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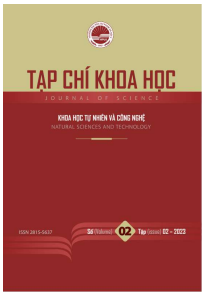

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Propagation of a U16 hybid Eucalyptus line by plant cell tissue culture

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Abstract

Eucalyptus is one of the important plant species for paper pulp, fuel wood, and timber. In this study, a hybrid Eucalyptus, U16 line, was investigated for rapid in vitro propagation. The apical and adventitious shoots served as initial explants. These were free-disease symptoms that were collected for surface sterilization. Different disinfectants including mercuric and Javel solutions at different concentrations and treated times were performed. The results showed that explants were cleaned under taping water with soap, rinsed in 70ºEtOH for 1 minute, and then immersed in 10% Javen solution for 15 minutes and 0.1% $HgCl_2$ for 10 minutes gave the highest disinfection rate that was 46.5%. The modified MS medium included basal MS, 30 g/L sucrose, and 7 g/L agar supplemented with 20 ml/L of coconut water (MS medium*) was the most suitable for the rapid multiplication of Eucalyptus lines U16, the shoot multiplication coefficient reached 1.56. In vitro multiplication of eucalyptus U16 was carried out by culturing disinfected shoot segments on the MS* medium supplemented with BAP 1.5 mg/L and kinetin 1.0 mg/L which gave the highest shoot multiplication coefficient (3.24 shoot per explant). The in vitro rooting medium of the U16 line was basal MS supplemented with 1.0 mg NAA/L, the rooting rate was 90.49%, the number of roots was 8.3, and the roots expressed strong growth.

Keywords: Eucalyptus, U16 line, tissue culture, in vitro propagation

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1. Introduction

Eucalyptus spp., be long to Myrtaceae are trees variety recognized for its remarkable traits including rapid growth and superior wood quality [1]. Them have cultivated spanning over 20 million hectares across more than 90 nations, with focal points situated predominantly in Brazil (covering 5.7 million hectares), India (spanning 3.9 million hectares), and China (encompassing 4.5 million hectares) [2]. Its status as one of the most widely cultivated hardwood trees globally remains prominent.

Cultivated eucalyptus provides important raw materials for the production of pulp, paper, chipboard, construction timber, furniture, biomaterials and bioenergy [1, 3], while helping to reduce bare land and hills. bald, protect the ecological environment [4]. In addition, eucalyptus essential oil is now being produced in large quantities and has high biological activity, widely used in the field of medicine and industry [5].

In Vietnam, the majority of eucalyptus trees are cultivated in temperate and mountainous regions in substantial quantities. However, the propagation methods primarily involve sowing seeds or importing germplasm from foreign sources, resulting in high costs and unproven efficiency [6]. Within the scope of our nation's afforestation program aiming to establish 5 million hectares of new forests, as many as 3 million hectares are designated for productive forests. This allocation encompasses 1 million hectares for long-term industrial plants and 2 million hectares for forestry trees, of which up to 70% comprise rapidly growing species, including acacia, eucalyptus, and pine [7]. This drives a substantial demand for diverse germplasm, particularly those exhibiting high yield and exceptional quality. Every year, localities throughout the country produce about 650 million afforestation seedlings, of which 77% are nursed plants such as *Eucalyptus, Acacia mangium, Pinus massoniana, Illicium verum, Chukrasia tabularis, Cinnamon, Maglonia conifera,..* plants propagated from cuttings and tissue culture accounted for 23% such as: hybrid acacia, hybrid eucalyptus, uro eucalyptus.

Recently, a series of investigations have been undertaken concerning the swift propagation of eucalyptus through tissue culture techniques [8-11]. In Vietnam, similar endeavors regarding *Eucalyptus* tissue culture have also been conducted [12-15]. With the objective of enhancing seedling quality to align with the high demands of forestry production, this study presents the outcomes of Eucalyptus U16 line propagation through the employment of tissue culture methodologies.

