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Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids

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Abstract

Leaf explants of *Paphiopedilum phiIippinense* hybrids (hybrid PH59 and PH60) directly formed adventitious shoots from wound regions within 1 month, when cultured on modified Murashige and Skoog medium (1/2-strength macro- and full-strength micro-elements) free of plant growth regulator in darkness. The combinations of 2,4-dichlorophenoxyacetic acid ((2,4-D) acid (0, 4.52 and 45.25 μM) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) (0, 0.45, 4.54 and 22.71 μM) were used to test their effects on direct shoot bud formation from two types of explants (1.5-cm long intact leaf explants and 0.5-cm long leaf segment explants). In hybrid PH59, 4.54 μM TDZ increased mean numbers of shoots per explant with leaf segment explants. In hybrid PH60, 4.52 μM 2,4-D plus 0.45 μM TDZ promoted direct shoot bud formation from leaf segment explants. In addition, three treatments (4.52 μM 2,4-D, 22.71 μM TDZ, 4.52 μM 2,4-D plus 4.54 μM TDZ) gave a higher response than control on mean numbers of shoots per explant with intact leaf explants. Healthy plantlets each with one to three roots were obtained from leaf-derived shoots after transfer onto a hormone-free medium for 22 months. These plantlets were acclimatized in a greenhouse and grew well with 100% survival rate.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; MS – Murashige and Skoog (1962) medium; TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea

Paphiopedilum, is a genus of terrestrial orchids with some 70 species that are native to South and Southeast Asia, and a distribution that extends from the Himalayas and Burma into Indochina and the Asean region up to Papua New Guinea (Teob, 1989). They are popularly known as Slipper Orchids because of the resemblance of the pouch-shaped lip to a lady's slipper, and are often marketed as pot plants with high value.

There are only a few reports describing aseptic culture methods for propagating *Paphiopedilum* orhcids, because the explants are difficult to maintain in culture (see review by Arditti and Ernst, 1993). Shoot tips have been used as explants to induce the growth of axillary shoots and regenerated plantlets (Arditti and Ernst, 1993; Huang, 1988). Huang et al. (2001) further modified their medium (Huang, 1988), and multiplied shoots taken from *in vitro* grown seedlings. Lin et al. (2000) established callus cultures from protocorms, and successfully regenerated plantlets through protocom-like bodies from the subcultured callus. However, poor callus formation rate, slow growth of callus and low regeneration capacity are still the major impediments limiting the utility of *in vitro* culture for *Paphiopedilum* propagation. Because of the limited success in tissue culture protocols, *Paphiopedilum* propagation has still been entirely by asymbiotic germination. In this communication, we established an efficient protocol for regenerating *Paphiopedilum* through direct shoot bud formation from leaf culture.

Thirty-six-month-old *in vitro* grown seedlings of *Paphiopedilum philippinense* (hybrid PH59 and hybrid PH60) cultured on MS (Murashige and Skoog, 1962) medium free of plant growth regulators were

Table 1. Effects of 2,4-D and TDZ on direct shoot bud formation from 1.5-cm long intact leaf explants of two*Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60). Percentage of explants with shoots and mean number of shoots per explants were scored after 150 days of culture

2,4-D (μM)	TDZ	Percentage of explants with shoots		[a]Mean no. of shoots per explant ± S.E.	
		Hybrid PH59	Hybrid PH60	Hybrid PH59	Hybrid PH60
0 (μM)	0	25	25	2.0±1.0[b]	1.0±0.5
0	0.45	50	0	1.5±0.9	0
0	4.54	0	0	0	0
0	22.71	25	50	1.0±0.5	3.0±1.9
4.52	0	25	50	1.0±0.5	2.0±1.4
4.52	0.45	0	0	0	0
4.52	4.54	25	25	2.0±1.0	2.0±1.0
4.52	22.71	0	0	0	0
45.25	0	50	0	1.0±0.6	0
45.25	0.45	0	0	0	0
45.25	4.54	0	0	0	1.0±0.5
45.25	22.71	0	0	0	0

[a] Data based on four explants per treatment.
[b] Mean ± standard deviation.

used as donor plants. Two types of leaf explants (1.5-cm long intact leaf explants and 0.5-cm long leaf segment explants excised from intact leaves) were used in separate experiments. Explants were placed on the surface of a modified MS basal medium containing 1/2-strength macro- and full-strength micro-elements of MS salts supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20 000), and Gelrite^{TM} (2200). Plant growth regulators (for concentrations see Table 1 and 2) were added prior to autoclaving. The pH of the media was adjusted to 5.2 with 1 N KOH or HCl prior to autoclaving for 15 min at 121 °C. Explants were incubated in 20×150 mm culture tubes under a 16-h photoperiod at 28–36 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co.,Taipei) and 26 ± 1°C. Sixteen and 21 replicates were taken for each treatment in experiment 1 (see Table 1) and experiment 2 (see Table 2), respectively. Cultures were examined and photographed with a stereozoom microscope (SZH, Olympus). The percentage of explants with shoots and mean numbers of shoots per explant were determined for each trial.

