

Plant Growth, Antioxidative Enzymes, Lipid Peroxidation and Organic Solute Contents in Mulungu Seedlings (*Erythrina velutina*) Under Different Field Capacities

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Abstract

Erythrina velutina (mulungu) is an endemic species of caatinga found in Northeast Brazil. As a result of its rapid plant growth, the species may be an alternative for the recovery of degraded areas. Thus, the present study aimed to analyze the effects of irrigation with different field capacities (FC): 20, 50 and 80% on plant growth, antioxidative enzyme activities, membrane lipid peroxidation and organic solute contents in mulungu seedlings under greenhouse conditions. The experiment was carried out at Instituto Federal de Educação, Ciência e Tecnologia do Ceará (IFCE)-Campus Maracanaú, Ceará, Brazil. Under the presented experimental conditions, *E. velutina* plants showed higher growth variables (dry matter yield and leaf area) when submitted to daily irrigation of 50% of FC. Irrigation at 20% of FC caused a small water deficit. However, 80% of FC watering may have resulted in an excess of water. In general, despite the reduction in plant growth in plants irrigated at 20% of FC, the activities of the antioxidant enzymes did not differ substantially between treatments. In general, the lowest organic solute contents were detected in irrigations at 20 or 80% of FC.

Keywords: *Erythrina velutina*, organic solute, oxidative stress, antioxidative enzymes, water stress

1. Introduction

The northeastern region of Brazil has a semi-arid climate with irregular rainfall that is concentrated in a short period, resulting in a water shortage for the rest of the year. Thus, plants need to adjust physiologically to this condition (Trovão et al., 2007). Hence, water is one of the main limiting factors of agricultural production in the region, a water deficit can occur due to low availability in the root zone, or when the evapotranspiration rate becomes excessive (Ghobadi et al., 2013).

The water deficit can cause damage to the plant by the closure of the stomata as a way to avoid excessive loss of water by evapotranspiration, which may cause a reduction in the photosynthetic rate due to the lower availability of CO₂. Subsequently, this can cause a disturbance in the balance between reactive oxygen species (ROS) production and antioxidant defense, resulting in phytotoxicity (Cruz de Carvalho, 2008; Wang et al., 2013).

With the lower availability of CO₂ in the biochemical stage of photosynthesis, there is a reduction of NADPH oxidation in the Calvin-Benson cycle, which results in its low availability as NADP⁺ in the photochemical stage. In this way, this process may cause the transfer of electrons from the reduced ferredoxin in photosystem I to O₂, and, consequently, increase the production of ROS, promoting oxidative stress and damage to the plant (Ghobadi et al., 2013; Cerqueira et al., 2015).

Although these ROS are harmful when in higher concentrations, they are produced naturally by means of some organelles such as the mitochondria, chloroplasts and peroxisomes (Karuppanapandian et al., 2011), being formed the hydroxyl radical ($\cdot\text{OH}$), the superoxide radical ($\cdot\text{O}_2^-$), singlet oxygen ($^1\text{O}_2$) and hydrogen peroxide (H₂O₂) (Sharma et al., 2012).

The production of ROS should not be considered as a deleterious process in the seedlings, in low concentrations they can act as signaling agents in order to reach acclimation to adverse situations, such as limited water availability (Ren et al. 2016). However, at high concentrations, they can cause damage to proteins and nucleic acids, as well as lipid peroxidation of cell membranes. Therefore, a balance between ROS production and elimination is needed (Miller et al., 2010).

The organism produces enzymatic and non-enzymatic defense mechanisms (antioxidants of low molecular weights), whose function is to eliminate the harmful effects of ROS. In the enzymatic system, the ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and superoxide dismutase (SOD) enzymes act to eliminate ROS (Deuner et al., 2008; Barbosa et al. 2014).

