Research paper

Micropropagation of *Quercus aliena* Blume var. *aliena* from Explants of Mature Trees

Yue-Ken Liao,[1,2] Miao-Ching Chuang[1]

【Summary】

This study established a micropropagation method for the tissue culture propagation of mature *Quercus aliena* Blume var. *aliena*. The method was modified from procedures successfully applied to other species of the same genus, and it was tested for its efficiency on mature explants of the target species. Stem nodal segments collected from trunk sprouts were used as the original explants. The newly induced axillary shoots (≥ 1.5 cm long) from the explants were defoliated, decapitated, and horizontally cultured on GD_1 medium supplemented with 0.88 μM 6-benzylaminopurine (BAP) and various concentrations of $AgNO_3$. Axillary shoot proliferation was obtained through an 8-wk cyclic culture in which the concentration of BAP gradually decreased. Decapitation and horizontal culture of explants together with the addition of 17.67 μM $AgNO_3$ proved beneficial for shoot proliferation and elongation; however, the efficiency of this method diminished when activated charcoal was added. The elongated shoots were rooted with indole-3-butyric acid (IBA, 123 μM) pulse stimulation for 48 h, after which the shoots were transferred to IBA-free root expression medium with 17.67 μM $AgNO_3$, and they achieved a better rooting percentage (87.5%). Rooted plantlets were acclimatized and successfully grown in a greenhouse, with a survival rate of 72.3%.

Key words: activated charcoal, micropropagation, *Quercus aliena* Blume var. *aliena*, silver nitrate.

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研究報告

以成熟林木培殖體進行槲櫟微體繁殖

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摘 要

本研究建立槲櫟(*Quercus aliena* Blume var. *aliena*)成熟林木組織培養微體繁殖之流程，採用並修改在同屬樹種中已經成功應用之培養條件，測試其在槲櫟之適用效果。微體繁殖的步驟以收集樹幹基部萌芽之節莖為培殖體，切取新誘導形成之側芽(長度≥ 1.5 cm)、去葉及截頂後橫放培養於GD_1培養基中並添加0.88 μM 6-benzylaminopurine (BAP)及不同濃度的硝酸銀，以8週為一培養週期並逐次遞減BAP濃度，可促進側芽的誘導及增殖。試驗中截頂之培殖體水平橫放培養及添加17.67 μM的硝酸銀可促進芽體增殖及抽長，若添加活性碳則否。抽長芽體利用123.0 μM indole-3-butyric acid (IBA)進行48 h的發根誘導，接著將誘導後之芽體培養在不含IBA，但添加17.67 μM硝酸銀的培養基中，有較佳發根率(87.5%)。發根芽體移盆健化時存活率為72.3%，並有多數小植株已能在溫室中正常生長。

關鍵詞：活性碳、微體繁殖、槲櫟、硝酸銀。

廖宇賡、莊妙菁。2014。以成熟林木培殖體進行槲櫟微體繁殖。台灣林業科學29(2):117-31。

INTRODUCTION

Quercus aliena Blume var. *aliena* is a shade-intolerant tree species of the Fagaceae family. Its natural habitat is primarily in temperate deciduous forests of Northeast Asia. However, in addition to China, Japan, and Korea, the species also grows widely in various parts of Southeast Asia, such as Thailand, Laos, Burma, and India (Phengklai 2006). An examination of habitats where it grows shows that the most favorable environment for growing this species appears to be either at high latitudes that are low in elevation or vice versa (K.-C. Yang, personal communication). In outdoor field conditions in Taiwan, the species only has a limited population (100~200 individuals) on the windward slopes of Niu-Kou-Lin (24°52’40”N, 120°58’12”E) in Hsinchu County (at an elevation of about 100~120 m) (Su et al. 2003), which notably disobeys the distribution rule of “low latitude, high elevation” expected for this species. Yang (2003) mentioned this unusual circumstance and recommended further studies of this species.

The population of *Q. aliena* var. *aliena* in Taiwan was classified as aged, declining, and relict-distributed (Cheng 2009). This isolated and vulnerable population has already reached stand maturity and is now under a high level of competitive stress due to other broadleaf trees. These features weaken the population, resulting in thin crowns and crown dieback with fungus-infected damage. Natural regeneration through seed germination rarely occurs due to the dense canopy coverage of neighboring competitive trees, such as *Schefflera octophylla* (Lour.) Harms and *Acacia confusa* Merr. (Liao and Chao, unpublished data). Natural regeneration is also affected by highly unstable annual seed production (commonly known as intervals of abundant and lean years) exhibited by this genus (Koenig et al. 1994).

