GENETIC TRANSFORMATION AND REGENERATION OF TRANSGENIC PLANTS FROM COTYLEDON OF *EUCALYPTUS UROPHYLLA* VIA *AGROBACTERIUM*

Bui Van Thang

Vietnam National University of Forestry

SUMMARY

A protocol of the *Agrobacterium* - mediated transformation of *Eucalyptus urophylla* cotyledon was successfully developed. The cotyledon explants from *in vitro* 7 day old seedlings were precultured for three days in dark on the induction medium MS* (reduced 1/3 total nitrogen content) supplemented with 1.0 mg/l benzylaminopurine (BAP), 0.5 mg/l naphthalene acetic acid (NAA), 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel, and 50 µM acetosyringone (AS). Those cotyledon explants were infected with *Agrobacterium tumefaciens C58* strain that harbored the binary vector pBI21 carrying *uid*A and *neomycin phosphotransferase II (npt*II*)* genes for ten minutes, then co-cultured on the MS with 100 µM AS for 72 hours in dark. Subsequently, the infected cotyledon explants were washed off the *Agrobacterium*, and transferred to the MS supplemented with 400 mg/l cefotaxime (without kananamycin) for four days of culture. In the next step, those cotyledon explants were transferred to the selective MS supplemented with 400 mg/l cefotaxime and 150 mg/l kanamycin for three weeks of culture. After three passages on the selective medium, the kanamycin-resisted shoots were transferred to the rooting MS** (reduced 1/2 total nitrogen content) supplemented with 0.3 mg/l NAA, 0,2 mg/l Indolebutyric acid (IBA), 20 g/l sucrose, 8 g/l agar, 300 mg/l cefotaxime, and 75 mg/l kanamycin; the shoots were rooted after two week of culture. The rooted shoots (plantlets) on the selective medium which were transformed were detected by histochemical GUS assay and PCR with specific primers. Six plantlets of the 162 co-cultured explants were successfully genetically transformed. The frequency of genetic transformation using markers *uidA* and *npt*II for *Eucalyptus urophylla* was 3.7%. The developed protocol can be applied in successful transformation of the genes of interest into *Eucalyptus urophylla.*

Keywords: *Agrobacterium tumefaciens, Eucalyptus urophylla*, genetic transformation, regeneration.

I. INTRODUCTION

Eucalyptus are important hardwood trees. The estimated plantation area covered by eucalyptus is 20 million hectares worldwide (GIT Forestry, 2008). The genus *Eucalyptus* is comprised of more than 700 species and hybrids, some of which bear the economic importance as a source of paper pulp, wood, firewood, mining timber, and essential oils (Eldridge et al., 1994). Because of the economic importance of *Eucalyptus*, great investments were made in breeding programs and in the development of vegetative propagation techniques. However, there are other techniques, such as genetic transformation, that can be incorporated in a genetic breeding program in order to increase the yields, shorten the production cycles and promote the development of high quality plantations (Diouf, 2003). The genetic improvement of plants through transgenic technology enables the introduction of specific traits of interest into a desirable genotype. Also, the genetic modification based on transgenic technology enables the transfer of selected genes across genera and kingdoms. Furthermore, the transfer of selected genes in a single generation through transgenic technology is especially important for *Eucalyptus*, as its improvement by conventional breeding approach is limited by long breeding cycles, high levels of heterozygosity and incompatibility barriers (Machado et al., 1997).

For successful introduction of desirable traits, an efficient regeneration protocol as well as a gene delivery system needs to be developed by *Agrobacterium* mediated gene transfer method. Teulières et al., (1991) observed the transient expression of reporter genes in polyethylene-glycol-treated or electroporated *Eucalyptus gunnii* protoplasts.

Chirqui et al., (1991) demonstrated the susceptibility of *E. gunnii* and *E. globules* to *Agrobacterium*. Adam et al., (1992) obtained the expression of *uid*A gene from neoformed meristems arising from cotyledons after the transformation via *Agrobacterium*. Successful transformation of *E. camaldulensis* was demonstrated by Ho et al., (1998) using *A. tumefaciens*. Sarrano et al., (1996) obtained the stable transformation of *E. globules* using the biolistic gene delivery system. *Agrobacterium tumefaciens*-mediated transformation is preferred to biolistic procedures for long living tree species as it does not produce chimeric plants and the insertion of large number of gene copies, which may lead to gene silencing (Mullins et al., 1997).