When faced with water stress or saline stress, plants need to maintain the water potential gradient between the soil and the root environment. In this manner, some species absorb ions and/or accumulate low molecular weight organic solutes such as N-aminosoluble, carbohydrates and proline (Sperdoui & Moustakas, 2012; Szabados & Saviouré, 2010). Furthermore, it is believed that these solutes act as osmoprotectants helping to maintain the integrity of the macromolecules and the elimination of several ROS (Mohammadkhani & Heidari, 2008; Szabados & Saviouré, 2010; Abid et al., 2017).

Erythrina velutina, known as mulungu in northeastern Brazil, is one of the few large leguminous plants of the region, endemic to the caatinga, with a large treetop and rapid growth. The plants are classified as angiosperms and are members of the family Fabaceae, they are tall spiny trees with a large bole size and deciduous foliage. The plant is hermaphroditic, with its pollination being made mainly by bees and has as main vehicles of dispersion seeds, the wind and birds (Carvalho, 2008). The tree is ornamental, mainly used for its flowers, and its wood has a low economic value, being used for stakes and matchstick production (Sales Guedes et al., 2009; Pereira et al., 2014).

Although the species does not have a great economic worth, its wood is used for the handicraft of the region and commonly used in popular medicine (Sousa et al., 2008). Moreover, there are studies that have demonstrated the presence of calming properties in the species that help in insomnia and other disorders of the central nervous system (Agra et al., 2007), with similar effects to the drug Diazepam (Ribeiro et al., 2006). It also has potential uses in treatment of inflammation-related disorders, disseminated intravascular coagulation and cancer (Monteiro, 2011).

Increasing deforestation, together with the difficulty of regenerating natural areas and erosion, have intensified environmental problems. These factors increase due to the fact that the deforested area no longer has the mechanisms to retain the water in the soil, a task which was once performed by the plants. In addition, erosion can be increased by the higher incidence of solar rays reaching the soil and the leaching of nutrients by rainfall, making the soil inhospitable (Nogueira et al., 2007; Brown et al., 2013; Lawrence & Vandecar, 2015).

Due to the advanced degree of degradation of the biomes of the Northeast region of Brazil (especially the caatinga) and the presence of particular characteristics for the growth, development and maintenance of the flora and fauna (de Albuquerque et al., 2012; da Silva et al., 2016), research using species of native plants, such as mulungu, for the recovery of degraded areas that may be subject to water stress is needed. In this way, it is believed that mulungu could develop in a situation of water stress. Though, it is necessary to identify adequate irrigation for seedlings produced in a greenhouse, which could later be used in the field for the recovery of degraded areas.

Thus, the objective of this work was to analyze the effects of irrigation with different field capacities on the activities of antioxidative enzymes, membrane lipid peroxidation and organic solute contents in mulungu seedlings under greenhouse conditions.

2. Method

The experiment was conducted under greenhouse conditions at Instituto Federal Educação, Ciência e Tecnologia do Ceará (IFCE)-Campus Maracanaú, Ceará, Brazil from August to September 2015, totaling 38 days. The mean values of temperature and relative humidity were, 32 °C and 52%, respectively.

The seeds of *E. velutina* were obtained by means of manual harvest in the year 2015 in the municipality of Tianguá, Ceará. After the harvest, seed selection was carried out, and then the dormancy was broken by manual scarification using a sandpaper number 10.

Initially, the seeds were placed in a styrofoam agricultural grid with 3 × 3 × 3 (cm) cells containing vermiculite as substrate, adding one seed per cell and irrigating daily at 80% of the field capacity. After the germination and establishment period (7 days), the most uniform seedlings were transplanted to plastic vases (5L) filled with sand

and comercial earthworm húmus (18.5 gN/Kg:26.2 gP/Kg:18.4 gK/Kg) in a 2:1 ratio, two plants were added per pot.

After transplanting, the seedlings were submitted to 3 treatments: daily watering at 20 (T20), 50 (T50) or 80% (T80) of the field capacity. On each occasion, each vase had its mass recorded so that the water lost by evapotranspiration was replenished daily. The field capacity was calculated based on the total weight of the vase when the substrate was completely filled with water.

