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MICROPROPAGATION OF *AERIDES MACULOSUM* LINDL. (ORCHIDACEAE)

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Summary

Young leaf segments from plants growing both *in vivo* and *in vitro* were cultured on Murashige and Skoog (MS) medium supplemented with auxins [naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D)], cytokinins [kinetin (KN) and N^6-benzyladenine (BA)] and coconut liquid endosperm (CW). The explants from mature leaves did not show any growth and turned necrotic, while those obtained from juvenile leaves growing *in vitro* developed protocorm-like bodies (PLBs) at their cut surfaces within 4–8 wk depending on the growth medium. An optimum of 18 PLBs developed from leaf explants on medium supplemented with 2.0 mg l^{-1} (8.87 μM) BA. Upon subculture in basal MS medium, the PLBs differentiated into plantlets within 6–8 wk. The resulting plantlets were successfully transferred to vermiculite initially and subsequently to potting mixture; 84% of the plantlets survived after 3 mo. of transplantation.

Key words: orchid; protocorm-like bodies (PLBs); *Aerides maculosum*.

Introduction

Tissue culture methods have been extensively exploited, not only for rapid and large-scale propagation of orchids, but also for their *ex situ* conservation. Protocols have been developed for the large-scale propagation of a number of orchid species through *in vitro* culture of various plant parts (Arditti and Ernst, 1993).

Aerides maculosum is one of the most important orchids, valued for its beautiful inflorescence/flowers. This species is endemic to South India and its natural populations are dwindling due to over exploitation (Rao, 1998). Multiplication of this species in nature is through seeds, and only 0.3% of seeds germinate in the presence of suitable mycorrhiza. Since vegetative propagation methods are not available, development of *in vitro* methods are essential for the conservation and commercialization of this species.

This present paper describes successful experiments performed for the regeneration of *Aerides maculosum* Lindl. from leaf explants.

Materials and Methods

Plant material. This consisted of young leaves, up to 2 cm in length, produced from mature greenhouse-grown plants and 5-mo.-old axenic seedlings. The axenic seedlings of *Aerides maculosum* were obtained by culturing the mature seeds on Vacin and Went (1949) medium with 15% coconut liquid endosperm and the leaves from the resulting seedlings were used as explants. Leaves were cut into 0.5–1.0 cm long segments (including leaf bases and leaf tips) and used as explants. These were surface-disinfected for 15 min in 0.1% mercuric chloride, washed thoroughly with sterile distilled water and cultured on the medium.

Culture medium. MS (Murashige and Skoog, 1962) basal medium and MS medium supplemented with growth regulators like 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), N^6-benzyladenine (BA), and kinetin (KN) (0.5–5.0 mg l^{-1}) were added to the medium singly and in several combinations. Coconut liquid endosperm (5–15%) was also tested with basal medium. Sucrose (3% w/v) was the carbon source, pH was adjusted to 5.6 and 1% agar (Bacteriological, Hi-Media, Bombay, India) was used as a solidifier.

Culture conditions. Cultures were maintained at 25 ± 2°C under 12 h photoperiod of 40 μmol m^{-2} s^{-1} (PAR). There were 15 replications for each treatment and experiments were repeated three times. Results are presented as mean±standard error.

Results and Discussion

The morphogenetic response of the leaf explants of *Aerides maculosum* to various concentrations of cytokinin and/or auxin is shown in Table 1. The explants from mature leaves did not show signs of growth up to 6–7 wk, during which they released excessive phenolic compounds to the medium. Frequent subculturing of explants to fresh medium and medium containing 2% charcoal (activated, Hi-Media, Bombay, India) have not yielded results and they perished in 9–10 wk. On the other hand, explants from juvenile leaves showed organogenetic changes on the medium supplemented with growth regulators. In the medium containing 2,4-D (0.5–2.0 mg l^{-1}) the explants showed swelling at the cut ends (in 4–6 wk) and subsequently developed callus. Of the various concentrations of NAA tested, only in the medium with 1.0 mg l^{-1} NAA have explants induced protocorm-like bodies (PLBs) within 6 wk.