When 1.5-cm long intact leaf explants and 0.5-cm long leaf segment explants of both hybrids of *Paphiopedilum philippinense* were cultured on modified 1/2-strength MS medium free of plant growth regulator in darkness, the wound regions of explants directly formed adventitious shoots within 1 month (Figure 1a,b). In light, almost all the shoots turned green and became plantlets have two to three leaves and one to two roots after 1 month of culture (Figure 1c). The regenerated plantlets were ready to be transplanted to pot after 22 months of culture on the same medium (Figure 1d). The regenerated plantlets were potted in sphagnum mss, and acclimatized well in greenhouse with 100% survival rate.

As shown in Table 1, on a growth regulator-free medium, 25% intact leaf explants of hybrid PH59 formed two shoots and 25% intact leaf explants of hybrid PH60 formed one shoot after 5 months of culture. In hybrid PH59, all concentrations of 2,4-D and TDZ and their combinations did not affect the percentage of shoot bud formation of intact leaf explants. However, except for 4.52 μM 2,4-D plus 4.54 μM TDZ, all combinations retarded mean numbers of shoots per explant on intact leaf explants. In hybrid PH60, the percentage of shoot-forming explants with intact leaf explants was not affected by 2,4-D and TDZ, but three treatments (22.71 μM TDZ, 4.52 μM 2,4-D, 4.52 μM 2,4-D plus 4.54 μM TDZ) gave higher response than control on mean numbers of shoots per explant (Table 1).

Table 2. Effects of 2,4-D and TDZ on direct shoot bud formation from 0.5-cm long leaf segment explants of two*Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60). Percentage of explants with shoots and mean no. of shoots per explants were scored after 170 days of culture

2,4-D	TDZ	Percentage of explants with shoot buds		[a] Mean no. of shoot buds per explant ± S.E.	
(μM)		Hybrid PH59	Hybrid PH60	hybrid PH59	Hybrid PH60
0	0	42.9	0	5.6±2.6[b]	0
0	0.45	28.6	0	3.5±2.2	0
0	4.54	14.3	0	7.0±2.6	0
0	22.71	0	0	0	0
4.52	0	28.6	0	2.5±1.3	0
4.52	0.45	14.3	28.6	2.0±0.8	1±0.5
4.52	4.54	0	0	0	0
4.52	22.71	0	0	0	0
45.25	0	14.3	0	3.0±1.1	0
45.25	0.45	14.3	0	3.0±1.1	0
45.25	4.54	0	0	0	0
45.25	22.71	14.3	0	1.0±0.4	0

[a] Data based on 21 explants per treatment.
[b] Mean ± standard deviation.

On plant growth regulator-free medium, 42.9% intact leaf explants of hybrid PH59 formed a mean of 5.6 shoots after 5 months of culture (Table 2). However, no shoots could be obtained from hybrid PH60 leaf cultures in the same conditions. In hybrid PH59, the percentage of bud-forming explants was not affected by any treatments. However, the shoot numbers per explant could be promoted or retarded by addition 2,4-D and TDZ. TDZ (4.54 μM) promoted shoot number per explant on leaf segment explants. In this treatment, 7 shoots could be obtained from one single leaf segment explant. In hybrid PH60, only one treatment (4.52 μM 2,4-D plus 0.45 μM TDZ) could promote direct shoot bud formation from leaf segment explants, and there was 28.6% of explants could form one shoot.

Leaf explants taken from two hybrids of *Paphiopedilum philippinense* were used in the experiments, and the results showed that their responses on shoot bud formation are quite different. On plant growth regulator-free medium, 42.9% leaf segment explants of hybrid PH59 formed shoots, but explants of hybrid PH60 did not form shoots. This indicated that the regeneration capacities of the explants vary with cultivar genotypes. Two types of explants were used in the separate experiments, and the results showed that explant type highly affected the response on shoot bud formation.

In hybrid PH59, leaf segment explants have a higher regeneration capacity than intact leaf explants on the hormone-free medium. However, in hybrid PH60, intact leaf explants have a higher response with shoot bud formation. Depending on the concentrations used, 2,4-D and TDZ may promote or retard leaf explants forming shoots. It was also found that plant growth regulator treatments (2,4-D, TDZ and the combinations) had different effects on the two hybrids. In hybrid PH59, 2,4-D and TDZ both retarded shoot bud formation from leaf segment explants. However, in hybrid PH60, 2,4-D plus TDZ promoted leaf segment explants to form shoots. TDZ was effective to induce *in vitro* morphogenesis in several orchids, such as shoot regeneration and proliferation (Chen and Piluek, 1995; Nayak et al., 1997; Chen and Chang, 2000a) and direct somatic embryogenesis (Chen et al., 1999; Chen and Chang, 2000a, 2001, 2002). Moreover, TDZ combined with 2,4-D were required for callus induction in *Cymbidium* (Chang and Chang, 1998), *Oncidium* (Chen and Chang, 2000a, b), *Palaenopsis* (Chen et al., 2000) and *Paphiopedlium* (Lin et al., 2000). However, Huang et al., (2001) reported that TDZ inhibited shoot proliferation and rooting in *Paphiopedlium*. In this re-

