A new and rapid micropropagation protocol for *Eucalyptus grandis* Hill ex Maiden

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**Abstract**

**Aim of the study:** We developed a faster micropropagation protocol specifically designed for *Eucalyptus grandis*. Eucalyptus breeding programs use micropropagation protocols to obtain high quality cloned seedlings, but current protocols are excessively time consuming.

**Area of the study:** The protocol has been developed in Argentina, but it can be applied anywhere.

**Materials and methods:** We used nodal segments as initial explants to obtain micropropagated shoots, which were then simultaneously rooted ex vitro and acclimated in a hydroponic system. Nodal segments were cultured in a MS medium supplemented with 1 mg l⁻¹ 6-benzylaminopurine, 30 g l⁻¹ sucrose, 1 g l⁻¹ active charcoal and 8 g l⁻¹ agar and incubated for four weeks at 25 ± 2°C under 16 h day photoperiod. Then, micropropagated shoots were exposed 15 seconds to 5000 ppm indol-butyric acid prior to being transferred to a hydroponic system, allowing simultaneous ex vitro rooting and acclimatization.

**Main results:** 73 ± 9% of nodal segments grew to generate 1.73 ± 1.03 shoots per explant (length: 0.76 ± 0.44 cm). After four weeks in hydroponic system, 46 ± 4 % of micropropagated shoots developed roots, which represents an acceptable and intermediate rate of success, compared to the reported in vitro rooting rates.

**Research highlights:** Our protocol allowed to obtain micropropagated seedlings in a total timespan of 8 weeks. Our results show that, by utilizing a hydroponic system, traditional protocols to micropropagate *Eucalyptus* can be substantially enhanced, allowing for improved production dynamics and potentially resulting in better organized seedling manufacturing facilities.

**Key words:** Woody plants; silviculture; nursery seedlings; rooting methods; hydroponics; acclimatization.

**Author contributions:** DGAV, LLE and RJJ obtained micropropagated eucalyptus shoots. DGAV, LLE and RJJ rooted shoots in hydroponics system. CJM designed hydroponic systems. TE and RJJ wrote the manuscript. TE, AG and RJJ experimental manuscript design. PASI and AG critical revision of manuscript. PASI English revision of manuscript. TE, AG and RJJ had the initial idea of the manuscript.

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**Supplementary material:** Table S1 and Figure S1 accompany the paper on FS’s website.

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Introduction

_Eucalyptus_ is a genus of woody plants which has gained great relevance in the last decades as a rapid source of cellulose pulp and solid wood (Albaugh et al., 2016). _Eucalyptus_ species currently lead the hardwood production market globally, including the countries of the Southern region of South America. Specifically, in the case of Argentina, of the 1.1 million hectares forested, 22% corresponds to _Eucalyptus_ (MA GyP, 2014), with an upward trend, being _Eucalyptus grandis_ Hill ex Maiden the most cultivated _Eucalyptus_ species in the area (Frangi et al., 2016).

The traditional method for obtaining seedlings is the germination of seeds originated in a seed orchard (Skolmen & Ledig, 1990). However, nurseries have obtained -through crossbreeding- elite specimens whose special traits should be kept (de Assiss, 2001). With this objective, the forestry industry is in a process of transition towards the cultivation of clones. Thus, rapid vegetative propagation techniques for producing _E. grandis_ clones are required by forestry companies nationally and worldwide in a strategic path towards enhancement of overall productivity (FAO, 2008).

As conventional macropropagation schemes limit the productivity of eucalyptus plantations due to inefficient rooting rates or excessive rooting timespans (Trueman et al., 2018), _in vitro_ propagation techniques are replacing the macropropagation protocols (Nakhhooda & Jain, 2016). Shoot culture is probably the most common technique for _E. grandis_ micropropagation (Nakhhooda & Jain, 2016; Trueman et al., 2018,) and comprises the following stages, each of which takes between 2 and 6 weeks: shoot culture, shoot multiplication, shoot elongation, root formation and acclimatization (Trueman et al., 2018), with an average time required of around 6 months. A possible way to shorten the duration of these protocols is the condensation of some stages in the same time period. For example, rooting of micropropagated shoots could be combined with the acclimatization in a hydroponic system.

The culture of _Eucalyptus_ in hydroponic systems has been consistently reported for decades with different purposes: phytoremediation (Iori et al., 2017), drought or salinity stress studies (Nawaz et al., 2016), fertilization experiments (Niu et al., 2015) or in combination with different microorganisms (Egerton-Warburton, 2015). Interestingly, hydroponic systems have never been employed for rooting of micropropagated _Eucalyptus_ shoots and, to our knowledge, it has not been reported in other tree species either. It has been described, however, for rooting micropropagated shoots in shrub species such as _Gypsophila paniculata_ (Wang et al., 2013), _Rubus fruticosus_ (Clapa et al., 2013), _Rosa hybrida_ (Clapa et al., 2013), or _Rhododendron sp._ (Zaytseva et al., 2018) and herbaceous species such as _Spathiphyllum sp._ (Dewit et al., 2005) or _Solanum tuberosum_ (Piao et al., 2004).