2. Materials and methods

2.1. Materials

Plant materials:

U16 hybrid *Eucalyptus urophylla* line, was provided by the Research Institute of Paper Plants. The apical and lateral shoots of 5-6 months old U16 were free-disease symptoms that were selected and used as *in vitro* initial explants. All experiments were carried out at the Laboratory of Biotechnology of the Institute for Scientific Research and Application (Hanoi Pedagogical University 2).

Chemicals and media:

The commercial plant growth regulators (PGRs) 6-Benzylaminopurine (BAP), Kinetin, and α-naphtalene acetic acid (NAA) were provided by Dulchefa, Netherlands. The macronutrients, trace minerals and vitamins used in tissue culture were provided by Xilong (China). Sucrose and agar were supplied by I Sugar Company (Vietnam) and Hai Long Co., Ltd., (Vietnam). MS added 30 g/L sucrose and 7 g/L agar, (pH 5,8) as the basal medium for all experiments [9, 16], for each *in vitro* propagation stage, components of the basal or concentration and/or type of PGRs were adjusted.

2.2. Methods

2.2.1. In vitro explants sterilization

The initial explants were the apical and lateral shoots of the U16 eucalyptus line, which were cut into segments with a length of 3-4cm, bearing from 2, 3 leaf axils. Explants were treated by cleaning with taping water with soap, rinsing in 70^{o} ethanol solution (EtOH) for 1 minute, then surface double sterilizing by 0.1%

$HgCl_2$ disinfectants and Javen solution (NaClO 5.3%) at different concentrations and different processing times (Table 1). The starting explants were cultured on the MS medium. The percentage of survival and non-infected explants (%) after 30 days were recorded.

Table 1: Surface sterilization formulas for apical and axillary shoots of eucalypts for tissue culture

Treatments	ConC. of disinfectants	Disinfection time (minute)
CT1	Javel 10%	15
	$HgCl_2$ 0.1%	5
CT2	Javel 20%	15
	$HgCl_2$ 0.1%	10
CT3	Javel 10%	15
	$HgCl_2$ 0.1%	10
CT4	Javel 20%	15
	$HgCl_2$ 0.1%	5

2.2.2. Rapid in vitro multiplication of Eucalyptus U16 line

- Culture medium:

The shoots were separated from the in vitro sample when the shoots reached a height of 3 - 4 cm, had 2, 3 leaves, and then were inoculated on 03 media namely MS, ½ MS, MS* (MS* is MS + 20 ml medium) coconut water/L). The experiment was arranged in a completely randomized design, with three replications, with n≥ 30 samples. Shoot multiplication coefficient after 30 days of culture was collected.

- Plant growth regulators:

In this experiment, the BAP and Kinetin were used to investigate the effect PGRs on in vitro shoot regeneration. Firstly, BAP was individually added at concentrations (0; 0.5; 1.0; 1.5; 2 mg/L) to determine suitable concentrations for multiplication. Secondly, BAP (at 1.5 mg/L) was combined with kinetin at various concentrations (0; 0.5; 1.0; 1.5; 2 mg/L) to promote shoot growth in the in vitro culture. Shoot multiplication coefficient and morphological characteristics of shoots after 30 days of culture were obtained.

2.2.3. In vitro rooting of Eucalyptus U16 line shoots

The shoots reaching a height of 4-5 cm, with 3 or more leaves, were cultured on MS medium supplemented with NAA (0; 0.5; 1.0; 1. ,5; 2 mg/L) to find the favourable rooting medium. Rooting shoot rate (%), number of roots/buds, root length, and root growth were collected.

2.2.4. Experimental construction and statistical analysis

All experiments were constructed by completely randomized design (CRD) with 3 repetitions. Data were statistically analyzed using SPSS software (v. 11.09). The data shown in the table are average values. Test the difference between the mean values using Fisher's Least Significant Difference ($LSD_{0.05}$).

3. Results and discussion

3.1. Surface sterilization of Eucalyptus U16 line for in vitro culture

In this experiment, apical and lateral shoots were used as explants which were disinfected by the following procedure: cleaning under tap water with soapy, rinsing in 70^o ethanol (EtOH) for 1 min, then immersing in javel solution and 0.1% $HgCl_2$ at various concentrations for various interval times. The obtained results are shown in Table 2.