For the evaluations of the activities of the antioxidant enzymes, lipid peroxidation (malondialdehyde contents) and organic solutes contents (soluble proteins, soluble carbohydrates, N-aminosoluble and proline), two harvests were performed: at 31 and 38 days after sowing (DAS). The following enzymatic activities were evaluated: SOD, APX, and GPX.

The leaf area was measured with a scanner type meter (model AM350, ADC “BioScientific” Ltd.). For the determination of dry matter, the plants were separated into roots, stems and leaves, then placed in an oven with forced circulation of air at 60 °C until a constant mass was obtained. Each sample was then weighed using an analytical balance.

In a mortar, 1 g of fresh leaves (FM) was macerated in liquid nitrogen to obtain a powder. Then, 4.0 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA was added. For determinations of APX activities, 2 mM ascorbate was added to the extraction buffer. The macerate sample was filtered on fine mesh nylon fabric and centrifuged at $12,000 \times g$ for 15 min.

The activities of APX, GPX and SOD enzymes were determined spectrophotometrically. The activity of the APX by the method of Nakano and Asada (1981), as the oxidation of ascorbate measured by the decrease in absorbance at 290 nm. The GPX activity by the method of Kar and Mishra (1976), being the reaction accompanied by the increase of the absorbance at 470 nm due to the formation of tetraguaiacol. Finally, SOD activity was determined by the method of Beauchamp and Fridovich (1971), the reaction being measured by the increasing absorbance at 560 nm, due to the production of blue formazan from the photoreduction of p-nitrobluetetrazolium (NBT). The activities of APX and GPX enzymes were expressed in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}$ FM, and that of SOD in UA g^{-1} FM, where: UA (unit of enzyme activity) was defined as the amount of enzyme required to cause 50% inhibition of the photoreduction of NBT; and FM represents fresh matter. Each extract was tested in duplicate.

Lipid peroxidation was estimated by quantification of the malondialdehyde (MDA) contents produced by reaction with thiobarbituric acid according to the method of Buege & Aust (1978). The MDA content was expressed in nmol MDA g^{-1} FM using the extinction coefficient $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$.

The organic solute contents were also determined: soluble carbohydrate content occurred according to Dubois et al. (1956); N-aminosoluble compounds, by the method of Yemm and Cocking (1955); proline, according to Bates et al. (1973); and soluble proteins, according to the method described by Bradford (1976). The contents of the organic solutes were expressed in $\mu\text{mol g}^{-1}$ FM except for those of proteins that were expressed in mg Protein g^{-1} FM. Each extract was tested in duplicate.

The experimental design was a completely randomized, with three treatments: irrigation at 20, 50 and 80% of the field capacity, with 10 replications, each consisting of a bucket with two plants. The data were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test ($P \leq 0.05$) through the statistical software Assisat 7.7, and the graphs plotted using Sigma Plot 11.0 software.

3. Results

Table 1 shows the growth variables analyzed in mulungu plants irrigated at 20, 50 and 80% of the field capacity (T20, T50 and T80). The highest values were found in the T50, while the lowest were in the T20. T80 presented intermediate values.

Table 1. Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM) and leaf area (LA) of mulungu plants under different field capacities at 31 and 38 days after sowing. Different letters in the same column indicate significant differences in relation to the type of irrigation 20, 50 and 80% of the field capacity (T20, T50 or T80) according to Tukey's test ($P \leq 0.05$). Statistical analyses were performed independently for each harvest.

Treatment	SDM (g plant ⁻¹)		RDM (g plant ⁻¹)		TDM (g plant ⁻¹)		LA (cm ² plant ⁻¹)	
	31DAS	38 DAS	31 DAS	38 DAS	31 DAS	38 DAS	31 DAS	38 DAS
T20	1.25c	2.15c	0.18b	0.29c	1.4c	2.33c	233.22c	327.28c
T50	2.5ba	4.93a	0.34a	0.93a	2.74a	5.82a	471.3a	789.93a
T80	1.72b	2.52b	0.34a	0.55b	2.22b	3.39b	376.6b	401.5b

At 31 days after sowing (DAS), no significant differences were detected between treatments for SOD activity (Figure 1A). At 38 DAS, lower SOD activity was observed in the irrigated treatment at 50% of the field capacity (T50). In addition, treatments with 20% (T20) and 80% (T80) of the field capacity showed a slight increase in SOD activity that differed significantly from the 50% treatment.

