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An Efficient Protocol of Protocorm–Like Bodies Regeneration from Callus Cultures of *Gastrodia elata* Blume and the Further Associations with Mycorrhizal Fungi

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In this study, we reported a reliable protocol of PLBs regeneration from embryogenic callus cultures of *Gastrodia elata*, a fully mycoheterotrophic orchid and an endangered herbal medicinal plant. The continuous immersion cultures with TDZ (0.5 and 1 mg·L⁻¹) and BA (10 mg·L⁻¹) improved PLBs regeneration frequency and rate. Histological studies indicated that starch grains were abundant within in the cytoplasm of embryogenic callus and developing PLBs. The formation of PLBs from cell aggregates in the periphery region of embryogenic callus was supposed to be the multicellular origin pathway. After inoculated with suitable *Armillaria* isolates, PLBs enlarged further and resulted in the formation of tubers. The results of phylogenetic analysis and inoculation experiments indicated that the asymbiotic PLBs could establish the appropriate association with *Armillaria mellea* complex, including *A. mellea* and *A. gallica*. Thus, this protocol of PLBs regeneration from callus cultures is beneficial for the multiplication of selected elites, and could be used as an experimental system for the molecular investigations of the mycoheterotrophic orchid – mycorrhizal fungi interactions.

Key words: *Armillaria*, liquid culture, mycoheterotrophic orchid, mycorrhizal fungi, somatic embryo

INTRODUCTION

Gastrodia elata Blume is a fully mycoheterotrophic orchid species and has a wide distribution, ranging from China, Taiwan, Korea and Japan (Hsu, 2008). The dried tuber of *G. elata* is known as 'Tian Ma' and has been used as traditional Chinese medicines for human diseases such as vertigo, blackout, headache and hemiplegia (Chang and But, 1986). Under natural conditions, *G. elata* relies on two groups of mycorrhizal fungi to complete its life cycle. For germination, *G. elata* seeds associate with *Mycena*, and then the further growth of protocorms into tubers requires symbiotic association with *Armillaria mellea* (Kusano, 1911; Guo and Xu, 1990; Xu and Fan, 2001).

Like many herbal medicinal plants, the tubers are over–collected from wild stocks, making *G. elata* an endangered species. Fortunately, the artificial cultivation of *G. elata* by mimicking the inoculation of the two mycorrhizal fungi under the natural condition has been successfully established (Guo and Xu, 1990; Kikuchi *et al.*, 2008a, b). For the production of *G. elata* tubers, the supply of tuber relies on the production of protocorms derived from symbiotic seed germination. However, the plant of *G. elata* only blooms and produces seeds once a year, the continual production of tubers in an environmental controlled chamber is hampered. If a somatic regeneration system of protocorm–like bodies (PLBs) from an embryogenic callus culture could be established, it is helpful to stabilize the supply end of *G. elata* tubers for the herbal medicinal market.

An efficient regeneration procedure through PLB formation from callus cultures is beneficial in the mass production. The regeneration of PLBs from the embryogenic callus culture has been documented in a number of orchids, such as *Cymbidium* (Chang and Chang, 1998; Huana *et al.*, 2004), *Cypripedium* (Lee and Lee, 2003), *Oncidium* (Jheng *et al.*, 2006), *Paphiopedilum* (Lin *et al.*, 2000) and *Phalaenopsis* (Ishii *et al.*, 1998; Tokuhara and Mii, 2001; Tokuhara and Mii, 2003). So far, most orchids for tissue culture propagation are autotrophic species with green leaves. The information of micropropagation of non–photosynthetic orchids is scarce. Although the protocorms *G. elata* derived from symbiotic germination with *Mycena* has been successfully used for symbiotic culture with *Armillaria* to produce tubers, it is still unknown that if PLBs from asymbiotic micropropagation can be used for symbiotic culture with *Armillaria*. In this paper, we describe a reliable protocol of PLB regeneration from embryogenic callus culture for *G. elata*. The effects of continuous immersion culture, cytokinin types and concentrations on the efficiency of PLB regeneration were carried out. Furthermore, the association of *Armillaria* isolates with PLBs for the development of tubers was investigated. Histological studies on the origin and development of PLBs from cal-

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lus culture, and the invasion of *Armillaria* hyphae in developing tubers were also detailed.