Table 2: Effect of javel solution and 0.1% $HgCl_2$ for *in vitro* sterilization on the Eucalyptus U16 line

Treatments	Conc. (%) of disinfectants	Treated time (min)	Rate of disinfected and survival explants (%)
CT1	Javel 10%	15	$26.9^{d} \pm 1.08$
	$HgCl_2$0.1%	5	
CT2	Javel 20%	15	$35.3^{b} \pm 1.00$
	$HgCl_2$ 0.1%	10	
CT3	Javel 10%	15	$46.5^{a} \pm 0.88$
	$HgCl_2$ 0.1%	10	
CT4	Javel 20%	15	$29.5^{c} \pm 0.73$
	$HgCl_2$ 0.1%	5	
CV%			2.70
$LSD_{0.05}$			1.75

In the column, the different letters such as a, b, c... were expressed significantly difference at the α = 0.05 level.

The results showed that, in CT1 treated with 10% Javen solution for 15 minutes and 0.1% $HgCl_2$ for 5 minutes, the survival rate of explants was 26.9 (%). When the concentration of Javen increased to 20% and the sterilization time of 0.1% $HgCl_2$ to 10 minutes, the rate of survival-uninfected explants reached 35.3%. In CT3, using 10% Javen solution for 15 minutes and 0.1% $HgCl_2$ for 10 minutes, the survival rate of explants was 46.5% (Fig. 1a). When continuously increasing the concentration of Javen solution up to 20%, sterilization time for 15 minutes, and sterilization with 0.1% $HgCl_2$ solution for 5 minutes, the percentage of uninfected explants decreased, to 29.5% (Table 2).

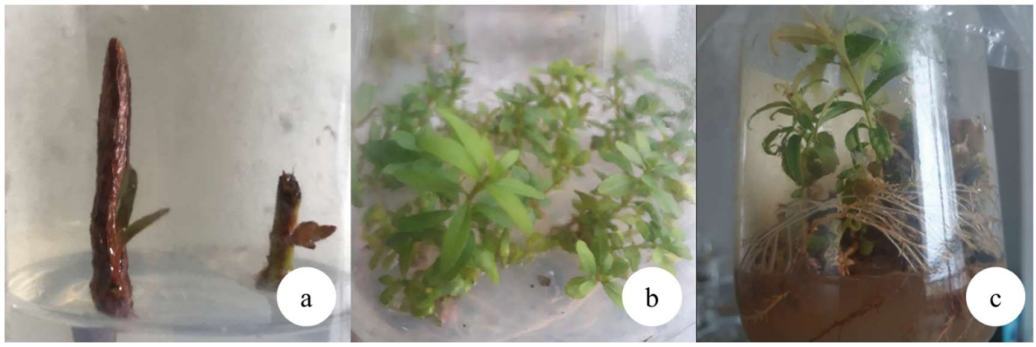

Figure 1: Some stages of *in vitro* propagation of *Eucalyptus* U16 line

(a) In vitro explants of U16 Eucalyptus treated at CT3 (10% Javen solution for 15 minutes and 0.1% $HgCl_2$ for 10 minutes); (b) Shoot clusters of U16 cultured on the MS* medium supplement with BAP 1.5 mg/l and kinetin 1.0 mg/l; (c) Root formation of in vitro Eucalyptus U16

Thus, the concentration of Javel and $HgCl_2$ at CT3 was suitable for removing surface microorganisms, and the percentage of survival and free- infections explants reached 46.5%.

3.2. Rapid multiplication of U16 Eucalyptus line

3.2.1. Multiplication media

The shoot propagation medium plays important for the survival and growth of shoots during multiplication. There are many types of basic media used in tissue culture but based on laboratory conditions, 03 different media: MS, ½ MS, MS* (in which MS* was standard MS the medium supplemented with 20 ml of coconut water/1 liter of culture medium). The obtained results are presented in Table 3.

