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Efficient *in vitro* Plant Regeneration from Protocorm of *Bletilla formosana* (Hayata) Schltr.

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An efficient mass propagation method for *Bletilla formosana* (Hayata) Schltr. was successfully established through direct shoot organogenesis. Multiple shoots were induced from protocorm explants on half–strength Murashige and Skoog (1/2–MS) basal medium containing 2 mg/L N–phenyl–N'–1,2,3–thiadiazol–5–yl urea (TDZ) and 0.5 mg/L 1–naphthaleneacetic acid (NAA) with the highest shoot formation rate of 100% and a maximum average shoot number of 5.2. The application of either NAA or 2,4–dichlorophenoxy (2,4–D) significantly induced root induction from shoots, and 2 mg/L NAA provided the highest root formation rate of 100% and a maximum number of roots (4.4 per shoot). Supplementation with kinetin and benzylaminopurine (BAP) of 0.5~2.0 mg/L in 1/2–MS basal medium significantly promoted plantlet growth. To optimize the *in vitro* development of plantlets, 1/2–MS basal medium with modified 1/4–strength nitrogen content, and 20 g/L sucrose was used. Well–developed plantlets were successfully acclimatized in a greenhouse with over a 90% survival rate. This effective protocol of *in vitro* plant regeneration through direct shoot organogenesis can be utilized for the mass propagation and germplasm conservation of *B. formosana*.

Key words: *Bletilla formosana*, multiple shoots, nitrogen content, plant growth regulators, protocorm

INTRODUCTION

Species of the genus *Bletilla*, temperate terrestrial orchids, belong to the Orchidaceae family, with only five species distributed in China, Japan, Taiwan, Vietnam, Thailand, and Myanmar. In addition to their attractive ornamental value, *Bletilla* species possess therapeutic effects against pneumonia, pulmonary tuberculosis, and gastric and duodenal ulcers (Wang and Meng, 2015). They also exhibit antibacterial, antifungal, and anti–tyrosinase activities and cytotoxic effects against several cancer cell lines (Lin *et al*., 2016). Bletillae Rhizoma, the dried tuber of *B. striata* (Thunb.) Reichb. f., is officially documented in the Chinese Pharmacopoeia as a traditional Chinese medicine.

Bletilla formosana (Hayata) Schltr., an endangered medicinal orchid species native to Taiwan, is mainly distributed along sunny slopes from sea level to an elevation of 3000 m. *Bletilla formosana* has greater antioxidant activity and higher contents of pharmacologically active components, such as militarine, cinnamic acid, and phenanthrenes, than do commercial crude drugs of *B. striata* (Wu *et al*., 2010). *Bletilla formosana* was also found to be useful against *Helicobacter pylori* infection (Wang and Huang, 2005) and to have anti–inflammatory properties (Lin *et al*., 2016). Wild populations of *B. formosana* are now threatened with extinction due to over–collection and habitat destruction (Wu and Lay, 2013). The optimal harvest time for vegetative tubers of *B. formosana* is in September and October, but the efficiency of vegetative propagation through dividing mature tubers is low (Wu and Lay, 2013). Asymbiotic seed germination is considered an efficient propagation method for orchids; however, the seed germination rate of *B. formosana* is significantly affected by the seed development stage and seed storage or cryopreservation conditions (Chen *et al*., 2009; Wu *et al*., 2013).

In vitro plant regeneration has been successfully applied to the mass propagation and germplasm conservation of several endangered orchids (Ahamed Sherif *et al*., 2016; Bhattacharyya *et al*., 2016; Roy *et al*., 2011; Zeng *et al*., 2012). Nevertheless, no effort has been made to develop an efficient propagation method for *B. formosana*. The characteristics of seedlings propagated by vegetative means are not uniform; therefore, propagation through tissue culture is desirable. Various tissue culture techniques have already been developed for orchids, and shoot tips or apical meristems are commonly used explants for *in vitro* culture, but the removal of meristematic tissues greatly damages the mother plant, and the available explants are limited.

There are no reports on plantlet regeneration from protocorm explants of *B. formosana* or optimization of sucrose and inorganic nitrogen compositions in *in vitro* culture media. The objectives of this work were to screen optimal medium and culture conditions for *in vitro* plant regeneration of *B. formosana*, and establish an efficient mass propagation scheme to increase the population size and conserve this exquisite orchid. The effects of plant growth regulators (PGRs) on multiple–shoot induction were evaluated, and an efficient regeneration method with protocorms as ideal explants for the large–scale production of *B. formosana* was estab-

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lished. In addition, the effects of different concentrations of sucrose and inorganic nitrogen on plantlet development, chlorophyll content, and the DPPH–scavenging activity of *B. formosana* are illustrated.

MATERIALS AND METHODS

Plant materials

Flowering plants of *B. formosana* (Hayata) Schltr. were harvested at Da–Lun–Wei Mt. (121°34'E, 25°06'N; Taipei, Taiwan), and authenticated by Prof. Chen Chang, at the Department of Horticulture, National Chung Hsing University, Taichung, Taiwan. These harvested plants were then potted and cultivated in a greenhouse at the Department of Horticulture and Biotechnology, Chinese Culture University (CCU, 121°32'21.3"E, 25°08'00.6"N). Voucher specimens were maintained in the corresponding author's laboratory at CCU. Mature capsules were collected at 10 weeks after hand pollination for asymbiotic germination. After a thorough washing with running tap water followed by 75% ethanol for 45 s, the capsules were then surface–sterilized with 1% sodium hypochlorite (containing 0.05% Tween–20) for 15 min and rinsed six times with sterilized distilled water. Disinfected capsules were cut open longitudinally with a sterile scalpel, and seeds were carefully sown on basal medium. The medium contained 1/2–Murashige–Skoog (MS) basal medium (Murashige and Skoog, 1962) consisting of half–strength MS salts, full–strength MS vitamins, 100 mg/L *myoinoisitol*, and 20 g/L sucrose, and was supplemented with 1 g/L peptone, 6 g/L potato powder (*Phyto*Technology Laboratories™, Shawnee Mission, KS, UDA), 150 ml/L coconut milk (KOH, Bangkok, Thailand), 1 g/L activated charcoal, and 3.2 g/L gelrite (Chen *et al*., 2009). The pH value was adjusted to 5.5, and medium was dispensed as 100–mL aliquots into orchid bottles. The medium was autoclaved at 121°C and 1 kg/cm^2 for 15 min. Seeds were cultured in a growth chamber (CH–202–A, Chin–Hsin, Taipei, Taiwan) at 25 ± 1°C with a light intensity of 50 μmol·m^{-2}·s^{-1} provided by cool white fluorescent lights, a 12–h photoperiod, and 75% relative humility. The same culture conditions in growth chambers were applied in subsequent experiments, including multiple–shoot induction, rooting of shoots, and plantlet growth and development.

