



Received for publication, December, 20, 2018

Accepted, December, 9, 2019

Original paper

Influence of water deficit on morphological, physiological and growth of Balsa (*Ochroma pyramidale*)

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Abstract

Water is one of the main factors that influence the growth and development of plants. Balsa (*Ochroma pyramidale*) is a fast-growing tropical tree species with a great demand in the international market. The influence of soil, water deficit on morphological, physiological, and the growth of Balsa (*Ochroma pyramidale*) with and without irrigation was investigated. For this study, four random repetitions were carried out in a randomized complete block design. The plants were irrigated regularly with furrow irrigation. The timing of irrigation was based on the soil, water potential, according to treatment criteria. All pots were irrigated at 15, 30, 45, 60 and 75 days. Genomic DNA of *O. Pyramidale* was identified by ERIC-PCR and RAPD, while phylogenetic tree was determined using the UPGMA method. The results showed that leaf number, shoot number, stem elongation and stem diameter were affected significantly ($P < 0.05$) between the plant without irrigation compared to control (with irrigation) during the days of assessment. Regarding stomata number, no significant differences ($P > 0.05$) were observed, while stomata length remained high ($> 20 \mu\text{m}$) and stomata diameter was decreased during evaluation days in plants without irrigation compared to control. DNA of eight plants isolated showed different band profiles. In conclusion, the parameters evaluated can be used as a measure of water stress, growers should be careful when using *O. Pyramidale* in both nursery and orchards where water scarcity is present.

Keywords *Ochroma pyramidale*, water potential, drought, growth.

To cite this article: VERA P, ASTORGA-ELÓ M, SEVERE R, MORANTE L, MORANTE J, MONTENEGRO J, DUMORNÉ K. Influence of water deficit on morphological, physiological and growth of Balsa (*Ochroma pyramidale*). *Rom Biotechnol Lett.* 2020; 25(6): 2148-2155. DOI: 10.25083/rbl/25.6/2148.2155

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Introduction

Balsa (*Ochroma pyramidale*) is a fast growing, early successional, tropical tree species characterized by very low wood density (SEABRA & al [1]). It is a forest species that has great demand in the international market. *O. pyramidale* is cultivated naturally and by reforestation, especially in the sub-tropical jungle of Ecuador, and is one of the forest resources of economic interest to the country (GONZÁLEZ-OSORIO & al [2]) (VLEUT & al [3]). The increase of urbanizations in arid zones has promoted the development of urban afforestation programs that seek to use specifically with low water requirements to achieve the sustainability of the urban forest (MARTINEZ & al [4]).

The water deficit is one of the main environmental factors that affects the development of plants, mainly their growth. In plants, low humidity conditions in the soil have had a negative influence on growth and development, affecting their productivity (DAI & al [5]) (LI & al [6]). Water stress affects protein synthesis, photosynthesis, respiration, and nucleic acid synthesis, which can be affected by plant performance. In addition to phenotypic variation, the effect of drought on a plant depends on the timing, duration, and severity of stress (CALVO-POLANCO & al [7]) (DEL POZO & al [8]). Water stress is one of the most important constraints in the growth and development of plants. Water deficit stress, in particular, is a major problem in agriculture and most crop plants show a higher sensitivity to this kind of stress than any kind of biotic constraint conditions. Crop plant growth and yields are severely impacted by inadequate supply of water, which result in decreased carbon assimilates contents (MANGENA & al [9]).

Water stress drives important losses in the crop market. Excess or deficit of water produces a cascade of physiological responses that ends-up in decreased production and reduced crop quality. Numerous factors are involved in plant responses to water stress. (SALAZAR & al [10]). Several studies indicated that the effect of water stress varies according to plants and growth stage (PETROPOULOS & al [11]). Plants exposure to water stress during the vegetative growth exhibit a decrease in plant height and leaf area dry matter production and number of leaves (ABBASZADEH & al [12]) (ALIABADI & al [13]) (NAJLA & al [14]).

