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Nitrate (NO_3^-) and ammonium (NH_4^+) ratios for propagation of *Eucalyptus* hybrid in two different *in vitro* cultivation systems

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Abstract

Eucalyptus have been cultivated around the world for timber and pulp. Production of seedlings for cropping is one of the key stages for their cultivation which requires strict control of material nutrition. Plant tissue culture techniques have been a useful tool in understanding factors that affect plant growth and can be used to determine conditions that may improve plant development. *In vitro* studies of specific nutrients such as Nitrogen have been particularly important for identifying productivity gains since this element may affect plant growth. This study was aimed at identifying which are the most effective ratios between nitrate (NO_3^-) and ammonium (NH_4^+) as a nitrogen supply for a commercial hybrid *Eucalyptus grandis x Eucalyptus urophylla* under two *in vitro* cultivation systems (a temporary immersion bioreactor and a semisolid medium). The evaluated ratios of (NO_3^-):(NH_4+) were 3:1, 2:1, 1:1, 1:2 and 1:3, respectively, in order to assess their effects on number of shoots, length of the largest shoot, fresh weight and number of leaves on the largest shoot during *in vitro* cultivation in both systems. These ratios were delivered in a modified MS medium supplied with IAA 5.7 μM and BAP 0.14 μM. According to the assessments, no hyperhydricity symptom was observed in the shoots. However, a callus formation was verified on the basis of explants grown during 30 days in semisolid medium. The 3:1 ratio of (NO_3^-):(NH_4^+) provided the best results for the *E. grandis x E. urophylla* cultivated in both cultivation systems, but greater biomass shoots were obtained in bioreactor as compared to those from semisolid medium.

Keywords: Clonal forestry; *Eucalyptus grandis* x *Eucalyptus urophylla*; hyperhydricity; *in vitro* nutrition; nitrogen; temporary immersion bioreactor.
Abbreviations: ABRAF_Associação Brasileira dos Produtores de Florestas Plantadas; ANOVA_Analysis of variance; BAP_6-benzilaminopurine; CAPES_Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; CNPq_Conselho Nacional de Desenvolvimento Científico e Tecnológico; FAPEMIG_Fundação de Amparo à Pesquisa do Estado de Minas Gerais; IAA_3-indoleacetic acid; MS_Murashige & Skoog; N_nitrogen; NH_4^+_ammonium; NO_3^-_nitrate; TIB_Temporary Immersion Bioreactor; NR_nitrate reductase.

Introduction

Eucalyptus genus belongs to the Myrtaceae family and encompasses more than 700 species throughout the world. It is among the main sources of wood and is widely used for a range of industrial purposes. *Eucalyptus* value is related with its economically desirable characteristics, such as high growth rate, straight trunks, relatively high wood density, fiber quality, wide adaptability to various soils and climates, and high resistance to biotic stress (Oliveira et al., 2012).
In Brazil, *Eucalyptus* crops accounted for about 76.6% of total area (6.6 million hectares) of forest plantations in 2012. An increase on the species' cultivated area is expected with these species in coming years due to their use in various industrial sectors, including that of paper and cellulose production, lumber, mechanically processed wood, and charcoal for the steel industry (ABRAF, 2013). Within the group of eucalyptus species both *Eucalyptus grandis* and *E. urophylla* are worthy of mentioning. *E. grandis* is regarded as having high productivity and good wood features (Barreiros et al., 2007), whereas *E. urophylla* shows wide adaptation capability to different soils and resistance against biotic and abiotic stresses (Pinto et al., 2014). These properties have placed these species and its hybrids among the main ones employed in forest plantations. The increasing demand for products derived from production chain these species has stimulated research on clonal propagation as a tool in order to maximize the supply of quality seedlings

to market at a competitive cost. The main vegetative propagation type currently used in *Eucalyptus* clonal forestry is the minicutting which consists of rooting stem cuttings with sizes ranging from 5 – 8 cm (Xavier and Silva, 2010). However, this method may be inefficient due to low rooting rates caused by inhibitors accumulation by certain species or clones (Andrade et al., 2006). A potential alternative to the minicutting method is plant tissue culture through micro-propagation. This technique has been a valuable tool in the propagation of several species, resulting in rapid genetic gains and increased productivity of higher plants in a shorter period (Aggarwal et al., 2012; Pereira and Fortes, 2003). Despite being useful this technique has not been extensively used for commercial seedling production because of chemical costs and skilled labor demand. Recent advances in *in vitro* cultivation using temporary immersion bioreactors (TIB) have the potential to reduce production costs by allowing greater biomass gain for shoots and reducing the period required for propagation (Dutra et al., 2009). Biomass gains are possible because of higher surface contact of shoots with the culture medium into TIB leading to a better nutrient absorption (Pereira and Fortes, 2003). Moreover, this technology is fully automated, hence decreasing the requirements (and costs) for skilled labor (Oliveira et al., 2011a). Besides, some studies have already demonstrated advantages of using bioreactor systems to *Eucalyptus* cultivation (Castro and González, 2002; Hajari et al., 2006; McAlister et al., 2005; Oliveira et al., 2011b).

