Paphiopedilum cloning in vitro
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Abstract
A mericloning protocol that enables shoot multiplication and rooting of Paphiopedilum orchids in one step or in the same medium was developed by systematically assessing and modifying the nutrient supplements employed earlier for shoot proliferation by Huang [Am. Orchid Soc. Bull. 57 (1988) 274]. Shoots of aseptically established seedlings obtained from a commercial breeder were used as explants. The new combination of supplements to the MS [Phyto. Plant. 15 (1962) 473] based medium consists of MT [Proc. First Intl. Citrus Symp. 3 (1969) 1155] vitamins, glycine and inositol; 13 µM BA (N6-benzyladenine); 1.6 µM NAA (α-naphthaleneacetic acid); 0.15 mM adenine sulfate 2H2O; 1.23 mM NaH2PO4. H2O; 0.18 M sucrose; and 15% (v/v) coconut milk from very young fruits. The coconut milk can be substituted with 1 g l−1 casein hydrolysate or 10 g l−1 potato tuber sections. The investigation also disclosed that TDZ (N-phenyl-N-1,2,3-thiadiazol-5-yl urea) inhibited shoot proliferation and rooting, and maltose depressed rooting. The protocol enables doubling the number of Paphiopedilum plants every 12 weeks. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Mericloning; Micropropagation; Orchid

1. Introduction

Paphiopedilum orchids continue to gain popularity in the flower industry, but slow plant growth and inefficient propagation by cuttings or divisions have kept them in short supply. Their increase has been by aseptic germination of seeds. Mericloning has not taken hold because easily followed protocols are not available. Indeed, Paphiopedilum species and hybrids remain as the only

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commercially grown orchids that are not cloned. A major obstacle to the
development of tissue culture procedures has been the great difficulty of
removing bacterial and fungal infections from explants originating from
greenhouse plants (Bubeck, 1973; Stewart and Button, 1975; Huang, 1988).
Huang (1988) reported that most bacteria and fungi could be excluded by
utilizing explants that were considerably smaller than those usually employed for
mericloning other orchids; Huang’s explants were 2–3 mm tall shoot tips.
Nevertheless, the survival incidence of the smaller explants was low and the plant
multiplication rate remained slow. Greenhouse plants are also too valuable to be
sacrificed in the numbers required for rigorous experimentation.

Aseptically, established seedlings of hybrids that grow relatively uniformly
are obtainable in large quantities and less expensively from commercial
breeders. In this investigation, the major obstacles were circumvented by
employing the seedlings as the explant source. Our objective was to develop a
protocol that enables plant increase in one step, i.e., multiplying shoots and
having them rooted in the same medium. This was pursued by re-evaluating and
modifying the nutritional supplements recommended by Huang (1988) for shoot
multiplication. The unusually high prescribed BA concentration, 100 mg l⁻¹,
was critically evaluated. Also, another cytokinin, TDZ, as well as the sugar,
maltose, was considered. Substitutes were explored for the coconut milk,
especially since its source was stipulated as immature fruits that are not widely
available.

2. Materials and methods

2.1. Explants and culture environment

Flasks of aseptically germinated seedlings of Paphiopedilum hybrids were
purchased from a commercial breeder in Taiwan. The hybrid, Paphiopedilum
philippinense × P. Susan Booth, had the largest number of seedlings; thus,
this hybrid was chosen for the experiments that are now reported. The final
findings were applied to two other hybrids, P. bellatulum ‘Big spot’ × P. Jo Ann’s
Wine and P. micranthum × P. glaucophyllum. Each flask contained up to 25
plants, with each plant containing a single, dominant 1.5–2.5 cm tall shoot.
Plants were removed from the flasks and replanted in the shoot multiplication
medium of Huang (1988) to increase the number of explants. Clusters of
three 4 cm tall shoots served as explants for experiments and for maintenance of
stock cultures. They were planted in culture flasks at the rate of two clusters, or
six shoots, per flask. All cultures were incubated at 26 ± 2°C and under 16 h
daily exposure to 25 μmol m⁻² s⁻¹ illumination from “cool white” fluorescent
lamps.
2.2. Nutrient medium modifications

The starting medium contained ingredients of the *Paphiopedilum* shoot multiplication step of Huang (1988): MS (Murashige and Skoog, 1962) salts; MT (Murashige and Tucker, 1969) vitamins, glycine and inositol; 444 μM BA; 0.54 μM NAA; 0.15 mM adenine sulfate-2H₂O; 0.22 M sucrose; and 15% (v/v) coconut milk.

