

Effects of Photomixotrophic Conditions on Plants of *Eucalyptus Urograndis* Propagated in Temporary Immersion Bioreactors

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Abstract— *Eucalyptus* is one of the crop, which has been investigated with commercial purposes in the world. There are more than 500 species, being the *Eucalyptus urograndis* one of the most important, because of its intensive use in the production of wood pulp to make papers. The multiplication by means of temporary immersion bioreactors (TIB) is among the present techniques to obtain higher productions of the pulp to satisfy the demands of the market. The effects of the photomixotrophic crop were studied during the elongation of the propagation of shoots in the TIB in order to increase the quality of them. This process consisted of the use of 30 g L⁻¹ combined with two concentration of CO₂ (350 and 1200 μmol mol) and two flows of photosynthetic photon flux (PPF= 80 and 250 μmol m⁻²s⁻¹). The higher percentage of suitable plants were found with the treatment of high PPF (250 μmol m⁻² s⁻¹) and 1 200 μmol mol of CO₂. The photosynthetic capacity of propagated shoots was of 64% of the adult plants. Also was observed that photomixotrophic conditions reduced the stressed environment that is imposed by the growing in vitro. The catabolic activity in the enzymes of the metabolism of carbon was also reduced, increasing the activity of the Sucrose Phosphate Synthase.

Keywords— *Eucalyptus*, micropropagation, photomixotrophism, vitroplants.

I. INTRODUCTION

According to predictions, the world population will increase annually in more than 80 million of people and it is estimated that in 2050 it will be about 7 000 millions of inhabitants, because of this, it is believed that the use of wood for building and for the paper industry will increase constantly. Whereas the global demand of wood products increase, the wood resources available in the world are decreasing dizzily due to the lack of knowledge and the

inappropriate use of them. It has permitted the destruction and the degradation of the forests in the tropics [1].

The *Eucalyptus* specie is one of the most investigated with commercial purposes in the world. This species is characterized by its easy management in tree nursery, its fast growth, its erectile shape and the properties of its wood, which is used for numerous applications as: the production of wood pulp for making papers, charcoal and also for construction. *Eucalyptus* is another specie of great commercial in forest because of its extensive use in the production wood pulp for making paper [2]

The vegetative propagation of some species of *Eucalyptus* by means of rooting presents some limitations as the change of the rooting ability among and the gradual decrease of the potentiality of rooting associated with the ontogenetic aging of trees. A method that is exploited in several clonal propagation programs. It has also been promoted the use of the micropropagation as a method to produce clones very fast [3, 4, 5].

The techniques of tissues culture have been used for propagating the clones with superior characteristics. More than 50 *Eucalyptus* species have been propagated using the techniques of tissue culture, most of them using semi-solid means [6]. Micropropagation of *Eucalyptus* clones is more expensive than the techniques of macropropagation using the shoot rooting. The operations that increase cost correspond to the manual subcultivation because of the repeated labor, which are carried out to obtain high rate of multiplications and the rooting of plantlets. These limitations can be solved with automatization of some of the phases of the organogenesis and the somatic embryogenesis to reduce labor and the cost of production [7, 8]. Starting from the first semi-automatized system of temporal immersion (e.g. RITA) some investigations about the use of this techniques in the proliferation of some crops have been carried [9, 10, 11, 12].

The acclimatization *in vitro* is the term used when carrying out photomixotrophism and autotrophism works in plants cultivated under *in vitro* conditions [13]. These works haven't been studied in depth in the cultivation of *Eucalyptus urograndis* when temporal immersion bioreactors are utilized. That's why the objective of this investigation is to establish the photomixotrophic conditions which increase the quality of *Eucalyptus urograndis* explants propagated in TIB for the acclimatization of plants under *ex vitro* conditions.

II. MATERIAL AND METHODS

2.1 General procedures:

All the experiments carried out during this investigation were fulfilled in the specialized areas of plant cell and tissue culture laboratory in Bioplant Center of Ciego de Avila University.

In the research was used vitroplants clone of shoots that come from elite trees of *Eucalyptus urograndis* (*E. grandis* Hill *ex* Maiden *x* *E. urophylla*) which were brought from the Biotechnology Center of the Universidad Católica de Oriente, Medellín, Colombia. The donating plants, from which the explants were taken to be inserted *in vitro* conditions, were kept in greenhouses under 80% relative humidity (RH), 25.5 °C temperature and photosynthetic photon flux (PPF) of 350 $\mu\text{mol m}^{-2}\text{s}^{-1}$ as average under natural photoperiods during eight months. The plants were planting with a combinations (1:1, v/v) of zeolite + filter cake (derived from sugarcane bagasse) as substrate. The top of these plants were pruned in several moments. They were applied foliar fertilizer (Bayfolan Forte® produced by Bayer CropScience) each week at a rate of 2.0 ml L⁻¹ in order that each plant takes 100 ml of final solution.