In addition to the cultivation system, the optimization of culture medium is important since the source and concentration of minerals supplied to explants will directly influence on plant growth rates and development. Nitrogen (N) is one of the major essential nutrients (Villa et al., 2009) present in culture medium. It is essential for biosynthesis of many compounds, such as amino acids, enzymes, proteins and pigments, all involved in a wide amount of metabolic pathways (Cruz et al., 2006; Kováčik and Bačkor, 2007; Manoli et al., 2014). High concentrations of N are required in plant tissues and its role in plant metabolism has been investigated in several crops. However, little information is available on the effect of this nutrient in *in vitro* forest species cultivation (Jesus et al., 2012). Nitrogen is available to plants either as nitrate (NO_3^-) or ammonium (NH_4^+) and depending on the form taken up by plants some changes might occur on its morphology (Kováčik and Klejdus, 2014). Generally, nitrate ions are more easily absorbed by plants in soil. Under *in vitro* conditions, preferences for certain nitrogen forms vary among species or even within genotypes of the same species (Allègre et al., 2004; Dominguez-Valdivia et al., 2008; Ivanova and Staden, 2009; Kintzios et al., 2004; Oliveira et al., 2011b). The work objective was to identify the optimal balance between nitrate and ammonium as a nitrogen supply during propagation of a commercial hybrid *Eucalyptus grandis x E. urophylla* in TIB system and in semisolid medium.

Results and Discussion

Shoots' performance in bioreactor

The 3:1 ratio of (NO_3^-):(NH_4^+) was the most effective at increasing values for all analyzed traits of propagated *E. grandis x E. urophylla* in TIB system. Nevertheless, the 1:3 ratio was always among the less efficient treatments in providing more suitable concentrations of nitrogen, thus showing that higher proportions of ammonium in culture medium are less effective at promoting shoot growth in TIB system (Fig 1). It was possible to obtain in average 165.69 mg fresh weight, 6.54 shoots per explant inoculated, with the largest shoot length reaching 1.49 cm and 9.45 leaves per shoot in presence of high ratios of nitrate in relation to ammonium during 15 days cultivation period in TIB system (Fig 1). When nitrate levels are higher than ammonium ones, the nitrate reductase (NR) activity there may be increased as a result of the enzyme induction by its substrate (Lea et al., 2006), enabling more effective nitrate absorption and promoting shoot growth. Our results are consistent with those obtained by *in vitro* propagation of another *E. grandis* x *E. urophylla* clone in systems using RITA® bioreactors. Here, 3:1 and 2:1 ratios of (NO_3^-):(NH_4^+) were effective in promoting improvements in fresh weight and total number of shoots (Oliveira et al., 2011b). Several mechanisms are involved in the absorption, assimilation and mobilization of N including complex regulatory systems of metabolic pathways which may vary in storage, remobilization, re-assimilation, and recycling during photorespiration and distribution between primary and secondary metabolic pathways (Stitt et al., 2002). In aerobic soil conditions, nitrate is the main source of N absorbed by plants. This anion acts as a signaling molecule that regulates expression of genes involved in root development and leaf expansion, which adjusts plant growth according to their availability (Ho and Tsay, 2010). Mimicry of soil conditions may have happened when nitrate availability was higher, allowing a more efficient use of this ion in TIB system.

Shoots growth in semisolid medium

After 15 days cultivation in the semisolid medium, only the length of the largest shoot showed similar means among treatments. The 3:1 ratio of (NO_3^-):(NH_4^+) produced the highest average number of shoots (6.37). As in TIB cultivation, a 1:3 ratio of (NO_3^-):(NH_4^+) was the least effective for promoting shoot growth (Fig 2). According to results the hybrid *E. grandis x E. urophylla* showed a positive response to higher nitrate levels (Fig 2). However, different species or even different genotypes of same species may vary in their responses to nitrate or ammonium levels accordingly to prior selective pressures and subsequent physiological adaptations (Allègre et al., 2004; Tercé-Laforgue et al., 2004). In a study with *Aloe polyphylla* grown in a semisolid medium it was observed that shoots propagated in 2:1, 1:1 and 1:2 ratios of (NO_3^-):(NH_4^+) had higher growth rates than those grown in the presence of either NH_4^+ or NO_3^- alone (Ivanova and Staden, 2009). These results indicate there are synergistic effects between these ions. Research on *in vitro* propagation of the same species in an MS semisolid medium showed that low NH_4^+ concentrations were effective to increase the number of shoots and avoid vitrification (Ivanova and Staden, 2008). There was no difference in fresh weight of explants between 3:1 and 2:1 ratios of (NO_3^-):(NH_4^+) treatments, with mean values of 155.99 and 135.94 mg, respectively (Fig 2C). These results indicate higher nitrate levels are effective to improve growth characteristics in *E. grandis x E. urophylla* despite the cultivation system.