The knowledge of the physiological, cellular, and molecular responses of plants to drought and waterlogging has already allowed great progress in plant breeding; however, few researchers have focused on *O. pyramidale*. The low production and adaptation of forest species is due to environmental factors such as low rainfall that affects plant development. *O. pyramidale* requires a warm and humid climate, the minimum amount of precipitation that it tolerates is around 1500 mm per year, which represents a great problem in the dry season, where rainfall is scarce, culminating in high mortality. More than 50% of the raft production in the establishment are affected by the lack of

water. Plants under stress are more susceptible to attack by pathogens and pests due to low expressions of resistance mechanisms. The objective of this study was to evaluate the effect of *O. pyramidale* with and without irrigation on morphological, physiological and growth of this plant.

Material and Methods

All the chemicals used in this study were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Plant material

The study was carried out at Quevedo State Technical University. *O. pyramidale* was collected at the experimental station of this University and were stored in optimal conditions. For this study, four random repetitions were carried out in a randomized complete block design. Therefore, 60 plants were used in total in this study (Fig. 1). Half of the plants were put under water deficit imposition by withholding water for ten weeks while another half were maintained in optimal water availability conditions at 15, 30, 45, 60 and 75 days. All plants were then placed on a bench in outside conditions. A polyethylene cover was also employed in case of rain during the experimental period.

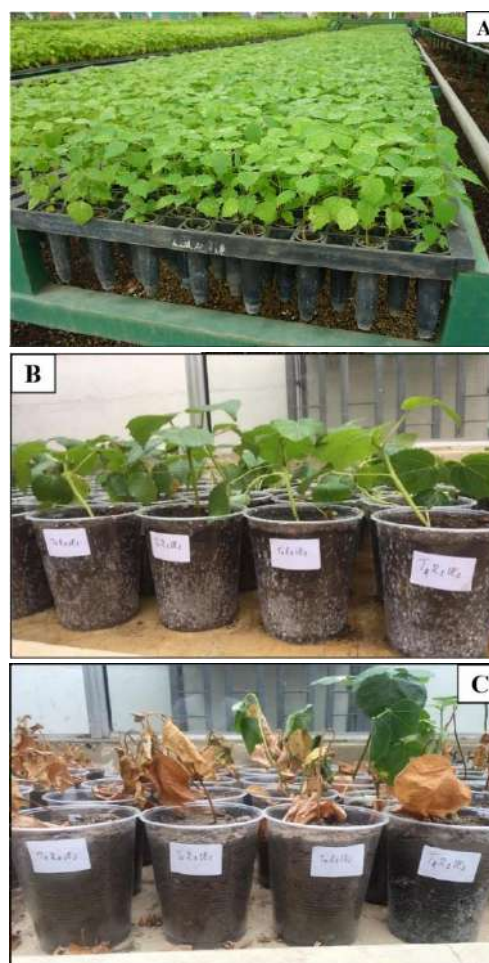


Figure 1. Plants treatment: A and B) with irrigation, C) without irrigation.

Plant measurements

Leaves number, shoots number, stem elongation and plant diameter were measured in without irrigation and with irrigation (Control). The evaluations were performed only in the morning. In addition, genomic DNA extractions were performed to validate the presence or absence of genes involved in tolerance to water stress.

Leaves number

Leaves number of each treatment was evaluated at 15, 30, 45, 60 and 75 days after being subjected to water deficit, taking as starting point 2-month-old plants. Only functional leaves that did not present pathogen symptoms or wounds caused by mechanical damage were evaluated.

Shoots number

Shoots number was evaluated periodically every 15 days, the evaluation was carried out in all the plants corresponding to the respective treatment directly without performing interventions such as shoot cutting or removal.

Stoma number

The morphophysiological condition of the stoma corresponding to each treatment was determined. The opening condition as a result of dehydration or reaction to water stress was also evaluated. Viable leaves that were covered with a thin layer of enamel were used; stoma condition was visualized using a microscope composed of a camera.

Stem diameter and elongation

Stem diameter and elongation were measured in periods of 15 days from the root base to the last insertion of the leaves, taking as reference the longitudinal advance of each plants evaluated.