Eucalyptus urophylla is the most economically important and productive forest crop in tropical regions. In Vietnam, *E. urophylla* is widely used for raising plantations because of its economic importance, such as providing wood and raw material for paper pulp. The development of genetic transformation and plant regeneration of *Eucalyptus urophylla*, which is an economically important pulp wood tree, allows the introduction of beneficial properties to this multipurpose tree species. However, in order to accomplish this objective and other genetic engineering ones, an efficient gene delivery and the subsequent regeneration of transformed plants need to be solved for this generally recalcitrant *Eucalyptus* species. This study aims to establish the procedure for transformation and regeneration of *Eucalyptus urophylla* cotyledon using *Agrobacterium tumefaciens*.

II. MATERIALS AND METHODS

Plant materials: The seeds of *Eucalyptus urophylla* were harvested from trees selected by Vietnamese Academy of Forest Sciences. The seeds were surface-disinfected with ethanol 70% for two minutes, then in sodium hypochlorite 8% for seven minutes, and washed thoroughly with sterilized distilled water for five times. Fifty seeds per plate were germinated aseptically in 6 cm diameter × 9 cm high Petri dishes that contained 20 ml of seed germination MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l kinetin, 0.2 mg/l 1-Naphthaleneacetic acid (NAA), 30 g/l sucrose, and 7 g/l agar. The seeds and all *in vitro* plant materials were incubated at 25 ± 2°C under a 16-hour photoperiod produced by cool white fluorescent lamps with an intensity of 30 $\mu Em^{-2} s^{-1}$.

Determination of antibiotic sensitivity, infection, and co-cultivation: In order to determine the appropriate concentration for selection of transgenic plants, the cotyledon explants were cultured on six concentrations of kanamycin (10, 50, 75, 100, 150, and 200 mg/l) on the regeneration MS medium (MS*, reduced 1/3 total nitrogen content) complemented with 1.0 mg/l 6-benzylaminopurine (BAP), 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, and 2.0 g/l phytagel. *Agrobacterium tumefaciens* C58 strain that harbors a binary vector pBI121 containing *uid*A and *npt*II genes was used for the transformation. The bacteria which were collected at late log phase were pelleted and resuspended in ½ MS basal medium (OD_{600} = 0.5). The cotyledon explants from 7 day old seedlings were separated and used as explants for the transformation experiments. The explants were pre-cultured on the MS* regeneration medium supplemented with 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel, and 50 µM acetosyringone (AS) for three days in dark. The pre-cultured cotyledon explants were gently shaken in the bacterial suspension for 10 minutes and blotted dry on a sterile filter paper. Subsequently, they were transferred to

the MS* co-cultivation medium supplemented with 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel, and 100 µM AS; and co-cultivated under conditions similar to the pre-culture period for three days in dark.

Selection of transformed shoots and rooting of transgenic plants: After co-cultivation time, the explants were washed in the ½ MS liquid medium containing 400 mg/l cefotaxime, blotted dry on a sterile filter paper, and transferred to MS* regeneration medium that contained 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel supplemented with 400 mg/l cefotaxime, and cultured for 4 days under a 16-hour photoperiod produced by white fluorescent lamps, and then the explants were cultured on the MS* selective regeneration medium supplemented with 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel, 150 mg/l kanamycin, and 400 mg/l cefotaxime. After 4 weeks of culture, the shoots which were regenerated from the explants were transferred to new selective medium (MS* medium supplemented with 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel, 200 mg/l kanamycin, and 400 mg/l cefotaxime). The elongated shoots (≥ 1.5 cm) were rooted in the MS (MS**, reduced 1/2 total nitrogen content) medium supplemented with 0.3 mg/l NAA, 0.2 mg/l Indolebutyric acid (IBA), 20 g/l sucrose, 8 g/l agar, 75 mg/l kanamycin, and 300 mg/l cefotaxime.

Histochemical GUS assay: β-glucuronidase *(*GUS) assay was carried out using kanamycin-resisted shoots and freshly infected cotyledon explants after three days of co-culture in order to score transient expression based on the method of Jefferson et al. (1987). Tissues were incubated overnight at 37^{o}C in 100 mM sodium phosphate buffer (pH 7.0) that contained 1 mM X-Gluc, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. Subsequently, tissue was cleared to remove chlorophyll by washing several times with 70% ethanol and then slowly increased to absolute ethanol. The tissue with blue colour was scored.