Table 3: Effect of medium on the microshoots regeneration of Eucalyptus U16 line

Shoot multiplication medium	Shoot multiplication	The quality of in vitro regeneration shoots
½ MS	$1.32^{c} \pm 0.03$	++
MS	$1.47^{b} \pm 0.05$	++
MS*	$1.56^{a} \pm 0.06$	+++
CV%	3.17	
$LSD_{0.05}$	0.09	

In the column, the different letters such as a, b, c... were expressed significantly difference at the α = 0.05 level. Notes: Symbol (+) showed the quality of shoots, +: low; ++: average; +++: good

The results showed that the shoot multiplication coefficient after 30 days of the ½ MS medium reached 1.32. In MS medium, the shoot multiplication reached 1.47, and in MS* medium, the highest shoot multiplication coefficient was 1.56. MS and MS* medium had different shoot multiplication coefficients because the MS* medium was supplemented with coconut water which was a good natural source of cytokinin for the formation of new shoots in plants in general and Eucalyptus lines when cultured in in vitro propagation (Table 3).

3.2.2. Plant growth regulators

BAP and Kinetin were two plant growth regulators belonging to the cytokinin group that has the effect of increasing the shoot multiplication factor in tissue culture plants. The effects of these substances on the shoot multiplication ability of the eucalyptus U16 line on MS* medium were investigated. The results are shown in Table 4.

Table 4: Effect of BAP on the newly micro shoots formation of the Eucalyptus U16 line

BAP concentration (mg/l)	Shoot multiplication coefficient	Characteristics of shoots growth
0.0	$1.74^{d} \pm 0.02$	Average
0.5	$1.89^{c} \pm 0.08$	Average
1.0	$1.99^{b} \pm 0.03$	Healthy
1.5	$2.21^{a} \pm 0.01$	Healthy
2.0	$1.76^{d} \pm 0.05$	Weak
CV%	2.0	
$LSD_{0.05}$	0.07	

The results in Table 4 show that the shoot multiplication coefficient reached 1.74 on the MS* medium without the BAP supplement. When the BAP concentration was increased to 0.5 mg/l, the shoot multiple coefficient increased to 1.89. The shoot multiplication coefficient was 1.99 on MS* medium supplemented with 1 mg/l BAP. The shoot multiplication coefficient reached the highest at 2.21 in the medium supplemented with

1.5 mg/l BAP with the characteristics of healthy shoots. When the BAP concentration increased to 2 mg/l, the shoot multiplication factor was only 1.76, and the shoot growth was weak. The MS* medium supplemented with BAP with 1.5 mg BAP/l gave the highest shoot multiplication coefficient (2.21 shoots) and healthy shoots. We used this medium supplemented with different concentrations of kinetin to find the best combinatorial formulation. The obtained results are shown in Table 5.

Table 5: Effect of BAP 1.5 mg/l and kinetin on shoot multiplication of *Eucalyptus* U16 line

Kinetin concentration (mg/l)	Shoot multiplication coefficient	Characteristics of shoots growth
0.0	$2.18^{b} \pm 0.07$	Healthy
0.5	$2.07^{c} \pm 0.09$	Healthy
1.0	$3.24^{a} \pm 0.05$	Healthy
1.5	$2.21^{b} \pm 0.05$	Average
2.0	$2.09^{c} \pm 0.03$	Average
CV%	1.77	
$LSD_{0.05}$	0.07	

The suitable medium for shoot multiplication was the MS medium supplemented with a combination of BAP 1.5 mg/l and kinetin 1 mg/l, the shoot multiplication coefficient reached 3.24 (Fig. 1b). When increasing the kinetin concentration to 1.5 g/l or 2 mg/l, the shoot multiplication coefficient reached 2.21 and 2.09, respectively. The shoot multiplication coefficient in this experiment was higher than that of some published studies [12-15].

A combination of the results from Tables 4 and 5, showed that rapid in vitro multiplication of the Eucalyptus U16 line was the best on MS* medium supplemented with BAP 1.5 mg/l and kinetin 1.0 mg/l, which expressed via shoot multiplication coefficient achieved the highest (3.24 buds).