TIB system and conventional cultivation in semisolid medium resulted in similar mean values for the traits, but

Table1. Ionic concentration of nitrate, ammonium and nitrogen total in five ratios of (NO_3^-):(NH_4^+) used to propagate hybrid *E. grandis* x *E. urophylla* in TIB and semisolid medium.

Ion	Ratio of N (NO_3^-):(NH_4^+) (mM)				
	3:1	2:1	1:1	1:2	1:3
$N(NO_3^-)$	45.0	40.0	30.0	20.0	15.0
$N(NH_4^+)$	15.0	20.0	30.0	40.0	45.0
N total	60.0	60.0	60.0	60.0	60.0

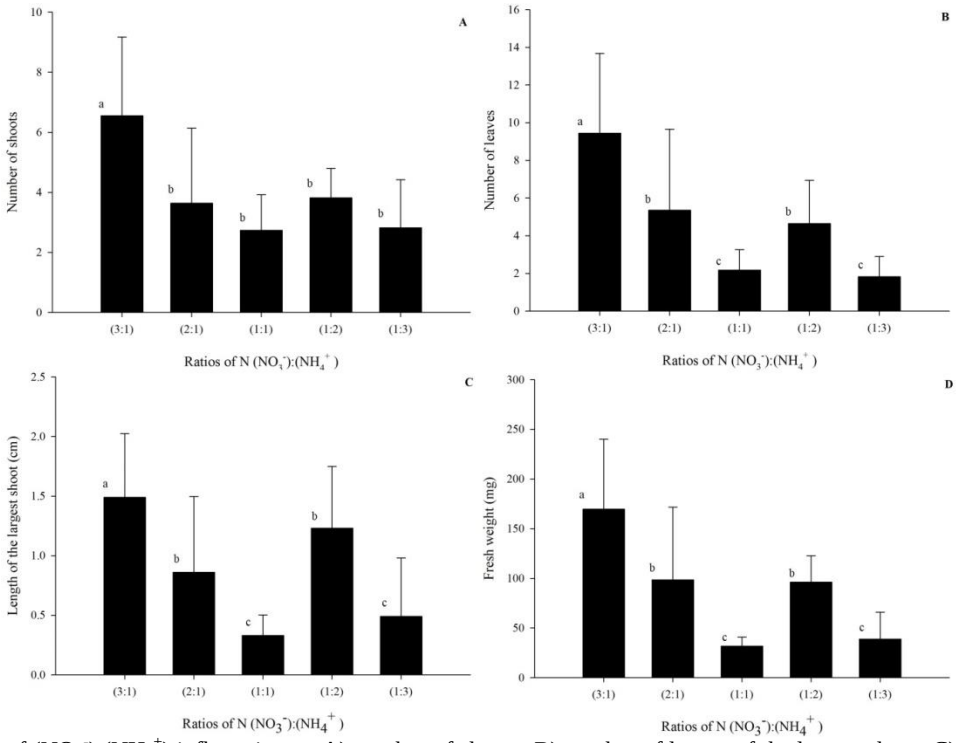

Fig 1. Ratios of (NO_3^-):(NH_4^+) influencing on A) number of shoots; B) number of leaves of the largest shoot; C) length of the largest shoot; and D) fresh weight in *E. grandis* x *E. urophylla* explants cultivated in TIB for 15 days. Different letters above columns represent statistical difference between treatments by the Scott-Knott test at 5% significance level.