Molecular analysis: Total genomic DNA from transformed and untransformed control plants were isolated using Plant/Fungi DNA Isolation Kit (Norgen – Canada). The presence of transgenes *uid*A was validated using forward primer uF1: 5′-TTC GCG TCG GCA TCC GCT CAG TGG CA-3′ and reverse primer uR1: 5′- GCG GAC GGG TAT CCG GTT CGT TGG CA-3′, which were designed to specifically amplify 530 bp of *uid*A gene (Prakash and Gurumurthi 2009). The presence of gene *npt*II was validated using forward primer nF1: 5'-GAG GCT ATT CGG CTA TGA CTG-3' and reverse primer nR1: 5'-ATC GGG AGC GGC GAT ACC GTA-3', which were designed to specifically amplify 700 bp of *npt*II gene. Vector pBI121 containing *uid*A and *npt*II genes was used as the positive control whereas DNA genome from the untransformed plants was used as the negative control. PCR reactions were carried out in 25 µl volume containing 20 ng of template DNA, 0.2 mM dNTPs, 1 µM of each gene-specific primer, and 2 unit of *Taq* DNA polymerase. PCR was carried out in a thermal cycler with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C denaturing for 1 min, 62°C annealing for 30 seconds and 72°C extension for 1 min, and 10 min final extension at 72°C for *npt*II gene detection. The PCR for *uid*A gene detection was 94°C initial denaturing for 5 min, followed by 35 cycles of 94°C denaturing for 1 min, 58°C annealing for 1 min and 72°C for 1 min, and 10 min at 72°C final extension. PCR product was electrophoresed on 1.2% agarose gel and then, detected by redsafe nucleic acid staining solution.

III. RESULTS

3.1. Determination of antibiotic sensitivity

The kanamycin sensitivity of *Eucalyptus* cotyledons was determined by culturing the cotyledon explants on MS medium that contained 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, and 2.0 g/l phytagel supplemented with six concentrations of kanamycin, including 10, 50, 75, 100, 150, and 200 mg/l. After four weeks of culture on the medium with 150 mg/l kanamycin and two weeks of culture on the medium with 200 mg/l kanamycin, chlorosis and eventual necrosis were observed in all explants (100%). Because the regeneration of our explants was completely inhibited at 150 mg/l kanamycin after four weeks of culture (figure 1), this concentration of kanamycin was used for the selection of genetic shoots in this study.

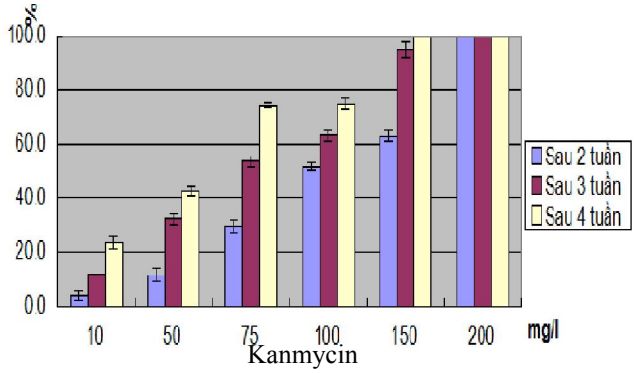

Figure 1. Kanamycin sensitivity of shoot regeneration in E. urophylla

(The cotyledon explants were cultured on MS* regeneration medium that contained 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, and 2.0 g/l phytagel supplemented with six concentrations of kanamycin (10, 50, 75, 150, and 200 mg/l). The lethality rate of explants was calculated after 2, 3, and 4 weeks of regeneration)

3.2. Genetic transformation and regeneration of transgenic plants

Prior to co-cultivation, the cotyledon explants were precultured for three days in the regeneration medium. Subsequently, healthy cotyledon explants were infected and co-cultivated with *A. tumefaciens* C58 strain that harbored a binary vector pBI121 containing *uid*A and *npt*II genes. It was found that when the cotyledons were pre-cultured on the regeneration medium for three days, the hypersensitivity response was reduced in comparison with the co-cultivation with *Agrobacterium* without preculture. During the initial four days in the non-selective regeneration medium, all co-cultivated and control explant materials maintained a healthy green color. After transferring to the selective medium with 150 mg/l kanamycin and 400 mg/l cefotaxime, control cotyledons (non – transformed) and some of the co-cultivated cotyledon explants became completely necrotic within three to four weeks. However, control cotyledon explants maintained on non-selective medium (without kanamycin) exhibited regeneration by the end of the fourth week of culture.