Based on the abovementioned facts, *Q. aliena* var. *aliena* in Taiwan is now in serious danger of becoming extinct. The unusual distribution within a limited habitat, the small, unhealthy population, poor seedling establishment, disease infection, and low competition in the field all make it particularly difficult to conserve the species or effectively enlarge its population *in situ*. Therefore, a simple vegetative propagation method is urgently needed to quickly establish planting stocks for *ex situ* conservation and preserve these genetic resources.

Fortunately, since many species in the *Quercus* genus are commercially important, well-developed vegetative propagation methods have been documented over the past 30 yr (Chalupa 1993, Vieitez et al. 2012). Propagation through rooted cuttings is preferable in young plants (Morgan and McWilliams 1976); however, this approach is not often used with aged plants due to their rapid loss of rooting capability (Morgan et al. 1980, Eshed et al. 1996). Axillary micropropagation and somatic embryogenesis are both workable tissue culture methods for *Quercus* spp. However, in previous studies, there was a low success rate when mature materials (explants) were used for somatic embryo initiation, thus limiting the efficiency and practicability of this method (Valladares et al. 2006, San-Jose et al. 2010). Conversely, axillary micropropagetion is relatively easy and adequate for producing propagules for *ex situ* planting.

The aim of this study was to establish a simple tissue culture method for the vegetative propagation of *Q. aliena* var. *aliena* in order to protect its genetic resources from disappearing, and produce enough planting stocks for *ex situ* conservation. We modified one of the micropropagation procedures successfully used by Vieitez et al. (2009) for several North American *Quercus* species and adapted the method to *Q. aliena* var. *aliena*. We report the results with respect to the induction and proliferation of axillary shoots from mature plant materials. We also examined the influence of medium supplements on adventitious rooting. Finally, evaluations of the adaptability of the modified method to *Q. aliena* var. *aliena* are given in this paper.

MATERIALS AND METHODS

Plant materials and sterilization

Epicormic shoots emerging from basal trunks of mature *Q. aliena* var. *aliena* were collected in 2012 from a wild population in Niu-Kou-Lin, Xinfeng Township, Hsinchu County, Taiwan. Actively growing shoots (about 30 cm long) were harvested, wrapped in moistened paper towels, securely sealed in a plastic bag, and transported in an ice bucket back to the laboratory. They were then defoliated and cut into short nodal segments of about 1~3 cm, with each segment including 1~3 nodes.

The nodal segments were first washed in diluted detergent (1~2 drops of commercial detergent in 200 mL of tap water) for 2~3 min and then soaked in 0.2% (v/v) Anti-A solution (China Chemical & Pharmaceutical, Hsinchu, Taiwan) for 10 min. They were then surface-disinfested in 20% (v/v) commercial bleach (Clorox®) for 3 min, after which they were soaked for 30 s in 70% (v/v) ethanol and 3 min in 0.1% (w/v) $HgCl_2$, both with regular shaking. Finally, they were rinsed with three 3-min washes of sterile double-distilled water.

Axillary shoot induction and proliferation

In order to induce initial axillary shoot formation, GD_1 medium (Gresshoff and Doy 1972) supplemented with 3% (w/v) sucrose and 0.75% (w/v) Difco Bacto agar was used

as the basal medium (pH 5.6). Sterilized nodal segments were individually incubated in a glass tube with 15 mL of basal medium containing 2.22 μM 6-benzylaminopurine (BAP) (Vieitez et al. 2009). In order to avoid damage caused by chemicals secreted by the explants, the nodal segment was removed from the medium and placed on the opposite side of the tube 1 d after being incubated. They were then subcultured in the same medium every 2 wk until axillary shoots emerged.

The initial axillary shoots (≥ 1.5 cm long) induced from the nodal segments were excised and defoliated. They were further cut and separated into apical shoot segments (cultured vertically) and basal stem segments (cultured horizontally). They were then incubated on shoot proliferation medium, which was GD_1 basal medium containing BAP with decreasing concentrations over an 8-wk culture period (according to Vieitez et al. (2009), with modifications). The time-frame for the decrease in BAP concentrations associated with subcultures was as follows: 0.88 μM (for 2 wk), 0.44 μM (for 2 wk), 0.44 μM (for 2 wk), and 0.44 μM (for 2 wk). At the 6th week (after completion of the 3rd subculture), the newly elongated axillary shoots (> 1.5 cm) from 2 different explants were recorded and harvested, either for rooting treatments or for the next 8-wk cycle of shoot proliferation. By the end of the 8th week, axillary shoot proliferation was determined by counting those > 1.5-cm (large) or ≤ 1.5-cm (small) shoots together, with previously harvested (large) shoots. The percentage of large-shoot formation, demonstrating the efficiency of producing usable shoots, was calculated as follows: (number of large shoots / total shoots proliferated) × 100%.