3.3. Rooting of in vitro Eucalyptus U16 line

NAA is one of the PGRs belonging to the Auxin group that has stimulating rooting of in vitro shoots. We used different concentrations of NAA added to MS medium to evaluate the rooting ability of the eucalyptus U16 line. The obtained results are shown in Table 6.

Table 6: Effect of NAA on rooting of Eucalyptus U16 line

Conc. of NAA (mg/L)	Rooting rate (%)	No roots/shoot	The length of roots (mm)	Root growth ability
0.0 (Control)	$31.69^{e} \pm 1,53$	$6.2^{b} \pm 0,5$	$15.5^{b} \pm 1,4$	Weak
0.5	$66.00^{c} \pm 1.50$	$6.5^{b} \pm 0.5$	$16.8^{b} \pm 1.0$	Average
1.0	$90.49^{a} \pm 3.33$	$8.3^{a} \pm 0.7$	$19.6^{a} \pm 1.2$	Healthy
1.5	$72.87^{b} \pm 2.54$	$9.2^{a} \pm 0.5$	$20.6^{a} \pm 1.0$	Healthy
2.0	$51.04^{d} \pm 2.42$	$8.9^{a} \pm 0.6$	$21.2^{a} \pm 1.2$	Average
CV%	3.79	7.5	6.4	
$LSD_{0.05}$	4.30	1.06	2.2	

In the MS medium without NAA added, *Eucalyptus* still took root with the rate of 31.69%. When using medium supplemented with 0.5 mg/l NAA, the rooting rate reached 66.0%. In the MS medium supplemented with 1 mg/l NAA, the rooting rate was 90.49%, the average number of roots/buds was 8.3 roots, the length was

19.6 mm (Fig. 1c).

When increasing the concentration of NAA to 1.5 and 2 mg/l, the rooting rate reached 72.9% and 51.0%, respectively; Root length ranges from 20.6 - 21.2 mm. In CT4 and CT5 due to the high concentration of NAA, it will inhibit the rooting process in plants in general and eucalyptus in particular. The results of the above experiment are higher than those of previous study, when eucalyptus H7 cultured on MS medium supplemented with 1.5 mg/l IBA and 0.1 mg/l ABT, the rooting rate was approximately 82.2% [12]. In our opinion, the results are different due to 2 factors: variety and rooting culture medium of the two studies using different.

4. Conclusions

- The sterilization process reveals that a combination of 10% Javen solution treatment for 15 minutes, followed by 0.1% $HgCl_2$ treatment for 10 minutes, leads to the most effective sterilization. The rate of non-infection explants was 46.5%.

- The modified MS medium (MS* medium) was basal MS supplemented with 20 ml/L coconut water was the most suitable for the rapid multiplication of *Eucalyptus* U16 line, the shoot multiplication coefficient reached 1.56. *In vitro* multiplication of the *Eucalyptus* U16 on the modified MS medium added BAP 1.5 mg/L and kinetin 1.0 mg/L had the highest shoot multiplication coefficient of 3.24 shoots.

- *In vitro* rooting medium for the *Eucalyptus* U16 line was basal MS supplemented with 1.0 mg NAA/L, the rooting rate reached 90.49%, the number of roots/shoots was 8.3 roots, *in vitro* plants showed strong growth.

Declaration of Competing Interest

The authors declare no competing interests.

Author contributions

"Media preparation and sample sterilization experiments: Ngoc Quynh-Tien Vien; writing original draft: Tien Vien-Thu Hien; review and editing: Tien Vien-Thu Hien; All authors have read and agreed to the published version of the manuscript."

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Additional notes; Appendix

References

[1]. Dhakad, A.K., V.V. Pandey, S. Beg, J.M. Rawat, and A. Singh. Biological, medicinal and toxicological significance of Eucalyptus leaf essential oil: a review. J Sci Food Agric (2018), 98 (3): 833-848.