2.2 Establishment and micropropagation of *E. urograndis* *in vitro*:

The methodology proposed by Grattapaglia *et al.* [14] was the one used but with some modifications. The shoots obtained after the pruning were utilized as source of explants for the establishment phase *in vitro*. The sections taken as explants were segments of stalk from one to two centimeters of length, from 1.5 to 3.0 mm of diameter and with one or two preformed shoots after being washed with commercial detergent and rinsed several times with much fresh water, under asepsis conditions they were submerged in bichloride of mercury on 0.05% during ten minutes.

After this; the shoots were washed three times with purified sterile water. The explants were planted in test tubes in liquid medium with zeolite previously sterilized as support.

As basic means; mineral salts and MS vitamins [15], 3.0% of sucrose and 0.1 mg L⁻¹ of BA were utilized. pH

solution was adjusted to 5.8 with 1 N NaOH and after this it was sterilized during 20 minutes, under 121 °C and a pressure of 1.2 kg cm². The test tubes were placed under a photoperiod of 16 hour-light with photosynthetic photon fluxes (PPF) about $\mu\text{mol m}^{-2}\text{s}^{-1}$, measured by means of a sensor of photon fluxes installed in a universal cuvette matched to a portable system of measuring photosynthesis (CIRAS-2) of PP system.

After 30 days, the shoots were subcultivated and multiplied in 250 ml flask in a semisolid MS medium enriched with BA (0.5 mg L⁻¹), L-glutamine (500 mg L⁻¹), MS vitamins, saccharose (3.0%) and solidified with agar (6.5 g L⁻¹) in all the cases; a volume of 25 ml was used. Five subcultivation were carried out, each one with a frequency of three weeks.

2.3 Establishment of the methodology for the propagation in the temporary immersion bioreactors (TIB):

A design of a temporal immersion bioreactor, modified by the Bioplant Center of Ciego de Avila University, Cuba [9], was used. As recipients flasks of Nalgene Company with a capacity of one liter, which were interconnected with couples using silicone hoses, in a flask the medium of liquid cultivation was placed and in the other the *Eucalyptus* explants, additionally each one was connected to an air entrance system coming from a compressor, which was turned on by an automatic programmer to control the frequency and the length of immersion, the light and the gas fluxes. All the entrance or outgoing of air fluxes was sterilized by hydrophobic filters of 0.2 μm , in such a way that each recipient was handled independently without risk of contamination.

The number of explants (13), the time (3 minutes) and the frequency of immersion (every 12 hours) were used during the experiments. The average volume corresponded to 750 ml and 13 explants, each with five shoots, were contaminated. The environment for cultivation consisted in the mineral salt of MS from which the nitrate of ammonium was reduced to 1 237.5 mg L⁻¹ also the organic (ingredients) which included: thiamine-HCl (0.4 mg L⁻¹), mio-inositol (100 mg L⁻¹), pyridoxine HCl (0.5 mg L⁻¹), nicotinic acid (0.5 mg L⁻¹), glycine (2.0 mg L⁻¹), L- glutamine (500 mg L⁻¹), saccharose (30 g L⁻¹), polyvinyl polypirrolidona (PVPP) in concentration of 250 mg L⁻¹ was used as a preventive agent of oxidation, and it was also supplemented with BA (0.5 mg L⁻¹). The medium was sterilized by autoclave (40 minutes, 121 °C), previously the pH was adjusted to 5.8 with Na OH. The cultivations were incubated to 24±1 °C with a photoperiod of 16 hours with artificial light helped supported by fluorescent lamps (PHILIPS TL 40 W/54) which irradiated an intensity of 80 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

2.4 Determination of photomixotrophic effects in elongation phase of *E. Urograndis* shoots propagated in temporary immersion bioreactors:

The experiment consisted of a bifactorial under a design completely random with three repetitions for treatments,

where the factors corresponded to different levels of light intensity and of CO₂ in the phase of elongation in TIB. The photomixotrophic conditions of *E. Urograndis* are shown on table 1.