We investigated the influence of explant type/orientation (vertically cultured apical segment vs. horizontally cultured basal segment) and $AgNO_3$ (0, 8.84, 17.67, 26.51, and 35.34 μM) supplementation on axillary shoot proliferation and large-shoot formation during the entire culture period. The experiment was repeated twice, with each treatment containing 10 explants.

Axillary shoot elongation

In order to enhance shoot elongation, the experiment followed culture procedures previously described for shoot proliferation. However, in order to better observe possible differences in shoot growth, the time-frame of BAP application/subculture was extended to 10 wk as follow: 0.88μM (for 2 wk), 0.44 μM (for 2 wk), 0.44 μM (for 2 wk), 0.44 μM (for 2 wk), and 0.44 μM (for 2 wk). Only defoliated and decapitated basal stem segments were used and horizontally cultured on GD_1 basal medium containing the abovementioned BAP and 17.67 μM $AgNO_3$ (as determined in the shoot proliferation trial) throughout the 10-wk culture period.

After completion of the 3rd subculture, the elongated large shoots were either excised (as they were in the prior experiment) or all remained as a shoot cluster on the stem explants. The explants were then subcultured on the same medium with the addition of activated charcoal (AC) at 0, 2.5, and 5 g L^{-1} for the last culture period (of 4 wk). Axillary shoot production was counted at the end of this experiment, including what was collected at the end of the 6th wk, as previously described. The influence of the early removal of large dominant shoots, as well as the addition of AC, on axillary shoot elongation (exhibited as the percentage of large-shoot formation), was studied. The experiment was repeated twice, with each treatment containing 10 explants.

***In vitro* rooting**

Actively proliferating large axillary

shoots (1.5~2.0 cm) were used as explants in the rooting experiments. The GD_1 basal medium with a reduced concentration of macro-inorganic elements (1/3 fold), supplemented with IBA (73.8, 123.0, 172.2, and 221.4 μM), was used as the root induction medium. Shoots were incubated in this medium for 48 h and then transferred to the same medium without IBA (root expression medium) for 5 wk. The rooted shoots were defined as those in which at least 1 root (≥ 0.5 cm) had developed at the basal part of the shoot.

In order to further improve rooting performance, shoots were cultured in GD_1 root induction medium containing 123.0 μM IBA (as determined in the prior rooting experiment) for 48 h and then transferred to root expression medium supplemented with 4 g L^{-1} AC (Vieitez et al. 2009) or 17.67 μM $AgNO_3$ for 5 wk. The GD_1 root expression medium without those supplements was used as a control. The data collection process for successful rooting was the same as that described for the prior rooting experiment.

Both experiments were repeated twice, with each treatment containing 40 explants (10 explants in 1 culture box), which gave the rooting percentage data on a per box basis.

Culture conditions

In the initial axillary shoot induction trial, a 22×115-mm glass tube was used as a culture vessel, while a 90×25-mm sterile Petri dish was used for the shoot proliferation and elongation experiments. However, the last 2-wk subculture for shoot elongation and the root induction experiments were carried out in a 90×104-mm orchid culture box (type CK-B, Ching Ke Enterprise, Taoyuan, Taiwan). A 16/8-h light/dark photoperiod of cool-white fluorescent light mixed with incandescent light at 68 μmol m^{-2} s^{-1} was administered for all experiments. The temperature in the culture room was set to 21±1℃. An additional water-agar plate was placed on the culture dish so as to prevent water condensation inside the vessel.

Acclimatization

For acclimatization, the basal part of rooted shoots was thoroughly washed in distilled water so as to remove any agar residues, and then transplanted into potting medium (peat: vermiculite: perlite = 1: 1: 1 by volume). They were planted individually in plastic pots, which were 6.0 cm in height and had diameters of 7.5 cm (top) and 5.3 cm (bottom). Under coverage of a small glass beaker, they were stored for 30 d in an acclimatization room with a 16/8-h light/dark photoperiod of cool-white fluorescent light mixed with incandescent light at 40~50 μmol m^{-2} s^{-1} (stage 1). The room temperature was 23±1℃. Afterwards, they were moved to a partially shaded greenhouse for another 30 d (stage 2). During this stage, the plantlets were subjected to a reduced amount of moisture by the gradual opening of the beaker and regular watering. The beaker was completely removed at the end of stage 2, and rooted plantlets were monitored for another 20 d (stage 3). The survival rate was recorded during each acclimatization stage of the 3 repeated experiments, each of which included at least 44 rooted plantlets. The average survival rate is shown for each respective stage.

Statistical analysis

The data regarding axillary shoot production and the percentage of large-shoot formation from the proliferation and elongation experiments, which were obtained using a completely randomized factorial design (2×5 and 2×3, respectively), underwent a two-way analysis of variance (ANOVA) and then a least significant difference (LSD) test.