Futhermore, at the same 31 DAS, the T50 presented higher APX activity (Figure 1B) in relation to the other treatments, being 178% higher than the T20. At 38 DAS, T50 presented APX activity lower than T80, however, it did not differ from T20.

GPX activities (Figure 1C) were higher in the T50 and T80 treatments at 31 and 38 DAS. On average, these treatments were 41 and 34% higher than T20 at 31 and 38 DAS, respectively.

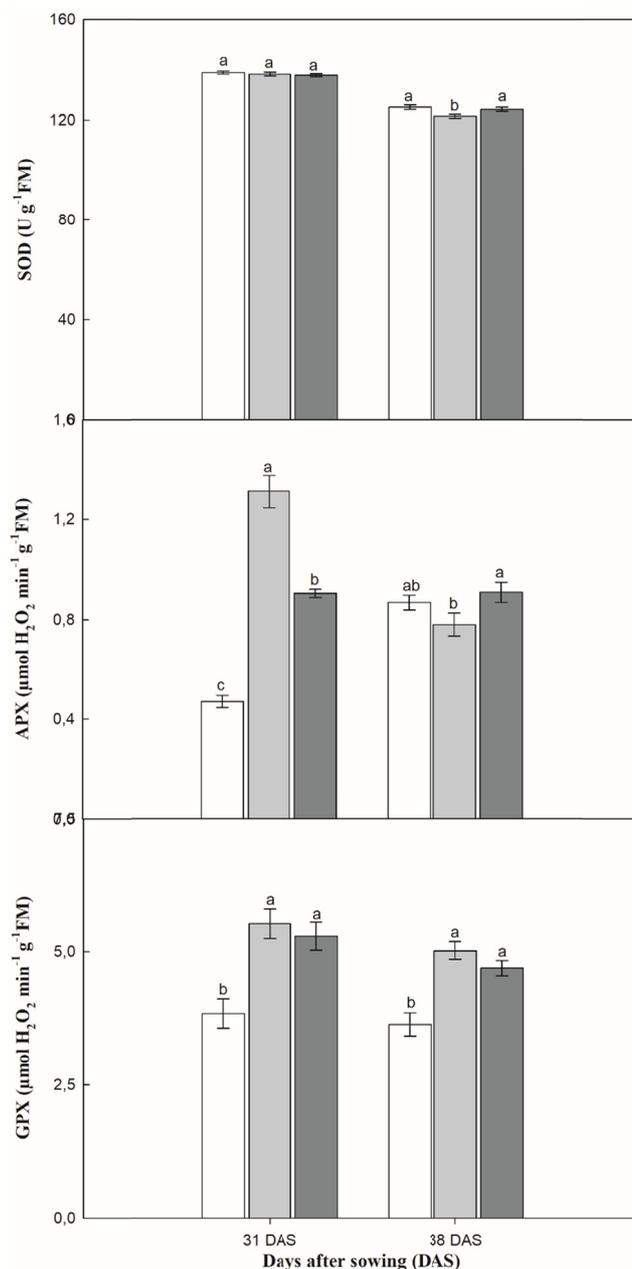


Figure 1. Activity of enzymes: superoxide dismutase-SOD (A), ascorbate peroxidase-APX (B) and guaiacol peroxidase-GPX (C) in leaves of mulungu plants at 31 and 38 days after sowing under 3 conditions irrigation: 20% of field capacity (FC) (white bars), 50% of FC (light gray bars) and 80% of FC (dark gray bars). Different letters indicate significant differences in relation to the type of irrigation according to Tukey's test ($P \leq 0.05$). Statistical analyses were performed independently for each harvest

The content of malondialdehyde (MDA), a subproduct of membrane lipid peroxidation, was evaluated as an indicator of oxidative and environmental damage (Figure 2). It was observed that T80 presented higher levels than the other treatments. At 38 DAS, the T80 presented increases of 66 and 214% in relation to T20 and T50, respectively.