[2]. Zamaloa, M.C. and M.A. Gandolfo. 52 million years old Eucalyptus flower sheds more than pollen grains. American Journal of Botany (2020) 107 (12): 1763-1771.

[3]. Sebei, K., F. Sakouhi, W. Herchi, M.L. Khouja, and S. Boukhchina. Chemical composition and antibacterial activities of seven Eucalyptus species essential oils leaves. Biol Res (2015), 48 (1): 7.

[4]. Madejón, P., T. Marañón, C.M. Navarro-Fernández, M.T. Domínguez, J.M. Alegre, B. Robinson, and J.M. Murillo. Potential of Eucalyptus camaldulensis for phytostabilization and biomonitoring of trace-element contaminated soils. PLoS One (2017), 12 (6): e0180240.

[5]. Mieres-Castro, D. and S. Ahmar. Antiviral Activities of Eucalyptus Essential Oils: Their Effectiveness as Therapeutic Targets against Human Viruses (2021). 14 (12):

[6]. Cuong, T., T.T. Chinh, Y. Zhang, and Y. Xie *Economic Performance of Forest Plantations in Vietnam: Eucalyptus, Acacia mangium, and Manglietia conifera*. Forests, 2020. **11**, DOI: 10.3390/f11030284.

[7]. Manh, H.D., D.T. Hue, N.T.T. Hieu, D.T.T. Tuyen, and O.T. Tuyet. The Mosquito Larvicidal Activity of Essential Oils from Cymbopogon and Eucalyptus Species in Vietnam. Insects (2020), 11 (2):

[8]. Ma, C., R. Deepika, A.A. Myburg, M. Ranik, and S.H. Strauss, *Development of Eucalyptus tissue culture conditions for improved in vitro plant health and transformability*. BMC Proc. 2011 Sep 13;5(Suppl 7):P153. doi: 10.1186/1753-6561-5-S7-P153. eCollection 2011.

[9]. Le Roux, J.J. and J.V. Staden. Micropropagation and tissue culture of Eucalyptus-a review. Tree Physiol (1991), 9 (4): 435-77.

[10]. Aumond, M.L., Jr., A.T. de Araujo, Jr., C.F. de Oliveira Junkes, M.R. de Almeida, H.N. Matsuura, F. de Costa, and A.G. Fett-Neto. Events Associated with Early Age-Related Decline in Adventitious Rooting Competence of Eucalyptus globulus Labill. Front Plant Sci (2017), 8 1734.

[11]. Zhang, Y., J. Li, C. Li, S. Chen, Q. Tang, Y. Xiao, L. Zhong, Y. Chen, and B. Chen. Gene expression programs during callus development in tissue culture of two Eucalyptus species. BMC Plant Biol (2022), 22 (1): 1.

[12]. Triệu Thị Thu Hà, C.T.L.. Nghiên cứu nhân giống bạch đàn lai UP (Eucalyptus urophylla x Eucalyptus pellita) bằng phương pháp nuôi cấy mô tế bào. Tạp chí Nông nghiệp và Phát triển nông thôn (2015), 6 124-130.

[13]. Đặng Ngọc Hùng, L.Đ.K.. Nhân giống dòng Bạch đàn lai UE35 và UE56 bằng phương pháp nuôi cấy mô tế bào. Tạp chí Khoa học và Công nghệ (2013), 108 (8): 47-55.

[14]. Nguyễn Thị Hường, N.V.V.. Xây dựng hệ thống tái sinh bạch đàn Uro (Eucalyptus urophylla S.T. Blake) từ mô sẹo phục vụ chọn dòng tế bào. Tạp chí Khoa học và Công nghệ Lâm nghiệp (2017), 10 26-33.

[15]. Trần Thị Lệ, H.T.T.G.. Nghiên cứu nhân giống bạch đàn U6 (Eucalyptus urophylla) bằng phương pháp nuôi cấy mô. Tạp chí Nông nghiệp và phát triển nông thôn (2012), 3 132-139.

[16]. Murashige, T. and F. Skoog. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum (2006), 15 473-497.