Of the two cytokinins tested, BA was more effective. The explants cultured on MS medium containing BA responded within 2 wk by the basal expansion and swelling. PLBs differentiated directly from swollen bases within 4 wk (Fig. 1A), without intervening callus and the differentiation continued until the 8th week. The frequency of

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TABLE 1

INFLUENCE OF DIFFERENT PHYTOHORMONES AND COCONUT WATER (CW) ON DEVELOPMENT OF PROTOCORM-LIKE BODIES (PLBS) FROM LEAF EXPLANTS OF *AERIDES MACULOSUM*

Phytohormone concentration (mg l^{-1})		Nature of response	PLB regeneration (%)	No. of PLBs per explant (*n*)
BA	0.0	No response	0	0
	0.5	No response	0	0
	1.0	Development of PLBs	45.6 ± 1.22	4.6 ± 0.15
	2.0	Development of PLBs	90.2 ± 1.26	18.2 ± 0.02
	5.0	Development of PLBs	40.3 ± 1.30	8.3 ± 0.12
KN	0.5	No response	0	0
	1.0	Development of PLBs	90.0 ± 1.29	20.6 ± 0.21
	2.0	Development of PLBs	56.8 ± 1.37	16.4 ± 0.27
	5.0	Development of PLBs	23.6 ± 1.40	2.1 ± 0.1
2,4-D	0.5	No response	0	0
	1.0	Callus development	0	0
	2.0	Callus development	0	0
	5.0	Callus development	0	0
NAA	0.5	No response	0	0
	1.0	Development of PLBs	12.2 ± 1.46	3.9 ± 0.17
	2.0	No response	0	0
	5.0	No response	0	0
CW (% v/v)	5.0	No response	0	0
	10.0	Development of PLBs	36.4 ± 1.62	4.9 ± 0.17
	15.0	Development of PLBs	38.8 ± 1.86	6.2 ± 0.15
NAA + BA	0.5 + 2.0	No response	0	0
	1.0 + 2.0	No response	0	0
NAA + KN	0.5 + 1.0	No response	0	0
	1.0 + 1.0	No response	0	0

Data (mean±SE) from three independent experiments each of 15 leaf explants, after 8 wk in culture.

PLB regeneration was optimal (18 PLB per explant) at 2.0 mg l^{-1} BA and in this medium 90% of explants developed PLBs. The frequency of PLB regeneration declined at higher concentrations of BA (Table 1). Of the various concentrations of KN tested, the best results were recorded on 1.0 mg l^{-1}. A total of 90% of explants cultured on a medium containing KN at 1.0 mg l^{-1} showed PLB regeneration in 6–8 wk. PLBs were also differentiated from explants cultured on medium with 2.0 and 5.0 mg l^{-1} KN. However, the frequency of PLB regeneration was decreased with increased concentration (Table 1).

Medium supplemented with coconut liquid endosperm (CW) (10% and 15%) induced PLB regeneration (36–38%) in 12 wk. The explants cultured on medium containing combinations of BA + NAA and KN + NAA did not respond and they perished after 8–10 wk (Table 1).

PLBs occurred only at the cut end of the leaf explants; they were not formed at the tip or on the surface of the explants. Anatomical studies revealed that differentiation of the leaf base started at the subepidermal cells. The meristematic region is then further differentiated to form a PLB. PLBs formed on the explants on BA- and KN-containing medium were subcultured into fresh medium, where they grew and multiplied (Fig. 1B).

Upon excision and transfer to basal medium the PLBs differentiated shoots and roots in 6–8 wk (Fig. 1C and D). The well-differentiated plantlets ready for transfer to community pots were obtained within 10–12 wk of subculture. The plantlets were initially transferred to vermiculite and subsequently to potting mixture (one part of brick pieces: three parts of charcoal: one part of chopped dried coconut husk) (Fig. 1E), and 84% of the plantlets survived after 3 mo. of transplantation.