Medium preparation and culture conditions with PGRs for multiple–shoot induction

Six–week–old protocorms (Fig. 1A) were used as explants and cultured on 1/2–MS basal medium containing different concentrations of N–phenyl–N'–l,2,3–thiadiazol–5–yl urea (TDZ; 0.5, 1, 1.5, and 2 mg/L) or benzylaminopurine (BAP; 0.5, 1, 1.5, and 2 mg/L) in combination with 1–naphthaleneacetic acid (NAA; 0, 0.5, 1.5, and 2 mg/L). The medium was cooled down after sterilization and dispensed as 20–mL aliquots into sterile Petri dishes (100 × 15 mm) in a laminar air flow hood. During the shoot induction period, protocorm explants secreted secondary metabolites into the medium. After 8 weeks of culture, explants were then subcultured onto the same induction medium supplemented with 0.1% activated charcoal for 4 weeks to prevent or reduce tissue browning. Multiple–shoot induction and growth were recorded 12 weeks after *in vitro* culture. Each treatment had five replicates of five explants each. The percentage of multiple–shoot formation was calculated as the number of explants with the formation of multiple shoots divided by the total number of explants. Shoot height was the average height of shoots induced from explants.

Establishment of *in vitro* rooting of shoots

Shoots with a height of approximately 2 cm were cultured in 1/2–MS basal medium supplemented with different concentrations of NAA (0.5, 1.0, and 2.0 mg/L) or 2,4–dichlorophenoxy acetic acid (2,4–D; 0.5, 1.0, and 2.0 mg/L) for *in vitro* rooting. Orchid bottles were used as containers with 100–mL aliquots of medium. Each treatment consisted of five replicates, and each replicate contained five explants. Cultures were incubated for 2 months.

Effects of cytokinins on plantlet development

Three–month–old plantlets of *B. formosana* were used as plant materials and subcultured in 1/2–MS basal medium supplemented with 0.5, 1.0, and 2.0 mg/L kinetin or BAP. Orchid bottles were used as containers with 100–mL aliquots of medium. Each treatment consisted of five replicates, and each replicate contained five explants. Cultures were incubated for 3 months.

Effects of sucrose and nitrogen contents on plant growth, chlorophyll contents, and DPPH–scavenging activities

Three–month–old plantlets of *B. formosana* were used as plant materials and subcultured in 1/2–MS basal medium with modified inorganic nitrogen contents and different concentrations of sucrose (20, 40, and 60 g/L). The three treatments of MS nitrogen contents included 825 mg/L NH_4NO_3 and 950 mg/L KNO_3, 412.5 mg/L NH_4NO_3 and 475 mg/L KNO_3, and 206.3 mg/L NH_4NO_3 and 237.5 mg/L KNO_3, which respectively denoted 1/2–, 1/4– and 1/8–fold inorganic nitrogen contents of MS basal medium (Murashige and Skoog, 1962). Each treatment consisted of three replicates, and each replicate contained 10 explants.

The relative chlorophyll (Chl) content of *B. formosana* seedlings was determined using a SPAD (Soil Plant Analysis Development) analyzer (SPAD–502 Chlorophyll Meter, Konica Minolta, Tokyo, Japan). The radical–scavenging activity of *B. formosana* seedlings was measured using a modified method of Brand–Williams *et al*. (1995). Briefly, each plantlet with 0.3 g of fresh weight was extracted with 1 mL of methanol, and 50 μL of the extract was mixed with 150 μL of 1,1–diphenyl–2–picrylhydrazyl (DPPH) solution for measuring the absorbance at 517 nm with a spectrophotometer (Powerwave XS2, BioTek, Friedrichshall, Germany). The same volume of methanol was used as a blank. The experiment was replicated five times. DPPH–scavenging activity (%) was defined as $[(A_{blank} - A_{extract}) / A_{blank}] \times 100\%$.