DNA extraction

Genomic DNA was isolated using the DNeasy Plant kit according to the protocol described by the manufacturer. Samples were suspended in 400 μ L Buffer AP1 and 4 μ L RNase and were incubated for 10 min at 65°C. Then 130 μ L AP2 was added and incubated for 5 min on ice. The samples were centrifuged for 5 minutes at 20.000 x g. The supernatants were removed and the pellets were suspended 1.5 mL of Buffer AP3, 650 μ L of the mixture was placed into the DNeasy Mini spin column placed in a 2 mL collection tube and was centrifuged for 1 min at \geq 6000 x g, 400 μ L Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 20.000 x g. The DNeasy Mini spin column was transferred to a 1.5 mL or 2 mL Microcentrifuge tube, and Pipet 100 μ L Buffer AE directly onto the DNeasy membrane. The sample was Incubated for 5 min at room temperature, and then centrifuge for 1 min at \geq 6000 x g to elute. DNA was evaluated by 1% agarose gel electrophoresis with ethidium bromide staining. Gels were prepared in 1X TAE (Tris, sodium acetate, EDTA) buffer containing: 40 mM Tris-base; 1 mM EDTA, 20 mM acetic acid. The gels were analyzed with a digital

photodocumentator (E-Gel Imager, Life Technologies) that incorporates a UVP ultraviolet transilluminator and a high-resolution digital camera.

Genomic PCR Screening

Coding sequence was amplified from genomic DNA by PCR. The probe sequence was PCR amplified using primers (OPA 10: 5'-GTGATCGCAG-3') (A01: 5'CAGGCCCTTC-3') (M 07: 5'-CCGTGACTCA-3'). Transcript amplification and detection were performed using the following thermal profile: 94°C for 3 minutes, followed by 40 cycles of 94°C for 1 minute, 37°C for 2 minutes, 72°C for 2minutes, 72°C for 7 minutes and 4°C for 5 minutes. The amplification products were separated by electrophoresis (ENDURO™ 300V power supply) at 60 volts for 5:30 hours / min, in 2.5% agarose gel, using TAE 1x buffer (Tris-base, glacial acetic acid, EDTA 0.5 M) and photographed with a digital UV light camera (Life Technologies).

Phylogenetic tree

Phylogenetic tree was determined using the UPGMA method with a branch length of tree = 2.18566002. The tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were calculated using the Kimura-2 parameters method. The analysis involved 17 nucleotide sequences. The included codon positions were 1st + 2nd + 3rd + non-coding. All positions containing voids and lost data were removed. There was a total of 67 positions in the final data set. Evolutionary analyzes were performed in MEGA6.

Stomatal density

To determine the stomatal density, epidermal cuts were made to each sample, and it was placed in the center of the engraved area of the Neubauer chamber, to avoid rapid dehydration of the epidermal tissue, 5 μ L of water was added, then the sample was covered with an object cover, stomatal density was observed using a phase contrast microscope (Carl Zeiss, Jena, Germany) at 400X magnification).

Statistical analysis

The data were expressed as mean \pm standard deviation (SD) and were analyzed using the statistical software GraphPad Prism®, version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were established using the Tukey's multiple comparison test. The level of significance was set at P<0.05.

Results

Morphophysiological evaluation of *O. pyramidale*

Leaves number

The results showed that there were significant differences between the control and plants without irrigation during evaluation days (P <0.05). At day 15 of evaluation

no significant differences ($P > 0.05$) were observed between the control and plants without irrigation for sheet number. However, at days 30, 45, 60 and 75 leaves number of plants without irrigation were decreased significantly ($P < 0.05$) compared to control, showing value 3.3 for day 60, leaves number for the control was increased with values > 5 (Fig. 2A).

Shoots number

Shoots number was affected significantly by the evaluation time in both treatment ($P < 0.05$). No significant

differences ($P > 0.05$) were observed on days 15 and 30 between the control and without irrigation. However, shoots number was decreased ($P < 0.05$) for the plant without irrigation showing value 0.95 for day 75, compared to control with 3.7 for the same day (Fig. 2B).