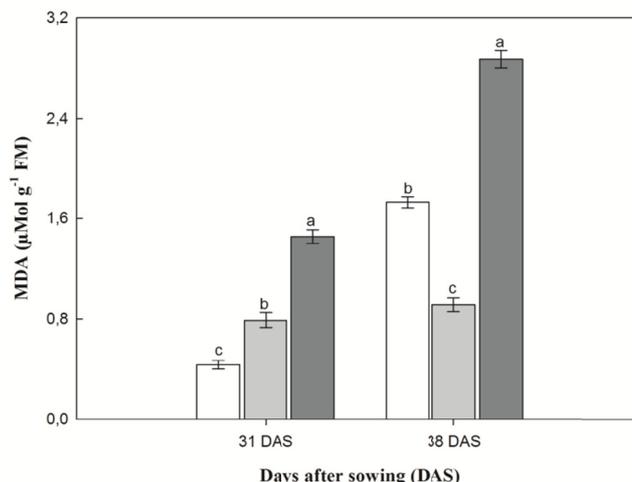


Figure 2. MDA contents in leaves of mulungu plants at 31 and 38 days after sowing under 3 irrigation conditions: at 20% field capacity (FC) (white bars), 50% FC And 80% of FC (dark gray bars). Different letters indicate significant differences in relation to the type of irrigation according to Tukey’s test ($P \leq 0.05$). Statistical analyses were performed independently for each harvest

At 31 DAS, the soluble protein contents (Figure 3A) were 47 and 34% higher in the T50 than in the T20 and T80, respectively, which differed from each other. At 38 DAS, T50 and T80 did not differ from each other and were 31% higher than T20.