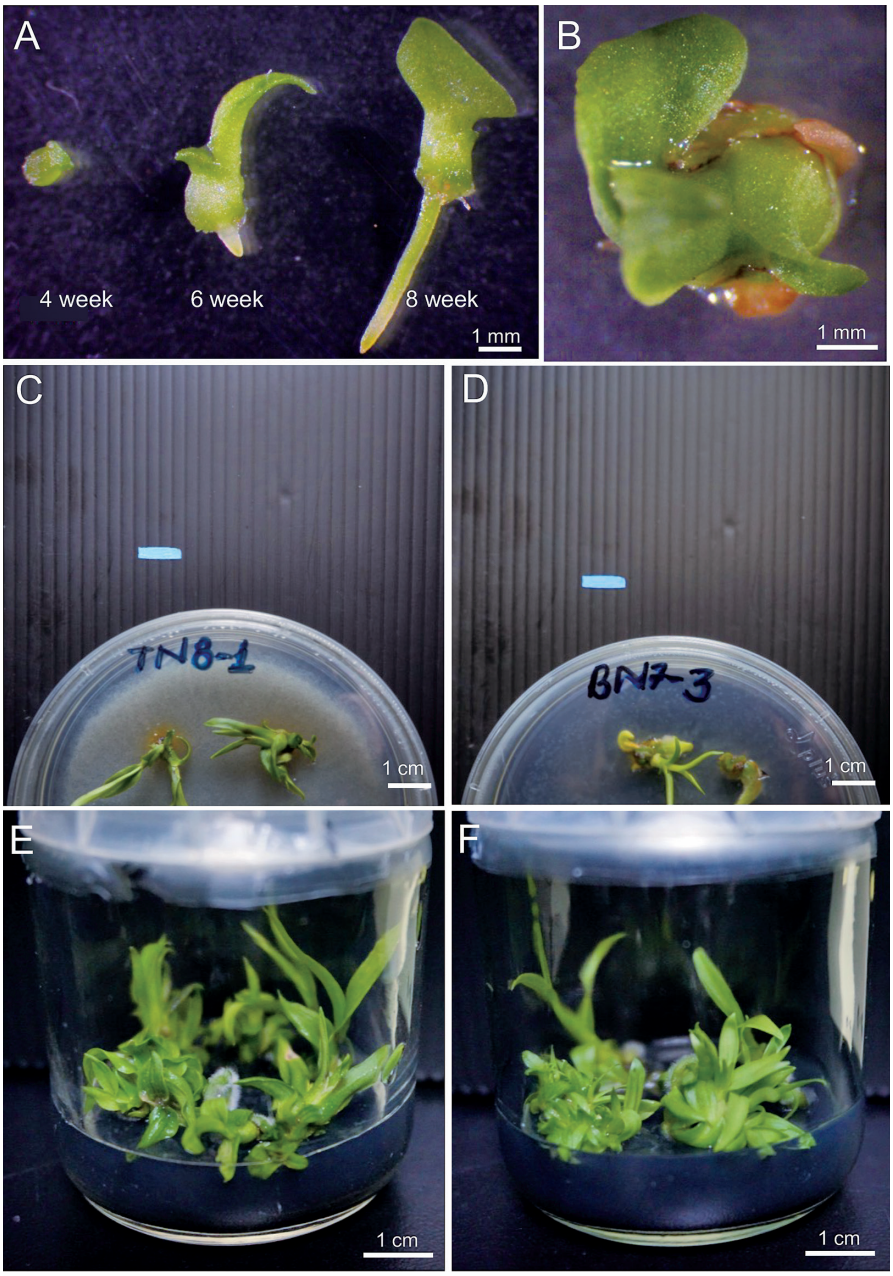

Fig. 1. Shoot proliferation and plant regeneration of *Bletilla formosana*. (A) Protocorms formed and developed after *in vitro* germination for 4, 6, and 8 weeks. Six–week–old protocorms were used as plant material for multiple–shoot induction. (B) Multiple shoots formed on 1/2–MS medium containing 2 mg/L TDZ and 0.5 mg/L NAA after 2 weeks of culture. After 6 weeks of culture, multiple shoots had formed and developed well on 1/2–MS basal medium containing 0.5 mg/L NAA and 2 mg/L TDZ (C) or 1.5 mg/L BAP (D). Multiple shoots were induced on 1/2–MS medium containing 0.5 mg/L NAA and 2 mg/L TDZ (E) or 1.5 mg/L BAP (F) after 8 weeks of subculturing on 1/2–MS medium containing 1 g/L active charcoal for 2 weeks.

Acclimatization of plantlets

For acclimatization, plantlets with well–developed roots were taken out from the *in vitro* condition and thoroughly washed under running tap water to remove medium. Plantlets were transplanted to a plug tray of 104 cells (50 mL for each cell) filled with sphagnum moss, and incubated in a plastic tray covered with a transparent plastic lid. Plantlets were cultured in a greenhouse and thoroughly irrigated every 2 days to maintain the relative humidity.

Statistical analysis

All experiments were arranged in a completely randomized design. Data were subjected to a one–way analysis of variance (ANOVA), with a significance level of $p < 0.05$ using CoStat statistical software (Cohort Berkeley, Monterey, CA, USA). Significant differences were determined using the Student–Newman–Keuls test. Effects of the MS nitrogen source and sucrose on plantlet growth were analyzed by a two–factor completely randomized ANOVA, which compared the MS nitrogen source and

sucrose. For significant values, means were separated by the least significant difference (LSD) test at $p \leq 0.05$, 0.01, or 0.001, using CoStat.

RESULTS

In vitro plant regeneration and influence of PGRs on multiple–shoot induction of *B. formosana*

The objectives of the present work were to develop a procedure for determining the effects of PGRs on multiple–shoot induction. *In vitro* protocorms were used as explants and were cultured in 1/2–MS medium supplemented with BAP (0.5, 1, 1.5, and 2 mg/L) and TDZ (0.5, 1, 1.5, and 2 mg/L) alone or combined with NAA (0.5 and 1 mg/L) for screening the optimal medium for multiple–shoot induction. Six–week–old protocorms were used as explants; however, explant browning was visible after 2 weeks of culture (Fig. 1B). After 6 weeks of culture, multiple shoots began to emerge; however, this was followed by the presence of medium browning caused by the release of phenolic exudates from the explants (Figs. 1C, D). Therefore, explants at 8 weeks of culture were subcultured in 0.1% activated charcoal–supplemented medium of the same composition as the original culture to avoid degenerative effects. After 4 weeks of subculture, the protocorm explants continued to exhibit regenerative potential and produced multiple shoots with exogenously applied PGRs (Fig. 1E, F).

