High-frequency Microtuberization by One-step Culture without Medium Renewal from Leaf Explants of *Pinellia ternata*, a Medicinal Plant

Jeong Ho Ahn[1] and Seung Yeob Lee[2]* ⓘD

[1]Division of Crop Research, Chungcheongnam-do ARES, Yesan 32418, Korea
[2]Institute of Life Science and Natural Resources, Wonkwang University, Iksan 54538, Korea

*Corresponding author: sylee@wku.ac.kr

Abstract

Pinellia ternata is an important medicinal herb in Northeast Asia, but its propagation rate is quite low in nature. This study was performed to develop a simple and rapid micropropagation method for applying to a plant factory system. In the regeneration process for the sustainable production of in vitro leaf explants, callus induction and propagation from leaf explants of *P. ternata* were excellent in MS medium containing 2.0 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA. The highest number of shoots (8.8 per callus) was regenerated from calluses cultured on a medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN. The callus proliferation with subsequent plant regeneration was achieved at the same time in regeneration medium, and the year-round production of in vitro leaf explants was possible in the regeneration cycle. The direct microtuberization from in vitro whole leaf explants was also achieved through the one-step culture without medium renewal. The highest frequency of microtuber formation (93.0%) as well as the highest number of microtubers per explant (6.1) was observed by one-step culture in MS medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA. Thus, the direct microtuberization from in vitro leaf explants was successfully achieved by the one-step culture protocol without medium renewal from microtuber induction to harvest. The adequately dried microtubers were well grown like an artificial seed in the pot culture containing a commercial soil. Accordingly, the in vitro microtubers through one-step culture could be commercially used as seed tubers for a plant factory system.

Additional key words: callus induction, growth regulators, plant regeneration, seed tubers, soil cultivation

Introduction

Pinellia ternata (Thunb) Breit is an herbaceous perennial plant belonging to the Araceae family and widely native in Northeast Asia. It is an important medicinal herb in Korea, China, and Japan. The dried tuber of original *P. ternata* (Pinellia Rhizoma, Korean name "Banha") has been used traditionally for curing coughing and dampness-phlegm, relaxing, dizziness, headache, and acute gastritis as a Korean herbal medicine. Pinellia Rhizoma has modern pharmacological effects, such as an anti-cancer effect, anti-obesity effect, emetic and anti-emetic effect, neuroprotective effect, and anti-stress effect (Kurata et al., 1998; Lim et al., 2009; Jie et al., 2015).

Recently, consumption of Pinellia Rhizoma has been increasing through mixed herbal formulas containing Pinellia Rhizoma extracts with the development of Asian medicine-derived systemic treatments (Park and Seo 2000; Lim et al., 2009). The artificially cultivated Pinellia Rhizoma is mainly circulating in herbal medicine markets because wild Pinellia Rhizoma is expensive. Nevertheless, products of Pinellia Rhizoma are insufficient compared to consumption in the Korean herbal medicine market. Most Pinellia Rhizoma is now being imported from China. Imports are increasing year by year. Also, the small rhizome of diverse plant species belonging to the other *Arisaema* species has been incorrectly used as Pinellia Rhizoma, which has greatly affected the quality of the herbal medicine (Lin et al., 2006; Jing et al., 2019).

P. ternata can be propagated both sexually seeds and asexually bulbils, but it is mainly depended on the bulbils in nature (Gu et al., 1994; Kim et al., 2005a; Luo et al., 2014). In natural conditions, the flowering rate is very low (Gu et al., 1994), because it is overlapping with the rainy season in Korea (Kim et al., 2005a). A mature plant of *P. ternata* produces only 7 – 8 bulbils per year (Peng et al., 2007). *P. ternata* is commercially cultivated using the small tubers without commercial value after harvesting. Tissue culture techniques has been used as an efficient protocol for vegetative propagation, because of poor reproduction in *P. ternata*. Callus induction and plant regeneration of *P. ternata* have been performed with explant sources, culture media, and combinations of plant growth regulators using a two- or three-step culture system by many researchers (Choi and Rha 1986; Kim et al., 1994; Kim et al., 2005a; Xu et al., 2005; Eun et al., 2015; Zhang et al., 2017). The two- or three-step protocol consists of as a first step, the induction of callus formation, the second step morphogenetic or embryogenetic induction and the third step organogenetic differentiation. Each phase require different hormone and takes long time to product in vitro organs (Komal, 2011). While the one-step protocol is that in vitro organs are directly induced from explant tissues. The one-step culture system reduces the cost and time of in vitro vegetative propagation. In vitro microtuber production of *P. ternata* has been accomplished with explant sources, gelling agents, and combinations of plant growth regulators by the one-step culture system (Xue et al., 2006; Kim et al., 2007; Peng et al., 2007). In particular, in vitro propagation of medicinal plants is essential to produce circularly without somaclonal variation. Somaclonal variation derived from tissue cultures can cause problems with the genetic uniformity of the regenerated plants and lead to subsequent negative efects on the use of tissue culture (Park et al., 2020). However, the protocols of previous researches were economically critical for use in the plant factory system. We designed a simple protocol to develop the new one-step culture system without medium renewal in the micropropagation of *P. ternata*.