Treatments in the 2 rooting experiments were arranged in a completely randomized experimental design. Significant differences between treatment means were also determined by means of an LSD test. The percentage data for large-shoot formation and rooting performance were subjected to arcsine transformation prior to analysis.

RESULTS

Axillary shoot induction and proliferation

During initial axillary shoot induction, development of the basal callus was first noted during the 2nd week of incubation. Most of the nodal segments that survived the high contamination rate (77~97%) were responsive to BAP stimulation producing 1 or 2 axillary shoots per explant after approximately 4~5 wk in culture (Fig. 1).

In our preliminary experiment, in which $AgNO_3$ was not applied for shoot proliferation, not all of the horizontally cultured stem segments corresponded to the BAP treatment. In addition, vertically cultured apical segments frequently exhibited shoot-tip necrosis and became dark brown, which demonstrated an unsuitable culture condition in the culture vessel. However, when $AgNO_3$ was added to the medium, the explants showed active shoot proliferation and a great improvement in growth performance. During the first 2 wk in medium containing 0.88 μM BAP, none of the explants showed any significant difference in shoot development. It was not until completion of the 6th week of culture (on 0.44 μM BAP for 4 wk) that shoot proliferation reached a level of significance (Fig. 2). Although excision of the large shoots at this stage enhanced additional dark secretion accumulation in the medium (which in turn deactivated the explant vigor in some of the explants), small shoots from those unaffected explants demonstrated further elongation.

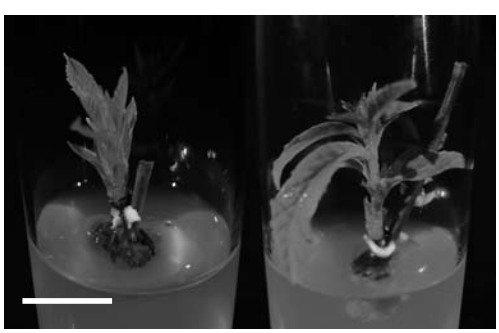

Fig. 1. Initial axillary shoot formation from nodal segments of *Quercus aliena* var. *aliena* cultured on GD_1 medium supplemented with 2.2 μM BAP for 4 wk. (bar = 1.0 cm).

Table 1 illustrates how shoot proliferation dramatically improved in horizontally cultured stem segments ($p < 0.05$). A 2-fold increase in shoot production was recorded for stem segments compared to vertically cultured apical segments. The medium supplemented with $AgNO_3$ also had a significant influence on shoot proliferation, especially at 17.67 μM, compared to other dosages used ($p < 0.05$). However, axillary shoot elongation exhibited a non-significant difference among treatments, which revealed how promotion of shoot growth was not effectively achieved by either of the 2 tested factors. Furthermore, only about 1/3 of axillary shoots thus induced achieved the desired elongation for further utilization. Although explants associated with different culture orientations and the addition of $AgNO_3$ did not enhance shoot elongation, a significant interaction was still detected between these 2 factors. A further analysis of the large-shoot formation data demonstrated that shoot elongation was more successful (40%) when horizontally cultured stem segments were incubated on medium containing 17.67 μM $AgNO_3$ (Table 2).

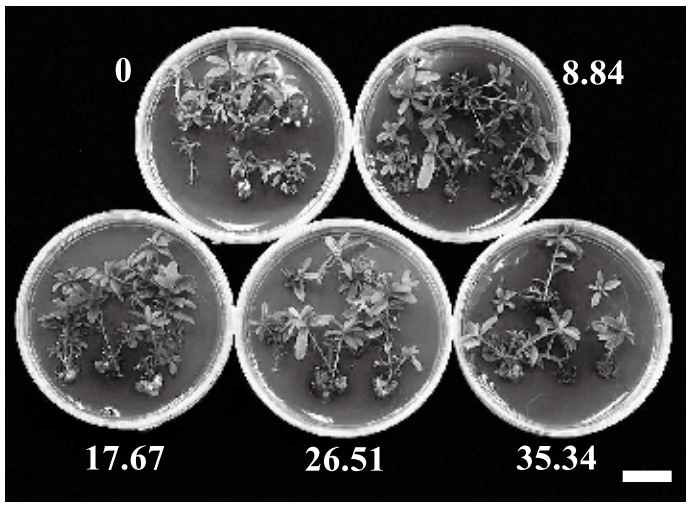

Fig. 2. Horizontally cultured basal stem segments of *Quercus aliena* var. *aliena* showing active growth of axillary shoots on shoot proliferation medium supplemented with 0.44 μM BAP and various concentrations (μM) of $AgNO_3$ (as marked) for 6 wk in the 8-wk cyclic culture period. (bar = 2.0 cm).