Stomata number

Regarding stomata number, no significant differences ($P > 0.05$) were observed between both treatments showing values > 6 for the plant without irrigation and > 8 for the control (Fig. 3C).

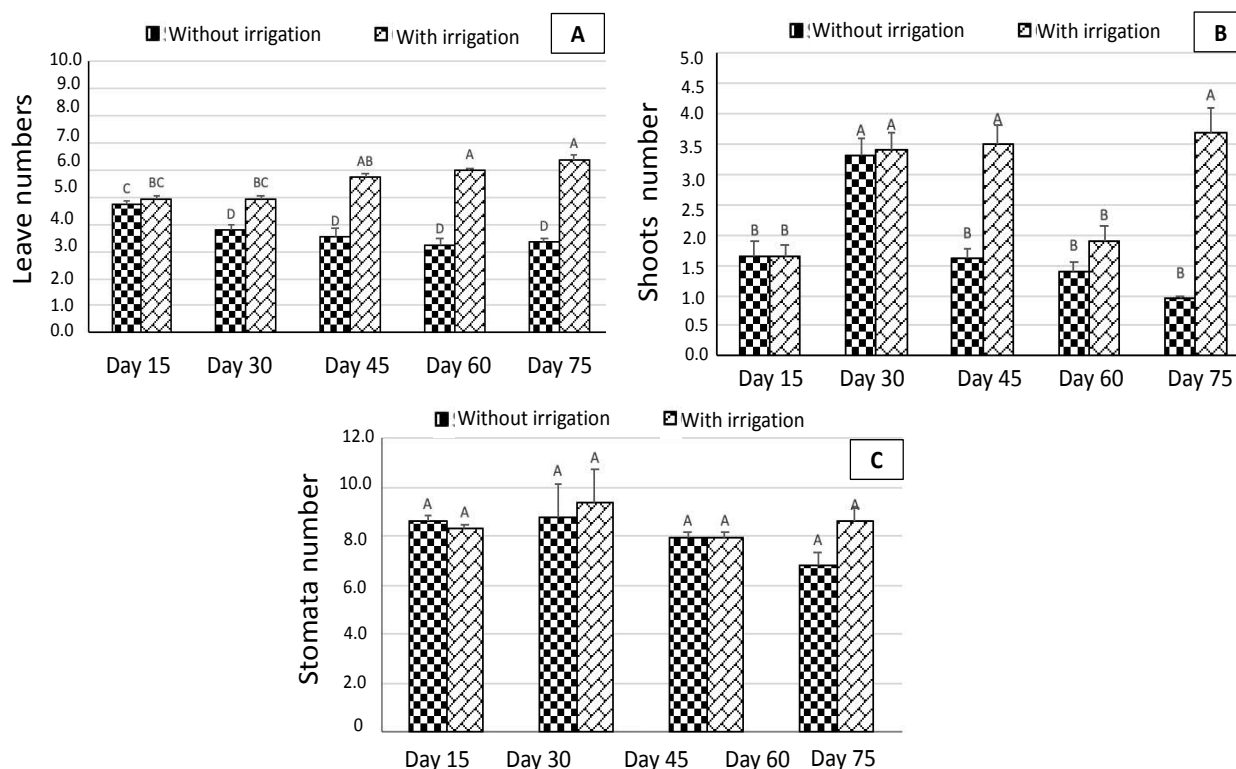


Figure 2. *O. pyramidale* plants subjected to water deficit. **A)** Leaves number; **B)** Shoots number; **C)** Stomata number. Data are presented as mean \pm SD. A, B, C, D. Different letters indicate statistically significant differences in the same group at different periods of treatment time. Different superscripts indicate different rates ($P < 0.05$).

Stem elongation

Stem elongation was affected significantly ($P < 0.05$) by the time of assessment in the control and plant without irrigation. Stem elongation in the plant without irrigation was decreased, showing a value 12.185 cm and 21.52 cm (control) at day 75, respectively (Fig. 3A)

Stem diameter

Significant differences between the control and plants without irrigation during evaluation days ($P < 0.05$). At day 15 and 30, no significant differences ($P > 0.05$) were observed at between the control and plants without irrigation. However, stem diameters for control was increased compared to plants without irrigation presented highest 5 cm for day 75 (Fig. 3B).