The present work aimed to develop a simple and rapid micropropagation method for applying the plant factory system. Firstly, we evaluated the effects of growth regulators on the sustainable production of in vitro leaf explants of *P. ternata*. Secondly, the effect of growth regulators on direct microtuberization of whole leaf explants was evaluated in one-step culture protocol without medium renewal. Thirdly, the growth of microtubers produced through one-step culture was estimated in soil cultivation.

Materials and Methods

Preparation of Leaf Explants

Tubers of *Pinellia ternata* were collected from the Medical Plant Garden at Wonkwang University in Korea, and tubers' removed leaves were grown under greenhouse conditions with 50% shade in plastic pots. Newly tender leaves were taken

as explant materials from regrown plants on May 25, 2018. The leaf explants were surface-sterilized for $10 - 15$ second in 70% EtOH (v/v), then rinsed twice with sterile distilled water on a clean bench. Furthermore, the leaf explants were sterilized for 20 min. in 0.5% NaOCl (v/v, Samchun Chemical Co, Ltd., Seoul, Korea) with 2 drops of Tween-20 (Daejung Chemical and Metal Co, Ltd., Korea), followed by rinsing three times with sterile distilled water. Finally, leaf explants were transferred to sterile Petri dishes and cut into small pieces (5×5 mm) with the removal of the main vein.

Callus Induction

For callus induction and proliferation, leaf segments were cultured on MS (Murashige and Skoog, 1962) media, gelled 0.3% (w/v) Phytagel (Sigma Chemical Co. Ltd., USA), and supplemented with different levels of 2,4-D (2,4-dichlorophenoxy acetic acid, Sigma Chemical Co. Ltd., USA) and BA (Benzyl amino purine, Sigma Chemical Co. Ltd., USA). The media pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. The leaf segments were plated in each Petri dish (100×15 mm), for a total of 50 explants per medium. The Petri dishes were sealed with parafilm (60×15 mm, Whatman Ltd.) and were incubated in the dark at 25 ± 1°C for 5 weeks. Callus induction frequency and fresh weight were investigated in media containing different concentrations and combinations of growth regulators at 5 weeks in the culture (Table 1).

Subsequent Plant Regeneration

For plant regeneration, the calluses propagated in the medium supplemented with 2 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA were transferred to 500 mL glass vessels containing MS media gelled 0.3% (w/v) Phytagel supplemented with different levels of NAA (Napthalene acetic acid, Sigma Chemical Co. Ltd., USA) and KIN (kinetin, Sigma Chemical Co. Ltd., USA). Six calluses were plated in each bottle (500 mL) for a total of 30 calluses per medium. The culture was incubated under a 16-h light photoperiod provided by cool white fluorescent lamps at a photon flux density of 27 µmol·m^{-2}·s^{-1}, at 25 + 1°C. The number of calluses producing shoots and the total number of shoots per callus were counted for each treatment at 6 weeks in the culture (Table 2). Also, the nature of callus proliferation was investigated in eight plant-regeneration media for subsequent callus proliferation and plant regeneration at the same time by a combination of plant growth regulators.

Direct Microtuberization by One-Step Culture

The leaves derived from the regeneration medium containing 2.0 mg·L^{-1} NAA, 0.2 mg·L^{-1} KIN were used as explant sources for mass production of microtubers by a one-step culture. Leaves of $10 - 15$ mm in size were excised and immediately cultured on MS media gelled 0.3% (w/v) Phytagel supplemented with different levels of NAA and BA (Table 3). Twenty leaf explants were plated in each Petri dish (100×15 mm) for a total of 60 leaves per medium. The culture was incubated under the same condition in a plant regeneration environment for 10 weeks until the media were naturally dried without a subculture. The number of explants producing microtubers and the total number of microtubers per leaf explant were counted for each treatment at 10 weeks in culture.