Table.1: Photomixotrophic conditions in elongation phase of *Eucalyptus Urograndis* shoots propagated in temporary immersion bioreactors (TIB).

| CO ₂ Concentration (μmol mol CO ₂) | Light intensity (μmol m ⁻² s ⁻¹) |
|---|---|
| 350 | 80 |
| | 250 |
| 1200 | 80 |
| | 250 |

A photoperiod of 16 hours light was used during the development of this experiment, the photosynthetic photons fluxes (μmol m⁻²s⁻¹) for the photosynthetic activity were measured with a sensor to measure photosynthesis (CIRAS-2), a portable PP system.

2.5 Photosynthetic, stomatic conductance and transpiration activity of *E. urograndis* plants during photomixotrophic conditions in TIB:

Net photosynthesis (A, μmol (CO₂) m⁻²s⁻¹), stomatic conductance (mmol (H₂O) m⁻²s⁻¹) and transpiration (μmol (H₂O) m⁻²s⁻¹) was measured at the ambient relative humidity and air temperature. The analyzer and cuvette were automatically calibrated before every measurement. Light was fixed at 600 μmol m⁻²s⁻¹ for the determination of the A/Ci curve, while the CO₂ was fixed at 600 μmol mol⁻¹, these values were obtained from saturation curve of light and CO₂ in *Eucalyptus* plants.

Measurement were always made on the youngest fully expanded leaves and between 9:00 am and 10:00 am. Five plants were measured with 10 repetitions each one for a total of 50 values.

2.6 Measurements of enzyme activity in *E. urograndis* plants during photomixotrophic conditions in TIB:

Enzyme extraction and assays. Leaf samples were taken before 10:00 am and immediately frozen in liquid N₂ and stored at -80 °C until use. A 250 mg frozen tissue was ground to a very fine powder with a mortar and pestle in liquid N₂. The sucrose phosphate synthase (SPS) enzyme (EC 2.4.1.14) activity was determined to pH 7.5 with 50 μL from the unsalted extract. The mixing was incubated for 20 minutes in 30 °C. The reaction stopped with the addition of 70 μL (5.35 mol L⁻¹) of KOH. For the white the reaction stopped with the addition of 70 μL (5.35 mol L⁻¹) of KOH in 0 minutes [16]. The formation of sucrose was determined by means of the antrone method [17].

The activity of invertase (EC 3.2.1.26) was determined in a mixing of reaction (reaction mixing) of 500 μL from the total volume that contains enzymes free of salt and 50 mmol L⁻¹ of saccharose, a tampon of acetate of sodium to

pH 4.5 was used. The reaction started with the addition of enzyme, previously incubated during 20 minutes to 30 °C. The formation of hexosa was determined enzymatically [18].

For the case of the phosphoenolpyruvate carboxylase enzymes (PEPC; EC 4.1.1.31) and pyruvate kinase (PK; EC 2.7.1.40) were extracted in 1 ml of tampon 50 mM Hepes-KOH containing 12 mmol L⁻¹ Mg Cl₂, 1 mmol L⁻¹ EGTA, 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ DTT, 10% glycerol, 2 mmol L⁻¹ benzamidine, 2 mmol L⁻¹ amino-n-caproic acid to pH 7.4 [19]. The catalysis of reaction was joined to the reacting of dehydrogenated malate to 25 °C for the decreasing of NADH used to 340nm in a spectrophotometer Pharmacia. The PQ activity was joined to the dehydrogenated lactate to 25 °C by monitoring the utilization of NADH to 340 nm.

2.6 Statistical analysis:

At each sampling date, six plantlets were randomly selected to measure photosynthesis and transpiration. For enzymatic determination, randomized plantlets were used. Three extraction in each evaluation moments and three repetitions for each extraction were performed. Twenty plantlets were used for morphological measuring. Analysis of variance was conducted using SPSS Program. Duncan's multiple range tests were used for mean separation at the p<0.05 level.

III. RESULTS AND DISCUSSION

3.1 Effects of photomixotrophic conditions on growing variables of *E. urograndis* plants during photomixotrophic conditions in TIB.

Light intensity showed the best results with the treatment in which the plants were submitted to a greater light intensity (250 μmol m⁻² s⁻¹), this shows statistical differences in all variables evaluated, with the exception in the dry mass. In the leaf number the plants developed five news leaves, which is in correspondence with the fresh dough found. Meanwhile CO₂ brought about significant differences just on the fresh mass variable,

where the higher values were got in plants submitted to treatments with high concentration of CO₂ (Table 2).

