Clonal Propagation In Vitro of Paphiopedilum Hybrids from Adult Plants

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Abstract. The aim of this study was to develop an efficient protocol for shoot tip culture from adult plants of Paphiopedilum Pfitzer. A considerable seasonal effect on explant collection was observed in the aseptic cultures established from adult plants, including the survival and microbial contamination of explants. The shoot tip explants excised from adult plants in February and May showed higher survival and had less contamination than those explants excised in August and November. Moreover, the season of explant collection also affected the subsequent shoot forming capacity and multiplication of axillary buds. In Paphiopedilum ‘In-Charm Silver Bell’, higher shoot forming capacity was observed in February and May, whereas higher shoot multiplication was observed only in February. In Paphiopedilum ‘Hsining Maudiae Leopard’, both February and May were optimal timing for shoot forming capacity and multiplication. We also demonstrated the effectiveness of transcinnamic acid (tCA), an antiauxin chemical in diminishing the apical dominance of shoot tip explant and thus improving the axillary bud outgrowth. In P. ‘In-Charm Silver Bell’, the addition of 100 μM tCA plus 13.3 μM 6-benzylaminopurine (BA) for 1 month promoted axillary shoot bud formation from shoot tip explants as compared with the control.

Paphiopedilum is commonly known as slipper orchid because of the likeness of the pouch-shaped lip to a lady’s slipper. It is a terrestrial orchid, containing ∼80 species that are native to habitats ranging from the Himalayas to southwestern China and southeast Asia (Cribb, 1998). Paphiopedilum is one of the most popular orchids that has been cultivated for more than 100 years, and a wide range of attractive hybrids and cultivars has been produced as potted plants or cut flowers in horticultural markets (Miguel et al., 2010). Micropropagation of Paphiopedilum is known to be intricate through tissue culture techniques (Arditti and Ernst, 1993; Stewart and Button, 1975), and for this reason the selected elites are usually propagated by vegetative division (Lee, 2018). Therefore, a rapid, reliable protocol for propagation is required for massive propagation of the selected elites.

Attempts to establish shoot tip culture in Paphiopedilum adult plants indicated that the major problems encountered are 1) getting aseptic explants from mature plants through the procedure of surface sterilization is difficult; and 2) explants from mature plants are obstinate to multiply or regenerate in vitro (Arditti and Ernst, 1993; Chugh et al., 2009). Plant regeneration and shoot multiplication through seed-derived explants in vitro has been reported in a number of Paphiopedilum species and hybrids (Chen et al., 2004; Hong et al., 2008; Huang et al., 2001; Lin et al., 2000; Long et al., 2010; Ng et al., 2010; Ng and Saleh, 2011; Nhu et al., 2007; Udondie et al., 2012); however, those seed-derived explants are not true-to-type and heterozygous. Until now, only a few cases of in vitro clonal propagation using the explant collected from adult plants has been reported (Huang, 1988; Stewart and Button, 1975). Recently, Liao et al. (2011) reported the shoot induction and plant regeneration in Paphiopedilum using flower buds as explants. These results imply that the protocol of starting aseptic culture from adult plants still has considerable room for improvement. In woody plants, the initial success of an aseptic culture is directly related to the timing of explant collection from adult plants (Evers et al., 1988; Kartonas and Papadoyiou, 2007). The seasonal influence in explant collection may affect the endogenous physiological state of explants and the surrounding composition of microorganisms. In our previous study, the timing of explant collection was critical for the initial success of aseptic culture in Cypripedium L. (a relative genus to Paphiopedilum) adult plants (Lee, 2000). Cypripedium occurs in the temperate region, whereas Paphiopedilum is native to tropical and subtropical Asia. It is not certain if the seasonal influence of explant collection also plays a role in establishing the aseptic culture of Paphiopedilum. Therefore, the objectives of this study were to investigate the effect of timing of explant collection from adult plants on micropropagation, and to examine the effect of transcinnamic acid (tCA), a potent inhibitor of auxin efflux, on in vitro shoot multiplication of Paphiopedilum.