Growth and Propagation of Microtubers in Soil

Microtubers produced by the one-step culture were overwintered at room temperature at around $10 - 15$°C. Fifteen microtubers (3 explants with 5 microtubers) were planted in plastic pots (Ø 110×100 mm) containing a commercial soil

(TKS2, N-P-K : 330 – 220 – 400 mg·L^{-1}, Korea Horticulture Materials Co. Ltd., Seoul, Korea) on April 20. In the greenhouse, the pots were cultivated under shade cloth (50% light reduction) with bottom watering every 3 days. Fertilizer was applied as a base dressing Urea (217 mg·L^{-1}), fused superphosphate (500 mg·L^{-1}), and potassium chloride (200 mg·L^{-1}). After 6 months, the plant height, number of tubers per pot, and tuber size were investigated using three size ranges (> 7, 4 – 7, and < 4 mm diameter) to evaluate the production of seed tubers for application in a plant factory system.

Statistical Analysis

Each experiment was conducted in a completely randomized design with 5 replications. The data were calculated using SAS software Version 9.1 (statistical analysis system, Version 9.1, SAS Institute Inc., USA). Significant differences between means were determined using Duncan's multiple range test ($p < 0.05$).

Results

Callus Induction

In plant tissue culture, the combination of auxin and cytokinin in the medium is vital for the commercial vegetative propagation of plants. Callus induction of *P. ternata* leaf explants was greatly influenced by growth regulators. After 2 weeks, inducing calluses could be observed at cross-sections of the leaf explants, and induced calluses were rapidly grown around 3 weeks after the culture. Thereafter, the growth of the calluses was affected by the combination of auxin and cytokinin. After 5 weeks, the percentage of callus induction was promoted in higher 2,4-D levels, and it was significantly higher in the combination media of 2,4-D and BA than with the application of 2,4-D alone (Table 1). The highest callus induction and propagation were produced in the MS medium supplemented with 2.0 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA. MS media added with other growth regulator combinations also showed excellent callus induction capacities, such as 2.0 mg·L^{-1} 2,4-D and 0.5 mg·L^{-1} BA as well as 1.0 mg·L^{-1} 2,4-D and 0.5 mg·L^{-1} BA. The highest callus fresh weights were obtained in media supplemented with 2.0 mg·L^{-1} 2,4-D and 0.2 – 0.5 mg·L^{-1} BA. A combination of 2,4-D and BA was also

Table 1. Effects of 2,4-D and BA on callus induction and growth from leaf explants of *P. ternata*

Growth regulators (mg·L^{-1})		No. of explants	Callus induction (%)	Fresh weight (mg·explant^{-1})
2,4-D	BA			
0.5	0.0	50	36.0 d^z	12.8 e
	0.2	50	70.0 c	24.8 cd
	0.5	50	84.0 b	25.8 bcd
1.0	0.0	50	46.0 d	20.6 d
	0.2	50	88.0 ab	28.2 bc
	0.5	50	92.0 ab	30.4 b
2.0	0.0	50	68.0 c	26.6 bc
	0.2	50	98.0 a	40.8 a
	0.5	50	94.0 ab	38.2 a
LSD 0.05			13.85	5.31

^{z}Means within each column followed by the same letter are not significantly different using Duncan's multiple range test ($p < 0.05$).

had a significantly higher fresh weight than 2,4-D alone. Further calluses were subcultured in the same MS media with 2.0 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA for increasing the number of calluses for uses in the experiment of plant regeneration. Thus, callus induction from leaf explants of *P. ternata* was achieved using MS medium with combinations of auxin (2,4-D) and cytokinin (BA).

Subsequent Plant Regeneration

The calluses propagated in MS media containing 2.0 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA were used as experimental materials for plant regeneration. Calluses were cultured in MS media containing the combinations of NAA and KIN under a 16-hour light photoperiod. After 1 week, many green spots appeared on the surface of light yellow- to light green-colored calluses, and nodular structures emerged soon from the green spots. Shoots were subsequently regenerated from the green nodules in 2 − 3 weeks, and leaves developed soon after shoot emergence. One to three roots per shoot were initiated in the media containing over 0.5 mg·L^{-1} NAA, and then shoots grew more vigorously. The percentage of shoot regeneration and number of shoots were estimated at 6 weeks after the culture (Table 2). Most of the cultured calluses showed well-developed shoots within 3 weeks. The combinations of NAA and KIN showed excellent shoot initiation in all tested regeneration media. The percentage of explants with shoots was 100% in all tested media. The number of plantlets per callus was affected by plant growth regulators, and significant differences in the number of plantlets per callus were found among the regeneration media tested.

