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Full Length Research Paper

Shoot multiplication of *Paphiopedilum* orchid through *in vitro* cutting methods

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Paphiopedilum **orchids are generally propagated through the division of axillary buds from mother plant, which limits commercial production due to its unproductive proliferation and time consuming. In this study, the effect of** ***in vitro*** **cutting methods and medium composition on efficient shoot multiplication of** ***Paphiopedilum*** **Hsinying Rubyweb was investigated. Among three different** ***in vitro*** **stem cutting methods used, vertical cutting was able to produce more new shoots than horizontal and cross cutting when cultured on Hyponex based medium. After 12 weeks of culture, plantlets regenerated from vertical cutting were able to produce new healthy and well rooted shoots higher than without cutting on the same medium. Moreover, the newly-formed shoots which were divided into single plantlets and subcultured onto half-strength Murashige and Skoog (MS) medium without growth regulators could remain higher shoot multiplication than in other media. The micropropagation procedure developed in this study provides a simple means to** ***in vitro*** **propagate** ***Paphiopedilum*** **plantlets which are able to produce large numbers of uniform plantlets in a shorter time compared to the conventional propagation method.**

Key words: Micropropagation, shoot multiplication, cutting, *Paphiopedilum*.

INTRODUCTION

Paphiopedilum is commonly known as ‘slipper orchid’ because of the resemblance of the pouch-shaped lip to a lady’s slipper. It contains over 70 species native to South and South-East Asia. Also, its distribution ranges from Himalayas, Burma into Indochina and the Asian region up to Papua New Guinea (Cribb, 1998). *Paphiopedilum* orchids have attained increasing demand in the flower industry. However, they suffer from less sufficient supply due to slow plant growth and difficulty of propagation. Generally, *Paphiopedilum* orchids are propagated through the division of axillary buds from mother plants. However, it is time consuming, extremely unproductive and unreliable for commercialization or conservation purposes (Liao et al., 2011; Ng and Saleh, 2011).

Micropropagation of *Paphiopedilum* through tissue culture is a prerequisite for the commercialization of slipper orchid by the trade regulation of the Convention on International Trade in Endangered Species (CITES) (Liao et al., 2011), which referring to wild and artificially propagated specimens can only be traded when subjecting to obtain the correct permits (CITES, 2001; McGough et al., 2006). The propagation of *Paphiopedilum* through *in vitro* shoot multiplication and direct shoot-bud formation and regeneration through protocorm-like body (PLB) formation using different explant sources such as seed derived seedlings, leaves, roots have been reported (Lin et al., 2000; Huang et al., 2001; Chen et al., 2002; Hong et al., 2008; Ng and Saleh, 2011). However, success in *Paphiopedilum* culture is

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Abbreviations: MS, Murashige and Skoog medium; **VW,** Vacin and Went medium; **PGR,** plant growth regulator; **PLBs,** protocorm-like bodies.

very limited due to insufficient information on culture media, microbial contamination, rapid necrosis of explants, low multiplication rate and rather expensive micro-plant cost (Long et al., 2010; Liao et al., 2011), all of which has reserved this procedure for laboratory-scale (Nhut et al., 2007). In addition, most published results used *in vitro* protocorm or seedlings as explant source without knowing their horticultural performance. The only available alternative commercial *Paphiopedilum* propagation by growers still depends entirely on asymbiotic seed germination (Hong et al., 2008). However, seed setting and germination rates of many hybrid cultivars may be very diverse in specific combinations and often affected by many unknown factors, in addition to genetic segregation of the surviving seedlings (Arditti, 2008; Lin et al., 2000).