The aim of the present work was to develop a new and faster micropropagation protocol for _Eucalyptus grandis_ that simultaneously would allow the direct rooting and acclimatization of the micropropagated shoots in a hydroponic system.

Materials and methods

_Eucalyptus grandis_ growth in a hydroponic system

Fifty mg of the seed mix of _Eucalyptus grandis_, provided by Paul Forestal (CO N°4081) from clonal seed orchard CIEF-Paul Forestal, were incubated in Petri dishes with a filter paper moistened with 10 ml of a nutritive solution (Javot et al., 2003), at 23 ± 2 °C under dark conditions during 7 days. Afterwards, the dishes were incubated under 16:8 h (L:D) photoperiod with a light intensity level of 150 ± 10 µmol photon m⁻² s⁻¹ and 60% humidity for three days. Then, 21 seedlings were transplanted into aerated hydroponic culture containers, which had styrofoam lids (36 x 15 x 2 cm) with circular holes, that were floating on a basin filled with 5L of the nutritive solution (seedlings were secured in position using 3 mm-thick polyurethane discs). Systems were incubated in a growth chamber for three months under the same conditions described above. After three months, seeding size was enough to extract the nodal segment to be cultured _in vitro_.

Shoot initiation of nodal segments

We cut branches from _Eucalyptus grandis_ seedlings grown in the hydroponic system as described above. First, we eliminated the leaves and the apical section of the branches, which were later carefully washed with a soap solution and distilled water, and were cut again into smaller branches with two or three nodal segments (Fig. 1A). The branches were disinfected in a solution with 24 g l⁻¹ of sodium hypochlorite for 5 min under aseptic conditions, followed by three 5 min rinses with distilled sterile water. Finally, the disinfected branches were cut into 3 cm long nodal segments and at least two axillary buds, removing a basal and apical section of 0.5 cm that could be possibly damaged during sterilization. The obtained nodal segments were cultured individually in test tubes that contained 20 ml of initiation medium. The shoot initiation medium consisted...
in MS (Murashige & Skoog, 1962) culture medium supplemented with 1 mg l⁻¹ 6-benzylaminopurine (BAP), 30 g l⁻¹ sucrose solidified with 8 g l⁻¹ of bacteriological agar (pH 5.74). As an antioxidant we used 1 g l⁻¹ of activated charcoal. The nodal segments were placed in an incubator model I-291PF (Ingelab) and incubated at 25 ± 2 °C under 16:8 h (L:D) photoperiod with a light intensity level of 40 µmol photon m⁻²s⁻¹ for four weeks. Thirty nodal segments were employed in each repetition and three independent experiments were performed. After these four weeks, we calculated the percentage of shoot initiation and measured the number and length of the micropropagated shoots.

Ex vitro rooting and acclimatization in hydroponic system

The micropropagated shoots were cut from the nodal segments and transferred into aerated hydroponic culture containers. The hydroponic system was similar to the already described one, but now the 20 mm-thick styrofoam lids were replaced by a high-impact polystyrene plaque (1 mm thick) while the micropropagated shoots were secured with 3 mm-thick polyurethane discs (Fig. S1 [suppl.]). To induce the ex vitro rooting, the micropropagated shoots were dipped for 15 seconds in a solution of 5000 ppm indol butyric acid (IBA) before being transferred to this hydroponic system and maintained for four weeks under the same conditions described before. The hydroponic system was wrapped with plastic film to maintain high humidity and avoid drying of micropropagated shoots. From the second week, holes were made in the plastic film to slowly reduce humidity until reaching the relative humidity of the culture chamber (60 %). Finally, at the end of the fourth week, the plastic wrap was removed and rooted shoots were counted. Three independent hydroponic culture containers were established with 24 micropropagated shoots in each case. The rooted eucalyptus continued its growth in a hydroponic system.

Statistical analysis

All data obtained in this manuscript were analyzed using SPSS software package (version 22.0; SPSS INC., Chicago, IL, USA). We compared the results between independent experiments to check that there were no significant differences between them. Initiation success rate of nodal segments and rooting rate, binomial variables, were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. The length and number of micropropagated shoots from each nodal segment, linear variables, were analyzed by one-way ANOVA.