MATERIALS AND METHODS

The initiation and subculture of embryogenic calluses

The initial embryogenic callus culture was established from protocorms obtained from asymbiotic seed germination. Mature seeds of *G. elata* were collected for asymbiotic germination. The capsules were taken to the laboratory, and were surface sterilized with a 1% (v/v) NaClO solution for 15 min and rinsed three times with sterile distilled water. After surface sterilization, the capsules were cut open, and then mature seeds were scooped out with forceps onto the culture medium. For asymbiotic seed germination, 1/2–strength macro–elements and full–strength micro–elements of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing $2\,mg\cdot L^{-1}$ glycine, $0.5\,mg\cdot L^{-1}$ niacin, $0.5\,mg\cdot L^{-1}$ pyridoxine HCl, $0.1\,mg\cdot L^{-1}$ thiamine and $100\,mg\cdot L^{-1}$ myo–inositol was used, and supplemented with $20\,g\cdot L^{-1}$ sucrose (Sigma Chemical Co.), $50\,g\cdot L^{-1}$ banana homogenate and $7\,g\cdot L^{-1}$ Agar. The pH value was adjusted to 5.6 prior to autoclaving at 121ºC for 20 min. Twenty milliliters of medium were placed into each Erlenmeyer flask (125 mL). After sowing, the flasks were placed in the dark at 23 ± 1°C. After 12 weeks of culture, embryos emerged from the testa and formed the young protocorms. At the same time, the appearance of primary callus from a few swelling embryos was observed. The granular callus with good proliferating rate was selected and evaluated. For the maintenance of calluses, 1/2 MS medium was used as described above, and supplemented with $100\,g\cdot L^{-1}$ potato homogenate, $20\,g\cdot L^{-1}$ sucrose, $6\,g\cdot L^{-1}$ Agar. The pH of the media was adjusted to 5.6 prior to autoclaving at 121ºC for 20 min. The callus cultures were incubated in darkness at 23 ± 1°C, and subcultured on the same medium every 4 months. After 12 months of continual subculture, the embryogenic callus (ECs) was selected for further studies.

Effect of culture conditions on PLBs regeneration

In this experiment, the continuous immersion culture (24 hours per day) was compared to the solid culture. Ten pieces of ECs masses (about 100 mg fresh weight) were transferred to the 125 mL flasks containing 25 mL of liquid or solid culture media as described below. In the continuous immersion culture, the 1/2 MS medium was supplemented with $20\,g\cdot L^{-1}$ sucrose, and $10\,mg\cdot L^{-1}$ N6–benzyladenine (BA). In the solid culture, the medium composition was the same as the continuous immersion culture, but solidified with $6\,g\cdot L^{-1}$ agar. The pH of the media was adjusted to 5.6 prior to autoclaving at 121ºC for 20 min. The cultures were incubated in darkness at 23 ± 1°C. After 7 days of treatments, the ECs masses were then transferred onto the growth medium containing 1/2 MS medium supplemented with $20\,g\cdot L^{-1}$ sucrose, $1\,g\cdot L^{-1}$ peptone, $1\,g\cdot L^{-1}$ activated charcoal, $100\,g\cdot L^{-1}$ potato homogenate, and solidified with $6\,g\cdot L^{-1}$ agar. One hundred milliliters of medium were placed into each flask (500 mL). The callus cultures were incubated in darkness at 23 ± 1°C for observation and further investigation. In this experiment, each flask contained ten pieces of ECs masses as one replicate, and five replicates were established for each treatment.

Effect of cytokinins on PLBs regeneration

Since the continuous immersion culture improved PLBs regeneration, the effects of different cytokinins on PLBs regeneration were evaluated using the continuous immersion culture. In this experiment, ten pieces of ECs masses (about 100 mg fresh weight) were transferred to the continuous immersion cultures with different cytokinins. A range of concentrations of N6–benzyladenine (BA; 1, 5 and $10\,mg\cdot L^{-1}$), kinetin (1, 5 and $10\,mg\cdot L^{-1}$), zeatin (1, 5 and $10\,mg\cdot L^{-1}$) and thidiazuron (TDZ; 0.1, 0.5 and $1\,mg\cdot L^{-1}$) were added to the cultures. In the control, the cytokinin was omitted. After 7 days of treatments, the ECs masses were transferred onto the growth medium and incubated in darkness at 23 ± 1°C. In this experiment, each flask contained ten pieces of ECs masses as one replicate, and five replicates were established for each treatment.