Since half–strength MS salts are a commonly used medium composition for protocorm growth and development of *B. formosana* (Chen *et al*., 2009), 1/2–MS basal medium was adopted and modified for acquisition of a well–defined *in vitro* medium composition in this study. To evaluate the efficiency of multiple–shoot induction from protocorms, 1/2–MS basal media containing BAP and TDZ alone or combined with NAA were used, and the effects of these PGRs on multiple–shoot induction of protocorm explants are shown in Table 1. Only 1/2–MS basal medium supplemented with 0.5 mg/L TDZ alone and without a PGR showed no multiple–shoot formation at all. Shoots were formed at a frequency of 8%~100% on MS medium supplemented with PGRs corresponding

Table 1. Effects of TDZ, BAP, and NAA on induction of multiple shoots from protocorms of *Bletilla formosana* after 12 weeks of culture

Plant growth regulators (mg/L)			Multiple shoots formation (%)	Shoot number	Shoot height (mm)	Leaf number	Root number
TDZ	BAP	NAA					
0	0	0	0.0 ± 0.0 g	1.0 ± 0.0 e	17.6 ± 1.0 bc	4.3 ± 0.2 b	1.3 ± 0.1 bc
0.5	0	0	0.0 ± 0.0 g	1.0 ± 0.0 e	15.4 ± 0.9 cd	3.8 ± 0.2 c	0.2 ± 0.1 f
1	0	0	36.0 ± 4.5 c	1.7 ± 0.2 cd	11.2 ± 1.1 ef	3.0 ± 0.2 de	0.0 ± 0.0 g
1.5	0	0	24.0 ± 6.0 d	1.6 ± 0.3 cd	11.0 ± 1.1 ef	3.2 ± 0.3 d	0.0 ± 0.0 g
2	0	0	8.1 ± 3.0 f	1.2 ± 0.1 d	10.5 ± 0.3 f	2.7 ± 0.2 f	0.2 ± 0.1f
0.5	0	0.5	44.0 ± 6.5 bc	2.1 ± 0.3 c	20.8 ± 1.0 b	3.2 ± 0.2 d	0.6 ± 0.1 d
1	0	0.5	12.0 ± 4.9 ef	1.6 ± 0.2 cd	14.0 ± 1.3 de	3.3 ± 0.2 d	0.7 ± 0.1 d
1.5	0	0.5	36.0 ± 4.5 c	2.2 ± 0.3 c	11.3 ± 1.1 ef	3.6 ± 0.3 c	0.4 ± 0.1 e
2	0	0.5	100.0 ± 0.0 a	5.2 ± 0.4 a	14.6 ± 1.2 de	2.6 ± 0.1 f	0.5 ± 0.1 de
0.5	0	1	12.0 ± 5.0 ef	1.5 ± 0.2 cd	11.9 ± 0.6 ef	3.7 ± 0.2 c	0.6 ± 0.1 d
1	0	1	20.0 ± 5.2 de	1.4 ± 0.1 cd	13.8 ± 0.8 de	3.5 ± 0.3 cd	0.6 ± 0.1 d
1.5	0	1	24.0 ± 6.8 d	1.5 ± 0.2 cd	13.2 ± 0.5 de	3.2 ± 0.2 d	0.9 ± 0.1 cd
2	0	1	24.0 ± 5.9 d	2.2 ± 0.5 c	12.0 ± 0.3 e	2.9 ± 0.2 e	0.9 ± 0.1 cd
0	0.5	0	20.0 ± 6.0 de	1.6 ± 0.4 cd	15.2 ± 1.7 cd	4.6 ± 0.4 ab	1.3 ± 0.1 bc
0	1	0	20.0 ± 6.3 de	1.3 ± 0.2 d	24.4 ± 1.3 a	4.7 ± 0.2 ab	1.7 ± 0.1 ab
0	1.5	0	16.0 ± 4.0 e	1.5 ± 0.2 cd	17.5 ± 1.7 bc	4.9 ± 0.3 a	1.2 ± 0.2 cd
0	2	0	12.0 ± 5.0 ef	1.2 ± 0.1 d	15.8 ± 1.5 cd	4.7 ± 0.2 ab	0.9 ± 0.2 cd
0	0.5	0.5	24.0 ± 4.7 d	1.3 ± 0.1 d	22.5 ± 1.8 ab	4.6 ± 0.4 ab	1.8 ± 0.2 ab
0	1	0.5	8.0 ± 2.9 f	1.1 ± 0.1 d	25.4 ± 1.6 a	5.0 ± 0.4 a	2.0 ± 0.3 a
0	1.5	0.5	48.0 ± 4.9 b	3.6 ± 0.7 b	17.6 ± 2.0 bc	3.8 ± 0.3 c	1.4 ± 0.2 b
0	2	0.5	32.0 ± 6.6 cd	1.4 ± 0.2 cd	19.7 ± 1.9 bc	4.7 ± 0.4 ab	1.4 ± 0.2 b
0	0.5	1	12.0 ± 3.9 ef	1.3 ± 0.1 d	18.0 ± 1.6 bc	5.0 ± 0.4 a	1.5 ± 0.2 b
0	1	1	16.0 ± 4.0 e	1.4 ± 0.2 cd	17.9 ± 1.9 bc	3.3 ± 0.5 d	1.2 ± 0.2 c
0	1.5	1	20.0 ± 6.3 de	1.3 ± 0.1 d	23.2 ± 1.7 ab	5.0 ± 0.2 a	2.0 ± 0.3 a
0	2	1	16.0 ± 5.7 e	1.4 ± 0.2 cd	24.0 ± 1.4 a	4.8 ± 0.2 a	2.0 ± 0.2 a

Values are means ± S.D. of 25 replicated samples for each treatment. Statistical analysis was according to the least significant difference (LSD) test ($p \leq 0.05$).

to the lowest (1.1±0.1) and highest (5.2±0.4) numbers of shoots per protocorm obtained on 1/2–MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA, and the combination of TDZ (2.0 mg/L) and NAA (0.5 mg/L), respectively. In addition, when BAP was used alone or combined with NAA (0.5 or 1 mg/L), it produced the longest shoot lengths ranging 22.5±1.8~25.4±1.9 mm. A significant difference in the highest leaf numbers (4.6±0.4~5.0±0.4) was detected in 1/2–MS basal medium containing BAP alone or mixed with NAA. The lowest leaf number (2.6±0.1) was observed on 1/2–MS basal medium supplemented with TDZ (2.0 mg/L) and NAA (0.5 mg/L). Roots formed at the highest numbers (2.0± 0.3 and 2.0±0.2) on 1/2–MS basal medium supplemented with BAP (1~2 mg/L) and NAA, but no roots were observed on 1/2–MS basal medium supplemented with TDZ (1~1.5 mg/L) alone.