Results and discussion

To develop our rapid micropropagation protocol, we used nodal segments of seed-derived *E. grandis* seedlings as primary explants (Trueman et al., 2018). Micropropagation using nodal segments is based on the existence of axillary buds at the base of each leaf axil and their ability to grow. We supplemented MS culture medium with 1 mg BA to promote the growth of axillary buds, obtaining a success rate of 73 ± 9% (Fig. 1B), shoots that grew. Concentrations between 0.1 and 1.5 mg l⁻¹ of BAP are normally used in the nodal segment culture of *Eucalyptus* (Trueman et al., 2018). Our initial success rate is strong compared to previous research: Watt et al., 2003 obtained success rates between 67% and 78% at the initiation of nodal segments of three different genotypes of *E. grandis*; in the case of hybrids of *E. grandis* with other *Eucalyptus* species, the success rate varied between 18% (*E. grandis* x *E. camaldulensis*) and 91.5% (*E. grandis* x *E. urophylla*) (Watt et al., 2003; Watt, 2014). Moreover, as *Eucalyptus* has multiple buds within each leaf axil (Jasrai et al., 1999), we obtained an average of 1.73 ± 1.03 micropropagated shoots in each nodal segment, with a length of 0.76 ± 0.44 cm (Fig. 1C). Other authors have obtained between 0,5 and 3 shoots per nodal segment in hybrids of *Eucalyptus grandis* (Watt et al., 1995; Borges et al., 2011). The next step among *Eucalyptus* micropropagation protocols, involves the multiplication and/or elongation of the obtained shoots (2-6 weeks and 3-4 week of duration respectively), followed by their rooting (Table S1 [suppl.]; Trueman et al., 2018). In our protocol, however, suppressing these two steps and directly moving forward to the rooting of micropropagated shoots (in a hydroponic system) reduced the extension of the whole protocol to 8 weeks. It should be noted, nonetheless, that a reduction in the number of total plants obtained was detected. Remarkably, there are no previous reports in the literature where *Eucalyptus* micropropagated shoots had been successfully rooted in a hydroponic system.

The rooting rate that we obtained was 46 ± 4 % after 4 weeks of incubation (Figs. 1D and 1E). Not surprisingly, the contact between the micropropagated shoots and the hydroponic solution was key to achieve a good shoot rooting. The short length of the micropropagated shoots (0.76 ± 0.44 cm, Fig. 1C) made this contact difficult, so the replacement of the styrofoam lid (20 mm) by a 1 mm thick high-impact polystyrene plaque was critical to the success of the rooting process.
With the aim of comparing the rooting rate obtained here with the rates published elsewhere, it is important to note that the latter ones tend to closely relate with the E. grandis or E. grandis hybrid genotypes utilized. From the gathered literature, the in vitro rooting rate varies between 100%, in genotypes of E. grandis (Na-

Figure 1. A) Initial nodal segment of Eucalyptus grandis used to obtain micropropagated shoots. B) Nodal segment after 4 weeks of in vitro culture. C) Micropropagated shoots obtained after 4 weeks in vitro culture. D) Hydroponic system used for ex vitro rooting and acclimatization of micropropagated shoots. E) Rooted plantlet after 4 weeks of the acclimatization. F) Rooted plantlet after 8 weeks of the acclimatization. G) Rooted plantlet after 12 weeks of the acclimatization.
Eucalyptus rapid micropropagation protocol

khodaa et al., 2011; 2014), to 35% in a hybrid of E. grandis x E. urophylla (de Oliveira et al., 2017). Other authors have obtained a range of varying in vitro rooting rates: 90% or 80% in genotypes of E. grandis (Hajari et al., 2006; Almeida et al., 2015), 36.7% to 90% in hybrids of E. grandis x E. nitens (Mokotedi et al., 2000; Watt, 2014), 35% to 90.0% in hybrids of E. grandis x E. urophylla (Jones & van Staden, 1994; Yang et al., 1995; Whitehouse et al., 2002; Hajari et al., 2006; Watt, 2014;) and 70% in a hybrid of E. grandis x E. camaldulensis (Whitehouse et al., 2002).

Hence, the rooting rate that we have obtained (46±4%) was lower-intermediate compared to the rates already published.

The main advantage of rooting in a hydroponic system is that the rooting and the acclimatization can be achieved simultaneously, while the Eucalyptus shoots rooted in vitro (conventional protocols) must be acclimatized in a further additional step. By performing these two steps in a combined manner, we reduced the overall duration of the protocol by 4 weeks. Indeed, the acclimatization success rates are also typically related to the micropropagated genotype utilized, varying between 20% to 100% (Trueman et al., 2018). Thus, the acclimatization not only prolongs the time of the micropropagation protocol, but also reduces the total number of seedlings obtained. In the case of our method, however, all the shoots rooted in the hydroponic system survived and continued their normal growth (Figs. 1F and 1G).

In conclusion, the use of a hydroponic system for ex vitro rooting of E. grandis micropropagated shoots allowed us to obtain seedlings faster than conventional protocols and with an acceptable success rate. This rapid micropropagation protocol has the potential of being useful for propagating elite genotypes with commercial purposes or for performing Eucalyptus physiological studies.

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