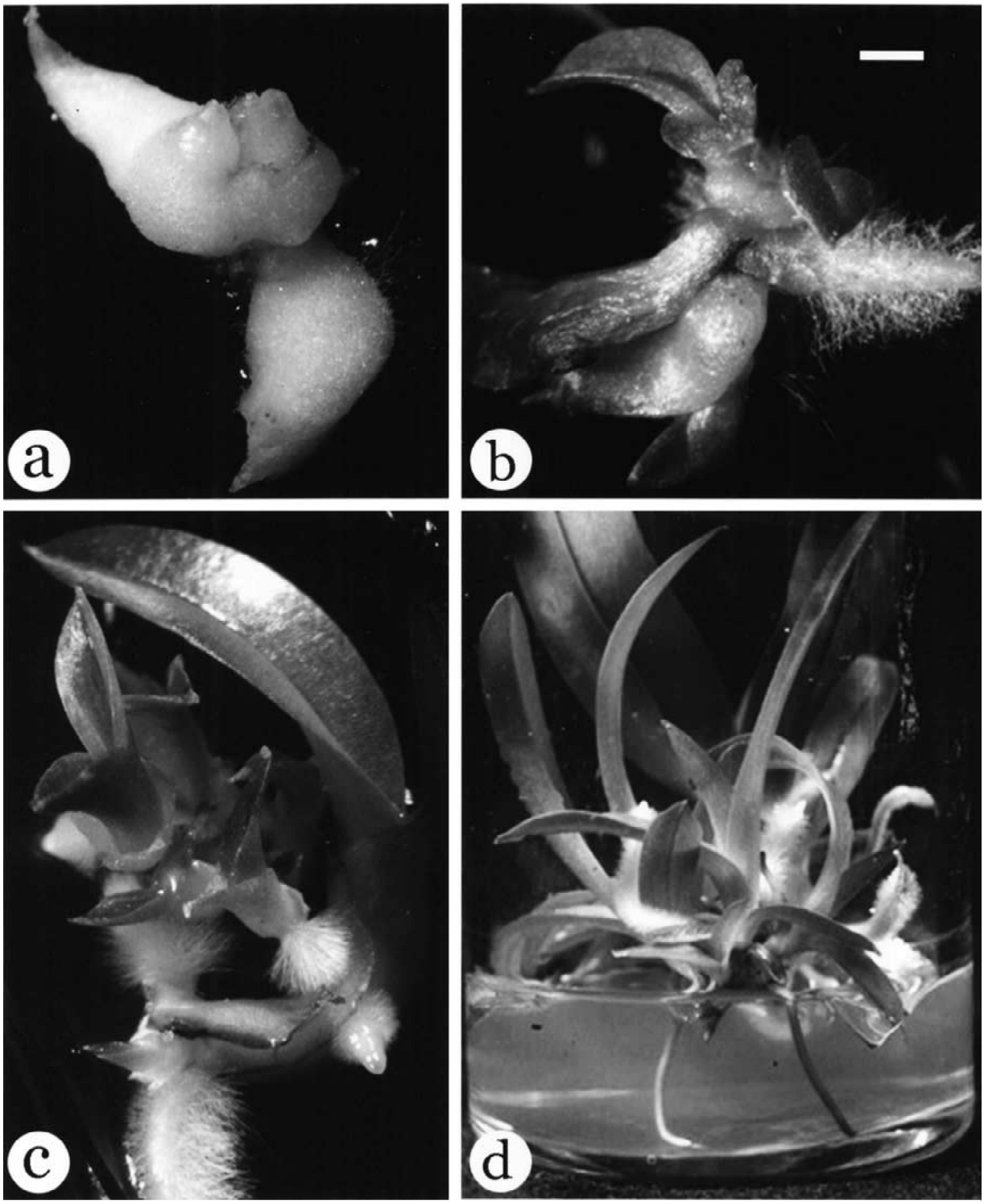

Figure 1. Direct shoot bud formation and plant regeneration from leaf explants of *Paphiopedilum* orchids. (a) Adventitious shoot buds formed directly from the wound region of leaf explants (bar: 1 mm). (b) Several shoots formed roots on an intact leaf explant (bar: 3 mm). (c) Several shoots formed roots on a leaf segment (bar: 2 mm). (d) Plantlets developed from leaf-derived shoots (bar: 6 mm).

port, TDZ alone or combined with 2,4-D were found to be effective in adventitious shoots induction from leaf explants of *Paphiopedilum.*

In conclusion, a reliable protocol via direct shoot formation for *Paphiopedilum* orchids propagation was established. In optimal conditions, almost half of leaf explants of hybrid PH59 and PH60 could form 5.6 and 3 shoots. Normal plantlets could be obtained from these regenerated shoots.

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