Materials and Methods

Plant material, the timing of explant collection, and culture condition. The mature plants of Paphiopedilum Maudiae-type hybrids (i.e., Paphiopedilum ‘In-Charm Silver Bell’ and Paphiopedilum ‘Hsining Maudiae Leopard’) were maintained in a greenhouse at the Horticulture Technology Center, National Chiayi University, Chiayi, Taiwan. Plants were grown in pine bark medium (Orchidta; Besgrow, Christchurch, New Zealand) under 70% shade. To investigate the seasonal effect on explant survival and shoot multiplication, the young shoot bud of 5 cm in length was collected from adult plants in early of February, May, August, and November. The young shoot bud was first washed under running tap water for 15 min, then immersed in 2% neutral liquid detergent Extraman 02 (Sigma-Aldrich Co., St. Louis, MO) for 30 min. In a laminar airflow, the outer young leaves were removed from the shoot bud, and then the shoot buds was immersed in 1% sodium hypochlorite solution with 0.1% Tween 20 (Sigma-Aldrich Co.) for 18 min. After three rinses with sterile distilled water, the shoot bud with one leaf attached was used as the explant (Figs. 1A and 2A). The culture medium used in this experiment was the modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), which contained one-fourth strength macro-elements with full-strength micro-elements and supplemented with 2 mg L⁻¹ glycine, 0.5 mg L⁻¹ nicin, 0.5 mg L⁻¹ pyridoxine HCl, 0.1 mg L⁻¹ thiamine, 100 mg L⁻¹ myo-inositol, 20 g L⁻¹ sucrose (Sigma-Aldrich Co.), 4 g L⁻¹ agar (Becton, Dickinson and Co., Sparks, MD) and 2 g L⁻¹ Gelrite (Sigma-Aldrich Co.). The plant growth regulator consisted of 13.3 μM 6-benzylaminopurine (BA; Sigma-Aldrich Co.), and the pH value was
Shoot multiplication procedure. After 5 months of culture, the formation of multiple shoots (more than 1 cm in length) from the initial explants were separated, and each single shoot was cut vertically into two explants and placed onto the same culture medium as described previously. The explants were subcultured every 30 d. After 3 months of subculture, as the multiple shoots (more than 1 cm in length) formed, they were separated and cut vertically for shoot multiplication. After 9 months of subculture (i.e., 3 times of multiplication), the number of new shoots (above 0.2 cm) produced from one initial explant were recorded.

Effects of tCA on shoot multiplication. To investigate the effect of tCA (Sigma-Aldrich Co.), a potent inhibitor of auxin efflux on shoot multiplication, the axillary buds collected in November were inoculated onto the one-fourth MS medium, as described previously, and supplemented with 100 μM tCA and 13.3 μM BA for 1 month. The medium without tCA was used as control. The cultures were maintained at 26 ± 2 °C under a 16/8-h photoperiod with daylight fluorescent lamps (20 W; China Electric Co.), at a light intensity of 30 μmol·m⁻²·s⁻¹. After 4 months of culture, the plantlets were 3–4 cm in height with three leaves and two roots that were ready for acclimatization in the greenhouse, then taking out of flasks for planting.

Results and Discussion

Seasonal effects on establishment of initial aseptic culture. After 2 months of culture, the shoot tip became swollen and the basal axillary buds started growing (Figs. 1B and 2B). The season of explant collection for the establishment of initial aseptic culture is critical in Paphiopedilum micropropagation. Of the two hybrids tested, the survival percentage of explants was higher in February and May as compared with those in August and November; and the contamination percentage of explants was reduced in February and May as compared with those in August and November. For the browning of explants in this study, there was no significant difference between seasons (Table 1). These results indicate that microbial contamination is the main cause contributing to the lower survival of explants in August and November. It has been reported that the challenge of starting aseptic culture in Paphiopedilum is the great difficulty of removing microbial contamination from explants collected from cultivated plants in greenhouses (Huang, 1988). Although reducing the explant size could be a possible solution, the explant size could not be reduced indefinitely. Furthermore, it is not practical to reduce explant size to the point of difficulty in handling.
lessen the ratio of microbial contamination, reducing the explant size also diminishes the survival of explants in the initial aseptic culture. In this study, we found the optimum timing for explant collection in February and May without reducing the explant size. The different contamination of explants could be attributed to seasonal fluctuations of microbial population and activity (Martini et al., 2013).