Light microscopy

The ECs, developing PLBs and small tubers were fixed in a solution of 2.5% glutaraldehyde (v/v) and 1.6% (v/v) paraformaldehyde in 0.1 M phosphate buffer at pH 6.8 overnight at 4ºC. After fixation, the samples were dehydrated using an ethanol series, and embedded in Technovit 7100 (Kulzer & Co., Germany). Sections of 3µm–thick were cut with glass knives using a Reichert–Jung 2040 Autocut rotary microtome. These sections were stained with Periodic acid–Schiff's reaction for total water insoluble carbohydrates, and counter–stained with either 0.05% (w/v) toluidine blue O (TBO) in benzoate buffer for general histology or 1% (w/v) amido black 10B in 7% (v/v) acetic acid for protein (Yeung, 1984). All bright field images were captured digitally using a CCD camera attached to a Zeiss light microscope (Axio Imager A2, Carl Zeiss AG, Germany).

Effect of *Armillaria* isolates on the growth of *G. elata* tubers

The *Armillaria* isolates were cultured on PDA medium (potato dextrose agar; $200\,mg\cdot L^{-1}$ potato homogenate, $20\,mg\cdot L^{-1}$ dextrose, $20\,mg\cdot L^{-1}$ agar) in darkness at 23 ± 1°C. After 2 weeks, the PDA agar plug (diameter 6 mm) with active growing mycelium from the colony margin was inoculated to a Magenta™ vessel (GA–7), containing autoclaved 100 g wood blocks of *Liqiudamber formosana*. After 1 month, two PLBs clumps (as Fig. 3D) were transferred on the surface of wood blocks with active growing mycelium, and then the PLBs clumps were covered by autoclaved sphagnum moss chips. The treatment without fungi was used as a control. In this experiment, each treatment was replicated on four GA–7 cultures. All treatments were placed in the culture room in darkness at 23 ± 1°C.

DNA extraction and PCR amplification of ITS rDNA

DNA was extracted from each *Armillaria* isolate by using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of the fungal nuclear ribosomal RNA gene was amplified using the primer combinations ITS1F/ITS4 or ITS1F/ITS4B (Gardes and Bruns, 1993). PCR amplification and sequencing were carried out as described by Ogura–Tsujita and Yukawa (2008). The sequences obtained in this study were identified using a BLAST search against the NCBI (National Center for Biotechnology Information) sequence database to find the closest sequence matches in the database, and then submitted to GenBank.

Phylogenetic analysis

For phylogenetic analysis, ITS sequences of *Armillaria* from GenBank were added to the analysis by referring Coetzee *et al.* (2003), and sequences of *Mycena* were used as outgroup taxa. DNA sequences were aligned using CLUSTALX (Thompson *et al.*, 1997), followed by manual adjustment. The alignment datasets were further analyzed by the Neighbor–joining (NJ) using MEGA 4 (Tamura *et al.*, 2007) with a Kimura two–parameter correction (Kimura, 1980). For assessing the relative robustness for branches, the bootstrap method (Felsenstein, 1985) was used with 1000 replicates. The trees obtained in these analyses were drawn with the TreeGraph 2 software (Stover and Muller, 2010).

Data calculation and statistical analysis

For the PLBs regeneration experiments, the number of PLBs per callus mass were observed and recorded from 4 to 8 weeks of culture on the growth medium. For the effects of *Armillaria* isolates on the growth of tubers, the numbers and fresh weights per culture were observed and recorded after 4 months of inoculation. In all experiments, the treatments were arranged in a completely randomized design. Data were analyzed by using analysis of variance (n=5) and means were compared by Fisher's protected least significant difference (LSD) test (P ≤ 0.05).

RESULTS

The PLBs regeneration in the liquid medium (88.6%) was much higher than those in the solid medium (5.5%) after 8 weeks of culture on the growth medium (Fig. 1). In the solid medium, only a few PLBs formations could be observed after 7 weeks of culture on the growth medium. Among four cytokinins tested, the percentages of PLBs regeneration improved as the cytokinins concentrations increased (Table 1; Fig. 2). The optimum percentages of PLBs regeneration was found in the media supplemented with TDZ (0.5 and 1 mg·L^{-1}) and BA (10 mg·L^{-1}). PLBs formation was first observed after 5 weeks of culture on the growth medium. As compared to the other cytokinins tested, the medium with BA (5 and 10 mg·L^{-1}) stimulated faster PLBs formation (Fig. 2).