TIB clearly shows several practical advantages: i) it uses liquid medium which is simple to prepare; ii) it does not need a gelling agent, and; iii) there is a reduced demand for skilled technicians. These factors significantly reduce production costs for TIB as compared to those of semisolid medium cultivation (McAlister et al., 2005; Pereira and Fortes, 2003). Shoots grown in TIB had 8.76% higher biomass (fresh weight) than those grown in semisolid medium. In the TIB system, there is a greater contact between explant and liquid medium, which increases absorption area and enhances the recovery of nutrients from the culture medium (Lemos et al., 2001). This does not happen in semisolid medium, since only the explant base is in contact with medium. An *in vitro* propagation study with a different *E. grandis* x *E. urophylla* clone also reported better results with the RITA® bioreactor system in comparison to the conventional method having agar as a gelling agent (Oliveira et al., 2011b). The leaf number of the largest shoot was the only trait that showed statistical differences among treatments for shoots grown in semisolid medium during 30 days. As in other experiments, higher ammonium levels resulted in the lowest values for the analyzed variable (Fig 3). Nevertheless, the average values for fresh weight and length of the largest shoot were higher than those found in shoots grown either TIB or semisolid medium during 15 days cultivation. This difference is likely caused by the longer period that shoots had to grow (30 days). Moreover, a greater fresh weight would be expected due to callus formation at the base of older explants. Some shoots grown in 1:2 and 1:3 ratios of (NO_3^-):(NH_4^+) exhibited abnormal morphology for all systems and cultivation periods. Shoots cultivated in TIB with higher ammonium levels produced fewer and shorter shoots (Fig 4, D and E); those cultivated for 15 days in semisolid medium also had decreased growth and early callus formation at the explant base, as well as a lower number of shoots compared with those cultivated in the 3:1 ratio (Fig 4, I and J); shoots grown for 30 days in semisolid medium produced an orange color across the entire stem region, chlorosis in leaves and it also formed calluses at the explant base (Fig 4, N and O).

Effect of nitrate/ammonium absorption in plant growth

The absorption of ammonium is more energetically economical than that of nitrate due to its reduced form, but higher ammonium levels in tissues may be toxic (Britto and Kronzucker, 2002; Ho and Tsay, 2010). Although toxicity responses of plants are not well understood, it is most likely that external environment acidification, acid / base balance disruption and energy loss caused by excessive export of ammonium may negatively affect plant growth and survival (Ho and Tsay,

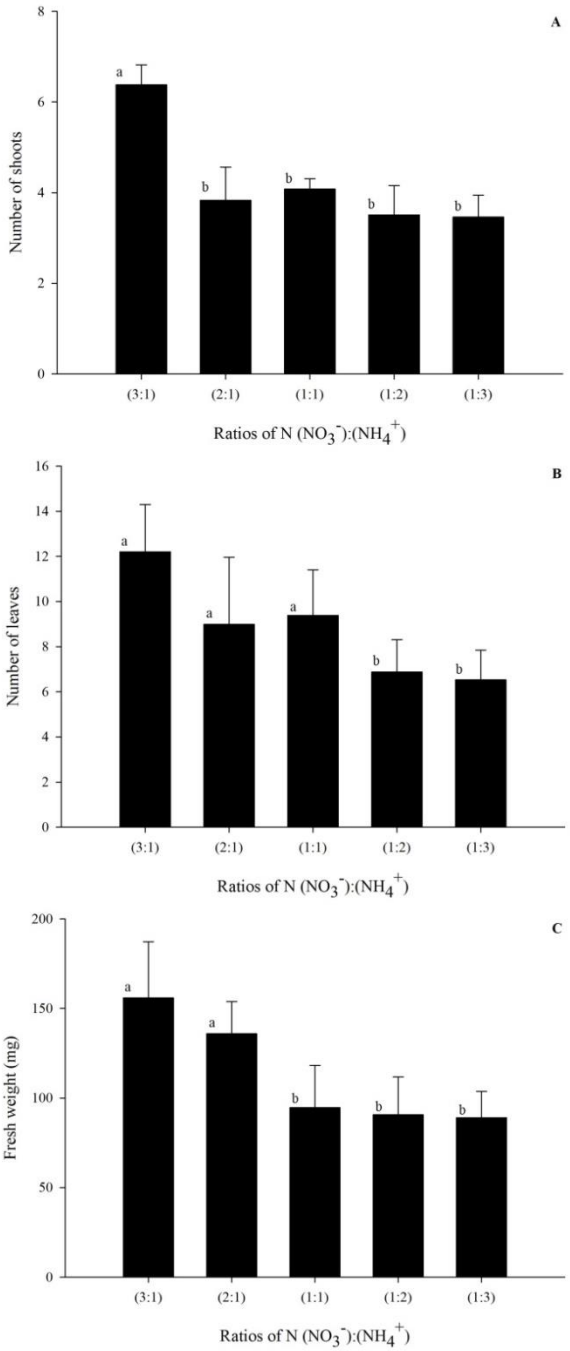

Fig 2. Ratios of (NO_3^-):(NH_4^+) influencing on A) number of shoots; B) number of leaves of the largest shoot; and C) fresh weight in *E. grandis* x *E. urophylla* explants cultivated in a semisolid medium for 15 days. Different letters above columns represent statistical difference between treatments by the Scott-Knott test at 5% significance level.