The highest number of shoots (8.8 per callus) was regenerated from calluses cultured on MS medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN. Other regeneration media also showed good shoot regeneration without significant differences, such as 1.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA as well as 0.5 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA. In vitro regenerated plants grew about 7 cm in height after 6 weeks (Fig. 1A). Shoot growth was similar in all media, but the proliferation and quality of calluses varied, as shown in Table 2. The proliferation and quality of calluses as well as plant regeneration were excellent in the medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN. Accordingly, the calluses proliferated in the regeneration medium were replaced in a fresh regeneration medium supplemented with NAA 2 mg·L^{-1} and KIN 0.2 mg·L^{-1} for using as the leaf explant source for a subsequent plant regeneration system.

Table 2. Effect of NAA and kinetin on plant regeneration from the callus derived from leaf explants of *P. ternata*

Growth regulators (mg·L^{-1})		No. of cultured calluses	% of explants with shoots	No. of plantlets per callus	Nature of callus propagation
NAA	Kinetin				
0.2	0.2	30	100	5.8 bcd[z]	− [y]
	0.5	30	100	6.4 bc	−
0.5	0.2	30	100	7.2 abc	+
	0.5	30	100	6.1 bcd	+
1	0.2	30	100	7.7 ab	+
	0.5	30	100	5.2 cd	++
2	0.2	30	100	8.8 a	+++
	0.5	30	100	4.2 d	+++
LSD 0.05				2.07	

[z]Means within each column followed by the same letter are not significantly different using Duncan's multiple range test ($p < 0.05$).
[y] −, +, ++, +++ ; poor, moderate, good, and excellent callus propagation.

Direct Microtuberization by One-Step Culture

Direct microtuberization of *P. ternata* by the one-step culture was conducted to develop a new micropropagation system. The leaf explants regenerated in vitro (more than 1cm length) were placed on MS media gelled 0.3% (w/v) Phytagel containing different levels of NAA and BA without cutting into a small size. Microtubers with pale yellow to greenish color were initiated around 3 weeks after the culture, and those were developed to about 3–4 mm in diameter at 6 weeks after culture. The percentage of microtuber formation and the number of microtubers per explant were estimated at 6 weeks after culture (Table 3). The highest frequency of microtuber formation (93.0%) was achieved in the medium with a combination of 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA. There was no callusing or sprouting in the microtubers derived from whole-leaf explants. The number of microtubers per explant was highest in the same medium (6.1 per explant). At the higher concentrations of BA media, there were significantly fewer of both the frequency of microtuber formation and the number of microtubers per explant.

The hormonal combination of BA and NAA was crucial for the direct microtuberization of whole-leaf explants of *P. ternata*. Whole-leaf explants were economically remarkable as an explant source for in vitro microtuber production of *P. ternata*. After 7 weeks, microtubers placed on the medium were spontaneously dried in the Petri dish removed Parafilm for 2 weeks and then preserved under room temperature for 4 months (Fig. 1B). Microtubers preserved in a Petri dish were used in the soil cultivation experiment.

Growth and Propagation of Microtubers in Soil

Microtubers produced in vitro were planted on April 20 in pots (Ø 110 × 100 mm), and the growth and propagation of microtubers after 6 months were investigated to evaluate the production of seed tuber for using in the plant factory system. Most microtubers were sprouted in 2–3 weeks after planting, and the average emergence rate was 81.5% after 1 month (Table 4). Plants sprouted in pots were vigorously grown without summer depression from July to August. The average plant height was 11.4 cm after six months (Fig. 1C). All plants had one to three leaves, and most of them developed one or two bulbils on the bases or tops of petioles in late September to early October. After 6 months, the average number of

Table 3. Effect of NAA and BA on direct microtuberization from in vitro leaf explants of *P. ternata*

Growth regulators (mg·L^{-1})		No. of cultured explants	Microtuberization (%)	No. of teberlets (no.·explant^{-1})
NAA	BA			
0.5	0.2	60	27.0 de[z]	1.0 e
	0.5	60	22.0 ef	0.8 e
	1.0	60	14.0 f	0.7 e
1.0	0.2	60	62.0 b	4.1 b
	0.5	60	41.0 c	3.2 c
	1.0	60	36.0 cd	2.8 d
2.0	0.2	60	93.0 a	6.1 a
	0.5	60	86.0 a	4.3 b
	1.0	60	63.0 b	3.5 c
LSD 0.05			10.64	0.38