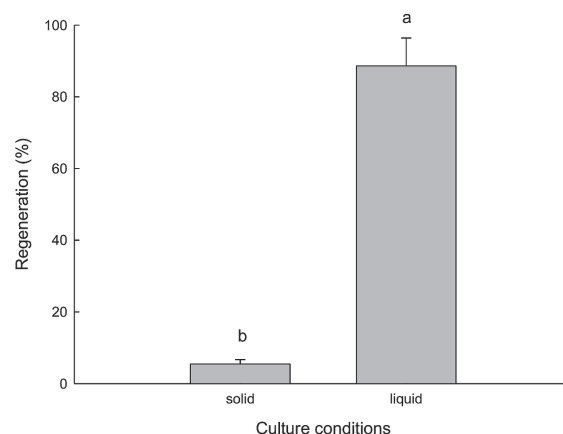The ECs masses of *G. elata* were yellowish–white

Fig. 1. Effect of culture conditions on PLBs regeneration of *G. elata*. Error bar is the standard error of mean (n = 5).

Table 1. Effect of different cytokinins on PLBs regeneration of *G. elata*[z]

Cytokinins (mg·L^{-1})		Number of PLBs per callus	PLBs regeneration (%)
Control	0.0	0.0	–
TDZ	0.1	10.6 d [y]	48.2 bc
	0.5	33.4 ab	88.6 a
	1.0	37.6 ab	94.1 a
BA	1.0	9.4 d	48.3 bc
	5.0	27.0 bc	78.7 ab
	10.0	41.0 a	88.5 a
Kinetin	1.0	6.6 d	36.0 c
	5.0	7.0 d	40.2 c
	10.0	6.2 d	40.4 c
Zeatin	1.0	10.0 d	42.1 c
	5.0	16.4 cd	46.9 bc
	10.0	17.4 cd	58.2 b

[z] Data were recorded after 8 weeks of culture.
[y] Means within each column followed by the different letter(s) are significantly different at P ≤ 0.05 by Fisher's protected LSD test.