Stomach morphological analysis of *O. pyramidale*

Length of stomata

The results showed that stomata length remained high ($> 20 \mu\text{m}$) in both treatments during the days of assessment. However, from day 30, 45, 60 of evaluation stomata lengths was significantly decreased ($P < 0.05$) for plants without irrigation regarding control (Fig. 3C).

Stomata diameter

The results showed that stomata diameter was decreased during evaluation days in plants without irrigation regarding control, however, no significant differences ($P > 0.05$) were observed. Significant differences in the control were observed ($P < 0.05$) during the evaluation days (Fig. 3D).

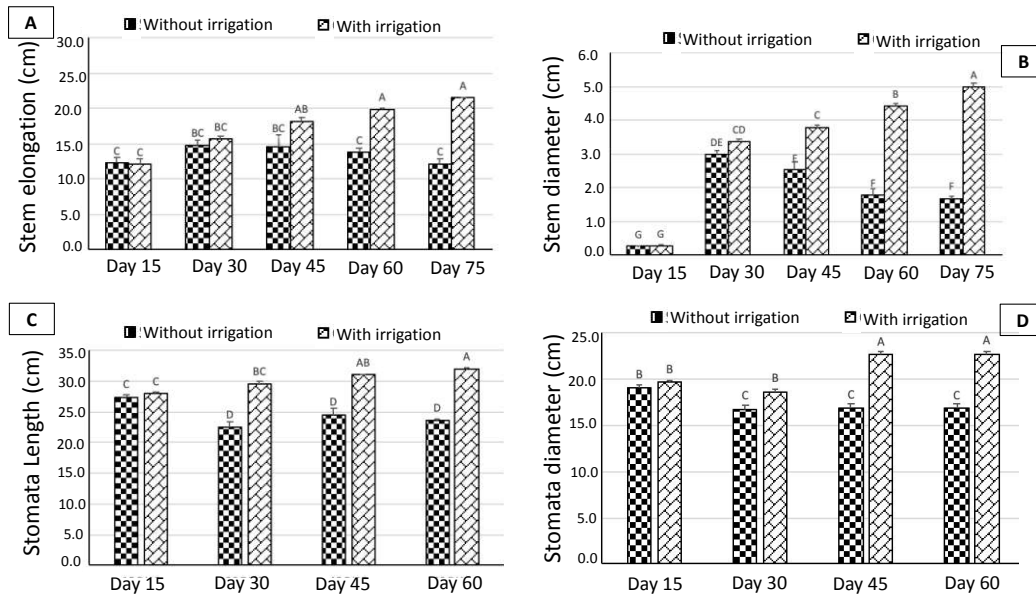


Figure 3. *O. pyramidale* plants subjected to water deficit. **A)** Stem elongation; **B)** Stem diameter; **C)** Stomata length; **D)** Stomata diameter. Data are presented as mean \pm SD. A, B, C, D. Different letters indicate statistically significant differences in the same group at different periods of treatment time. Different superscripts indicate different rates ($P < 0.05$).

Stoma status

Figure 4 showed stoma status of *O. pyramidale* visualized by microscopy subjected to water deficit: Semi-open stomatal (days 15) (Fig. 4A); Closed and semi-open stomatal (days 30) (Fig. 4B); C) Closed stomatal (days 45) (Fig. 4C); Closed stomatal (days 60) (Fig. 4D). At the

stomatal level, there is variation in the morpho-physical characteristics in plants with water deficit, after 30 days the length and diameter of stomata is less with 22.50 and 16.79 μm , while a plant with constant irrigation the length is significantly higher showing values of 29.55 and 18.68 μm , respectively.