Different protocols have been developed for the large-scale propagation of a number of orchid species through *in vitro* culture of various parts including shoot tips, flower stalk nodes, buds, root tips and rhizome segments. For mass propagation, regeneration from tissue cultured explants is advantageous to seed culture due to year round availability of explants and true to type. It is felt that due to tremendous uniformity in vegetatively propagated plants, the future mass-market orchids will most likely be explant propagated and not seed propagated (Samira et al., 2009; Arditti, 2008; George et al., 2008; Pierik, 1997; Kaewubon and Meesawat, 2007). Long et al. (2010) reported that *Paphiopedilum* hybrids are easier to micropropagate than native species due to their difficulty in maintaining explants in culture. However, there are only a few published reports available on the efficient micropropagation of *Paphiopedilum* hybrids. Stewart and Button (1975) used shoot apex of *Paphiopedilum* to induce callus, and occasionally, a few plantlets were obtained during subculture. However, the callus was difficult to maintain and eventually failed to survive. For this reason, most reports on *Paphiopedilum* tissue culture used seed-derived explants for practice to examine suitable media and methods, so that once the aseptic mother stocks are established, the system can be adopted readily. Lin et al. (2000) induced callus from seed-derived protocorms of a *Paphiopedilum* hybrid and a few plantlets were formed via protocorm-like-body (PLB) formation from the callus. Huang et al. (2001) established a clonal propagation protocol by multiplying shoots from geminated seeds of *Paphiopedilum* hybrids (*Paphiopedilum philippinense* × *Paphiopedilum* Susan Booth) in MS medium supplemented with 13 µM benzylaminopurine (BAP). Chen et al. (2002, 2004) also reported that induction of multiple shoot could be achieved from stem and leaf explants of *P. philippinense* (hybrids PH59 and PH60) cultured on MS medium supplemented with 4.52 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 4.54 µM thidiazuron (TDZ), respectively. In addition, Hong et al. (2008) have also re-established shoot multiplication from callus-derived plantlets of *Paphiopedilum* Alma Gavaert by culturing on Murashige and Skoog (MS) medium supplemented with 4.65 µM kinetin. There are several reports on the micro-propagation of *Paphiopedilum* using callus and PLB induction, but results indicated tissues did not survive upon subculture or low level of plant regeneration were achieved. Liao et al. (2011) induced shoots and regenerating plants from the flowering plants of a sequentially flowering *Paphiopedilum* Deperle and a single floral *Paphiopedilum* Armeni White using cross-sectioned young flower buds. However, to reach to the flowering stage of plants is time consuming, which makes the method less efficient. Moreover, *in vitro* propagation of *Paphiopedilum* orchids so far has demonstrated that genotypic difference dictates the response of the *Paphiopedilum* explants on different media formulations.

The aim of this study was to develop an efficient shoot multiplication by adopting *in vitro* cutting methods and medium composition. Since it is very difficult to obtain enough aseptic shoot tip explants for experimental purpose, we modeled the *in vitro* seedling of *Paphiopedilum* Hsinying Rubyweb as the explant source to test the regeneration system.

MATERIALS AND METHODS

Seeds of *Paphiopedilum* Hsinying Rubyweb were collected from greenhouse grown plants in National Pingtung University of Science and Technology, surface-sterilized with 0.6% sodium hypochlorite for 10 min, and then washed with sterile water for 3 times. Sterilized seeds were resuspended in sterile water and sowed onto medium which consist of 2 gL^{-1} Hyponex® (N:P:K = 7-6-19) (Taihe Horticultural Co. Ltd., Taiwan), vitamins and glycine as described in Murashige and Skoog (1962), supplemented with 10 gL^{-1} sucrose, 2 gL^{-1} activated charcoal (AC), 1 gL^{-1} peptone, 50 gL^{-1} potato extract, 25 gL^{-1} banana and solidified with 5 gL^{-1} Sigma agar (Sigma-Aldrich Co. LLC., Taiwan) combined with 1 gL^{-1} Gelrite (Cheng Hsin Tang Chemical Co. Ltd., Taiwan). Six months after germination, seedlings were transferred to P_2 medium. The P_2 medium composition is the same as described earlier, except the peptone was replaced with 1 gL^{-1} bacto-tryptone (HiMedia Laboratories Pvt. Ltd., India). The pH of both media was adjusted to 5.6 - 5.8 prior to autoclaving at 121°C and 1.21 $kg.cm^{-2}$ for 30 min. Cultures were maintained at 25 ± 2°C under a 12/12-h (day/night) photoperiod provided by cool white fluorescent lamps (Starcoat^{TM} F28W/T5/840,170 MA, Hungary) at photosynthetic photon flux (PPF) of 40 ± 10 $\mu mol.m^{-2}.s^{-1}$. Eighteen-month-old *in vitro* seedlings maintained on P_2 medium were used as explants (Figure 1a).