Table 2. Effect of *Armillaria* isolates on the growth of *G. elata* tubers [z]

Armillaria isolates	Number of tubers per culture	Fresh weight of tubers (g)
1	14.3 b[y]	1.3 b
2	82.0 a	9.7 a
3	0.0 c	– c
4	66.8 a	8.6 a
5	0.0 c	– c
6	0.0 c	– c
7	0.0 c	– c

[z] Data were recorded after 4 months of culture.
[y] Means within each column followed by the different letter(s) are significantly different at P ≤ 0.05 by Fisher's protected LSD test.
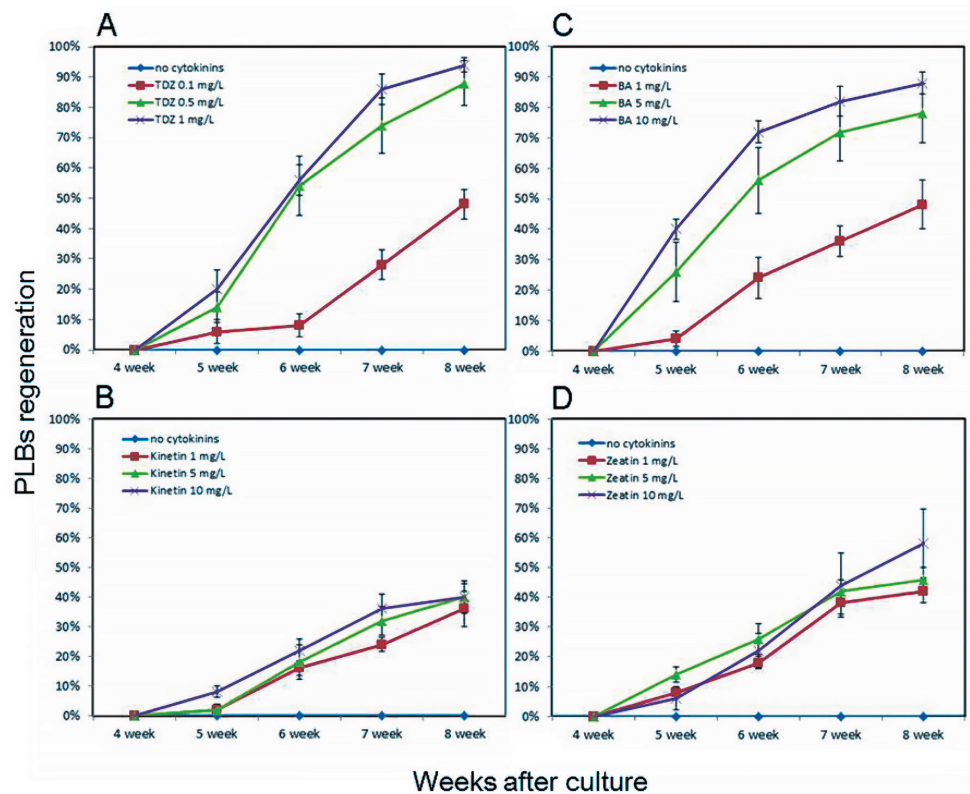
Fig. 2. Mean percentage of PLBs regeneration of *G. elata* in relation to the time in culture for different cytokinins treatments (A) TDZ, (B) Kinetin, (C) BA and (D) Zeatin. Error bar is the standard error of mean (n = 5).
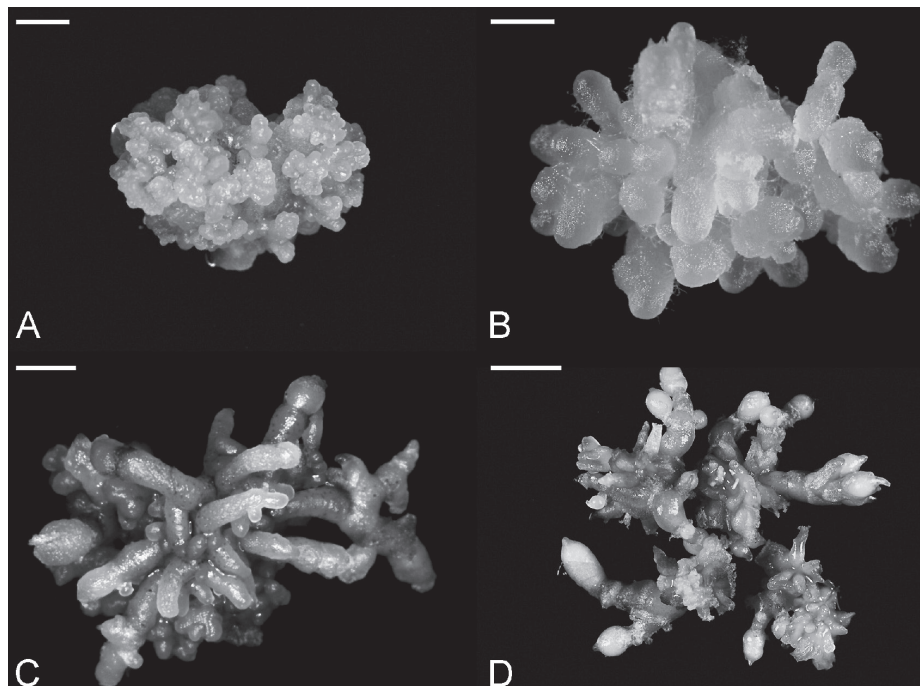