Table 1. Effects of explant type/orientation and $AgNO_3$ concentration during the 8-wk incubation on axillary shoot proliferation and large-shoot formation (%) in *Quercus aliena* var. *aliena*

Source factor	Level	Shoot proliferation[1)]	Large-shoot formation (%)[2)]
Explant type/Orientation	Apical segment/Vertically cultured	2.6 ± 0.3^{b}	28.24 ± 3.69
	Basal segment/Horizontally cultured	5.4 ± 0.3^{a}	30.50 ± 2.66
$AgNO_3$ (μM)	0	4.0 ± 0.6^{b}	25.50 ± 5.07
	8.84	4.2 ± 0.5^{ab}	31.29 ± 4.95
	17.67	5.5 ± 0.7^{a}	26.72 ± 4.09
	26.51	3.3 ± 0.4^{b}	31.76 ± 5.41
	35.34	2.9 ± 0.4^{b}	31.57 ± 5.82

Significance[3)]		
Source factor	Shoot proliferation	Large shoot formation (%)
Explant type/Orientation (EO)	*	n.s.
$AgNO_3$	*	n.s.
EO × $AgNO_3$	n.s.	*

[1)] Values of axillary shoot proliferation per explant (mean ± SE) corresponding to different levels for each source factor where mean values followed by different superscript letters significantly differ as determined by an LSD test at $p < 0.05$.

[2)] Percentage data of large-shoot formation (mean ± SE) were subjected to arcsine transformation before analysis.

[3)] Source factors and their interactive effects on shoot proliferation and large-shoot formation, where * represents a significant difference determined at $p < 0.05$ by the LSD test; and n.s. a non-significant difference ($p > 0.05$).

Axillary shoot elongation

Neither the removal of the quickly developed large shoots nor supplementation with AC during the last 4-wk culture period significantly influenced axillary shoot elongation. Shoot removal only resulted in a slight increase in the large-shoot percentage; however, this increase did not reach a level of significance. A similar result was observed in AC treatments as the large-shoot percentage showed no significant variance for any level of AC addition (Table 3). Although neither culture manipulation produced meaningful enhancement in shoot elongation, an overall

Table 2. Interactive effects exhibited by the combination of 2 source factors from Table 1 showing various levels of large-shoot formation in *Quercus aliena* var. *aliena*

$AgNO_3$ (μM)	Large-shoot formation (%)[1)]	
	Apical segment/Vertically cultured	Basal segment/Horizontally cultured
0	35.92 ± 8.98^{abc}	15.09 ± 3.69^{cd}
8.84	32.29 ± 8.93^{abcd}	30.28 ± 4.57^{abcd}
17.67	12.61 ± 4.11^{d}	40.83 ± 5.56^{a}
26.51	37.67 ± 9.34^{abc}	25.86 ± 5.41^{abcd}
35.34	22.71 ± 8.14^{bcd}	40.43 ± 8.03^{ab}

[1)] Percentages of large-shoot formation (mean ± SE) followed by different superscript letters significantly differ as determined by an LSD test at $p < 0.05$.

Table 3. Effects of large-shoot removal and activated charcoal during the last 4-wk culture of a 10-wk incubation on axillary shoot proliferation and large-shoot formation (%) in *Quercus aliena* var. *aliena*

Source factor	Level	Shoot proliferation[1)]	Large-shoot formation (%)[2)]
Explant	Shoots remained	8.6 ± 0.5	32.89 ± 1.99
	Large shoot removal	8.6 ± 0.5	39.56 ± 2.61
Activated charcoal (g L^{-1})	0	9.9 ± 0.7^{a}	36.71 ± 2.87
	2.5	8.5 ± 0.7^{ab}	37.58 ± 3.19
	5	7.4 ± 0.4^{b}	34.40 ± 2.61
		Significance[3)]	
Source factor		Shoot proliferation	Large-shoot formation (%)
Explant		*	n.s.
Activated charcoal (AC)		*	n.s.
Explant × AC		n.s.	n.s.

[1)] Values of axillary shoot proliferation per explant (mean ± SE) corresponding to different levels for each source factor where the mean values followed by different superscript letters significantly differ as determined by an LSD test at $p < 0.05$.

[2)] Percentage data of large-shoot formation (mean ± SE) were subjected to arcsine transformation before analysis.

[3)] Source factors and their interactive effects on shoot proliferation and large-shoot formation, where * represents a significant difference detected at $p < 0.05$ by the LSD test; and n.s. a non-significant difference ($p > 0.05$).

increase in axillary shoot production was observed. This increase may perhaps have been due to the prolonged cultured period (a total of 10 wk, compared to a total of 8 wk in the shoot proliferation experiment), meaning that the explants were still in a good physiological condition and capable of proliferating shoots during the extended culture period. Therefore, since shoot production dramatically dropped when AC was added during the prolonged culture period, this strongly suggests that an inhibitory effect was produced by the additionally loaded AC (Table 3).