Table.2: Effects of photomixotrophic conditions on growing variables of *E. urograndis* plants during photomixotrophic conditions in TIB.

| Factors | | Length (cm) | Leaf number | Mass (g) | |
|--|-------------|-------------|-------------|----------|--------|
| | | | | fresh | Dry |
| I - Light ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | 80 | 2.14 b | 11.17 b | 0.13 b | 0.0189 |
| | 250 | 2.86 a | 17.02 a | 0.21 a | 0.0184 |
| | Sign. | * | * | * | ns |
| | SE | 0.13 | 1.22 | 0.023 | 0.0018 |
| II - CO₂ ($\mu\text{mol mol}$) | 350 | 2.42 | 13.56 | 0.12 b | 0.0186 |
| | 1 200 | 2.58 | 15.14 | 0.22 a | 0.0188 |
| | Sign. | ns | ns | * | ns |
| | SE | 0.13 | 1.22 | 0.023 | 0.0018 |
| Interaccion I - II | 80 + 350 | 2.34 bc | 11.24 b | 0.14 b | 0.0197 |
| | 80 + 1 200 | 1.93 c | 12.16 b | 0.13 b | 0.0182 |
| | 250 + 350 | 2.49 b | 19.04 a | 0.11 b | 0.0174 |
| | 250 + 1 200 | 3.23 a | 14.96 ab | 0.32 a | 0.0194 |
| | Sign. | * | * | * | ns |
| | SE | 0.18 | 1.72 | 0.033 | 0.0025 |

Measures with different letters differ statistically (one-way ANOVA, Tuckey, $p < 0.05$). Each data represents the mean for $n=30$.

Effects of the interactions of the two factors showed that plants which were submitted to a higher light intensity and enriched environment with CO₂, highest values are obtained in the studied variables of plant quality, with the exception the dry mass and the number of leaves, which shows significant differences among the other interactions. We should point out that treatments submitted to the same conditions were the ones which obtained the greater percentage of optimal size plants, motivated too for the BIT ability to aerate plant tissue and provide contact between entire explants and the liquid medium.

The shoot length, the number of new nodal segments for explant and the multiplication coefficient were significantly higher in *Eucalyptus tereticornis* under photomixotrophic conditions than in photoautotrophic condition [20]. High significant differences too were found in fresh mass and net photosynthetic rate in *E. camaldulensis* plants when they were submitted to forced ventilation [21].

Growth of plantlets *in vitro* is often greater under photomixotrophic conditions than under heterotrophic

conditions, provided that the *in vitro* environment is properly controlled for promoting photosynthesis. In this case and in some others, it is speculated about the metabolic routes favored under the conditions established for cultivation, this information is too important for better interpretation of the results.

Some variables related to the photosynthetic activity, measures during the photomixotrophic treatments when finishing the phase of elongation of the *E. Urograndis* shoots propagated in temporary immersion bioreactors should be evaluated in these experiments.

3.2 Stomatic conductance, transpiration and photosynthetic activity of *E. urograndis* plants during photomixotrophic conditions in TIB:

E. urograndis plants showed a high stomatal conductance measured in terms of water flow. As the stomatal conductance is equivalent to the permeability and inverse to the resistance of water fluxes, it is inferred that transpiration in these leaves had to be proportional, however this reasoning is not always materialized (Table 3).

Table.3: Values of transpiration, stomatal conductance and net photosynthesis in *E. urograndis* shoots propagated under photomixotrophic conditions in TIB.

| Factors | Transpiration ($\text{mmol (H}_2\text{O) m}^{-2}\text{s}^{-1}$) | Stomatic Conductance ($\text{mmol (H}_2\text{O) m}^{-2}\text{s}^{-1}$) | Photosynthesis ($\mu\text{mol (CO}_2\text{) m}^{-2}\text{s}^{-1}$) |
|---------|--|--|---|
|---------|--|--|---|

| | | | | |
|---|-----------|--------|-----------|--------|
| I - Light ($\mu\text{mol m}^{-2}\text{s}^{-1}$) | 80 | 6.34 | 4067.32 a | 4.34 a |
| | 250 | 6.37 | 2633.33 b | 0.00 b |
| | Sign. | ns | * | * |
| | SE | 0.043 | 115,40 | 0.20 |
| II - CO₂ ($\mu\text{mol mol}$) | 350 | 6.78 a | 4261.63 a | 3.26 a |
| | 1 200 | 5.90 b | 2439.02 b | 1.12 b |
| | Sign. | * | * | * |
| | SE | 0.043 | 115,40 | 0.20 |
| Interaction I - II | 80+350 | 6.51 b | 4960.63 a | 6.52 a |
| | 80+1 200 | 6.17 c | 3174.00 b | 2.25 b |
| | 250+350 | 7.05 a | 3562.63 b | 0.00 c |
| | 250+1 200 | 5.63 d | 1704.03 c | 0.00 c |
| | Sign. | * | * | * |
| | SE | 0.06 | 163,20 | 0.28 |

Means with different letters differ statistically (one-way ANOVA, Tuckey, $p < 0.05$). Each data represents the means for $n=30$.