In vitro cutting methods, media and culture conditions

The purpose of this experiment was to determine the effect of *in vitro* cutting methods and media composition on shoot multiplication of *Paphiopedilum* hybrids. *In vitro* seedlings (2 to 4 cm in height) were cut directly by vertical, cross and horizontal cutting (Figure 1b) and cultured onto P_2 medium. For the effect of media composition, intact and vertically cut seedlings were placed onto three different media, including P_2, VW (Vacin and Went, 1946) and RB (Chen and Chen, 1998). All media were poured into 97 × 142-mm glass culture bottles containing 125 mL of medium. After 12 weeks of culture, new shoots with roots were divided into single plantlet with vertical

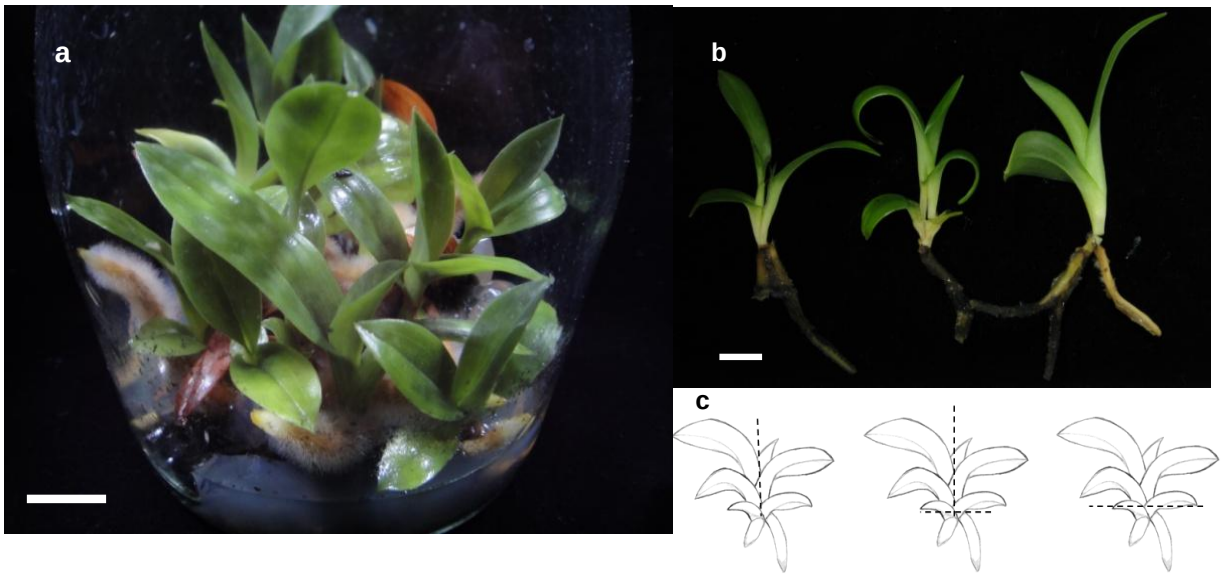

Figure 1. Eighteen-month-old *in vitro* seedlings of *Paphiopedilum* Hsinying Rubyweb maintained on P_2 medium were used as explants (**a**), with three different cutting methods, vertical, cross and horizontal, respectively through shoot tip (**b**); the corresponding schematic drawing of the cutting type is illustrated in (**c**) (bar = 10 mm).

Table 1. Effect of different types of cutting on shoot multiplication of *Paphiopedilum* Hsinying Rubyweb after 12 weeks of culture.

Cutting methods	Number of new shoots
Cross	2.5^b
Vertical	10.5^a
Horizontal	1.0^b

Treatments followed by the same letter are not statistically different at $p<0.05$ by Fisher's protected LSD test.

cutting and subcultured to both half- and full-strength MS, VW and P_2 media without activated charcoal (AC) for the comparison of shoot multiplication rate. The pH of the media was adjusted to 5.6 - 5.8 prior to autoclaving at 121°C and 1.21 $kg.cm^{-2}$ for 30 min. All cultures were maintained at 25 ± 2°C under a 12/12-h (day/night) photoperiod provided by cool white fluorescent lamps (Starcoat^{TM} F28W/T5/840,170 MA, Hungary) at PPF of 40 ± 10 $\mu mol.m^{-2}.s^{-1}$.