Shoots were formed directly from the cut end of the infected cotyledons after four weeks. The explants with shoots emerging from the edges were subcultured on the new selective medium for elongation of shoots. The transformed shoots, which attained more than 1.5 cm in length, were excised and then transferred to the MS** medium that was supplemented with 0.3 mg/l NAA, 0.2 mg/l IBA, 20 g/l sucrose, 8 g/l agar, 75 mg/l kanamycin, and 300 mg/l cefotaxime for

rooting. Shoots were rooted after two weeks of culture (Figure 2), and then the plantlets which were generated from these shoots were transplanted in greenhouse. The average transformation frequency into cotyledons of *Eucalyptus urophylla* was 3.7% (table 1).

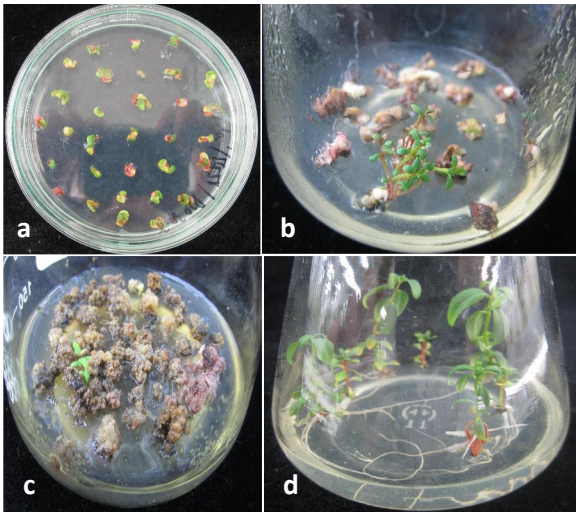

Figure 2. Regeneration of transgenic shoots and transgenic plantlets on selective medium
(a - cotyledons were infected and co-cultivated with *A. tumefaciens* harboring pBI121; b and c – shoots were regenerated from the transformed cotyledon explants on selective medium containing 150 mg/l kanamycin; d - The transformed shoots were rooted in selective medium containing 75 mg/l kanamycin)

Table 1. Transformation frequency of *E. urophylla* from pre-cultured cotyledon explants on MS* selective regeneration medium that contained 1.0 mg/l BAP, 0.5 mg/l NAA, 100 ml/l coconut water, 20 g/l sucrose, and 2.0 g/l phytagel supplemented with 150 mg/l kanamycin, and 400 mg/l cefotaxime

Experiment	Number of explants co-cultured	Number of regenerated shoots	Number of shoots responded (GUS^+)	Transformation frequency (%)
Expt 1	47	1	1	2.1
Expt 2	55	5	3	5.5
Expt 3	60	6	2	3.3
Total	*162*	*12*	*6*	*3.7*

3.3. Validation of transgenic plants

Histochemical staining of putatively transgenic lines which were resistant to kanamycin showed the expression of *uid*A gene (figure 3). The presence of the gene of interest was further confirmed by PCR analysis of total genomic DNA which was isolated from the leaf of transformed and un-transformed (control) plants using primers that were designed to amplify *uid*A and *npt*II genes as described in the "Materials and Methods" section.

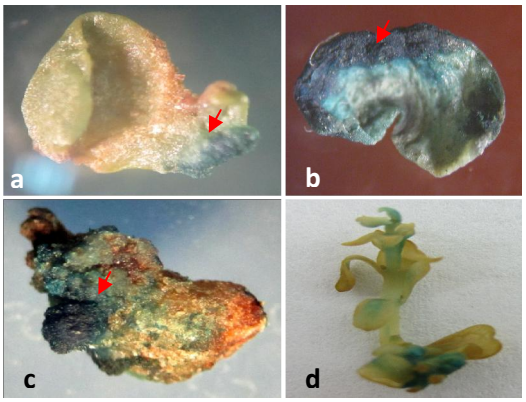

Figure 3. Cotyledon explants and shoot of transgenic plant showing GUS activity
(a, b, and c - cotyledon explants;
d – shoot of transgenic plant)

Six GUS-positive lines and one non-transgenic line (wild type) were used as the negative controls whereas the vector pBI121 containing *uid*A and *npt*II genes was used as the positive control. The 530 bp *uid*A and 700 bp *npt*II DNA fragments were amplified from the genomic DNA of all transgenic plants and the vector pBI121 containing *uid*A and *npt*II genes whereas the corresponding bands were not detected in the un-transformed control plants (figure 4).