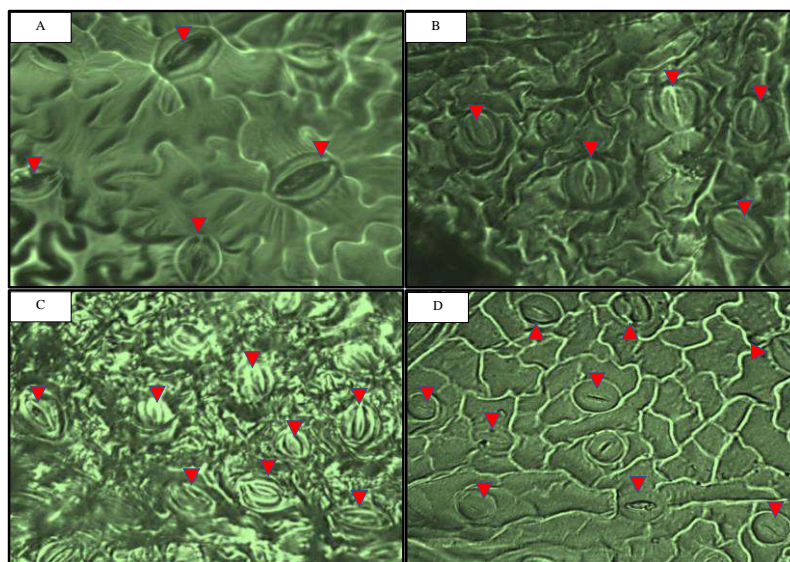


Figure 4. Leaves visualized by microscopy: **A)** Semi-open stomatal (days 15); **B)** Closed and semi-open stomatal (days 30); **C)** Closed stomatal (days 45); **D)** Closed stomatal (days 60).

DNA Extraction by ERIC-PCR and RAPD

The extraction of DAN was realized for evidencing the possible similarities among the plants using 8 *O. pyramidale*. The results showed identical bands, both in number and size as shown in Fig. 5A. However, Random

Amplified Polymorphic DNA (RAPD) technique and ERIC-PCR increased the resemblances degree amongst between the plants. It has been found that the largest amplified product was 1500 bp and the smallest was only 100 bp (Fig. 5B and C).

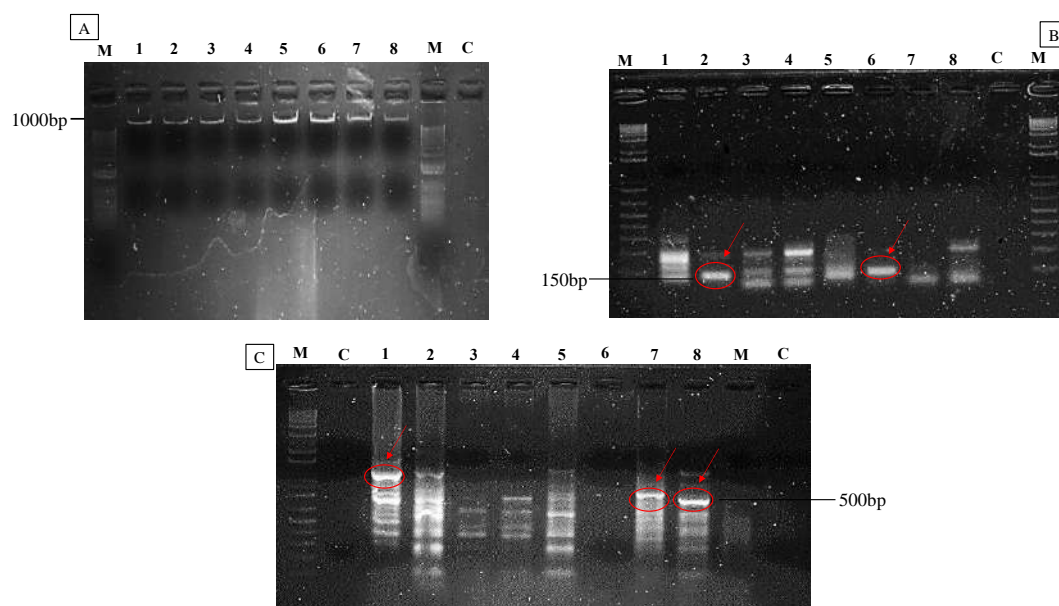


Figure 5. A) Genomic DNA identification of *O. pyramidale*, B) by ERIC-PCR, C) by RAPD. The number of scorable bands for responding primers ranging from 1 to 11 with an average of four bands, ranging from the largest amplified product, 1500 bp and the smaller, 100 bp. While 1-2 (15 days) 3-4 (30 days), 5-6 (45 days), 7-8 (60 days), M (molecular weight markers) and Control.