In vitro rooting

In the first rooting experiment, basal end swelling was observed in a majority of explants cultured in the root expression medium for 2 wk. A highly proliferating callus developed and extruded from the surface break of swollen tissue. Once the diameter of the callus exceeded 1.0 cm, adventitious rooting was barely observable in the explants (Fig. 3A). A small amount (or even the absence) of a basal callus was clearly favorable for adventitious root development (Fig. 3B). However, the roots did not vertically grow downwards to form a typical tap root system, but instead, grew horizontally into a lateral position. The IBA concentration tested in the range of 73.8~221.4 μM led to a minor promotion effect at the intermediate levels (123.0~172.2 μM). An average rooting percentage of 68.75±7.42% was achieved (Table 4).

Tests using $AgNO_3$ and AC supplements as rooting-enhancement agents showed that their influences on the rooting percentage considerably varied. The addition of $AgNO_3$ (17.76 μM) to the medium after the 48-h IBA pulse treatment resulted in a rooting percentage of 87.5% (Table 5). This was a great improvement in rooting performance compared to the control and with the addition of AC. The amount of AC (4 g L^{-1}) used in this experiment was likely harmful, as the rooting percentage significantly decreased (Table 5).

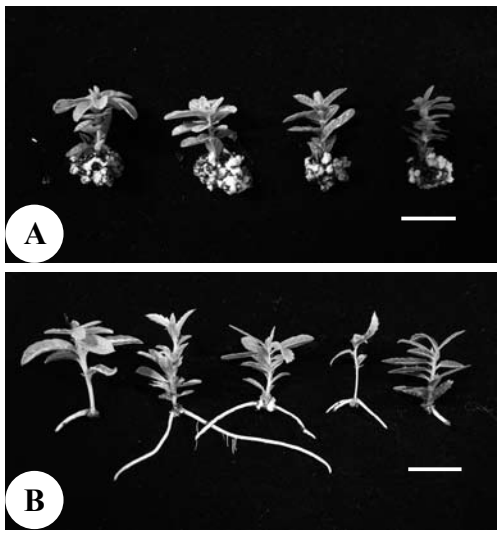

Fig. 3. *In vitro* adventitious rooting from axillary shoots of *Quercus aliena* var. *aliena*. (A) Shoots with overgrowth of basal calli showed difficulty in root formation; (B) root development from axillary shoots with a small amount or even the absence of basal calli. (bars = 1.0 cm).

Table 4. Comparison of *in vitro* adventitious rooting in axillary shoots of *Quercus aliena* var. *aliena* stimulated by different IBA levels

IBA (μM)	Adventitious rooting (%)[1)]
73.8	62.50±7.96
123.0	68.75±7.42
172.2	68.75±8.54
221.4	62.50±10.98

[1)] Percentage data of rooting (mean±SE) were subjected to arcsine transformation before analysis.

Acclimatization

During the first 3 wk of acclimatization, rooted plantlets showed new leaf development

Table 5. Comparison of *in vitro* adventitious rooting in axillary shoots of *Quercus aliena* var. *aliena* with the addition of activated charcoal or $AgNO_3$ to the root expression medium

Treatment	Adventitious rooting (%)[1)]
Control	75.00±6.55[ab]
Activated charcoal (4 g L^{-1})	60.00±8.24[b]
$AgNO_3$ (17.67 μM)	87.50±4.12[a]

[1)] Percentage data of rooting (mean±SE) were subjected to arcsine transformation before analysis. Mean values followed by different superscript letters significantly differ as determined by an LSD test at $p < 0.05$.

and internode elongation while they were still inside the glass beaker (Fig. 4A). A survival rate of 85.3±5.4% was recorded at the end of stage 1. Once the plantlets were moved to a shaded greenhouse with the gradual opening of the beaker, regular watering was provided based on the moisture conditions in the potting medium. A survival rate of 80.7±8.1% was recorded at this stage (stage 2). After the complete removal of the beaker for 20 d (Fig. 4B), the survival rate decreased to 72.3±9.7%, which suggests that greater protection and a further adjustment of moisture conditions are needed during the final acclimatization stage.