The BA concentration was repeatedly assessed first, followed by tests of TDZ, NAA, adenine sulfate-2H₂O concentrations in combinations with additional inorganic phosphate, sucrose and maltose, and coconut milk and other complex addenda. The BA was examined in concentrations ranging from 0.13 to 444 μM (the highest concentration approximated the 100 mg l⁻¹ of Huang (1988)). TDZ was bio-assayed in a 0.0014–0.14 μM range. The auxin NAA was tested at levels of 0.16–5.4 μM. Concentrations of 0.15–1.5 mM adenine sulfate-2H₂O were examined, with and without 1.23 mM NaH₂PO₄·H₂O as additional phosphate. The comparison of sucrose and maltose considered concentrations in the range 0.09–0.26 M. Complex addenda were tested as follows: coconut milk, 7.5–30% by volume; casein hydrolysate (ICN Biochemicals), 1–4 g l⁻¹; potato tuber sections, 10–30 g l⁻¹; and banana powder (Sigma), 20–60 g l⁻¹. Coconut milk (liquid endosperm of *Cocos nucifera*) was obtained from green fruits that weighed 1.2 kg or less at harvest. Large volumes were collected at each harvest, filtered through cheese cloth, mixed, divided into convenient aliquots, and frozen until needed. Potato tuber sections were obtained from freshly purchased potatoes. The tubers were diced into small cubes, weighed and added before incorporating 0.25% Gelrite™ (Kelco) and autoclaving.

The pH of media was adjusted to 5.7 prior to final dilution and the addition of Gelrite. Media were distributed in 500 ml Erlenmyer flasks at a rate of 125 ml per flask. Flasks were closed with MEICO gas-permeable stoppers (Microwave Enterprises, Taipei, Taiwan), and all media were sterilized by autoclaving for 10 min at 1.05 kg cm⁻² (121°C).

2.3. Experimental design

Ten flasks were employed per treatment. The *Paphiopedilum* cultures developed very slowly; thus, each experimental variable was monitored for at least two 6-week long passages, except those of cytokinins. The cytokinin effects were studied over four passages, or 24 weeks. The numbers of newly emerged shoots and roots were recorded after each passage and tissues were transferred to fresh media without dividing. Only data obtained after the second passage are presented in this report, except for those of BA and TDZ. Data of all four passages of cytokinin experiments are included. The data were evaluated for significance by calculating standard errors of means (Snedecor, 1957).
3. Results

3.1. Cytokinin effects

The cytokinin effects were first evident after two passages (Figs. 1 and 2). All BA concentrations showed the same numbers of new shoots and roots after one

![Graph showing shoot proliferation response to BA concentrations.](image)

Fig. 1. The shoot proliferation response of *Paphiopedilum* cultures to BA concentrations as related to passage number. The basal medium included 0.54 μM NAA, 0.15 mM adenine sulfate-2H₂O, 0.22 M sucrose, and 15% by volume of coconut milk. Missing standard errors in passages 1 and 2 are due to their values being too small for inclusion on charts.

![Graph showing rooting response to BA concentrations.](image)

Fig. 2. The rooting response of *Paphiopedilum* shoot cultures to BA concentrations as related to passage number. The basal medium included supplements as listed in Fig. 1.
6-week passage. Some repressive effects, especially on root number by concentrations of 44 μM and higher, were apparent after two passages. Also observed after two passages were slight inhibitions of shoot proliferation by the 132 and 444 μM levels. After the third and fourth passages, the rooting inhibition by the three higher concentrations was more pronounced. The lowest BA level (13 μM) continued to show no repression of rooting. Shoot multiplication was reduced slightly in the third passage by the highest level (444 μM). In the fourth passage, all BA concentrations decreased shoots, with severity increasing from 132 μM to higher levels. Even though the responses were mainly negative, BA was retained as the standard addendum at 13 μM, or the level that did not strongly depress rooting and shoot proliferation throughout all passages.

No advantages were evident by employing TDZ in place of BA. Data from the first passage revealed no differences in shoot proliferation or rooting in all concentrations of TDZ (Figs. 3 and 4). However, in subsequent passages rooting as well as shoot proliferation was clearly suppressed by the two higher concentrations, 0.014 and 0.14 μM. The lowest level, 0.0014 μM, neither inhibited nor promoted root or shoot formation throughout the four passages.

3.2. Auxin effects

The data after two passages revealed no beneficial effects on shoot formation by NAA, but a slight inhibition at its highest concentration (5.4 μM) (Fig. 5). Rooting was enhanced by the 1.6 and 5.4 μM levels; these levels of NAA produced 2–4 more roots than the lower levels. Based on its enhanced rooting, a 1.6 μM concentration was adopted for further inclusion.

![Graph showing shoot proliferation response to TDZ concentrations](image-url)

Fig. 3. The shoot proliferation response of *Paphiopedilum* cultures to TDZ concentrations as related to passage number. Basally included supplements were the same as described in Fig. 1.
3.3. Adenine sulfate and supplemental phosphate

The addendum combining the optimum concentration of adenine sulfate, 0.15 mM, with additional inorganic phosphate stimulated both shoot proliferation and rooting (Fig. 6). All supplements of adenine sulfate showed no adverse action on shoot proliferation, although the higher levels (0.5 and 1.5 mM) reduced rooting.
3.4. Sucrose and maltose addenda

Less rooting occurred in all maltose concentrations (Fig. 7). Sucrose possibly enhanced rooting at the 0.18 M level, but was suppressive at its highest concentration (0.26 M). No difference was observed in effectiveness of the two sugars on shoot proliferation, except perhaps a slight stimulation by sucrose at 0.18 M.
3.5. Addenda of natural complexes