When analyzing the effect of light, the first variation of this postulate is found. The plants submitted to a higher PPF reduce significantly the stomatal conductance but it doesn't modify the transpiration. As it is known a general behavior of replying of the stoma to the increasing of light intensity, turns out in a greater number of open stomas and in the increasing of transpiration.

In the case of concentration of CO₂, the behavior of the *E. Urograndis* shoots cultivated in TIB was similar to the most general tendency that plants flow. When air was enriched with CO₂, plants reduced their stomatal conductance associated to a reduction in the quantity of open stomas, and the transpiration decreased significantly. It is known that the effects of CO₂ are very powerful over the opening of stomas, the increase of its concentration in occlusive cell cause a partial closure of stomas and it can inhibit the assimilation of CO₂, just partially compensated when the photosynthetic activity increases.

In the integration of the effects of both factors, the concentration of factors, the concentration of CO₂ in the environment where the shoots were cultivated, had greater influences when comparing each level of light with the two levels of CO₂ the stomatal conductance was reduced and the transpiration too. However in the comparison of each concentration of CO₂ with the two levels of PPF evaluated, it is reiterated that the increase of light reduced the stomatal conductance, but transpiration of plants cultivated with 350 μmol (CO₂) mol of air increased.

The regulation of the opening of stomas is a complex process which does not depend only on the factors previously analyzed. Others as temperature, relative humidity, cytosolic concentration of calcium, hormones,

and enzymes which indicates the related metabolic routes, they also exert important influences and development. Some of these measurements should be considered in future investigations.

The effects of CO₂ over stomatal opening is adjusted in function of the demand of photosynthesis in plants. The measurement of this relative indicator demonstrated that in low levels of CO₂ and light the greater quantity of fixed CO₂ are registered.

The addition of sugar in the environment of cultivation showed a negative effect in the growing and photosynthesis in plants *in vitro* [22]. Besides the saccharose provoked the stimulations of the growth and the photosynthesis activity of tobacco plants *in vitro* [23] also in beet plants [24] and in potato plants [25]. These results indicate that the photosynthesis capacity depend mainly on the species and the environmental conditions and of cultivation that plants are submitted.

The integral analysis of the results showed on table 4 are not enough to justify the effects of treatments about the quality of plants cultivated in TIB, because the behavior of the physiological indicators is insufficient when they depend on some other factors, among them the metabolic routes.

3.23 Activity of enzymes of the carbon metabolism in the *E. urograndis* shoots propagated under photomixotrophic conditions in TIB.

In order to specify the results of the treatments under photomixotrophic conditions, in table 4 is shown the activity of important enzymes involved in the metabolism of carbon, measured in the *E. urograndis* shoots once the cultivation *in vitro* phase is finished in the TIB.

The enzymes of the catabolic routes of the metabolism of carbon were much more active than the metabolic means.

The activity of the acid invertase and pyruvate kinase surpass several times the saccharose phosphate synthase

and the phosphoenolpyruvate carboxylase (table 4).

Table.4: Activity of enzymes of the carbon metabolism, sucrose phosphate synthase (SPS) acid invertase (AI), pyruvate kinase (PK) and phosphoenolpyruvate carboxylase (PEPC) in *E. urograndis* shoots propagated under photomixotrophic conditions in TIB.

| Factors | | SPS ($\mu\text{mol g}^{-1} \text{FM h}^{-1}$) | AI ($\mu\text{mol g}^{-1} \text{FM h}^{-1}$) | PK ($\mu\text{mol g}^{-1} \text{FM h}^{-1}$) | PEPC ($\mu\text{mol g}^{-1} \text{FM h}^{-1}$) |
|--|-----------|--|---|---|---|
| I - Light ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | 80 | 7.04 | 24.34 a | 51.16 b | - |
| | 250 | 5.95 | 15.54 b | 223.48 a | - |
| | Sign. | ns | * | * | |
| | SE | 0.77 | 0.84 | 24.38 | |
| II - CO₂ ($\mu\text{mol mol}$) | 350 | 5.72 | 10.43 b | 112.90 | - |
| | 1 200 | 7.27 | 29.46 a | 161.74 | - |
| | Sign. | ns | * | ns | |
| | SE | 0.77 | 0.84 | 24.38 | |
| Interaction I - II | 80+350 | 8.22 a | 0.17 d | 31.63 c | 10.18 |
| | 80+1 200 | 5.86 ab | 48.52 a | 70.68 bc | 0.00 |
| | 250+350 | 3.21 b | 20.69 b | 194.16 ab | 0.00 |
| | 250+1 200 | 8.68 a | 10.40 c | 252.81 a | 0.00 |
| | Sign. | * | * | * | |
| | SE | 1.09 | 1.18 | 34.48 | |

Means with different letters are statistical different (one-way ANOVA, test TUCKEY, $p < 0.05$). Each data represents the means for $n=9$.