Effects of auxin on root induction from shoots of *B. formosana*

Table 2 reveals that both NAA and 2,4–D supplements resulted in different morphological responses in terms of the percentages of root formation, numbers of shoots, root lengths, and plant heights. Shoots cultured on auxin–free medium failed to produce roots, but after 8 weeks of culture, the rooting efficiency had increased up to 85.3% and 100% with concentrations of NAA or 2,4–D of 0.5~2 mg/L. The minimum (0.9±0.1) and maximum (4.4±0.5) numbers of root per explants were observed in 1/2–MS basal medium alone and that supplemented with 2 mg/L NAA, respectively. In addition, supplementation with 2,4–D (0.5~2 mg/L) was also found to produce 2.6±0.2~3.6±0.4 roots. Application of NAA produced significantly longer root lengths (14.6± 1.8~19.2±1.6 mm) than those (5.2±0.4~7.4±0.7 mm) with 2,4–D. Compared to auxin–free medium, both NAA and 2,4–D supplements produced significantly greater plant heights. Notably, the maximum plant height (44.0±2.7 mm) of explants was observed in treatment with 2 mg/L 2,4–D compared to other treatments.

Effects of cytokinins on plant growth of *B. formosana*

Different concentrations of kinetin and BAP supplements were tested to optimize plantlet growth. Table 3 shows that kinetin and BAP significantly increased plant heights (87.2±1.1~93.6±1.1 mm) compared to the control (78.1±4.7 mm). Notably, no significant differences were shown in plant heights in all treatments. Adventitious shoot numbers were significantly induced from plantlets as kinetin or BAP increased from 1.0 to 2.0 mg/L and ranged from 1.8±0.2 (1.0 mg/L kinetin) to 2.6±0.3 (2.0 mg/L BAP). However, root numbers and root lengths were unaffected by kinetin or BAP, except

Table 2. Effects of auxin on root formation from shoots of *Bletilla formosana* after 8 weeks of culture

Auxin	Concn (mg/L)	Root formation (%)	Root number	Root length (mm)	Plant height (mm)
	0	88.0 ± 8.0 bc	0.9 ± 0.1 d	14.6 ± 1.1 b	26.3 ± 1.0 c
NAA	0.5	85.3 ± 3.0 c	2.1 ± 0.4 c	14.2 ± 1.8 b	40.1 ± 2.5 ab
	1	100.0 ± 0.0 a	2.5 ± 0.3 bc	16.9 ± 1.5 ab	39.8 ± 1.9 ab
	2	100.0 ± 0.0 a	4.4 ± 0.5 a	19.2 ± 1.6 a	38.2 ± 3.3 ab
2,4–D	0.5	88.0 ± 4.9 bc	3.6 ± 0.4 b	7.4 ± 0.7 c	34.3 ± 2.4 b
	1	92.0 ± 2.9 b	2.7 ± 0.4 bc	5.2 ± 0.4 c	33.0 ± 1.2 b
	2	88.0 ± 8.0 bc	2.6 ± 0.2 bc	5.2 ± 0.4 c	44.0 ± 2.7 a

Values are mean± S.D of 5 replicated samples for each treatment. Statistical analysis was according to the least significant difference (LSD) test ($p \leq 0.05$).

Table 3. Effects of kinetin and BAP on plant development of *Bletilla formosana* after 12 weeks of culture

Cytokinin	Concn (mg/L)	Plant height (mm)	Shoot number	Root number	Root length (mm)	Leaf number
	0	78.1 ± 4.7 b	1.0 ± 0.0 e	3.4 ± 0.2 a	36.0 ± 1.3 a	6.3 ± 0.1 a
kinetin	0.5	93.6 ± 1.1 a	1.3 ± 0.1 c	3.8 ± 0.2 a	35.8 ± 0.4 a	6.3 ± 0.1 a
	1.0	91.2 ± 1.8 a	1.8 ± 0.2 b	3.7 ± 0.2 a	35.2 ± 0.7 a	6.2 ± 0.1 a
	2.0	93.5 ± 1.6 a	2.4 ± 0.4 a	3.4 ± 0.3 a	34.6 ± 0.8 a	5.6 ± 0.1 b
BAP	0.5	93.4 ± 1.1 a	1.2 ± 0.1 d	3.6 ± 0.1 a	35.5 ± 0.8 a	6.4 ± 0.1 a
	1.0	90.0 ± 1.3 a	2.3 ± 0.1 a	3.1 ± 0.1 a	29.6 ± 0.6 b	5.3 ± 0.1 c
	2.0	87.2 ± 1.1 a	2.6 ± 0.3 a	3.7 ± 0.4 a	37.2 ± 0.6 a	6.4 ± 0.1 a

Values are mean ± S.E of 25 replicated samples for each treatment. Statistical analysis was according to the least significant difference (LSD) test ($p \leq 0.05$).

that the shortest root length (29.6±0.6 mm) was found in treatment with 1.0 mg/L BAP. The highest (6.4±0.1) and lowest (5.3±0.1) numbers of leaves respectively were observed among treatments containing 2.0 and 1.0 mg/L BAP.

Effects of sucrose and the MS nitrogen contents on plant development, Chl contents, and DPPH–scavenging activities

In this experiment, a factorial experimental design with a completely randomized arrangement was used, and ANOVA results of the main effects and their interactions on plantlet growth, Chl contents, and DPPH–scavenging activities are summarized in Table 4. Sucrose treatments significantly differed at the 0.01% significance level, but nitrogen treatment showed negligible differences. On the other hand, the interaction showed a significant effect ($p < 0.001$). All of the tested parameters displayed significant differences ($p < 0.001$ or 0.01) for the main and interaction effects, with the exception of the DPPH–scavenging activity with nitrogen treatment and leaf number and fresh weight with S × N, which were all non–significant.

