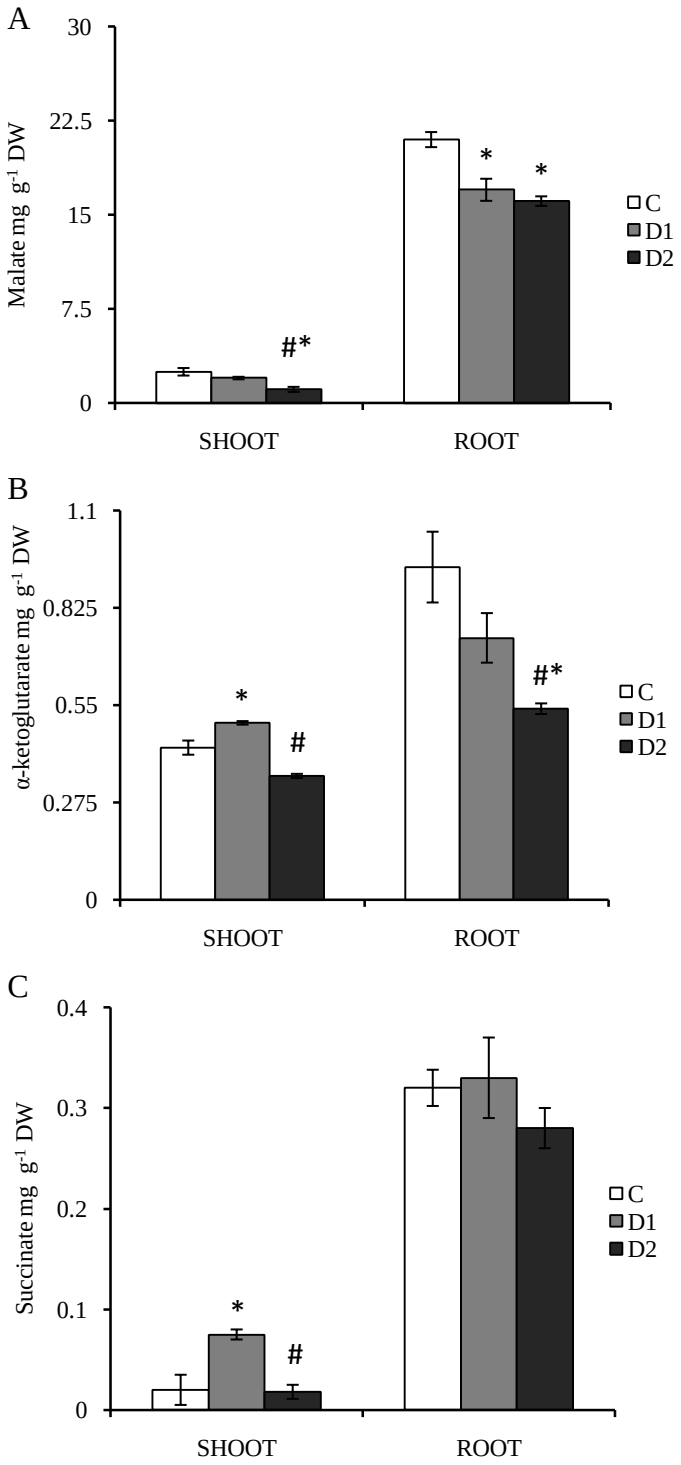

Figure 8: Malate (A), α-ketoglutarate (B) and succinate (C) content in shoot and root of *M. truncatula* plantlets exposed to C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54 MPa in root) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n= 3).

A lower activity of the Krebs cycle during respiration and the reduced availability of sucrose could be associated to the variation of these organic acids. Indeed, the slight but significant increment of glucose and fructose in shoot of D1 treatment may be related to the transient increase of α-ketoglutarate and succinate, suggesting an activation of the carbon metabolism under moderate stress conditions promoting root growth. Previous studies on salt stress effect showed a decrease in malate content in both shoot and root (Saha *et al*., 2012). However, studies dealing with Krebs cycle intermediates are scarce.