The PK registered the greatest activity in all the treatments with significant differences marked by the light factor. As it was previously analyzed (table 3) the increasing of light reduced the stomatal conductance, apparently the resistance of the flow of water increased and although it could be one thousand superiors to the entrance of CO₂, the closing of stomas should have reduced the capture of it. Nevertheless, the entrance of oxygen, necessary for the oxidized process, specifically the respiratory ones. The relevant activity of PK is an evidence that these were the favored process.

Anyhow the activity of AI is also big, it is supposed that they degraded the saccharose from the environment of cultivation (in all cases to 30 g L⁻¹) and the resulting hexoses were degraded in the glycolytic means which marks the catalytic action of PK. Probably the high concentrations of saccharose limited the photosynthetic fixation of CO₂ as it has been shown by [26, 27], because they were sufficient to guarantee the metabolic demands which improved the quality of plants propagated under a greater flux of photosynthetic photons (PPF).

Comparing the enzyme behavior of plants propagated in the treatments with greater intensity of light, which were the ones that obtained better plants, differences between IA and SFS were observed. The treatment with best results in the production of competent plants, those which

were elongated with greater levels of light and CO₂ in the air, registered lower activity of AI and higher activity of SPS. This shows that absorbed fewer saccharose from the means of cultivation and that synthesized more, favored by higher concentration of CO₂ in the atmosphere.

Activities of SPS and AI were increased much more in tomato plants cultivated in 3% of saccharose than without saccharose when they were submitted under PPF and lower concentration of CO₂, while it was observed a different behavior in the activity of SPS, when its increase was higher in conditions of high PPF and high concentration of CO₂ and in an environmental mean of cultivation without saccharose [28].

With this analysis, the interpretation of the best results in quality of plants, which were obtained under conditions of photomixotrophic cultivation, is completed. To the intense metabolic activity measured in those shoots (table 4) it is added the best management of the hydraulic related to transpiration (table 3). In *E. urograndis* plants propagated with high PPF and concentration of CO₂, these factors provoke the least stomatal conductance, the lowest loss of water and they obtained the highest values of length and fresh mass. The ones which are elongated with high PPF but with the lowest concentration of CO₂, duplicate the stomatal conductance and they registered the

highest loss of water, which showed the significant reduction of length and fresh mass.

In both treatments with higher light intensity, the null values of PEPC are corresponded with the increased activity of PK, because the two enzymes compete for the phosphoenolpyruvate and the data show that the breathing consumption prevailed because of the PK. Just with the control treatment, with the lowest levels of PPF and with CO₂, PEPC activity was detected in the *E. urograndis* shoots, the lowest activities of PK were also measured in them.

Finally, the increase of concentration of CO₂ with the lowest PPF resulted the worst combination to stimulate the elongation of the *E. urograndis* shoots in the TIB, because the percentage of plants non competent was increased to more than 20%. They were distinguished for the higher activity of the acid invertase in the catabolic process already analyzed, which together to the action of PQ, resulted determinant over the anabolic activity represented by SFS.

The interpretation of these results is complex, as it is the action of each enzyme evaluated. They go from the optimal levels enzymatic complexes to other particularities as the regulation of SPS by the CO₂ and the temperature [29] mediating other functions as the Rubisco activity and the nitrogenous metabolism, as the important relation between PEPC and the malic enzymes, the presence of isoforms in both, the regulation by light and temperature [30] as the integrated behavior for Rubisco, SPS, SS, AIC, PEPC associated to a fixation of CO₂, hormones and photoperiod [31]

Although some of the factor previously quoted were not included in this study, the results show the effects of the growth regulators in the multiplication and the photomixotrophic treatments in the elongation of *E. urograndis* shoots in TIB. The combination of high levels of PPF and of CO₂ in the air favored the quality of the propagated plants, in biochemical level approximations were obtained to reasons that justify these answers, based on the metabolism of carbon.

Nevertheless the treatments applied also influenced in the concentration of phytohormons and in its regulation [32] which are integrated to the system of signals that should have existed in this species in reply to the action of the regulators of growing, concentration of CO₂ in the air and levels of PPF, which include changes in the metabolism [33].