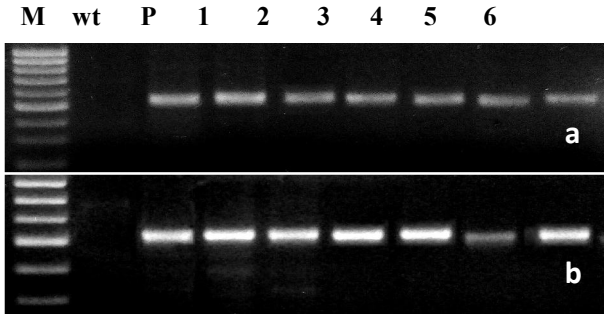

Figure 4. PCR analysis of genomic DNA isolated from transgenic and untransformed Ecalyptus urophylla plants by amplification of the uidA and nptII genes

(a- detection of the uidA gene; b - detection of the nptII gene; M - DNA marker 100 bp; wt: untransformed plant; P: plasmid pBI121 (positive control); 1 – 6: transformed plant lines)

DISCUSSION

Agrobacterium mediated transformation has been studied in different parts of *Eucalyptus*, for example, hypocotyls and cotyledons (Ho et al., 1998; MacRae and Van Staden, 1999; Prakash and Gurumurthi, 2009). In our study, the transgenic plantlets of *E. urophylla* were generated in which the cotyledons were used as the gene recipients. The result showed that the cotyledons from in vitro seedlings which were pre-cultured for three days before co-cultivation with *A. tumefaciens* reduced the hypersensitive response and increased the transformation efficiency. Moralejo et al., (1998) also found that the transgenic *E. globules* was obtained by using mature seeds as the gene recipients. The transformation frequency increased due to pre-culturing the explants. According to Prakash and Gurumurthi (2009), transgenic *E. tereticornis* were generated using hypocotyls and cotyledons which were pre-cultured for two days before co-cultivation with *Agrobacterium* that harbors a binary vector pBI121 containing *uid*A and *npt*II genes. The average transformation frequency of cotyledons and hypocotyls were 21.29% and 14.43%, respectively.

Kanamycin is seen as an effectively selective marker. Before establishing the procedure of transformation in which kanamycin resistance gene is employed, kanamycin sensitivity of the plant material should be determined (James et al., 1990). Different concentrations of kanamycin have been used to select several transgenic woody species, including *Leucaena leucocephala* (Jube and Borthakur, 2009), *Quercus suber* (Alvarez and Ordas, 2007), and *Melia*

azedarach (Bui Van Thang et al., 2013). After transforming the gene of interest into hypocotyl of *E. camaldulensis* via *Agrobacterium tumefaciens*, Ho et al., (1998) used 40 mg/l of kanamycin for the selection of the transformed plants. The same concentration of kanamycin was employed by Prakash and Gurumurthi (2009) for the selection of the transformants of *E. tereticornis*. Mullins et al., (1997) reported that 9 mg/l was enough for selection of select *E. camaldulensis* transgenic plants. In the current study, 150 mg/l kanamycin was used to select *E. urophylla* transgenic plants. According to Draper et al., (1988), roots are more sensitive to antibiotics and the root ability on selective medium could possibly avoid the shoots, which escaped from previous selection. In this study, the elongated shoots were transferred to rooting selective medium with 75 mg/l kanamycin and further confirmed the putative transgenic plants by GUS staining and PCR analysis with the specific primers for *uid*A and *npt*II genes.

IV. CONCLUSION

This study demonstrated that pre-culture of cotyledons *in vitro* seedlings was an important step for generating transgenic plants of *E. urophylla* using *Agrobacterium tumefaciens*-mediated transformation technique. Also, the transformation protocol which was developed by this study can be used effectively for the transformation of economically important desirable genes into *E. urophylla* which is quite difficult for gene transformation.

Acknowledgements

This study was implemented by the financial support of research project "Creation of transgenic *Eucalyptus urophylla* to improve growth rate" (Agriculture Biotechnology Program, Ministry of Agriculture and Rural Development, Vietnam).

REFERENCES

1. Adam S, Chirqui D, Caissard DJC (1992). Transformation genetique de bougeous transgenques chez *Eucalyptus gunnii*. AFOCEL (1991) Complement des annals ISSN 0398-494. Director of Publication J Sturn. 81–110.

2. Alvarez R, Ordas RJ (2007). Improved genetic transformation protocol for cork oak (*Quercus suber* L.). Plant Cell Tissue Organ Culture 91(1): 45–52.