[z]Means within each column followed by the same letter are not significantly different using Duncan's multiple range test ($p < 0.05$).
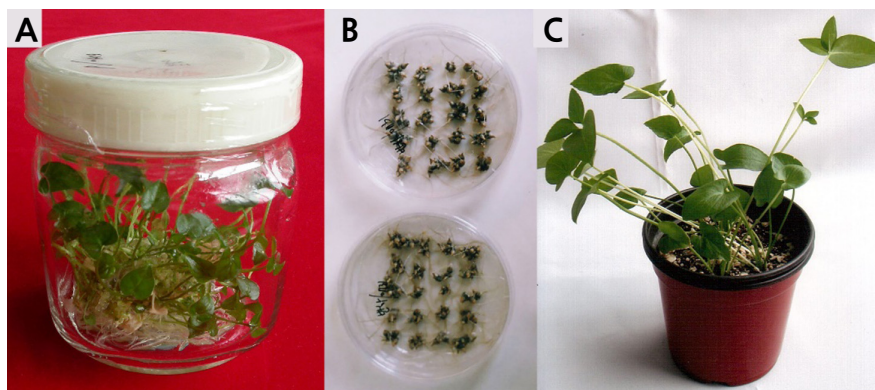
Fig. 1. Direct microtuberization from the leaf explants regenerated in vitro of *P. ternata* by one-step culture. Plants regenerated in the medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN (A), Direct microtuberization from leaf explants by one-step culture in the medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA (B), growth of in vitro microtubers under 50% shading in greenhouse (C).

Table 4. Growth and propagation of plants grown from in vitro microtubers of *P. ternata* in pot culture

Plant height (cm)	Emergence rate (%)	No. of tuber per pot (%)				Fresh weight (mg·tuber^{-1})				Propagation times
		< 4 mm	4 − 7 mm	7 mm <	Total	< 4 mm	4 − 7 mm	7 mm <	Mean	
11.4	81.5	18.0 (67.2)	8.8 (32.8)	0.0 (0.0)	26.8 (100)	96.3	305.8	0.0	165.5	1.5

harvested tubers was 26.8 per pot, and the average number of tubers with a diameter of below 4 mm was 67.2%. The total number of tubers propagated was about 1.5 times for 6 months in the pot culture. The mean fresh weight of tubers harvested was 165.5 mg per tuber, and the mean fresh weight was higher by over 3 times in tubers of 4-7 mm than in tubers below 4 mm. Thus, tubers harvested in soil cultivation could be used as seed tubers in the field or plant factory.

Discussion

In vitro micropropagation is an effective propagation method for large-scale industrial or commercial needs in asexual or vegetative propagation of plants. Micropropagation of *P. ternate* has been studied by many researchers (Choi and Rha, 1986; Kim et al., 1994; Kim et al., 2005a; Xu et al., 2005; Eun et al., 2015; Zhang et al., 2017). In vitro plant regeneration is very important as a key target in the process of micropropagation. Plant regeneration from explants can be achieved by direct or indirect organogenesis and embryogenesis. In the case of direct plant regeneration, in vitro organs are directly induced from explant tissues by one-step; in the case of indirect regeneration, in vitro organs are typically formed via callus induction by a two- or three-step process. One-step process of plant regeneration seemed to be a cost effective and shorter protocol (Tsay et al., 1989; Xue et al., 2006; Komal, 2011). One-step culture are widely used for spore germination, gametophyte proliferation, and sporophyte formation in *Reridophyta* (Jang et al, 2019a, 2019b). So we experimented to develop the simple protocol for direct microtuberization from leaf explants of *P. ternata* by one-step process without medium renewal.

Firstly, we evaluated the effects of growth regulators on the sustainable mass-production of in vitro leaf explants of *P. ternata*. We also tried to select the medium for subsequent callus proliferation and plant regeneration in the same medium.