Table 5 and Fig. 2 reveal that plant heights of *B. formosana* subcultured in 1/2–MS medium supplied with different contents of sucrose (20~60 g/L), NH_4NO_3 (206.3~825 mg/L), and KNO_3 (237.5~950 mg/L) ranged from 69.2±6.1 to 97.7±11.6 mm. Compared to 60 g/L sucrose supplementation of 1/2–MS medium, both 20 and 40 g/L sucrose displayed relatively greater plant heights. The greatest (97.7±11.6 mm) and least (67.3±7.6 mm) plant heights were respectively observed in 1/2–MS supplemented with 20 g/L sucrose, 412.5 mg/L NH_4NO_3, and 475 mg/L KNO_3 and with a mix of 60 g/L sucrose, 825 mg/L NH_4NO_3, and 950 mg/L KNO. The maximum number of adventitious shoots (4.3±0.1) was significantly stimulated by treatment with 40 g/L sucrose, 825 mg/L NH_4NO_3, and 950 mg/L KNO_3. Leaf numbers decreased as the concentration of sucrose increased, and application of 60 g/L sucrose, 206.3 mg/L NH_4NO_3, and 237.5 mg/L KNO_3 in 1/2–MS basal medium significantly reduced the leaf number to 8.0±1.1. Root numbers increased in 1/2–MS medium with 40 g/L sucrose compared to both 20 and 60 g/L sucrose, and a mixture with 412.5 mg/L NH_4NO_3 and 475 mg/L KNO treatment resulted in the highest number of roots (15.9±3.9). *In vitro* plantlets grew vigorously on 1/2–MS basal media supplemented with 20 or 40 g/L sucrose and 1/2– or 1/4–fold nitrogen contents of MS medium for 12 weeks (Fig. 2A, B). Table 5 also shows that the largest (5.5±0.4) and smallest (2.5±0.4) stem widths were observed in conditions of 825 mg/L NH_4NO_3 and 950 mg/L KNO combined

Table 4. ANOVA of sucrose (S), nitrogen (N), and their interaction (S×N) on plantlet development, chlorophyll contents, and DPPH–scavenging activities of 3–month–old plantlets of *Bletilla formosana* after 10 weeks of culture

Source of variance	Degree of freedom	*F* value and significance						
		Plant height	Shoot number	leaf number	Root number	Fresh weight	Chl content	DPPH scavenging activity
Sucrose (S)	2	29.4***	11.0***	30.3***	11.4***	21.8***	156.5***	6.3**
Nitrogen (N)	2	10.0***	20.2***	10.6***	5.9**	8.3**	18.2***	1.4^{NS}
S × N	4	5.4***	5.1***	2.3^{NS}	4.7**	2.4^{NS}	12.1***	12.8***

***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; NS: non–significant difference

Table 5. Effects of sucrose and nitrogen contents on plantlet development of *Bletilla formosana*

Sucrose (g/L)	NH_4NO_3 (mg/L)	KNO_3 (mg/L)	Plant height (mm)	Shoot number	Leaf number	Root number	Stem width (mm)	Fresh weight (g)	Chlorophyll content (mmol/g DW)	DPPH scavenging activity (%)
20	825	950	69.2± 6.1 c	2.6±0.2 bc	16.6±2.8 ab	10.4±1.0 b	2.5±0.4 c	1.0±0.1 a	4.6±0.6 c	72.3±1.1 cd
40	825	950	86.3± 3.7 b	4.3±0.1 a	16.0±2.4 ab	12.3±2.2 ab	5.5±0.4 a	1.2±0.1 a	3.5±0.4 d	84.6±1.7 b
60	825	950	67.3± 7.6 c	2.2±0.2 c	10.4±1.3 cd	11.2±2.2 ab	3.8±0.4 b	0.7±0.2 b	1.8±0.3 e	70.8±1.6 d
20	412.5	475	97.7±11.6 a	3.0±0.7 b	18.6±2.8 a	12.7±3.1 ab	3.8±0.2 b	1.2±0.0 a	8.1±0.6 a	84.9±1.4 b
40	412.5	475	93.4± 6.0 ab	2.5±0.2 bc	13.7±1.2 b	15.9±3.9 a	4.1±0.4 b	1.0±0.1 a	4.2±0.3 cd	75.0±0.7 c
60	412.5	475	69.4± 8.1 c	2.3±0.2 bc	12.5±0.1 bc	11.2±3.0 ab	3.5±0.4 b	0.7±0.0 b	1.6±0.3 e	71.0±1.8 d
20	206.3	237.5	90.7± 9.1 ab	2.2±0.1 c	14.9±1.3 b	12.1±2.4 ab	3.7±0.2 b	1.0±0.1 a	6.2±0.3 b	87.1±0.4 a
40	206.3	237.5	88.8± 3.7 ab	1.7±0.4 d	11.7±0.5 c	13.6±3.6 ab	3.8±0.3 b	0.7±0.1 b	1.6±0.1 e	68.9±0.4 d
60	206.3	237.5	70.5± 7.4 c	0.7±0.5 e	8.0±1.2 d	8.0±1.1 c	3.6±0.1 b	0.6±0.1 b	1.4±0.2 e	81.8±2.0 b

Values are mean ± S.E of 30 replicated samples for each treatment. The statistical analysis was according to the least significant difference (LSD) test ($p \leq 0.05$).