Fig. 3. Regeneration of PLBs from ECs cultures. (A) The ECs were yellowish–white that were composed of compact cell aggregates. Scale bar = 2 mm. (B) Several small globular PLBs appeared on the surface of ECs masses after 5 weeks of culture. Scale bar = 2 mm. (C) After 6 weeks of culture, the elongated PLBs of different sizes were visible. Scale bar = 2.5 mm. (D) The formation of small tuber–like structure from elongated PLBs was visible after 8 weeks of culture. Scale bar = 5 mm.
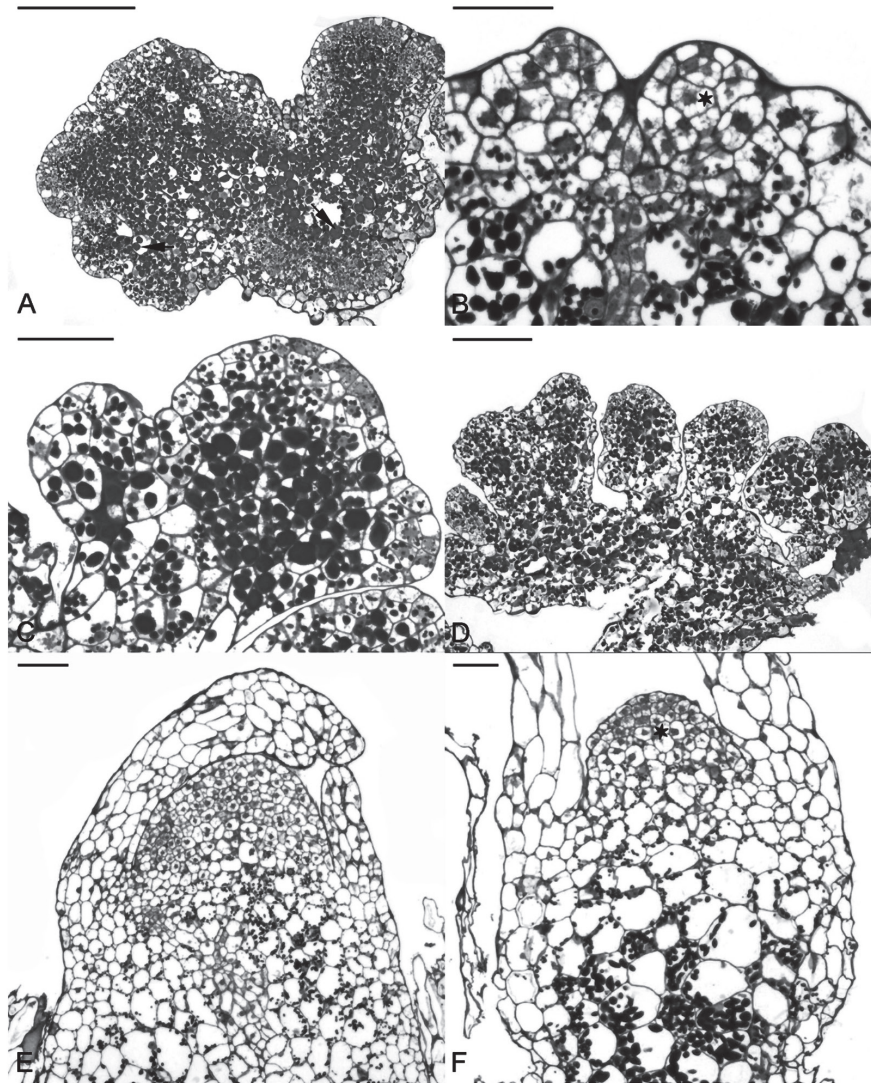
Fig. 4. Histological study on the regeneration of PLBs from ECs cultures. (A) Histological sections reveal that ECs masses were compactly organized. The cells at the periphery zone of ECs masses had a dense cytoplasm with small starch grains and vacuoles. The cells at the inner region of ECs masses were highly vacuolated and filled with a number of large starch grains (arrows). Scale bar = 1 mm. (B) After 4 weeks of culture, the formation of meristemoid aggregates (*) was visible in the surface of ECs masses. Scale bar = 10 μm. (C) The meristemoid aggregates were subsequently organized into growth centers that resulted in PLB formation. Scale bar = 60 μm. (D) After 6 weeks of culture, numerous granular PLBs were produced from ECs masses. Scale bar = 300 μm. (E) The anterior part of PLBs elongated and formed a shoot pole with scale leaves covered. Scale bar = 100 μm. (F) After 8 weeks of culture, further development of PLBs resulted in the formation of small tuber–like structure. The anterior part of small tuber–like structure had a distinct shoot pole (*) that consisted of large cytoplasmic zone. Scale bar = 100 μm.