Callus induction was greatly influenced by explant sources and growth regulators. The callus induction of *P. ternata* was successfully achieved from various explants such as leaf, petiole, root, and tuber (Kim et al., 2005a; Xu et al., 2005; Jie et al., 2015). Callus induction from leaf explants was also higher than that from petiole (Kim et al., 2005b; Xu et al., 2005). Although Tsay et al. (1989) and Xu et al. (2005) reported that the best explant of *P. ternata* for promoting tuberization or callus induction was a tuber segment among leaf, petiole, and tuber, we found the leaf explant was more economic as an explant source because leaves can be cut in many segments and more readily collect in nature and in vitro. In this work, the callus induction and proliferation of *P. ternata* were investigated on several MS basal media containing different concentrations and combinations of 2,4-D and BA. Callus induction from leaf explants of *P. ternata* was associated with the best combination in the medium containing 2.0 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} BA. Similar results had been reported in *P. ternata* by other researchers (Choi and Rha 1986; Kim et al., 2005a; Xu et al., 2005; Kim et al., 2007). Kim et al. (1994) reported that callus induction of the leaf explant was better in the media containing 2,4-D than NAA or IAA. Peng et al. (2007) also reported that no visible callus appeared in a medium without 2,4-D. The effective induction and proliferation of callus in *P. ternata* was achieved in the presence of 2,4-D, and showed more excellent results in the combination of 2,4-D and BA than 2,4-D alone. Thus, a suitable combination of 2,4-D and BA is required for callus induction and proliferation from leaf explants of *P. ternata.*

In the plant regeneration medium, a suitable combination of auxin and cytokinin was also required for organogenesis of *P. ternata.* This combination medium was important for cycling a subsequent callus proliferation and plant regeneration at the same time. BA, KIN, or zeatin added to the nutrient medium might stimulate shoot formation (Kim et al., 2005b; Xu et al., 2005; Jie et al., 2015). In this work, the subsequent callus proliferation and plant regeneration of *P. ternata* was associated with the best combination in the medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN (Table 2). In another study, the addition of NAA alone in the regeneration medium was found to inhibit regeneration (Jie et al., 2015). Also, 2,4-D was ineffective for induction of embryogenesis from leaf and petiole explants *P. ternata*, but a combination of NAA and BA promoted the frequency of embryogenesis (Kim et al., 2005b). We tried to establish the subsequent callus proliferation and plant regeneration system at the same time, and we successfully gained the leaf explants of plants regenerated in vitro year-round. Propagated calluses were possible maintaining the plant regenerating potential over 18 months after the subculture by Xu et al. (2005). In this study, high-frequency shoot regeneration and the maximum number of shoots per callus were obtained on MS medium containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN. The regenerated plants were used as the source of disease-free explants for direct microtuberization.

Secondly, we tried to establish the simple and rapid propagation method using direct tuberization of *P. ternata* by a one-step culture system. In vitro microtuberization of *P. ternata* has also been done with explant sources, gelling agents, and combinations of plant growth regulators by many researchers (Xue et al., 2006; Kim et al., 2007; Peng et al., 2007; Jie et al., 2015; Kim et al., 2020). The results of previous research were, however, transplanted the plants regenerated from in vitro microtubers into the soil. The regenerated plantlets demand a long time for acclimatization and growth in soil. We were established the production protocol of in vitro dried microtubers like an artificial seed by one-step culture without a renewal culture medium. The direct tuberization from whole-leaf explants of *P. ternata* was successfully achieved in a combination media of BA and NAA. In nature, the bulbil of *P. ternata* was formed in the lower part of petiole, and it started from the parenchyma cells in young leaves (Luo et al., 2014). The highest frequencies of microtuber formation (93.0%) and microtubers per explant (6.1) were achieved in the medium with a combination of 2.0 mg·L^{-1} NAA and 0.2

mg·L^{-1} BA. Although the direct organogenesis of *P. ternata* was possible in a medium containing NAA alone (Peng et al., 2007), the direct tuberization from explants without callus induction was largely affected by a higher cytokinin concentration than auxin (Kim et al., 2007; Eun et al., 2015). Jie et al. (2015) explained that cytokinins are the primary effector, and auxin plays a secondary role. In this work, the microtuberization from whole-leaf explants occurred through direct organogenesis without sprouting or callusing in the medium containing higher NAA concentration (2.0 mg·L^{-1}) than BA (0.2 mg·L^{-1}). We assumed that this result was affected by the endogenous hormones of leaf explants derived from the plant regeneration medium (containing 2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} KIN). The number of microtubers per explant was high at 8.8 per explant in the same medium (2.0 mg·L^{-1} NAA and 0.2 mg·L^{-1} BA), and the efficiency of the microtuber production was able to produce about 150 − 170 microtubers per Petri dish during 7 weeks (Fig. 1). Tsay et al. (1989) reported that the explants directly produced adventitious buds in a solid medium containing 2 mg·L^{-1} BA and 0.2 mg·L^{-1} NAA, and claimed that 1.7×10^{27} plantlets in one year could be produced from a single bulbil through protocom-like bodies and adventitious shoots without callus induction. Microtubers induced in vitro did not sprout for up to 6 weeks. Microtubers were spontaneously dried from 7 weeks, and the adequate dried microtubers were used as a seed tuber in soil cultivation.