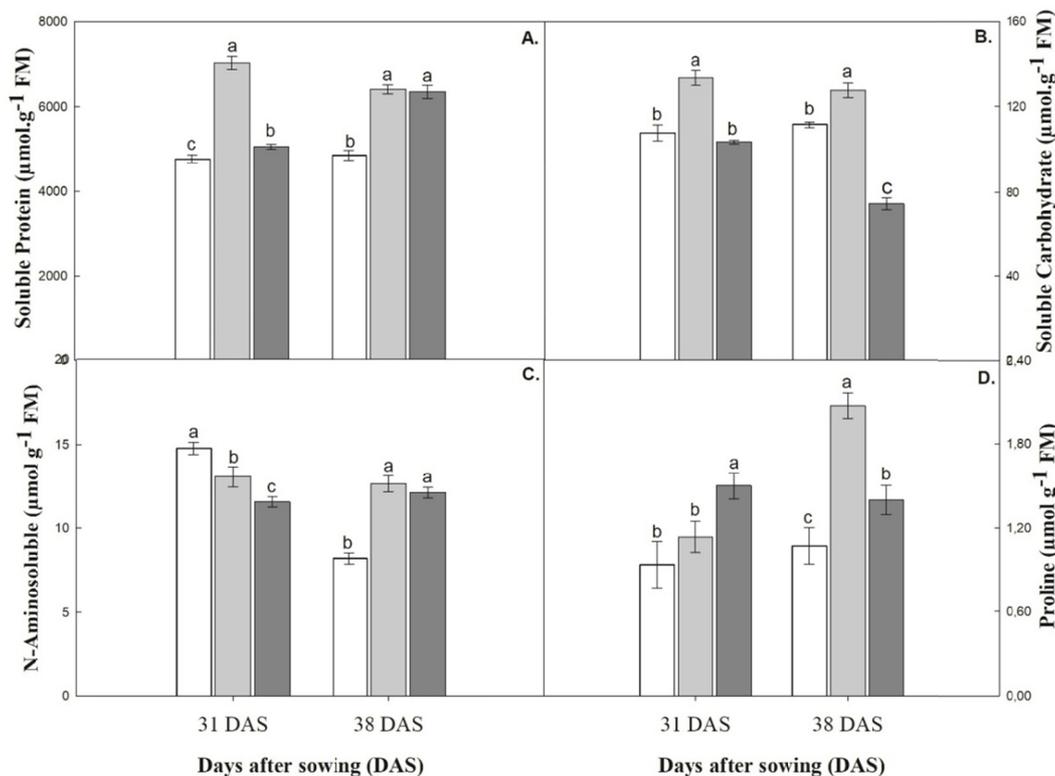


Figure 3. Soluble protein (A), soluble carbohydrate (B), N-aminosoluble (C) and Proline (D) contents of mulungu leaves at 31 and 38 days after sowing under 3 irrigation conditions: at 20% field capacity (FC) (white bars), 50% FC (light gray bars) and 80% FC (dark gray bars). Different letters indicate significant differences in relation to the type of irrigation according to Tukey’s test ($P \leq 0.05$). Statistical analyses were performed independently for each harvest

For the soluble carbohydrate contents (Figure 3B), higher values were observed in T50 during the periods of 31 and 38 DAS. T80 presented the lowest values, mainly at 38 DAS.

At 31 DAS, the lowest levels of N-aminosoluble (Figure 3C) were detected in T80. At 38 DAS, the highest values were observed in T50 and T80, which were on average 50% higher than T20.

Still at 31 DAS, the levels of proline (Figure 3D) were higher in T80, presenting respective significant increases of 91 and 59% in relation to T20 and T80, respectively. However, at 38 DAS, the highest values were observed in T50.

4. Discussion

The irrigations at T20 and T80 caused reductions in plant growth due to a lack of water and an excess of water, respectively (Table 1). The T80 conditions may have caused hypoxia, which may reduce the absorption capacity of water and nutrients (Taiz & Zeiger, 2009). Moreover, some authors report that hypoxia can reduce the uptake of nutrients in roots due to the limited available energy (Bailey-Serres & Voesenek, 2008; Wegner, 2010; Marschner, 2011).

In general, treatments T50 and T80 showed higher activities of the antioxidative enzymes at 31 and 38 DAS compared to T20 (Figure 1). In contrast to the present study, other authors observed increases in the activities of antioxidative enzymes in plants under conditions of water deficit (Abedi et al., 2010; Gunes et al., 2008; Yang et al., 2009). The increase in the activity of these enzymes is important, since it could act as a defense mechanism of the plant in response to stress situations, such as water deficit or hypoxic conditions as a result of excess water (as may have occurred in T80). However, even with reduced APX and GPX activities, especially at 31 DAS, T20 plants were able to withstand the water scarcity and low growth (Table 1), as well as membrane lipid peroxidation (Figure 2). It should be noted that the mulungu plants are found in the caatinga biome, consequently, they are adapted to the low water availability in the soil rather than excess water, which can result in hypoxia-inducing conditions in the root medium.

In the present work, an increase in GPX activity in the T50 and T80 plants compared to the T20 plants was observed. Bin et al. (2010), observed increases in SOD, APX and catalase activities in genotypes of flood tolerant maize plants. Similarly, Yin et al. (2010), in *Dendranthema* spp., also observed increases in the activities of antioxidative enzymes. However, Hossain et al. (2009) demonstrated that SOD and catalase activities increased rapidly in tolerant organisms, whereas APX showed a delayed response to the same hypoxic condition.

Ribeiro et al. (2014) observed inhibition of APX and GPX activities in mulungu seeds with decreasing osmotic potential. This behavior may have been observed because APX and GPX act as a secondary mechanism in the elimination of ROS when compared to catalase. Though, in the present work, it was not possible to detect catalase activity in mulungu seedlings by the methodologies employed.

Malondialdehyde is the first product of membrane lipid peroxidation and is considered one of the key indicators of oxidative damage (Bai et al., 2010). In the present study, even with moderately high enzymatic activities when compared to T20, high lipid peroxidation was detected in T80 (Figure 2). Thus, it is suggested that the irrigation at T80 was excessive for the mulungu plants, which are not well adapted to these conditions. The same behavior was observed in other studies in plants subjected to flooding (Bin et al., 2010; Zheng et al., 2009; Hossain et al., 2009).