that were composed of compact cell aggregates (Fig. 3A). After 5 weeks of culture, small globular PLBs appeared on the surface of ECs masses (Fig. 3B). After 6 weeks of culture, elongated PLBs of different sizes could be observed (Fig. 3C). After 8 weeks of culture, further development at anterior part of PLBs resulted in the formation of small tubers (Fig. 3D). Histological sections revealed that ECs masses of *G. elata* were compactly organized (Fig. 4A). The cells at the periphery zone of ECs masses had a dense cytoplasm with small starch grains and vacuoles. The cells at the inner region of ECs

masses were highly vacuolated and filled with a number of large starch grains. After 4 weeks of culture, the formation of meristemoid aggregates was visible in the surface of ECs masses (Fig. 4B). After 5 weeks of culture, the meristemoid aggregates were subsequently organized into growth centers that resulted in PLB formation (Fig. 4C). After 6 weeks of culture, numerous granular PLBs were produced from embryogenic callus masses (Fig. 4D). The PLBs developed further and formed a shoot pole with scale leaves covered (Fig. 4E). After 8 weeks of culture, the formation of small tuber with a distinct
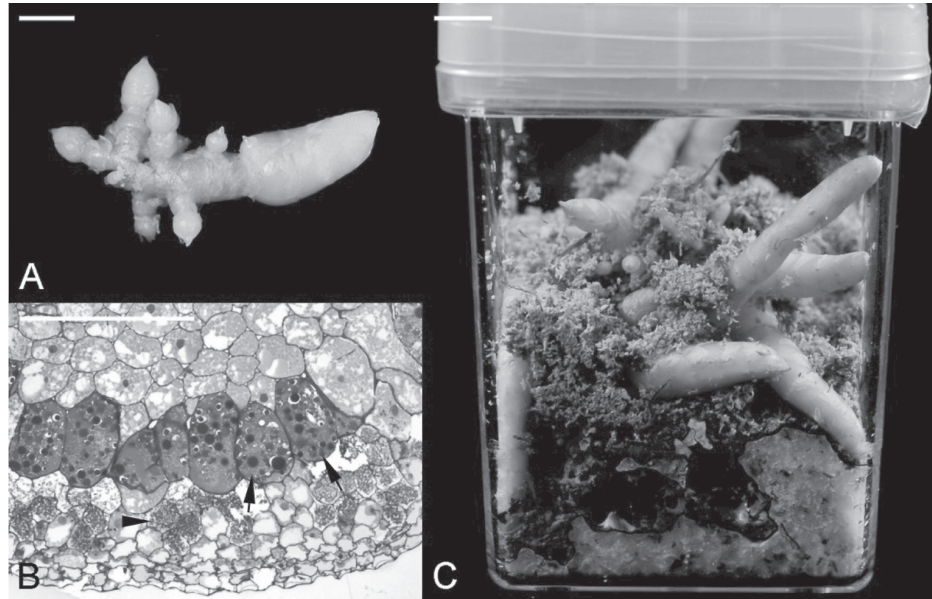
Fig. 5. The growth of *G. elata* tuber after the inoculation with *Armillaria isolates*. (A) After 2 months of inoculation, the growth of tubers was stimulated by *Armillaria* isolates. Scale bar = 5 mm. (B) Light micrograph showing the hyphae (arrowhead) invaded the cortical region at the basal part of tuber after 2 months of inoculation with *Armillaria* isolates. The large digestive cells (arrows) are visible. Scale bar = 500 μm. (C) A number of tubers enlarged and elongated after 4 months of inoculation. Scale bar = 10 mm.
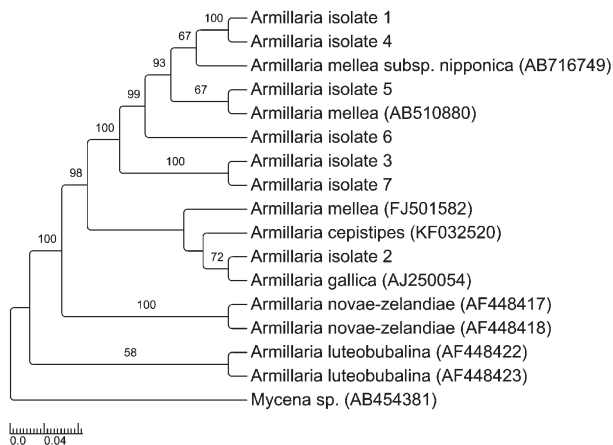

Fig. 6. Phylogenetic tree of ITS rDNA sequences of *Armillaria* isolates for symbiotic cultures with *G. elata* tubes in this study. *Mycena* sp. was used as the outgroup. Phylogenetic analysis was conducted using Neighbor-joining method with 1000 bootstrap replicates (values of more than 60% are shown at each branch).

shoot pole that consisted of large cytoplasmic zone was visible (Fig. 4F).

Among *Armillaria* isolates tested, the isolates 1, 2 and 4 improved the further growth of tubers as compared to the other isolates (Table 2). In the cultures with the isolates 3, 5, 6 and 7, the invasion looked pathogenic. The PLBs did not develop and finally became dead. In the control, no further growth of PLBs was found without the symbiotic mycorrhizal fungus. In the culture with the isolates 1, 2 and 4, the obvious growth of tubers had occurred after 2 months of inoculation (Fig. 5A). Histological sections revealed that the invasion of

Armillaria hyphae was restricted at the basal part of tuber. The hyphae existed in the outer and middle cortical regions, while the hyphae was absent in the inner cortical region (Fig. 5B). After 4 months of inoculation, a few larger tubers had reached 1 cm in width and 7 cm in length (Fig. 5C).

The internal transcribed spacer (ITS) and 5.8S rDNA sequences of seven *Armillaria* isolates showed high similarities (99%) to those ITS and 5.8S rDNA sequences of *Armillaria* species. from the results of the BLAST searches. Neighbor–joining (NJ) phylogenetic analysis using the ITS and 5.8S rDNA sequences indicated that *Armillaria* isolates 1, 3, 4, 5, 6 and 7 clustered together with the *A. mellea* with a bootstrap support of 100 % (Fig. 6). Among the *Armillaria* isolates tested, only the isolate 2 grouped with *A. gallica* with a bootstrap support of 72%.