2010). This would explain some symptoms observed in shoots, such as reduced growth, lower number of shoots and the chlorotic appearance of leaves. At higher nitrate levels, shoots displayed a normal aspect, with green leaves, no hyperhydricity, and an average length of shoots over 1.4 cm in both TIB cultivation system and semisolid medium after 15 days cultivation, mainly for cultivation at 3:1 ratio (Fig 4, A and F). On the other hand, shoots cultivated for 30 days in semisolid medium were characterized by callus formation at explant base for

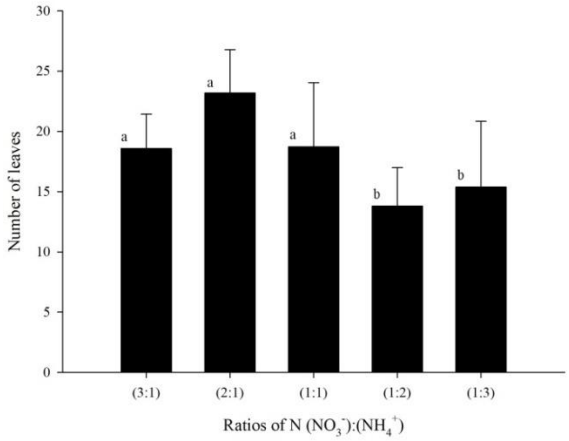

Fig 3. Ratios of (NO_3^-):(NH_4^+) influencing on number of leaves of the largest shoot in *E. grandis* x *E. urophylla* explants cultivated in a semisolid medium for 30 days. Different letters above columns represent statistical difference between treatments by the Scott-Knott test at 5% significance level.

every treatment (Fig 4, K-O). Generally, callus formation requires more potassium, phosphorus and calcium than organogenic cultures, and tends to consume more nitrogen from the medium (Kintzios et al., 2004). The increased amount of potassium nitrate added to culture medium, especially at ratios with higher nitrate levels, likely increased nitrogen and potassium availability. Accumulation of this compound at the basal region of explants can be the cause of callus growth. Callus growth at the explant base is very common in woody species and is considered to be undesirable in micropropagation because it may affect root quality. Specifically, callus formation may impair the vascular connection, thus negatively influencing nutrient absorption by plants from culture medium (Erig and Schuch, 2005). Ammonium nitrate is relatively expensive, difficult to purchase (Fráguas et al., 2003; Villa et al., 2009) and is the only compound among MS medium minerals which provides NH_4^+ to explants. Moreover, the effect of this cation on cultivation of *E. grandis x E. urophylla* clone was not beneficial. It might therefore be interesting to replace ammonium nitrate with another, less harmful compound. One possibility is urea (CH_4N_2O), an organic nitrogen compound that is less expensive than ammonium nitrate (Villa et al., 2009). However, its biological effectiveness for *Eucalyptus* propagation is unknown and further studies would be required to evaluate its effect on plant development.

Materials and Methods

Plant material

Plant material was obtained from a commercial hybrid matrix *E. grandis x E. urophylla* produced at a nursery located in Lavras, Minas Gerais, Brazil. Shoots from the clonal matrix were excised and sterilized with paraformaldehyde tablets for 40 minutes. In a horizontal laminar-flow chamber, shoot tips were isolated and inoculated on Petri dishes (90 x 15 mm) with 25.0 mL MS (Murashige and Skoog, 1962) culture medium added with 20.0 $g.L^{-1}$ sucrose gelled with 1.7 $g.L^{-1}$ Phytagel® (SIGMA) and pH adjusted to 5.8. Explants were kept in