The importance of leaf/foliar explants as an effective alternative to shoot meristems for micropropagating orchids is being increasingly realized. They are easy to obtain, do not require the sacrifice of the mother plant and offer exciting opportunities to raise large numbers of true-to-type plants. Their regenerative potential has so far been successfully tested in many species representing diverse taxonomic affinities, habits and habitats (Churchill et al., 1970, 1971; Vij et al., 1984; Vij and Pathak, 1990; Seeni and Latha, 1992; Nayak et al., 1997). Presently, foliar explants have been successfully used for micropropagation of *Aerides maculosum in vitro*. The explants from mature leaves did not respond to any culture medium, while proliferation occurred in those from juvenile leaves, which developed PLBs. The differential responses of the explants from mature and juvenile leaves under identical nutritional conditions seem to indicate the importance of their source and physiological age of the explant. Physiological age of an explant is an important factor for regeneration and in accord with the observations of Mathews and Rao (1980), Vij et al. (1984, 1986), and Vij and Pathak (1990).

In the present study, regeneration of PLBs occurred only at the basal cut end of the explants. Similarly, in *Cattleya* (Champagnat et al., 1970), *Vanda coerulea* (Seeni, 1988), *Neofinetia falcata*, *Satyrium nepalense*, *Vanda cristata* and *Vanda testacea* (Vij and Pathak, 1990) regenerative responses remain restricted to the leaf base, in agreement with the earlier suggestion of Zimmer and Peiper (1975) that the leaf base is generally meristematic in monocots, and upon isolation and culture it can differentiate into plantlets.

The efficiency of plant growth regulators in activating proliferative loci in foliar explants and regulating their subsequent development into plantlets is specific in orchids (Vij and Pathak,

FIG. 1. Micropropagation of *Aerides maculosum* Lindl. A, Development of protocorm-like bodies (PLBs) from the base of leaf explants on MS + BA (2.0 mg l^{-1}) after 8 wk culture (*bar* = 0.25 cm). B, Multiplication of PLBs on MS + BA (2.0 mg l^{-1}) after 6 wk of subculture (*bar* = 1 cm). C and D, Development of shoots and roots from PLBs on MS basal medium after 8 wk of subculture (*bar* = 1 cm). E, A regenerated plant in pot containing brick pieces, charcoal and chopped coconut husk (*bar* = 1.8 cm).

1990). The combined effect of cytokinins and auxins proved useful in foliar cultures of hybrid Vandas (Mathews and Rao, 1980), *Vanda coerulea* (Seeni, 1988), *Vanda cristata* and *Vanda testacea* (Vij and Pathak, 1990), *Pholidata chinensis* (Yam and Weatherhead, 1991), and *Spathoglotis plicata* (Teng et al., 1997). In the present cultures auxin alone induced PLB formation, but it was inhibitory when in combination with BA and/or KN; whereas BA (2.0 mg l^{-1}) and KN (1.0 mg l^{-1}) alone have induced PLBs in 90% of cultures. Explants have also developed PLBs in medium supplemented with CW (15%) and utility of CW has already been demonstrated in promoting regeneration in *Phalaenopsis* leaf cultures (Tanaka and Sakanishi, 1977).

In summary, an attempt was made in this investigation to develop an *in vitro* propagation system for the rare/endemic orchid species, *Aerides maculosum*. Juvenile leaf explants cultured on MS medium with cytokinins (BA/KN) and coconut liquid endosperm developed protocorm-like bodies. Optimal results were noticed on the medium supplemented with 2.0 mg l^{-1} BA, and on this medium explants developed 18 protocorm-like bodies per explant in 6–8 wk. The protocorm-like bodies were subcultured to MS basal medium, wherein they developed into plantlets after 6–8 wk in culture. The plantlets were successfully transplanted to community pots and 84% of plantlets survived after 3 mo. of transplantation.

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