DISCUSSION

The current study demonstrated an efficient protocol for *G. elata* micropropagation through PLB regeneration from ECs culture. One of the most notable findings of this study is that continuous immersion culture enhances the regeneration frequency and rate (Figs. 1 and 2). In *G. elata*, the continuous immersion culture had 16–fold higher PLBs regeneration frequency as compared to the solid culture. The advantages of liquid medium cultures, e.g. temporary immersion system for improving somatic embryo development have been reported in several species (Alvard *et al.*, 1993; Cabasson *et al.*, 1997; Etienne–Barry *et al.*, 1999). The lack of a gelling agent in liquid medium cultures may increase availability of water and nutrients to explants (Debergh,

1993). Besides, the11 liquid cultures generate potential stress conditions for explants that may enhance somatic embryogenesis (Zimmerman, 1993; Martre *et al*., 2001). It is worthy to note that the ECs of *G. elata* are not friable. During subculture operation, the tactile impression of callus mass was like having a rigid shell. Histological observations also demonstrated that the cells at the peripheral zone of embryogenic calluses were all tightly packed, forming a strict boundary (Fig. 4A). The lower regeneration frequency in the solid medium culture was probably due to the slow accessibility of nutrients and plant growth regulators hampered by the rigid callus surface.

In this study, TDZ and BA were found to be more effective than kinetin and zeatin in PLBs regeneration. TDZ at low concentrations (0.5 and $1\,\mathrm{mg}\cdot\mathrm{L}^{-1}$) induced the high frequency of PLB regeneration (Table 1). TDZ is a phenylurea derivative that has high biological active at lower concentrations than the adenine–type cytokinins (Capelle *et al*., 1983; Murthy *et al*., 1998). In orchids, the promotive effect of TDZ on PLBs regeneration has been reported in *Oncidium* (Chen and Chang, 2001), *Phalaenopsis* and *Doritaenopsis* (Ernst, 1994; Chen and Piluek, 1995; Park *et al*., 2002).

From the histochemical staining results, it is worthy to note the distinctive accumulation of starch grains in the cells of embryogenic callus and developing PLBs (Fig. 4). As the PLBs regeneration process commenced, starch grains decreased dramatically in developing meristemoid aggregates (Fig. 4B). The presence of abundant carbohydrate reserves, e.g. starch grains has been reported in the embryogenic calluses (Yeung, 1995; Puigderrajols *et al*., 2001). The rapid hydrolysis of starch when embryogenesis starts provides not only energy and carbon sources for proliferation, but may also play a regulatory role. Somatic embryogenesis through the multicellular pathway occurred frequently in the compact callus mass of cork oak (Puigderrajols *et al*., 2001). In the tightly packed callus mass of *G. elata*, PLB formation derived from cell aggregates in the periphery region is supposed to be the multicellular origin. Similar regeneration process is observed in *Phalaenopsis* orchid, PLB formation of from the tightly packed callus mass is considered as the multicellular origin (Lee *et al*., 2013).

In the inoculation experiments with *Armillaria* isolates, three isolates 1, 2 and 4 promoted the tuber formation, while the others caused PLBs rotted (Table 2). *Armillaria* comprises a number of species and strains that show great differences in pathogenicity (Coetzee *et al*., 2003; Mohammed *et al*., 1994). With the suitable *Armillaria* isolates association, the tuber enlarged further, and the cortical layers of the tuber had prominent digestive cells that hosted the *Armillaria* hyphae (Fig. 5B). From the phylogenetic analysis, the isolates 1 and 4 are grouped with *A. mellea* subsp. *nipponica* (Fig. 6). Recently, a fully mycoheterotrophic orchid, *Cyrtosia septentrionalis* was reported to associate with three *Armillaria* species, i.e. *A. gallica*, *A. mellea* subsp. *nipponica* and *A. tabescens* (Umata *et al*., 2013). This group (isolates 1, 4 in this study and *A. mellea* subsp.

nipponica) appeared to be less pathogenic and more compatible to orchids as compared to other isolates tested. Besides, the isolate 2 is grouped with *A. gallica* (Fig. 6), indicating that *G. elata* can recruit the less pathogenic *Armillaria* species for the appropriate mycorrhizal association as reported by Kikuchi *et al*. (2008 a, b).

In conclusion, we first reported a reliable protocol of PLB regeneration from callus culture for a fully mycoheterotrophic orchid – *G. elata*. The continuous immersion culture with TDZ and BA significantly improved the PLB regeneration. After inoculated with suitable *Armillaria* isolates, PLBs enlarged further and resulted in the formation of tubers. This high efficient PLBs regeneration system from callus culture is beneficial for the multiplication of selected elites and the molecular investigations of the mycoheterotrophic orchid–mycorrhizal fungi interactions.

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