3.6. Drought stress markers

In order to characterize the drought effect in our experimental system, several drought stress markers were analyzed. Malondialdehyde (Figure 9), proline and amino acid content (Figure 10) were determined as drought markers in the different treatments.

MDA, the final product of plant cell membrane lipid peroxidation, indicate the peroxidation degree due to stress. As it is shown in figure 9, shoot MDA content increases significantly in both drought treatments. These increments were around 50 % in D1 and 100% in D2 compared to controls. Conversely, root MDA content decreases significantly in D1 showing a significant increase in D2. However, the changes of MDA content observed at root content were much more moderate than those observed at the shoot. Most of the studies approaching lipid peroxidation are carried out in leaves or aerial parts of the plants and scarce information about MDA response at root content has been found. Zabalza *et al*. (2007) explored lipid peroxidation in roots after application of Imazethapyr, an imidazolinone herbicide, observing also a decreased content of MDA. These authors suggested that this may be related to changes in fatty acid composition in this tissue, but the lack of response of this stress marker, would not avoid an oxidative stress response.

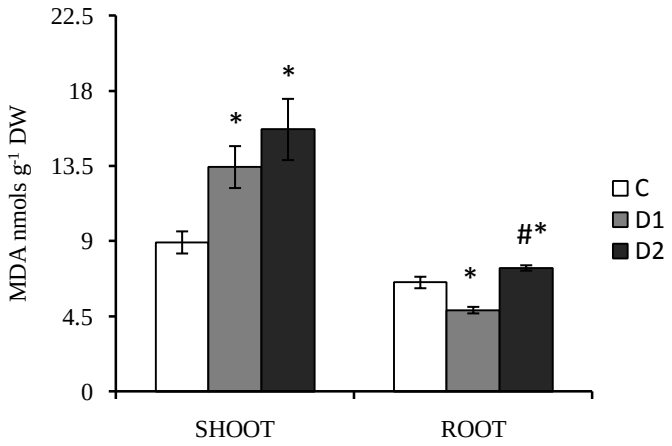

Figure 9: MDA content in shoot and root of *M. truncatula* plantlets exposed to C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54 MPa in root) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n= 3).

Either proline or amino acids content increased in shoot and root both D1 and D2. This accumulation of amino acids and proline corresponds to the osmotic adjustment that takes places with the objective to maintain the cellular turgor and promote water uptake in drought conditions (White *et al*., 2000; Chaves *et al*., 2003).

Proline content increase in shoot and root both in D1 and D2 (Figure 10A). No increase in proline content as drought level increased was observed, in fact in shoot D2 decreases this content respect D1. In root, this increase in proline content remained constant in both D1 and D2. Proline content is frequently measured in drought stress studies. Proline level increases as much as a hundred-fold in primary roots of maize under water deficiency conditions (Voetberg & Sharp, 1991) Mohammadkhani & Heidari, (2008) (Mohammadkhani & Heidari, 2008) showed an increment in free proline content in response to drought stress simulated with PEG and this increment was higher in shoots than in roots. A greater increase was not observed in D2 possibly due to proline content increase very quickly under stress conditions but then usually remains constant.

An increase in amino acids content was observed in shoot and root both in D1 or D2 (figure 10B). Whereas this increase in shoot remained constant, an increase of amino acids content as drought level increased was observed. This increase in amino acids content was also shown in others *Medicago truncatula* studies (Usadel *et al*., 2008; Lugan *et al*., 2010). Gil-Quintana *et al*, (2013) (Gil-Quintana *et al*., 2013) showed this

increment of amino acids content under water stress conditions where plants were grown with a split-root system and exposed to gradual water deprivation. This accumulation of amino acids has a beneficial effect during stress acclimation and does not indicate cell damage as it occurs in some species (Widodo *et al.*, 2009).