Additionally, at 38 DAS, high levels of MDA were also observed in T20. Other studies also detected an increase in lipid peroxidation in plants under water stress conditions. For example, Huang et al. (2013) in hybrid ramie (*Boehmeria nivea* L.) and Deeba et al. (2012) in cotton (*Gossypium herbaceum* L.).

Thus, it was found that the conditions of deficit (T20) or excess water (T80) caused oxidative damages with consequent peroxidation of membrane lipids. Furthermore, the T50 irrigation was found to be the most suitable.

In some studies involving salinity and water stress, reductions were observed in soluble protein contents (Gondim et al., 2011; de Oliveira et al., 2013; Vardharajula et al., 2011). This behavior was observed in T20.

Under hypoxia-inducing as may have occurred in T80 plants, the synthesis of many proteins are suppressed, whereas the synthesis of a specific group of proteins increases. Important metabolic routes include pyruvate as a substrate to produce lactate through lactate dehydrogenase (LDH), and a second route produces ethanol through two subsequent reactions catalyzed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Shingaki-Wells et al., 2011; Borella et al., 2013). Therefore, the degradation of certain proteins occurs in parallel with the production of others to aid in the fermentation process under hypoxic conditions, such as the increased activity of ADH and PDC enzymes (Christianson et al., 2009).

Additionally, since fermentation requires a glucose substrate, or an adequate supply of fermentable sugars, via increased starch degradation or by using storage carbohydrates as single sugars, the process may be an important mechanism for surviving flood conditions (Peña-Fronteiras et al., 2008), which could explain the resulting reduction of carbohydrates in T80 plants.

One of the mechanisms to support plants with a water deficit is the accumulation of osmotically active organic solutes in the cellular cytosol, such as proline and soluble carbohydrates. Thus, the leaf water potential decreases and increases the water absorption of the plants and attenuate the deleterious effects of water scarcity (Mohammadkhani & Heidari, 2008; Szabados & Saviouré, 2010; Abid et al., 2017). However, no changes of these variables were observed in mulungu T20 plants, while the T50 plants presented higher concentrations, which may indicate an attempt to increase water absorption. Similarly, Maia et al. (2007), observed this behavior in maize cultivars under water stress.

In relation to the accumulation of proline, Pirzad et al. (2011), worked with *Matricaria chamomilla* under different irrigation levels, the authors noticed that proline levels were not affected by the irrigation regimes. They inferred that proline may act as a regulatory or signaling molecule in order to activate multiple responses as part of the adaptation process. Moreover, the function of proline in the fight against water stress may simply be a general response of the plant to stress.

Similar to the result found by Ribeiro et al. (2014), proline contents remained constant or had a slight increase with a decrease in osmotic potential, however, with potentials lower than -0,2MPa there was a reduction of proline relative to its initial contents. It is inferred that in mulungu plants, which are from a dry region, proline production seems to be associated with this situation to a certain extent as a form of plant osmoprotective mechanism (Kishor et al., 2005).

5. Conclusions

E. velutina plants presented higher plant growth variables (dry matter yield and leaf area) when submitted to daily irrigation of 50% of the field capacity.

Irrigation at 20% of the field capacity caused a small water deficit. Though, 80% watering may have caused excess water, due to the fact it triggered reductions in plant growth.

In general, despite the reduction in growth found in T20 plants, the activities of the antioxidative enzymes did not differ substantially between treatments T20, T50 and T80, except for GPX.

In general, the lowest organic solutes contents were detected in T20 or T80.

Plants irrigated at 80% of the field capacity may have been subjected to hypoxia in the roots, as demonstrated by the damage due to the increase in lipid peroxidation. Reduced carbohydrate levels corroborate the hypothesis of hypoxia, possibly due to degradation as a response to supply the energy requirements in the plants.

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