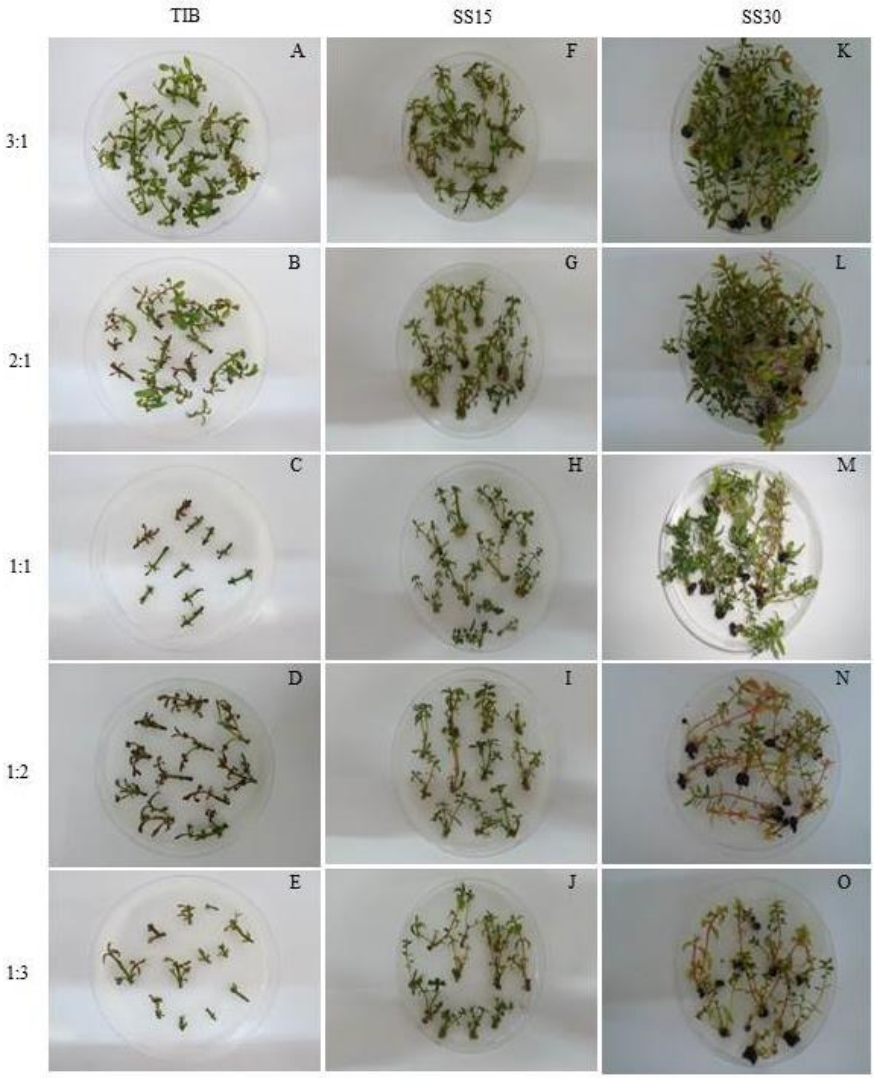

Fig 4. Shoots cultivated at different ratios of (NO_3^-):(NH_4^+) and under different conditions. A to E: cultivation in TIB; F to J: cultivation in semisolid medium for 15 days (SS15); and K to O: cultivation in semisolid medium for 30 days (SS30). Shoots grown in the 3:1 ratio: A, F and K); in the 2:1 ratio: B, G and L); in the 1:1 ratio: C, H and M); in the 1:2 ratio: D, I and N); and in the 1:3 ratio: E, J and O), respectively.

the dark for five days and subsequently transferred to a modified MS medium supplemented with 5.7 µM of indole-3-acetic acid (IAA) and 0.14 µM of 6-benzylaminopurine (BAP). Plant material was kept in a growth chamber with a 16-hour photoperiod, 40 $\mu mol.m^{-2}.s^{-1}$ irradiance provided by white fluorescent light at 26 ±2 ºC, remaining under these conditions for 40 days.

Temporary immersion bioreactor

A temporary immersion bioreactor (TIB®) coupled to a pneumatic chassis model MFE – 1001 (Fitoclone, Viçosa, MG, Brazil) was used to conduct the experiments. The temporary immersion cycle was controlled by an electronic system (temporizer) configured as follows: switch of culture medium in flasks every 2 hours, contact of culture medium with explants for 10 seconds and air injection for renewal of the *in vitro* atmospheric environment every 1 hour. Air injection was achieved by passing the air through filters (sterilized) with 0.20 µm pores for sterilization. Culture flasks were chemically sterilized remaining 12 hours in a commercial sodium hypochlorite solution diluted to 0.036 % (m/v) active chlorine. All culture media used in the bioreactor had pH adjusted to 5.8. Bioreactor with plant material was kept in a growth room at 26 ± 2 ºC, 16-hours photoperiod and 40 $\mu mol.m^{-2}.s^{-1}$ irradiance provided by white fluorescent light bulbs controlled by an electronic system.

Assessment of nitrate and ammonium ratios as nitrogen supply

Shoots previously established *in vitro* were employed as explant source. Each shoot consisted of a stem region with two nodal segments in order to evaluate N (NO_3^-):(NH_4^+) ratios using TIB. Explants were inoculated in bioreactor flasks containing 400.0 mL basal liquid MS medium modified with different NO_3^- and NH_4^+ ratios (3:1, 2:1, 1:1, 1:2, 1:3), respectively (Table 1). The culture medium was supplied with IAA 5.7 µM, BAP 0.14 µM and 20 $g.L^{-1}$ sucrose. Inoculation and maintenance of plant material followed the conditions previously described. After 15 days of cultivation, the following variables were analyzed: i) number of shoots; ii) length of the largest shoot; iii) fresh weight, and; iv) number of leaves on the largest shoot. The experimental