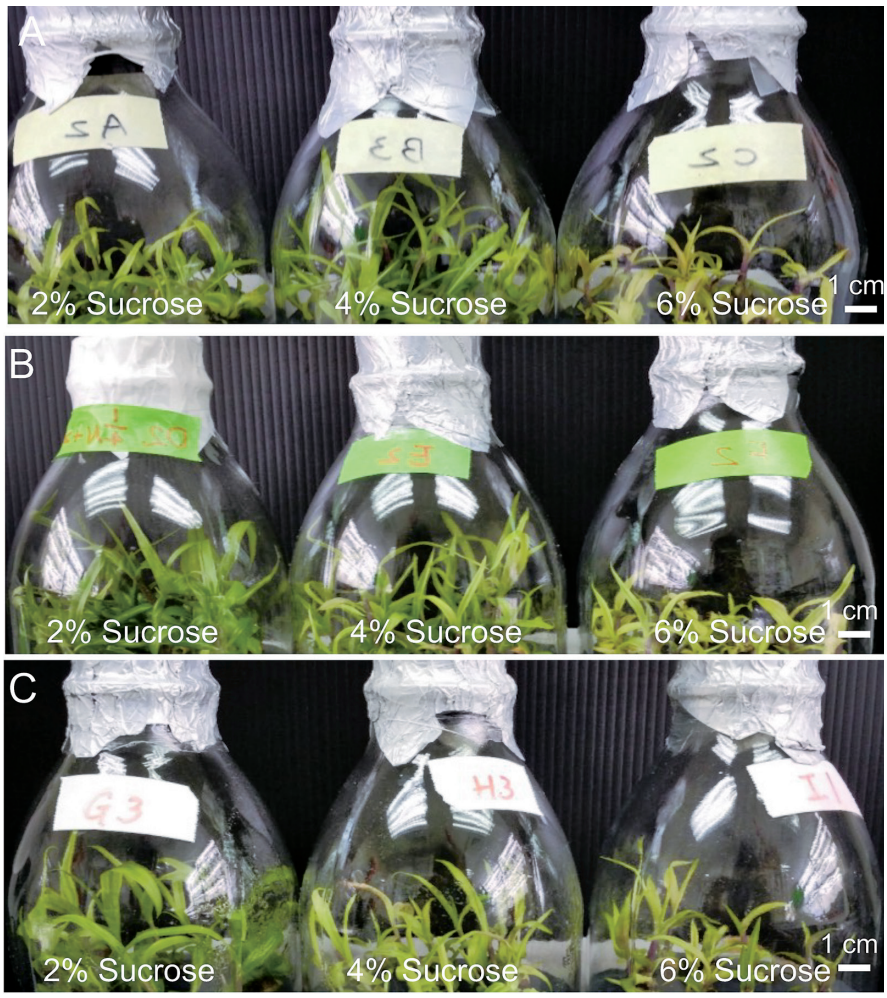

Fig. 2. Effects of sucrose and MS nitrogen contents on plant development of *Bletilla formosana*. Three–month–old seedlings were cultured on 1/2–MS medium with different sucrose contents (20~60 g/L) and nitrogen contents of 825 mg/L NH_4NO_3 and 950 mg/L KNO_3 (A), 412.5 mg/L NH_4NO_3 and 475 mg/L KNO_3 (B), and 206.3 mg/L NH_4NO_3 and 237.5 mg/L KNO_3 (C) after 12 weeks of culture.

with 40 and 20 g/L sucrose, respectively, but the other conditions showed no significant differences. Biomass markedly decreased in treatments with 60 g/L sucrose. Supplementation with 20 or 40 g/L sucrose was effective in biomass accumulation as shown by the fresh weight. Chl contents of *B. formosana* seedlings significantly decreased when sucrose increased from 20 to 60 g/L combined with various nitrogen concentrations, and the Chl content in 1/2–MS treated with 20 g/L sucrose, 412.5 mg/L NH_4NO_3, and 475 mg/L KNO_3 was the highest (8.1±0.6 mmol/g dry weight) compared to other treatments. In general, 1/2–MS basal medium containing 60 g/L sucrose, 206.3 mg/L NH_4NO_3, and 237.5 mg/L KNO_3 showed lower plant development and Chl contents compared to other treatments. The significantly higher DPPH–scavenging activity (87.1%±0.4%) of plantlets was achieved in 1/2–MS medium containing with 20 g/L sucrose, 206.3 mg/L NH_4NO_3, and 237.5 mg/L KNO_3, but an increase in the sucrose concentration to 40 g/L resulted in a significant decrease in the DPPH–scavenging activity to 68.9%±0.4%.

***Ex vivo* acclimatization**

Well–developed plantlets were acclimatized for 4 weeks (Fig. 3A) and then transplanted to plastic pots (5.5 cm diameter × 7.0 cm height) filled with sphagnum moss in a greenhouse (Fig. 3B). More than 90% of the acclimatized plants grew well, blossomed the second year (Fig. 3C, D), and formed white or green pseudobulbs (Fig. 3E).

DISCUSSION

The success of rapid and direct shoot regeneration from protocorm explants offers an efficient way to mass–propagate *B. formosana*. The requirement of exogenous auxins and/or cytokinins for regeneration of shoot and plantlet development was reported for many orchid species (Malabadi *et al.*, 2008). However, the combinations, concentrations, and ratios between auxin and cytokinin for shoot formation vary from species to species. In our study, the highest shoot formation (100%) and number (5.2±0.4) were achieved from protocorm explants using 1/2–MS supplemented with TDZ (2 mg/L)

Fig. 3. Plantlet development, cultivation, flowering, and pseudobulb formation of *Bletilla formosana*. (A) Healthy plantlets after acclimation for 4 weeks. (B) Vigorous plants after cultivation for 1 year. (C) Flowering plants of the second year. (D, E) Well-developed root systems and compressed spindle-shaped pseudobulbs of a white color.

and NAA (0.5 mg/L) and differed significantly from other treatments. Most protocorm explants cultured on 1/2–MS medium containing TDZ or BAP alone showed low efficiency of multiple–shoot regeneration, whereas a combination of TDZ or BAP with NAA significantly increased the formation of multiple shoots, and a high concentration of TDZ was found to be the most effective. Newly emerging shoot buds appeared as small green protuberances, covering the protocorms, and gradually developed into multiple shoots during 12 weeks of culture (Fig. 1C–F). NAA seems to be indispensable for inducing multiple shoots, and the addition of NAA (0.5 mg/L) proved synergistic. Moreover, activated charcoal was very important in the *in vitro* culture of *B. formosana* because the phenolic exudate around the explants appeared after 6 weeks of culture, which damaged the explants (Fig. 1C, D). Nevertheless, leaf growth was promoted on 1/2–MS medium containing BAP alone or combined with NAA. TDZ seemed to inhibit leaf growth since lower leaf numbers (2.6±0.1~3.8±0.2) were mostly recorded in treatment with TDZ alone or combined with NAA.