3. Bui Van Thang, Do Xuan Dong, Le Van Son, Chu Hoang Ha (2013). The high frequency of transformation procedure for expression of GUS gene in chinaberry tree (*Melia azedarach* L.) using *agrobacterium. Vietnam Academy of Science and Technology,* Journal of Biology 35(2): 227 – 233.

4. Chirqui D, Adam S, Caissard JC, Noin M, and Azmi A (1991). Shoot regeneration and *Agrobacterium* mediated transformation of *Eucalyptus globules* and *E. gunnii*. In: Schsnau APG (ed) The role of *Eucalyptus*, IUFRO Symposium on Intensive Forestry. Pretoria, South Africa: Southern African Institute of Forestry. 70–80.

5. Diouf D (2003). Genetic transformation of forest trees. African Journal of Biotechnology 2(10): 328-333.

6. Diwakar A, Anil K, Reddy Sudhakara M (2011) *Agrobacterium* tumefaciens mediated genetic transformation of selected elite clone(s) of *Eucalyptus tereticornis*. Acta Physiol Plant 33:1603–1611.

7. Draper J, Scott R, Hamil J (1988). Plant genetic transformation and gene expression: A Laboratory Manual. Blackwell Science Inc, Oxford, 103.

8. Eldridge K, Davidson C, Harwood C, Van Wyk G (1994). *Eucalyptus* domestication and breeding. New York: Oxford University Press.

CHUYỂN GEN VÀ TÁI SINH CÂY CHUYỂN GEN TỪ LÁ MẦM BẠCH ĐÀN NÂU (*EUCALYPTUS UROPHYLLA*) THÔNG QUA *AGROBACTERIUM*

Bùi Văn Thắng

Trường Đại học Lâm nghiệp

TÓM TẮT

Quy trình chuyển gen vào cây Bach đàn nâu (*Ecalyptus urophylla*) sử dụng mảnh lá mầm thông qua vi khuẩn *Agrobacterium tumefaciens* đã được nghiên cứu hoàn chỉnh. Các mảnh lá mầm của cây hạt 7 ngày tuổi in vitro được tiền nuôi cấy cảm ứng trên môi trường tái sinh MS* (giảm 1/3 nitơ tổng số) bổ sung 1,0 mg/l benzylaminopurine (BAP), 0,5 mg/l naphthalene acetic acid (NAA), 100 ml/l coconut water, 20 g/l sucrose, 2.0 g/l phytagel và 50 µM acetosyringone (AS), 3 ngày trong tối. Các mảnh lá mầm được nhiễm *Agrobacterium* trong thời gian 10 phút và đồng nuôi cấy trên môi trường tái sinh có 100 µM AS, 72 giờ trong tối. Sau thời gian đồng nuôi cấy, rửa khuẩn và nuôi cấy mẫu trên môi trường tái sinh bổ sung kháng sinh diệt khuẩn 400 mg/l cefotaxime, không bổ sung kanamycin, nuôi trong 4 ngày; sau đó, chuyển mẫu sang môi trường tái sinh chọn học bổ sung kháng sinh 150 mg/l kanamycin và 400 mg/l cefotaxime, nuôi trong 3 tuần cấy chuyển sang môi trường chọn lọc mới. Các chồi tái sinh và sống sót sau 3 lần sàng lọc, được cấy chuyển sang môi trường ra rễ chọn lọc MS** (giảm 1/2 nitơ tổng số) bổ sung 0,3 mg/l NAA, 0,2 mg/l Indolebutyric acid (IBA), 20 g/l sucrose, 8 g/l agar, 300 mg/l cefotaxime và 75 mg/l kanamycin, chồi ra rễ sau 2 tuần nuôi. Các chồi ra rễ trên môi trường chọn lọc được được xác định là chuyển gen bằng nhuộm GUS và PCR với cặp mồi đặc hiệu. Kết quả thu được 6 chồi chuyển gen trên 162 mẫu biến nạp gen. Hiệu suất chuyển gen chỉ thị (gen *uid*A và *npt*II) vào Bạch đàn nâu là 3,7%. Quy trình này có thể được áp dụng để chuyển thành công các gen có giá trị vào Bạch đàn nâu, cải thiện giống.

Từ khóa: *Agrobacterium tumefaciens,* chuyển gen, *Ecalyptus urophylla*, tái sinh chồi.

Reviewer : Dr. Dao Quoc Hung
Received : 11/7/2016
Revised : 20/8/2016
Accepted : 20/9/2016