Thirdly, we tried the soil cultivation of the dried microtubers produced by one-step culture like an artificial seed. The dried microtubers were seeded in plastic pots (Ø 110 × 100 mm) containing a commercial bed soils. The microtubers sprouted in 2-3 weeks after planting in soil, and the average emergence rate was 81.5% after 1 month (Table 4). After 6 months, the total number of tubers propagated was about 1.5 times that in the pot culture, and harvested tubers could be used as seed tubers for field cultivation in the next year. Although the fresh weight of harvested tubers was low in the pot culture (mean 165.5 mg), we assumed this was because of the small size of the pot (Ø 110 × 100 mm) culture and the high temperature in the greenhouse. If in vitro dried microtubers were cultivated in the plant factory, and this protocol could be produced the high-quality seed tubers of *P. ternata* for commercial applications.

In a typical tissue culture experiment, the protocol consists of, as the first step, the callus induction, the second step, morphogenetic/embryogenetic induction, and the third step, plant differentiation and transplanting into soil. Each step requires different growth regulators and takes about 8 weeks to achieve a sufficient result (Komal, 2011). We guessed that this one-step protocol was more simple and economic results for the commercial applications of *P. ternate.*

In conclusion, in vitro micropropagation by one-step culture could reduce the cost and time in the seed tuber production of *P. ternata* with low propagation in nature. We conclude that the one-step microtuberization protocol without medium renewal by in vitro cycling system was established for the mass production of seed tubers from *P. ternata*. In this cycling system, the main step was to establish the direct microtuberization system without medium renewal during a short period of time. The direct microtuberization from whole-leaf explants of *P. ternata* could be successfully achieved in the combination media of BA and NAA. This system needed a process of callus proliferation and plant regeneration to achieve a sustainable supply of disease-free leaf explants. The sustainable supplying of in vitro leaf explants was possible in the plant regeneration medium containing NAA and KIN. Thus, we established the subsequent callus proliferation and plant regeneration system in the same medium, and the in vitro whole leaf explants with no disease were successfully supplied for microtuberization all year-round. Furthermore, the direct microtuberization by the one-step culture without medium renewal is suitable for mass producing seed tubers of *P. ternata* in the plant factory system. The results could lead to reduced cost and time to farmers in the commercial production of *P. ternata*.

Literature Cited

Choi JS, Rha ES (1986) Studies on the mass propagation of *Pinellia ternata* (Thunb.) breit in vitro. Korean J Crop Sci 31:30-42

Eun YJ, Ryu YB, Choi SA, Ahn MS, Liu JR, Min SR, Kim SW (2015) Mass propagation of microtubers from suspension cultures of *Pinellia ternata* cells and quantitative analysis of succinic acid in *Pinellia* tubers. Plant Biotechnol Rep 9:331-338. doi:10.1007/s11816-015-0369-0

Gu DX, Li YX, Xu BS (1994) A study on the reproductive biology of *Pinellia ternata* (Thunb.) Breit. J Plant Res Environ 3:44-48

Jang BK, Cho JS, Lee CH (2019a) Propagation methods for gametophyte proliferation and sporophyte formation in silver cloak fern (*Cheilanthes argentea*). Hortic Environ Biotechnol 60:435-442. doi:10.1007/s13580-019-00128-6

Jang BK, Cho JS, Kwon HJ, Lee CH (2019b) Optimal conditions for spore germination and gametophyte and sporophyte production in the autumn fern *Dryopteris erythrosora*. Hortic Environ Biotechnol 60:115-123. doi:10.1007/s13580-018-0097-9

Jie EY, Ryu YB, Choi SA, Ahn MS, Liu JR, Min SR, Kim SW (2015) Mass propagation of microtubers from suspension cultures of *Pinellia ternata* cells and quantitative analysis of succinic acid in *Pinellia* tubers. Plant Biotechnol Rep 9:331-338. doi:10.1007/s11816-015-0369-0