Rooting of *in vitro* regenerated orchid plants is important for successful acclimatization in the field. Shoots derived from protocorm explants of *Dendrobium aqueum* Lindley produced maximum numbers of roots on 1/2–MS medium supplemented with 3–indolebutyric acid (IBA) 5 mg/L (Parthibhan *et al.*, 2016). Supplementation with 4 μM TDZ and 0.1 μM IBA increased root formation from shoots of *Herminium lanceum* (Singh *et al.*, 2016). In the present study, both NAA and 2,4–D of 0.5~2 mg/L induced regenerated shoot rooting, and 2 mg/L NAA produced a higher efficiency of root formation and growth in *B. formosana* after 8 weeks (Table 2).

The vigor of *in vitro* plantlets of orchids is largely influenced by the presence of PGRs and the nutritional composition of the culture medium. In addition to adventitious shoot induction, exogenous kinetin and BAP of 0.5~2 mg/L significantly promoted plantlet growth of *B. formosana* plants (Table 3). Explants and *in vitro* plantlets are considered to have limited photosynthetic efficiency and require sucrose as a major carbon and energy source for their heterotrophic or mixotrophic growth (Kozai, 1991). Sucrose concentrations of 10~40 g/L and full– to 1/4–strength MS salts were used for *in vitro* plantlet growth of *Caularthron bicornutum* Raf. (Pivetta *et al.* 2010), *Cypripedium macranthos* Sw (Huh *et al.*, 2016), and a *Phalaenopsis hybrid* (Zahara *et al.*, 2017). In our study, compared to 40 or 60 g/L sucrose, 20 g/L sucrose combined with 1/4–fold of MS nitrogen content proved to be excellent not only for plantlet growth and development but also for biomass accumulation and Chl contents (Table 5). Supplementation with 20 g/L sucrose plus 1/8–fold of MS nitrogen content increased the DPPH–scavenging activity of *in vitro* plantlets. Furthermore, 1/2–MS basal medium supplemented with a higher sucrose concentration of 40 g/L was superior for adventitious shoot formation. The inhibitory effect of a high concentration of sucrose on *in vitro* plantlet growth may have been caused by cessation of the cell cycle (Wu *et al.* 2006) and a problem with nutrient uptake due to a lower water potential (Shim *et al.*, 2003) and high osmotic stress

(Shohael *et al*, 2006) of the culture medium. Nitrogen is supplied by ammonium, nitrate, and organic forms in culture medium as constituents of amino acids, enzymes, and proteins, and its bioavailability and absorption are related to plant growth and development. Cardoso and Ono (2011) reported that *in vitro* seedlings of *Brassocattleya* exhibited a greater growth in height on 1/2–MS basal medium supplemented with 1/4–fold of MS nitrogen content. Similarly, in our study, 1/2–MS basal medium supplemented with 20 g/L sucrose and 1/4–fold of MS nitrogen content might provide an optimal osmotic potential and nutrient bioavailability for *in vitro* plantlet growth of *B. formosana*. Moreover, DPPH is a stable free radical, which has been widely used for studying the free radical–scavenging activities of natural antioxidants. Higher DPPH radical–scavenging activity of *in vitro* plantlets of *B. formosana* was observed on 1/2–MS basal medium with 20 g/L sucrose and 1/8–fold of MS nitrogen content, suggesting that minimal nitrogen could increase the antioxidant capacity of *B. formosana*.

The last stage of the mass propagation process, following the shoot multiplication and/or rooting stage, is *ex vitro* acclimatization, which is particularly important for *in vitro* plantlets to overcome the stressful period from the transition of heterotrophic to autotrophic growth. After *ex vitro* acclimatization for 4 weeks, more than 90% of regenerated plantlets had successfully adapted to the *ex vitro* conditions (Fig. 3A). The healthy potted plants grew vigorously with no morphological abnormalities (Fig. 3B) and flowered in the second year with white to pale–pink corollas (Fig. 3C), well–developed root systems (Fig. 3E), and compressed spindle–shaped pseudobulbs of a white color (Fig. 3E).

Conclusions

The efficiency of multiple–shoot formation from protocorms of *B. formosana* was greatly dependent upon the PGRs in the medium. The highest shoot formation from protocorms of *B. formosana* was observed in 1/2–MS basal medium supplemented with 2 mg/L TDZ and 0.5 mg/L NAA, suggesting that *in vitro* culture can be successfully employed for rapid mass propagation. After 12 weeks of *in vitro* culture, regenerated shoots had successfully formed roots in 1/2–MS basal medium supplemented with 2 mg/L NAA. In addition, exogenous kinetin or BAP and modification of the MS nitrogen content to 1/4–fold improved the growth and development of *in vitro* plantlets. Well–developed plantlets were acclimatized with over a 90% survival rate. To our knowledge, this is the first report of *in vitro* plant regeneration of *B. formosana* plants through protocorms, and the efficient protocol can be applied to the large–scale mass propagation and germplasm conservation of this endangered orchid species, endemic to Taiwan.

AUTHOR CONTRIBUTIONS

MKN performed the overall experiments and analyzed the data. KHL participated in the statistical analysis of the study and discussed on the results. LRC designed the study and wrote the paper. TCH and YO supervised the work and edited the paper.

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