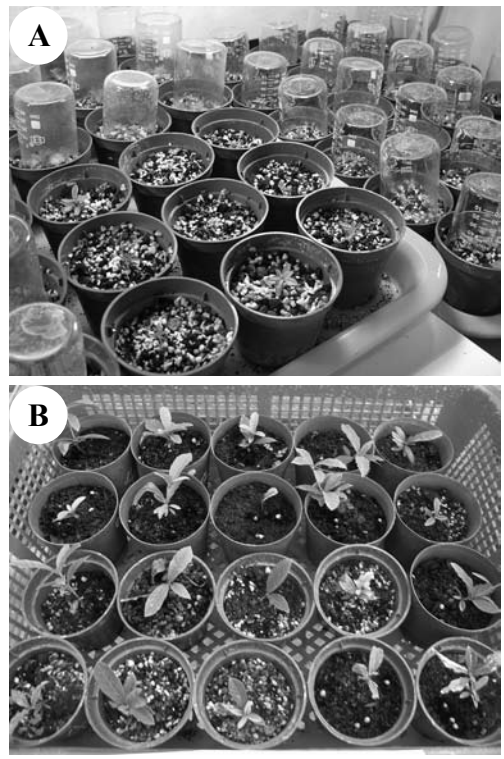

Fig. 4. Acclimatization of rooted plantlets of *Quercus aliena* var. *aliena* at different stages. (A) Plantlets maintained in the acclimatization room (stage 1) individually covered by glass beakers for moisture control. Shown in the front are specimens without coverage for temporary viewing of plantlet growth; (B) plantlets which had completed acclimatization (stage 3) in a partially shaded greenhouse.

DISCUSSION

The method of *in vitro* axillary micropropagation has been well developed in *Quercus* spp., and optimized culture conditions were documented. In studies of *Q. rubra* L., *Q. robur* L. (Vieitez et al. 1993, 1994, Sanchez et al. 1996, Vidal et al. 2003), *Q. alba* L., and *Q. bicolor* L. (Vieitez et al. 2009), the authors claimed that the placement of nodal stem explants during the shoot proliferation stage was one of the crucial factors affecting the efficiency of shoot production. They suggested that the stem explant, decapitated and horizontally placed on the solidified medium, was a superior form of culture manipulation for enhancing axillary shoot proliferation. Zimmerman and Fordham (1989), McClelland and Smith (1990), Orlikowska et al. (2000), and Debnath (2005) also made that same recommendation when working with other plant species. However, previous studies produced

satisfactory results, which demonstrated how the vertically cultured stem explant with an intact shoot tip was also applicable to *Quercus* spp. (Vieitez et al. 1985, Favre and Juncker 1987, Romano et al. 1992). In our study of *Q. aliean* var. *aliean*, in which 2 explant types associated with their specific culture placement on medium were examined, horizontally cultured explants were more responsive to BAP stimulation and achieved a 2-fold increase in axillary shoot production (Table 1). However, the formation of large and usable shoots in horizontally placed explants did not significantly increase.

Theoretically, as apical stem explants include more closely condensed axillary buds, their potential for performance in shoot proliferation under suitable cytokinin stimulation should improve. However, the apical dominance may perhaps trigger the inhibition of axillary bud growth located at a lower point on the explant; hence, in a decapitated nodal stem explant, axillary buds would have more opportunity to develop after they are free from apical dominance (San-Jose et al. 1988). This enhancement may be further improved when explants are horizontally placed on the medium that allows direct contact of each node of the explant with cytokinin stimulation (Jaiswal and Amin 1987).

Ethylene is known to be a plant growth regulator (PGR) that affects shoot regeneration and induces developmental abnormalities in *in vitro* culture systems (Trujillo-Moya and Gisbert 2012). However, $AgNO_3$ effectively inhibits the action of ethylene thanks to Ag+ binding to the ethylene receptor protein on the cell membrane. It is not surprising that $AgNO_3$ would therefore show promotive effects on shoot organogenesis in culture (Roustan et al. 1992, Mohiudddin et al. 1997, Brar et al. 1999, Anantasaran and Kanchanapoom 2008). Vieitez et al. (2009) first reported the *in vitro* use of $AgNO_3$ for axillary shoot production in *Q. rubra.* According to their conclusions, a medium supplemented with $AgNO_3$ (17.67~35.34 μM) demonstrated a positive effect on shoot growth appearance, including the production of straight and vigorous shoots with fully expanded dark-green leaves and a reduction in shoot tip necrosis in elongated shoots. More importantly, the addition of $AgNO_3$ tended to increase shoot production, and it significantly promoted shoot elongation. All of these growth features are crucial for improving the culture efficiency of tested species. The same manipulation was well adapted to *Q. aliean* var. *aliena* in the present study. After 17.67 μM $AgNO_3$ was applied to the culture medium, the explants produced the best result in terms of shoot proliferation (Table 1), and shoot tip necrosis also diminished (data not shown). However, an improved effect on shoot elongation was not seen when the percentage data for large-shoot formation from the 2 different explant culture types were pooled for analysis (Table 1). The beneficial effect was only obtained in horizontally cultured nodal stem explants in conjunction with the incorporation of 17.67 μM $AgNO_3$ (Table 2), which is in agreement with the results of a study conducted by Vieitez et al. (2009).