Seasonal effects on shoot formation and multiplication. After 5 months of culture, a number of axillary buds arisen from the basal part of explants were visible (Figs. 1C and 2C). Of the two hybrids tested, the shoot forming capacity of explants was higher in February and May as compared with those in August and November (Table 2). The newly formed axillary shoots (above 1 cm) were excised and further subcultured on the same fresh medium. After 3 months of subculture, multiple shoots were induced (Figs. 1D and 2D). The axillary shoots were excised and subcultured again. After another 3 months of subculture, the increased shoot multiplication rate was evident (Figs. 1E and 2E). For the shoot multiplication, explants of *Paphiopedilum* ‘Hsinying Maudiae Leopard’ produced more shoots in February and May as compared with those in August and November (Table 3). In *Paphiopedilum* ‘In-Charm Silver Bell’, the number of shoots formed per explant was reduced in May, as compared with those in February, and reached the lowest level in August and November. In studies with woody plants, the season of explant collection from adult plants is important on the initial aseptic culture and shoot multiplication (Kartsonas and Papafotiou, 2007; Kumar et al., 2005; Hohtola, 1988; Martini et al., 2013; Thomas and Ravindra, 1997). Such seasonal variation also has been observed previously for explants excised from adult plants of temperate orchids, such as *Cypris pedium formosanum* Hayata (Lee, 2010) and *Pleione formosana* Hayata (Chang and Lee, 1992). In both cases, collecting shoot buds during the cold season significantly improved the survival of explants, shoot formation, and multiplication. The high viability and multiplication of the explant collected during the cold season may reflect the natural course of endogenous metabolism. A study by Lee and Teng (1987) reported the dramatic increase of endogenous gibberellins and indole-3-acetic acid (IAA) levels in *P. formosana* corms after cold storage. Most temperate orchids are deciduous, and their underground organs (e.g., rhizomes and corms) are covered by snow during the winter, whereas *Paphiopedilum* species are evergreen and inhabit subtropical and tropical areas (Cribb, 1998). However, in the natural habitat, such as the subtropical monsoon climate region or a high-altitude area, *Paphiopedilum* plants still experience noticeable changes of temperature and rainfall yearly (Averyanov et al., 2003). In this study, it seems that the season-dependent metabolites, such as plant hormones, are retained in the explants under the in vitro conditions.

**Table 1.** Seasonal effects on initial establishment of aseptic culture of two *Paphiopedilum* hybrids.

| Hybrid                | Contamination (%) | Browning (%) | Survival (%) |
|-----------------------|-------------------|--------------|--------------|
| *P. In-Charm Silver Bell* | 24.58 ± 2.17      | 4.58 b       | 70.83 a      |
| *P. Hsinying Maudiae Leopard* | 37.5 ± 2.12      | 7.50 ab      | 55.00 b      |

**Table 2.** Seasonal effects on shoot formation in two *Paphiopedilum* hybrids.

| Hybrid                  | Shoot forming (%)          |
|-------------------------|----------------------------|
|                         | February | May | August | November |
| *P. In-Charm Silver Bell* | 97.92 ± 0.97 | 97.62 ± 0.86 | 62.64 ± 1.92 | 48.33 ± 0.97 |
| *P. Hsinying Maudiae Leopard* | 80.48 ± 0.97 | 92.62 ± 0.86 | 11.57 ± 1.92 | 10.19 ± 0.97 |

Fig. 2. Micropropagation of *Paphiopedilum* ‘Hsinying Maudiae Leopard’. (A) After the surface sterilization, the lateral bud with one leaf attached was used as the explant. Bar = 1 cm. (B) After 2 months of culture, the basal axillary buds (arrow) started growing. Bar = 1 cm. (C) After 5 months of culture, a number of axillary buds had arisen from the basal part of explants. Bar = 1 cm. (D) The newly formed axillary shoots (as indicated in C) were excised for multiplication. After 3 months of subculture, several new shoots were visible. Bar = 1 cm. (E) The newly formed axillary shoots (as indicated in D) were excised again for multiplication. After 3 months of subculture, more shoot multiplication can be observed. Bar = 1 cm.