Experimental design and data analysis

All experiments were performed in a completely randomized design (CRD). Each experiment included five replicates, with five plantlets per treatment. The number of new shoots per explants was determined for each experiment after 12 weeks of culture. Analysis of variance (ANOVA) was performed by using SAS version 8.2 and mean separation with the LSD test. A significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

The eighteen-month-old germinated seedlings maintained on P_2 medium were used as explants. For each explant the roots were kept intact so that after the cut and upon culture, the explants could grow readily in the media without growth regulators. The different types of *in vitro* cutting method showed significant difference on shoot multiplication of *Paphiopedilum* Hsinying Rubyweb. The highest number of new shoots was observed when the stems were cut vertically through shoot tips as compared to other cutting types. After 12 weeks in culture, the vertically cut explants produced an average of 10.5 new shoots, whereas those in cross and horizontal method showed less shoots (Table 1). The new shoot emerged from tightly grown and wounded axillary buds around the cutting area (Figure 2b) whereas intact plantlet without cutting produced new shoots only at ordinary axillary bud (Figure 2a). The newly formed young shoots could grow readily on the P_2 medium without extra growth regulators. Moreover, the original explants of vertical cut was able to survive after cutting, whereas probably due to heavy damage of wounding the explants, other cutting methods turned dark brown and died later with only new shoots developing from the lower nodes.

The explants produced more new shoots when using vertical cutting method than using intact shoot without cutting when cultured on VW, P_2, and RB media (Table 2). In addition, there was no significant difference in shoot multiplication between three media. Besides, all plantlets developed normal roots with white root hairs and the average number of root was about 2.4 - 2.9, while the lowest number of root was 1.8 on VW medium with vertical cut. However, after 3 months of culture, only plantlets grown on P_2 medium showed green and well developed leaves, whereas plantlets cultured on RB and VW media had yellowish leaves, and thick and crispy

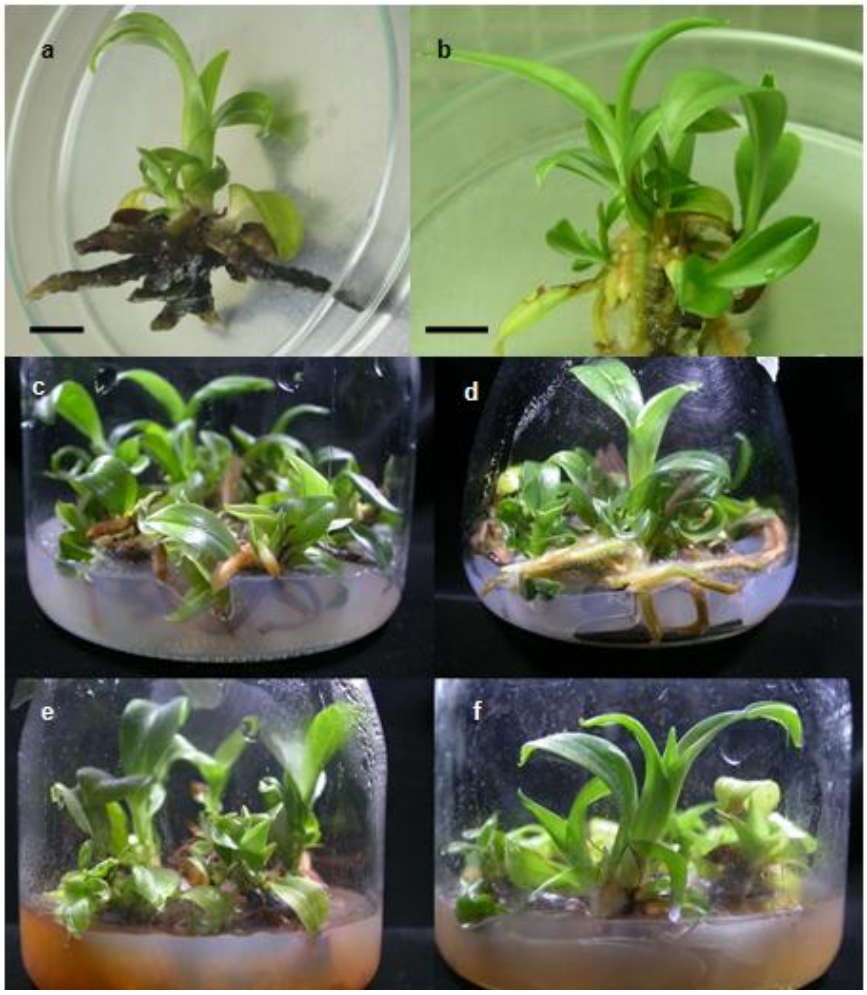

Figure 2. Formation of multiple shoots of *Paphiopedilum* Hsinying Rubyweb on P2 without cutting (a), with vertical cutting method (b), and vertical cutting on MS (c), ½ MS (d), VW (e) and P2-AC (f) after 12 weeks of culture (bar = 10 mm).