design was completely randomized with 11 replicates per treatment; each replicate consisted of one stem explant with two nodal segments. The N ratio experiment using the semisolid medium was carried out according to the previous experimental conditions done for TIB cultivation. Modified MS medium supplied with IAA 5.7 μM, BAP 0.14 μM, 20 $g.L^{-1}$ sucrose and different ratios of (NO_3^-):(NH_4^+) (3:1, 2:1, 1:1, 1:2, 1:3) were used (Table 1). Culture media was gelled with 1.7 $g.L^{-1}$ Phytagel® (SIGMA) and pH was adjusted to 5.8. After inoculation, plant material was kept under the previously described conditions. After 15 and 30 days of cultivation the same response variables (previously described) were evaluated. The experimental design was completely randomized with 4 replicates per treatment and each replication consisting of a flask containing 6 explants (stem regions bearing 2 nodal segments), totaling 20 flasks for each growing period.

Statistical analysis

Statistical analysis was performed via analysis of variance (ANOVA) and means compared by Scott-Knott test at 5% probability. All tests were performed using the statistical software Sisvar (Ferreira, 2014).

Conclusions

The 3:1 ratio of (NO_3^-):(NH_4^+) was the most effective one in promoting increased productivity after 15 days cultivation in TIB and in a semisolid medium. Higher ratios of ammonium present in culture medium are toxic for the cultivation of a commercial hybrid of *E. grandis x E. urophylla*, resulting in decreased productivity and abnormal growth patterns of shoots for both cultivation systems. Although TIB and semisolid cultivation systems generated similar results for the number of shoots, the fresh weight of explants tends to be higher in the TIB system. The current findings will help determine which nitrogen source as well as its proportion would be more suitable to improve the *in vitro Eucalyptus* cultivation using bioreactor system to produce healthy seedlings.

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References

Aggarwal D, Kumar A, Sharma J, Reddy MS (2012) Factors affecting micropropagation and acclimatization of an elite clone of *Eucalyptus tereticornis* Sm. In Vitro Cell Dev Biol – Plant. 48(5):521-529.

Allègre A, Silvestre J, Morard P, Kallerhoff J, Pineli E (2004) Nitrate reductase regulation in tomato roots by exogenous nitrate: a possible role in tolerance to long-term root anoxia. J Exp Bot. 55(408):2625-2634.

Andrade WF, Almeida M, Gonçalves NA (2006) Multiplicação *in vitro* de *Eucalyptus grandis* sob estímulo com benzilaminopurina. Pesqui Agropec Bras. 41(12):1715-1719.

Associação Brasileira dos Produtores de Florestas Plantadas (ABRAF). Anuário estatístico: ano base 2012. Brasília, 2013. 148p. Available in: < http://www.bibliotecaflorestal.ufv.br/handle/123456789/3910>. Access in: January, 09, 2015.

Barreiros RM, Gonçalves JLM, Sansígolo CA, Poggiani F (2007) Modificações na produtividade e nas características físicas e químicas da madeira de *Eucalyptus grandis* causadas pela adubação com lodo de esgoto tratado. Rev Árvore. 31(1):103-111

Britto DT, Kronzucker HJ (2002) NH4+ toxicity in higher plants: a critical review. J Plant Physiol. 159(6):567-584.

Castro DR, González JO (2002) Micropropagación de Eucalipto (*Eucalyptus grandis* Hill ex Maiden) en el sistema de inmersión temporal. Agric Tec. 62(1):68-78.

Cruz JL, Pelacani CR, Araújo WL (2006) Efeito do nitrato e amônio sobre o crescimento e eficiência de utilização do nitrogênio em mandioca. Bragantia. 65(3):467-475.

Dominguez-Valdivia MD, Aparicio-Tejo PM, Lamsfus C, Cruz C, Martins-Loução MA, Moran JF (2008) Nitrogen nutrition and antioxidant metabolism in ammonium-tolerant and -sensitive plants. Physiol Plantarum. 132(3):359–369.

Dutra LF, Wendling I, Brondani GE (2009) A micropropagação de Eucalipto. Pesqui Florest Bras. edição especial:49-59.

Erig AC, Schuch MW (2005) Tipo de luz na multiplicação *in vitro* de Framboeseira (Rubus idaeus L.) 'Batum'. Rev Bras Frutic. 27(3):488-490.

Ferreira DF (2014) Sisvar: a Guide for its Bootstrap procedures in multiple comparisons. Cienc Agrotec. 38(2):109-112.

Fráguas CB, Chagas EA, Ferreira MM, Carvalho JG, Pasqual M (2003) Micropropagação de gloxínia em diferentes concentrações de nitrato de amônio e uréia. Cienc Agrotec. 27(4):811-815.