Phylogenetic grouping of *O. pyramidale*

The phylogenetic tree obtained by ERIC-PCR was performed according to the Maximum Composite Likelihood model, formed by two main groups (I and II) and four subgroups (A, B, C, and D). Group I, brought together a sub group (A) consisting of Balsa 1, Balsa 5, Balsa 4 and Balsa 8, and subgroup (B) consisting by Balsa 2 and Balsa 3 plants, all these plants belonging to the treatments of water deficit. Group II, brought together the sub group (C) consisting of the Balsa 6 and Balsa 7 plants, belonging to the water deficit treatments, there is

also the sub group (D) made up of the Balsa 9 plants, which was as a control (Fig. 6a). The phylogenetic tree obtained by RAPDs also presented two groups (I and II).

Group I brought together a sub group (A), consisting of the Balsa 2, 3, 8, 9, 6 plants and sub group (B) consisting of the Balsa 5 plants, all of these plants belonging to the water deficit treatments at the exception of Balsa 9 which was as a control. Group II brought together a sub group (C) consisting of the Balsa 4 plant and sub group (D) consisting of the Balsa 10, 1 and 7 plants, also belonging to the water deficit treatments (Fig. 6b).

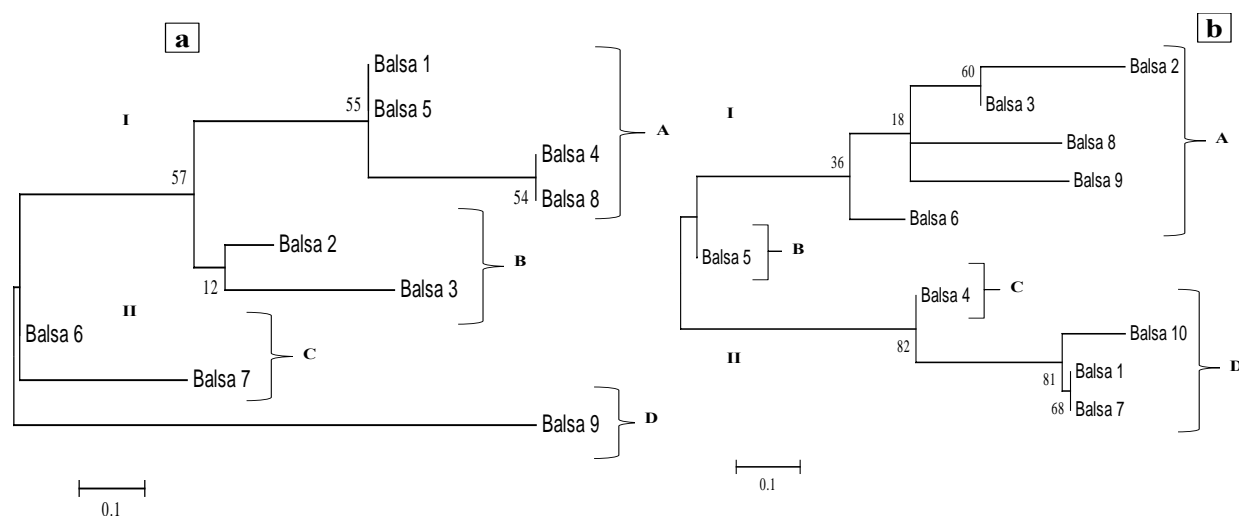


Figure 6. Phylogenetic clustering of *O. pyramidale* by ERIC-PCR (a) and RAPD (b) constructed from plant DNA. The evolutionary distance was deduced using the Neighbor-Joining method. The phylogenetic tree was constructed using Maximum Composite Likelihood. The numbers represent percentages of bootstrap.