Table 2. Effect of medium and vertical cutting method on shoot multiplication of *Paphiopedilum* Hsinying Rubyweb after 12 weeks of culture.

Medium	Vertical cutting method	No. of new shoots	No. of root per explants
VW	No	3.0^{b}	2.7^{a}
	Yes	13.3^{a}	1.8^{b}
P_2	No	1.3^{b}	2.9^{a}
	Yes	11.0^{a}	2.4^{ab}
RB	No	1.7^{b}	2.9^{a}
	Yes	16.7^{a}	2.6^{a}

Treatments followed by the same letter within columns are not statistically different at $p<0.05$ by Fisher's protected LSD test.

leaves observed on RB medium. Furthermore, shoot multiplication was higher in half strength MS medium than in full strength MS, P_2 without AC and VW three months after subculture, with an average of 28.8 shoots per explants (Table 3, Figure 2c to f).

Micropropagation of orchids via multiple shoot

Table 3. Effect of different media on shoot multiplication of *Paphiopedilum* Hsinying Rubyweb after 12 weeks of culture.

Media	Number of shoots
MS	16.6[b]
½ MS	28.8[a]
VW	13.0[b]
P_2 -AC	16.0[b]

Treatments followed by the same letter are not statistically different at $p<0.05$ by Fisher's protected LSD test.

formation is an alternative means for the production of uniform clones of *Paphiopedilum* orchids (Ng et al., 2010). However, the success of micropropagation has been limited mainly due to the difficulties in decontaminating the explants and plant regeneration (Liao et al., 2011). In this study, the *in vitro* seedlings were used to monitor the possibility of shoot multiplication and in the mean time, avoid the contamination problem before clean stock is available. The aseptic nature of mother plants will ensure lack of microbial contamination and protect their meristem-derived juvenility status, rendering them responsive to tissue culture regeneration (Liao et al., 2011). Furthermore, direct shoot regeneration without going through the callus stage can avoid somaclonal variation problems. Besides, clonal plantlets are expected to have the same genetic traits as their mother plants, which are important for commercial mass production. In this study, the vertical cutting method using *in vitro* seedlings showed the highest number of shoot regeneration and multiplication. The apical meristem might lose its dominance by cutting through the shoot tip (vertical cut) and allowing the development of lateral meristems from each dormant axillary bud. Subsequently, young shoots emerged efficiently by this cutting method to give higher multiplication rate than others (Table 3; Figure 2).

Success in micropropagation of *Paphiopedilum* orchids through direct shoot regeneration depends on optimizing culture media to a large extent. Huang et al. (2001) reported that doubling of plantlet numbers occurred only after 12 weeks with a 3-shoot cluster in modified MS medium supplemented with both coconut water and high concentration of BAP. Cytokinin has been implicated for the release of apical dominance to produce more multiple shoots in monocots (Hussey, 1976). Hong et al. (2008) reported that an average of three shoots were obtained from a single young shoot of *Paphiopedilum* Alma Gavaert after 60 days of culture on modified half-strength MS medium supplemented with kinetin. The regeneration response was also genotype-dependent, and may not be applicable to other cultivars.

In this study, we demonstrated the efficient formation of multiple shoots by vertical cutting plantlet explants and cultured on half-strength MS medium without supplementation of plant growth regulator. Since no exogenous plant growth regulator (PGR) was involved, it is expected that the occurrence of somaclonal variation would be relatively low in these cultures compared to multiple shoots induced in the presence of exogenous PGR. The method developed in this study provides a simple and efficient means to *in vitro* propagate *Paphiopedilum* orchids, which are able to produce large numbers of uniform plantlets in a relatively short time in comparison to the conventional propagation method since normal plantlets could be obtained from these regenerated shoots within 3 months. Furthermore, *in vitro* cutting method may be applicable for commercial production of large quantity of young plants for the market.

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