Jing Y, Lai Y, Chen H, Li M, Zhou J, Lan Z (2019) Study on the identification of *Pinelliae rhizoma* and *Pinelliae pedatisectae rhizoma* based on the characteristic component triglochinic acid. RSC Adv 9:11774-11780. doi:10.1039/C9RA01626K

Kim HS, Kim TC, Lee SY (2005a) Callus induction and plant regeneration from root explants of *Pinellia ternata*. Hortic Environ Biotechnol 46:87-92

Kim, SW, In DS, Tae KH, Liu JR (2005b) Somatic embryogenesis and plant regeneration in leaf and petiole explant cultures and cell suspension cultures of *Pinellia tripartita*. Plant Cell Tiss Organ Cult 80:267-270. doi:10.1007/s11240-004-1018-4

Kim TS, Park MS, Park HK, Kim S, Jang YS (1994) Plant regeneration and in vitro tuber enlargement from callus in *Pinellia ternata* (Thunb.) Breit. Korean J Med Crop Sci 2:246-250

Kim YG, Komakech R, Choi JE, Lee KY, Lee TK, Kim KH, Moon BC, Kim SW, Kang Y (2020) Mass production of *Pinellia ternata* multiple egg-shaped micro-tubers (MESMT) through optimized growth conditions for use in ethnomedicine. Plant Cell Tiss Organ Cult 140:173-184. doi:10.1007/s11240-019-01720-5

Kim YK, Lee JY, Kim EH, Cho DH, and Park SU (2007) Effect of plant growth regulators, media, and gelling agents on in vitro microtuber production of *Pinellia ternata* Breit. Korean J Med Crop Sci 15:210-214

Komal R (2011) One step method of plantlet regeneration in *Trichosanthes dioica* Roxb.: An approach towards cost effective and shorter protocol. Afr J Biotechnol 10:9-12. doi:10.5897/AJB10.031

Kurata K (1998) Quantitative analysis of anti-emetic principle in the tubers of *Pinellia ternata* by enzyme immunoassay. Planta Medica 64:645-648. doi:10.1055/s-2006-957539

Lim YK, Park JH, Seo BI, Roh SS, Ku SK (2009) Single oral dose toxicity study of *Pinelliae Rhizoma* aqueous extract in ICR mice. Toxicol Res 25:147-157. doi:10.5487/TR.2009.25.3.147

Lin J, Zhou X, Gao S, Wu W, Liu X, Sun X, Tang K (2006) Authentication of *Pinellia ternata* and its adulterants based on PCR with specific primers. Planta Med 72:844-847. doi:10.1055/s-2006-931605

Luo R, Du YS, Sun YY, Cao ZJ (2014) Morphological observation and anatomical study on bulbil development of *Pinellia ternata*. Acta Botanica Boreali-Occidentalia Sinica 34:1776-1781

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco culture. Physiol Plant 15:473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

Park JH, Seo BI (2000) A philoligical study on poisoning of *Pinellia rhizoma*. J East-West Med 25:47-55

Park JS, Park JH, Kim SJ, Park YD (2020) Genome analysis of tissue culture-derived variations in regenerated *Brassica rapa* ssp. pekinensis plants using next-generation sequencing. Hortic Environ Biotechnol 61:549-558. doi:10.1007/s13580-020-00237-7

Peng ZS, Luo CK, Cai P, Mao ZC, Kang CY, Yang J (2007) Rapid propagation of the medicinal plant *Pinellia ternata* by in vitro leaves culture. Bulg J Agric Sci 12:1-6

Tsay HS, Gau TG, Chen CC (1989) Rapid clonal propagation of *Pinellia ternata* by tissue culture. Plant Cell Rpt 8:450-454. doi:10.1007/BF00269046

Xu T, Zhang L, Sun X, Tang K (2005) Eficient in vitro plant regeneration of *Pinellia ternata* (Thunb) Breit. Acta Biol Crac Ser Bot 47:27-32

Xue JP, Zhang AM, Sheng W, Zhai ZJ (2006) Effects of potassium salts on direct induction of microtubers in *Pinellia ternata*. China J Chinese Materia Medica 31:546-548

Zhang B, Hu Y, Jia M, Jin L, Xu D, Chen J (2017) Micropropagation of *Pinellia ternata* (Thunb.) Berit. Plantlets using temporary immersion bioreactors. J Biobased Materials and Bioenergy 11:59-65. doi:10.1166/jbmb.2017.1619