Hajari E, Watt MP, Mycock DJ, McAlister B (2006) Plant regeneration from induced callus of improved *Eucalyptus* clones. S Afr J Bot. 72(2):195-201.

Ho CH, Tsay YF (2010) Nitrate, ammonium, and potassium sensing and signaling. Curr Opin Plant Biol. 13(5):604-610.

Ivanova M, Staden JV (2008) Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of *in vitro* regenerated shoots of *Aloe polyphylla*. Plant Cell Tiss Org. 92(2):227-231.

Ivanova M, Staden JV (2009) Nitrogen source, concentration, and NH_4^+:NO_3^- ratio influence shoot regeneration and hyperhydricity in tissue cultured *Aloe polyphylla*. Plant Cell Tiss Org. 99(2):167-174.

Jesus GL, Barros NF, Silva IR, Neves JCL, Henriques EP, Lima VC, Fernandes LV, Soares EMB (2012) Doses e fontes de nitrogênio na produtividade do eucalipto e nas frações da matéria orgânica em solo da região do cerrado de Minas Gerais. Rev Bras Cienc Solo. 36(1):201-214.

Kintzios S, Stavropoulou E, Skamneli S (2004) Accumulation of selected macronutrients and carbohydrates in melon tissue cultures: association with pathways of *in vitro* dedifferentiation and differentiation (organogenesis, somatic embryogenesis). Plant Sci. 167(3):655-664.

Kováčik J, Bačkor M (2007) Changes of phenolic metabolism and oxidative status in nitrogen-deficient *Matricaria chamomilla* plants. Plant Soil. 297(1-2):255-265.

Kováčik J, Klejdus B (2014) Induction of phenolic metabolites and physiological changes in chamomile plants in relation to nitrogen nutrition. Food Chem. 142(1):334-341.

Lea US, Leydecker M-T, Quilleré I, Meyer C, Lillo C (2006) Posttranslational regulation of nitrate reductase strongly affects the levels of free amino acids and nitrate, whereas transcriptional regulation has only minor influence. Plant Physiol. 140(3):1085-1094.

Lemos EEP, Ferrei MS, Alencar LMC, Oliveira JGL, Magalhães VS (2001) Micropropagação de clones de Banana cv. Terra em biorreator de imersão temporária. Rev Bras Frutic. 23(3):482-487.

Manoli A, Begheldo M, Genre A, Lanfranco L, Trevisan S, Quaggiotti S (2014) NO homeostasis is a key regulator of early nitrate perception and root elongation in maize. J Exp Bot. 65(1):185-200.

McAlister B, Finnie J, Watt MP, Blakeway F (2005) Use of temporary immersion bioreactor system (RITA®) for production of commercial Eucalyptus clones in Mondi Forests (SA). Plant Cell Tiss Org. 81:347-358.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum. 15(3):473-497.

Oliveira LA, Breton MC, Bastolla FM, Camargo SS, Margis R, Frazzon J, Pasquali G (2012) Reference genes for the normalization of genes expression in *Eucalyptus* species. Plant Cell Physiol. 53(2):405-422.

Oliveira ML, Xavier A, Filho RMP, Otoni WC, Teixeira JB (2011a) Efeitos do meio de cultura e da relação BAP/ANA na multiplicação *in vitro* de clones de *Eucalyptus grandis* x *E. urophylla* em biorreator de imersão temporária. Rev Arvore. 35(6):1207-1217.

Oliveira ML, Xavier A, Penchel RM, Santos AF (2011b) Multiplicação *in vitro* de *Eucalyptus grandis* x *E. urophylla* cultivado em meio semissólido e em biorreator de imersão temporária. Sci For. 39(91):309-315.

Pereira JES, Fortes GRL (2003) Protocolo para produção de material propagativo de batata em meio líquido. Pesqui Agropecu Bras. 38(9):1035-1043.

Pinto DS, Resende RT, Mesquita AGG, Rosado AM, Cruz CD (2014) Seleção precoce para características de crescimento em testes clonais de *Eucalyptus urophylla*. Sci For. 42(102):251-257.

Stitt M, Müller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible W-R, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot. 53(370):959-970.

Tercé-Laforgue T, Mäck G, Hirel B (2004) New insights towards the function of glutamate dehydrogenase revealed during source-sink transition of tobacco (Nicotiana tabacum) plants grown under different nitrogen regimes. Physiol Plantarum. 120(2):220-228.

Villa F, Pasqual M, Pio LAS, Fráguas CB, de Rezende JC (2009) Utilização de nitrato de amônio e de uréia como fontes de nitrogênio na micropropagação de amoreira-preta. Sci Agrar. 10(5):365-370.

Xavier A, Silva RL (2010) Evolução da silvicultura clonal de *Eucalyptus* no Brasil. Agron Costarric, 34(1):93-98.