Our further attempts at improving shoot elongation did not produce significant results (Table 3). The data presented in Table 3 indicate that the early removal of fast growing large shoots did not enable the remaining shoots to experience greater follow-up growth. This feature may rule out the possibility of growth inhibition induced by apical dominance from large shoots, and could also exclude the hypothesized absence of space and nutrient supplies required for shoot elongation. With the incorporation of 2 levels of AC in the final subculturing period, axillary

shoot proliferation progressively decreased, and no further shoot elongation was obtained. This suggests that possible BAP overloading, which inhibits shoot growth, might not exist since the decrease in shoot proliferation could be seen as an indicator that too much BAP was possibly withdrawn from the subcultures. The existence of AC in the culture medium did not necessarily enhance shoot multiplication or elongation (Paek and Hahn 2000, Liu et al. 2007), since it also led to an inhibition of shoot multiplication (Li et al. 2005). Thus, it is clear that more studies are needed to fully optimize the working dosage for culture conditioning through AC incorporation.

The *in vitro* rooting of axillary shoots was tested with various concentrations of IBA (73.8~221.4 μM) which showed no significant difference (Table 4). Based on the consideration of the minimum use of a rooting stimulator to obtain an acceptable rooting efficiency (68.75%), 123.0 μM IBA was determined to be a proper concentration and was routinely used thereafter. Similar operations (123.0 μM IBA pulsed for 24 h) were also reported for rooting shoots from mature explants of *Q. robur* and *Q. rubra* (Sanchez et al. 1996, Vidal et al. 2003). This is further evidence demonstrating the high adaptability of *Q. aliean* var. *aliena* to culture protocols previously used in other *Quercus* shoot cultures. In addition, the rooting of axillary shoots in *Q. aliean* var. *aliena* seemed to have been more acutely affected by basal callus formation on the shoot explants. This relationship was also mentioned by Vidal et al. (2003) as the difficulty of rooting shoot cultures from mature *Q. robur* coincided with the formation of calli proliferating from cortex cells. Vengadesan and Pijut (2009) proposed that basal calli may interrupt vascular connections between the adventitious roots and stem explants, thereby leading to the death of rooted plantlets during acclimatization. In the present study, wherein the survival rate of rooted shoots notably dropped by about 10% during stage-3 acclimatization, the failure may be partially attributed to this interruption. A careful adjustment of treatment times and working concentrations when using an auxin pulse to enhance *in vitro* rooting of *Quercus* spp. is therefore essential to avoid overgrowth of basal calli.

Supplements of $AgNO_3$ and AC in the medium were tested for their influence on rooting percentages. One of the supplements ($AgNO_3$) was noted to have beneficial effects on *in vitro* rooting of shoot cultures (Table 5). This is in agreement with results obtained for *Decalepis hamiltonii* Wight & Arn. (Bais et al. 2000), *Rotula aquatica* Lour. (Chithra et al. 2004), and *Gentiana lutea* L. (Petrova et al. 2011). Upon addition of an ethylene precursor (1-aminocyclopropane-1-carboxylic) (Ma et al. 1998) or ethephon (an ethylene-releasing agent) (Bais et al. 2000) to the rooting medium as a reverse control, researchers demonstrated that the promotive effects of $AgNO_3$ on *in vitro* rooting were also attributed to an inhibition of ethylene's action. However, incorporation of AC (4 g L^{-1}) in the medium for *in vitro* rooting was not as effective as previously suggested by Vieitez et al. (2009). AC is a non-specific absorbent used in tissue culture to remove undesirable chemicals from the medium and maintain a non-harmful culture environment for explant development. As we discussed above, overloading of AC may induce growth problems as essential PGRs are over-withdrawn. Since greater or lower dosages of AC (incorporated either during or after rooting stimulation) were previously reported to enhance explant rooting *in Quercus* spp. (Volkaert et al. 1990, Sanchez et al. 1996, Ostrolucka et al. 2007), it seems that more-precise adjustments of the timing and dosage of AC are necessary in fu-

ture studies. This is the only component in the recommended protocols outlined by Vieitez et al. (2009) that was not well adapted to the target species and requires further modification.

The present study has established a workable tissue culture method for *Q. aliean* var. *aliena* to prepare adequate planting materials for genetic resource preservation and for the immediate use in *ex situ* conservation. We have already maintained more than 10 clones of this species in the laboratory for sequential axillary shoot production. However, 2 of the original donor trees perished in the habitat due to local environmental stress. This unfortunate event strongly emphasizes the value of our efforts in protecting this species in Taiwan. A follow-up project that focuses on propagating the trees is underway. It is hoped that such efforts will rapidly mitigate the risk of the population disappearance of *Q. aliena* var. *aliena* in Taiwan.

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