To enable comparisons among addenda, the data on coconut milk, casein hydrolysate, potato tuber, and banana powder are shown as relative rates of organogenesis, instead of actual numbers of shoots and roots obtained per culture (Fig. 8). The rates were calculated by assigning the value 1.0 to shoot and root numbers in media lacking addenda. A relative rate above 1.0 denoted enhancement of organogenesis and that below 1.0 indicated suppression. Coconut milk and casein hydrolysate enhanced both shoot and root formation, whereas potato and banana stimulated proliferation of shoots only. The potato addendum showed no significant effects on rooting, whereas banana was repressive, particularly at its two higher concentrations (40 and 60 g l\(^{-1}\)). The addendum optima were 15% (v/v) for coconut milk; 1 g l\(^{-1}\) for casein hydrolysate; and 10 g l\(^{-1}\) for potato tuber sections. Banana powder promoted shoot proliferation at 20 g l\(^{-1}\).

Fig. 8. Shoot proliferation and rooting effects of coconut milk, casein hydrolysate, potato tuber sections, and banana powder on *Paphiopedilum* cultures. The relative rates were calculated by assigning 1.0 to shoot and root numbers of cultures in media without the addendum. Biossays were conducted with a basal medium containing 13 μM BA, 1.6 μM NAA, 0.15 mM adenine sulfate-2H₂O, 1.23 mM NaH₂PO₄·H₂O, and 0.18 M sucrose.
4. Discussion and conclusions

The investigation has resulted in a *Paphiopedilum* cloning protocol that enables shoot increase and rooting to occur in one step. The earlier protocol by Huang (1988) required two steps: multiplication of shoots in a high cytokinin medium followed by rooting of shoots in the absence of exogenous cytokinin. The new procedure is, thus, simpler and shortens the time required for obtaining plants. A representative culture after two 6-week passages in this medium can be seen in Fig. 9. The medium contains MS salts (Murashige and Skoog, 1962); MT vitamins, glycine and inositol (Murashige and Tucker, 1969); 13 μM BA; 1.6 μM NAA; 0.15 mM adenine sulfate·H₂O; 1.23 mM NaH₂PO₄·H₂O; 0.18 M sucrose;

Fig. 9. A sample culture showing simultaneously multiplied and rooted shoots of *P. philippinense*×*P. Susan Booth* after two 6-week passages in the medium with revised supplements.
and 15% by volume of coconut milk from immature fruits. The levels of adenine sulfate and coconut milk are unchanged from Huang’s shoot multiplication medium (Huang, 1988). However, BA has been reduced substantially, NAA has been increased threefold, and sucrose is slightly higher. The NaH₂PO₄ is additional phosphate over that contained in the MS medium. The investigation revealed that coconut milk of the quality as stipulated, when unavailable, can be substituted with casein hydrolysate (1 g l⁻¹) or potato tuber sections (10 g l⁻¹). Banana powder does not appear to be a satisfactory substitute, although all four natural complexes are employed for many orchid tissue cultures (Arditti, 1977).

The investigation clearly demonstrated that when working with species, the tissue cultures of which develop very slowly, experimental variables must be monitored over two or more successive passages for accurately assessing treatment effects. Thus, the high BA concentration (444 μM) of the earlier shoot multiplication medium (Huang, 1988), although neither promotive nor inhibitory of shoot and root organogenesis during the first passage, was found to be clearly repressive in succeeding passages. Similarly, TDZ showed no significant effects in the first passage, but severely repressed organogenesis in subsequent passages. TDZ was investigated because it is reportedly the most effective cytokinin for shoot proliferation of recalcitrant species (Huettmann and Preece, 1993).

Maltose has been reported as being superior to sucrose for organogenesis of cereal cultures (Xie et al., 1995). Our investigation found it less effective for *Paphiopedilum*.

The examination of adenine sulfate and NaH₂PO₄·H₂O combinations was prompted by a report of their beneficial effects on shoot differentiation in tobacco callus culture (Murashige, 1977); our findings concurred.

The one-step protocol produces an average of 12 new plants per culture in two passages, or 12 weeks, which extrapolates to 100 plants from each culture per year. Since each culture begins with two 3-shoot clusters, a doubling of plants may be expected every 12 weeks. This cloning rate is quite substantial for the *Paphiopedilum* orchids.

Although unverified by histological examination, the new shoots appeared to have originated as axillary buds. There was no callus development. Nevertheless, excessive crowding in cultures caused internode elongation of some shoots, resulting in their development into rhizome-like structures that are atypical of *Paphiopedilum*. Normal shoots developed when their nodal and terminal sections were transferred to fresh medium. Nevertheless, being mindful of somaclonal variants that can arise in tissue culture, we are continuing to monitor plant development in the greenhouse.

The protocol of Huang (1988) began with 2–3 mm tall shoot tips excised from greenhouse-grown plants. The small explants enabled effective exclusion of bacteria and fungi and possibly also of viruses and related pathogens. We,
therefore, applied our protocol to small explants. As anticipated, their development was initially very slow, but in time they grew into shoots that were large enough for plant multiplication by our protocol.

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References