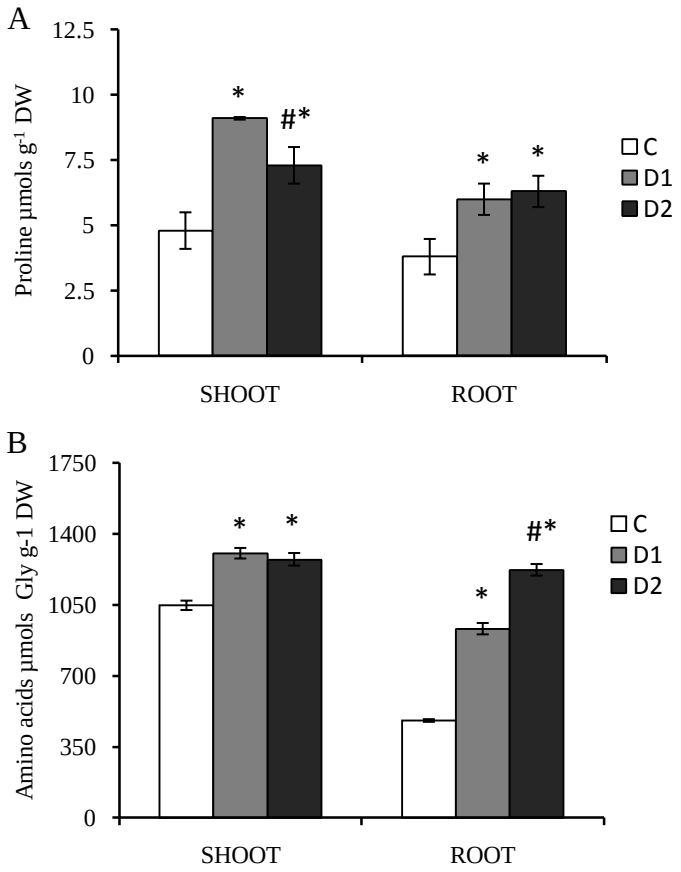

Figure 10: Proline (A) and amino acids (B) content in shoot and root of *M. truncatula* plantlets exposed to C: Control (-0.77 MPa in shoot and -0.82 MPa in root), D1: Drought 1 (-0.82 MPa in shoot and -1.38 MPa in root) and D2: Drought 2 (-1.38 MPa in shoot and -1.54 MPa in root) treatments. Asterisks (*) indicate significant differences ($P \leq 0.05$) between control and drought treatments; hash (#) indicates significant differences between moderate and severe drought. Values represent mean ± se (n= 3).

4. Conclusions

The use of different agar concentrations was useful to simulate different drought stress levels in Petri dishes, providing a standardized protocol for easy characterization of root response to drought. The drought response in *Medicago truncatula* plantlets has been characterized by applying this methodology by measuring the response of several mechanisms known to be involved in this response.

Mild drought level (D1) provoked an induction in root length that did not occur under moderate drought conditions (D2). This root length induction was associated to an

enlargement of the upper root and root hair sections, being the meristematic root tip zone not affected. Despite this root length induction at D1, root area and volume were progressively reduced together with the root diameter concomitantly with the increase of the drought treatment severity.

Respiration rate was significantly reduced in roots of moderate drought (D2) conditions. Cytochromic respiratory capacity was not modulated by any drought treatments. However, a transient decrease of the alternative respiratory capacity was observed at D1, together with progressive reduction of the main carbon metabolites what could be associated to the drought response of carbon metabolism. A progressive increase of the residual respiration capacity was observed concomitantly with the increase of drought severity. This carbon limitation of carbon metabolism at the root level occurs independently of the root growth strategy, which differed among the two drought stress levels.

The typical drought stress markers analysed reflected the two drought stress levels imposed by using this protocol. Despite proline increased rapidly in both drought levels, other stress markers as amino acid content increased progressively in root and shoots indicating that a progressive from mild to moderate drought stress level was obtained.

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