**Significance of two-way ANOVA.**

1. *NS, * , **, ***Non-significant or significant at 5%, 1%, or 0.1%, respectively.
2. **Data were recorded after 2 months of culture.
3. **Means having the same letter within a column are not significantly different at 5% according to least significant difference test.
4. **Means having the same letter within a column (in small letter), and within a row (in capital letter) are not significantly different at 5% as scoring to least significant difference test.
Table 3. Seasonal effects on shoot multiplication in two Paphiopedilum hybrids.

| Hybrid                        | Shoot multiplication (number of new shoots/explant) |
|-------------------------------|-----------------------------------------------------|
|                              | February | May    | August | November |
| P. In-Charm Silver Bell       | 6.32 ± 0.3 aA | 3.80 ± 0.2 bA | 1.15 ± 0.6 cC | 0.92 ± 0.4 bC |
| P. Hsinying Maudiae Leopard  | 4.68 ± 0.3 bA | 4.50 ± 0.2 aA | 0.90 ± 0.6 bB | 0.50 ± 0.4 bB |

Significance of two-way ANOVA

Hybrid ***
Season ***
Hybrid × Season ***

*Means having the same letter within a column (small letter), and within a row (capital letter) are not significantly different at 5% according to least significant difference test.
***Significant at 0.1%.
ANOVA = analysis of variance.

Table 4. Effects of transcinnamic acid (tCA) on shoot multiplication of Paphiopedilum In-Charm Silver Bell.

| Treatments | Shoot >1 cm | Shoot <1 cm | Total shoot number |
|------------|-------------|-------------|--------------------|
| Control    | 1.35 ± 0.19 b | 0.67 ± 0.19 b | 2.00 ± 0.19 b |
| 1 mo cultured with tCA | 2.67 ± 0.51 a | 3.78 ± 0.48 a | 6.44 ± 0.91 a |

*Means having the same letter within a column (small letter), and within a row (capital letter) are not significantly different at 5% according to least significant difference test.
*Significant at 0.5% or 0.1%, respectively.

Fig. 3. Rooting and acclimatization of plantlets. (A) Shoots were excised and cultured on the rooting medium in the Magenta GA-7 plant culture box. Bar = 1 cm. (B) After 4 months of culture, plantlets with well-developed roots were ready to take out of flasks. Bar = 2 cm. (C) After 3 months of culture in the greenhouse, the vigorous plantlets with newly emerged leaves could be observed. Bar = 2 cm.

The lateral shoot buds were collected in November, and data were recorded after 8 months of culture.

Rooting and acclimatization of plantlets.

In the multiplication medium, shoots of more than 2 cm in length were excised and transferred onto the rooting medium (Fig. 3A), and after 4 months of culture, the plantlets with well-developed roots were ready for acclimatization in the greenhouse (Fig. 3B). After taking out of flasks for 3 months, the plantlets were vigorous in the potting mixture with newly emerged leaves (Fig. 3C). In conclusion, we demonstrate an efficient procedure of Paphiopedilum micropropagation for explants collected from adult plants. Collecting the explants in February and May for initial aseptic culture gives optimal survival, shoot formation, and multiplication. The addition of tCA in combination of BA promoted multiple shoot formation by releasing shoot apical dominance. Once the aseptic culture established, the shoots can be multiplied repeatedly. The technique described here makes it possible to propagate the selected elites of Paphiopedilum in quantity for the horticultural market.

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