IV. CONCLUSION

In conclusion, the results of the present study indicated that during the photomixotrophic cultivation the light improved the quality of *E. urograndis* shoots, and that the saccharose limited the photosynthetic assimilation of CO₂

while the high PPF and concentration of CO₂ provoked less stomatal conductance, lower-loss of water and higher values of length and fresh mass. Nevertheless the catabolic activity was intense, but the combination of high levels of light and CO₂ reduced the activity of AI and increased the activity of SPS.

REFERENCES

- [1] ABRAF (Brazilian Association of Forest Plantation Producers), Yearbook statistical of ABRAF 2013, ABRAF, Brasília, 150p, (2013).
- [2] Anguenot, R. (2003). Protocolos, sucrose synthase. ([http://www.agrobiotheque.ca/Protocolos/enzymo/sucrose synthase.html](http://www.agrobiotheque.ca/Protocolos/enzymo/sucrose%20synthase.html)), accessed December 2007.
- [3] Arya, I.D., Sharma, S., Chauhan, S., and Arya, S. (2009). Micropropagation of superior eucalyptus hybrids FRI-5 (*Eucalyptus camaldulensis* Dehn x *E. tereticornis* Sm) and FRI-14 (*Eucalyptus torelliana* F.V. Muell x *E. citriodora* Hook): a commercial multiplication and field evaluation. African Journal of Biotechnology 8: 5718–5726.
- [4] Casati, P., Lara, M.V., and Andreo, C.S. (2000). Induction of a C₄-like mechanism of CO₂ fixation in *Egenia densa*, a submersed aquatic species. Plant Physiol, 123(4): 1511-1622.
- [5] Castro. D., and González-Olmedo, J. (2002). Micropropagación de eucalipto (*Eucalyptus grandis* hill ex maiden) en sistema de inmersión temporal. Agricultura Técnica 62:68–78.
- [6] Cournac, L., Dimon, B., Carrier, P., Lohou, A., and Chagvardieff, P. (1991). Growth and photosynthetic characteristics of *Solanum tuberosum* plantlets cultivated in vitro in different conditions of aeration, sucrose supply, and CO₂ enrichment. Plant Physiol. 97: 112-117.
- [7] Dutra, L.F., Wendling, I., and Brondani, G.E.A. (2009). Micropropagação de eucalipto. Pesquisa Florestal Brasileira, n. 58, p. 49-59.
- [8] Escalona, M., Lorenzo, J.C., Gonzalez, B., Daquinta, M., Gonzalez, J.L., Desjardins, Y., and Borroto, C.G. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Rep., 18: 743-748.
- [9] FAO (Food and Agriculture Organization of the United Nations). (2013). Summary report of the 3rd International Congress on Planted Forests. accessed March 2015, Rome.
- [10] Gonzales, R., Ríos, S., Avilés, F., and Sánchez-Olate, M. (2011). *In vitro* multiplication of *Eucalyptus globulus* by temporary immersion system. Bosque. 32:147-154.