Discussion

CHEN & al ([15]) have reported that a plant adapts to prolonged drought stress via changes in the root system morphological conformation, photosynthetic product allocation to above ground, and underground portions, and more efficient utilization of the limited soil water. JALEEL & al ([16]) have reported that drought stress is a very important limiting factor during early seedling growth and establishment.

It affects both elongation and expansion growth. Water-deficit stress is considered as one of the most important growth-limiting factors that decrease plant growth. Injury of plants takes place under water shortage at any vegetative growth stage (TÁTRAI & al [17]). Results from our study indicate that water-deficit stress reduced the growth of *O. pyramidale*. Several studies have reported similar effects caused by water-deficit stress (LENHARD & al [18]) (AVRELLA & al [19]). In this study, stem elongation in *O. pyramidal* without irrigation was decreased, showing a value 12.185 cm compared to control 21.52 cm at day 75. A similar situation was reported by AVRELLA & al ([19]) for stem elongation *O. pyramidal* showing a value 29.59 cm with irrigation for 35 days and 53.96 cm at 70 days. One explanation of this reduction could be that plants grown with constant irrigation of water, it has more capacity to attract nutrients from the soil. The results obtained for height and diameter throughout the time of conducting the experiment in high levels of water deficiency, is the sensitivity of the physiological process to water deficit, which is a strategy of the plant in dealing with the range of variation of water availability.

AVRELLA & al ([19]) reported that some plants show a water stress adaptive mechanism by restricting their growth while water shortages in the rhizosphere persists. Accordingly, we might suggest that *O. pyramidal* plants conserve their growth and sacrifice the vegetative development for gaining a long-term survival under drought stress condition. In our study, the plants subjected to more than 45 days of water deficit showed up to 50% less leaf area, which means that their morpho-physical characteristics were altered. Well-developed root system is considered as a strategy for desiccation avoidance in natural vegetation. Morphological root components play a major role in root hydraulics that can reflect different responses to drought stress (BRAMLEY & al [20]).

Physiologically, the water content is probably the most appropriate for a plant CALVO-POLANCO & al [7]) (KOLENC & al [22]). Our results showed that significant differences between the control and plants without irrigation during evaluation days in all parameters evaluated. Several studies have demonstrated that species of high-resource environments take advantage of the water supply increase, while species adapted to stress are less plastic. Severe stress caused reductions in length, number of leaves, suggesting that *O. pyramidale* is vulnerable to water stress.

Water stress, in general, leads to the accumulation of solutes in the cytoplasm and in the vacuole of plant cells, thus allowing the cells to maintain the pressure even at a low water potential. This positive pressure is essential for plant growth, as well as increasing the mechanical stiffness of cells and tissues. Studies show that plants subjected to severe water deficit, invest more in root elongation than in the shoot, which increases the potential to absorb water from the deeper layers of the soil profile (SOARES & al [23]) (VENIER & al [24]). According to these studies, to achieve a high level of budding success, plant materials should not be watered deficit during the bidding period. In general, the results obtained in this study suggest that *O. pyramidale* can be used in management plans and restoration of areas, as well as its commercial production in areas with water stress.

Conclusions

In this present study, it can be concluded that water deficit affected plant growth. The water deficit resulted in reduction in growth parameters by decreasing relative shoot length and diameter of the plant. The effect of water deficit conditions was more visible in the plant without irrigation compared to control. This should be taken into account where the water deficit is a limiting factor in *O. pyramidale* nursery and orchards.

According to the analysis of morphological and physiological, it is concluded that *O. pyramidale* plants were not able to tolerate conditions of low water availability. A further detailed study is also needed to elucidate the underlying biochemical processes and anatomical and genetic parameters which are responsible for differential responses of *O. pyramidale*.

Acknowledgments

This research was supported by Universidad Técnica Estatal de Quevedo, Quevedo, Ecuador and University of La Frontera/Postdoctoral (KD) 2019.

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