- [11] Grattapaglia, D., and Machado, M.A. (1990). Micropropagacao. En: A. C. Torres; L. S. Caldas (eds). Técnicas e aplicacoes da cultura de tecidos de plantas. Brasilia: ABCTP/EMBRAPA- CNPH. 433 p.
- [12] Hussain, M.W., Allen, L.R., and Borves, G. (1999). Up-regulation of sucrose phosphate synthase in rice grown under elevated CO₂ and temperature. *Photosynthesis Reserch* 60: 1999-208.
- [13] Jova, M.C., Kosky, R.G., Cuellar, E.E., and Cuellar, A.E. (2012). Efficiency of semi- automated culture systems on microtubers formation of yam (*Discorea alata* L.). *Biotechnol. Agron.Soc. Environ.* 16:45-47.
- [14] Kirdmanee, C., Kitaya, C., and Kozai, T. (1995). Effects of CO₂ enrichment and supporting material in vitro on photoautotrophic growth of *Eucalyptus* plantlets *in vitro* and *ex vitro*. *In vitro Cell Develop. Biol. Plant.* 31: 144-149.
- [15] Kotvun, T., and Daie, J. (1995). End-product control of carbon metabolism in culture-grown sugar beet plants. *Plant Physiol.* 108: 1647-1636.
- [16] Li, C.R. Gan, L.J. Xia, K., and Hav, C.S. (2001). Responses of carboxylating enzymes, sucrose metabolizing enzymes and plant hormones in a tropical epiphytic CAM orchid to CO₂ enrichment. *Plant Cell Environ.* 25: 369-377.
- [17] Lorenzo, J.C., Ojeda, E., Espinosa. A., and Borroto, C.G. (2001). Field performance of temporary immersion bioreactor-derived sugarcane plants. *In Vitro Cell Dev. Biol. Plant.*37: 803-806.
- [18] Miron, D., and Schaffer, A.A. (1991). Sucrose phosphate synthase, sucrose synthase and invertase activities in developing fruit of *Lycopersicon hirsutum* Humb and Bonpl. *Plant Physiol.* 27: 643-665.
- [19] Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays whit tobacco tissue cultures. *Plant Physiol.* 15:473-497.
- [20] Nguyen, Q.T., and Kozai, T. (2001). Growth of *in vitro* banana (*Musa* spp.) shoots under photomixotrophic and photoautotrophic conditions. *In Vitro Cell. Dev. Biol Plant* 37: 824-829;
- [21] Pinto, G., Araújo, C., Santos, C., and Neves, L. (2013). Plant regeneration by somatic embryogenesis in *Eucalyptus* spp.: current status and future perspectives. *Southern Forests* 2013, 75(2): 59–69.
- [22] Quiala, E., Cañal, M.J., Meijón, M., Rodríguez, R., Valledor, L., Chávez, M., De Fera, M., and Barbón, R. (2012). Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. *Plant Cell. Tiss. Organ Cult.* 109:223–234. DOI 10.1007/s11240-011-0088-3.
- [23] Rodríguez-Escriba, R.C., Rodríguez, R., López. D., Lorente. G.Y., Pino, Y., Aragón, C.E., Garza, Y., Podestá, F.E., and González-Olmedo, J.L. (2015). High light intensity increases CAM expression in ‘MD-2’ micropropagated pineapple plants at the end of the acclimatization stage. *American Journal of Plant Sciences*, 2015. 6, 3109-3118 <http://dx.doi.org/10.4236/ajps>.
- [24] Serret, M.D., Trillas, M.I., Matas, J., and Araus, J.L. (1996). Development of photoautotrophy and photoinhibition of *gardenia jasminoides* plantlets during micropropagation. *Plant Cell Tiss. Organ Cult.* 45: 1-16.
- [25] Siegel, G., and Stitt, M. (1990). Partial purification of two forms of spinach leaf sucrose-phosphate synthase which differ in their kinetic properties. *Plant Sci.* 66: 205-210
- [26] Sha Valli Khan, P.S. Kozai, T., Nguyen, Q.T., Kubota, C., and Dhawan, V. (2002). Growth and net photosynthetic rates of *Eucalyptus tereticornis* Smith under photomixotrophic and various photoautotrophic micropropagation conditions. *Plant Cell, Tissue and Organ Culture* 71: 141–146.
- [27] Tichá, I., Pacovska, F.D., Hofman, P., Haisel, D., Capkova, V., and Schafer, C. (1998). Culture on sugar medium enhances photosynthetic capacity and light resistance of plantlets grown *in vitro*. *Plant Physiol.* 99: 155-162.
- [28] Van Handel, E. (1968). Direct microdetermination of sucrose. *Anal. Biochem.* 22:280-283.
- [29] Van Quy, L., Samson, V., and Desjardins, Y. (2001). Opposite effects of exogenous sucrose on growth, photosynthesis and carbon metabolism of *in vitro* plantlets of tomato (*L. esculentum* Mill.) grown under two levels of irradiances and CO₂ concentration. *J. Plant Physiol.* 158: 599-605.
- [30] Watt, M.P. (2012). The status of temporary immersion system (TIS) technology for plant micropropagation. *African Journal of Biotechnology*, vol. 11, no. 76, 14025-14035, ISSN 1684-5315, DOI 10.5897/AJB12.1693.
- [31] Ziv, M. (2015). Bioreactor Technology for Plant Micropropagation. *Horticultural Reviews*, Volume 24, Edited by Jules Janick ISBN 0-471-33374-3 © 2000 John Wiley & Sons, Inc. DOI: 10.1002/9780470650776.ch
- [32] Zobayed, S.M.A. (2005). Ventilation and micropropagation. In: Kozai T, Afreen F, Zobayed SMA, editors. Photoautotrophic (sugar-free) medium micropropagation as a new

micropropagation and transplant production system.
Springer. The Netherlands. pp. 147-186.

- [33] Zobayed, S.M.A., Kubota, C., and Kozai, T. (2000).
Mass propagation of *E. camaldulensis* in a scale-up
vessel under *in vitro* photoautotrophic condition.
Annals of Botany, vol. 85, no. 5, 587–592,
<https://doi.org/10.1006/anbo.1999.1106>