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High frequency multiplication of *Phalaenopsis gigantea* using trimmed bases protocorms technique

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Abstract

This study was conducted to determine the effects of coconut water (CW) and activated charcoal (AC) on multiplication of *Phalaenopsis gigantea* protocorms. The protocorms used for this study were obtained by germinating seeds *in vitro*. Protocorms with trimmed and untrimmed bases were cultured on XER basal medium containing 0, 10, 15 or 20% (v/v) CW; and 0, 1, 2 or 2.5 g AC l^{-1}. Trimmed protocorms exhibited the highest percentage of proliferation on a medium containing 15% (v/v) CW and 2.5 g AC l^{-1} (56.82 ± 38.86%) with an average of 4.24 ± 2.89 pro-protocorms formed per protocorm. Untrimmed protocorms cultured on a medium containing 20% (v/v) CW without AC produced the highest percentage of new protocorms (6.93 ± 6.28%) with an average of 0.72 ± 0.57 per protocorm. When CW was added to a medium singly, 10% (v/v) CW induced a higher degree of proliferation on trimmed protocorms (5.68 ± 10.14%) with an average 0.50 ± 0.84 new protocorms per protocorm. Untrimmed protocorms proliferate to a much lower extent (2.57 ± 2.74%) with an average of 0.72 ± 0.57 protocorms per protocorm when cultured on a similar medium. A high concentration of CW enhanced proliferation on untrimmed protocorms, but increased mortality of trimmed protocorms. The addition of CW with AC to media increased protocorm proliferation and survival of both trimmed and untrimmed protocorms. When cultured on all media, trimmed protocorms produced a higher number of new protocorms (an average 0.5–7.0) as compared to untrimmed protocorms (0.3–1.9). Comparative studies showed that trimmed protocorms produced up to 10 times more new protocorms than untrimmed ones. Altogether this study showed that trimmed protocorms cultured on a medium containing CW and AC can be used for high-frequency multiplication of *P. gigantea* seedlings.

Keywords: Protocorms; Trimmed and untrimmed; Protocorms; New per protocorm; *Phalaenopsis gigantea*

1. Introduction

Phalaenopsis gigantea, known as an elephant's ear orchid due to its huge leaves, occurs in Sabah (Malaysian part of Borneo) and Kalimantan (Indonesia). This species is endemic to Borneo. It is classified as an endangered species in Appendix II of the Convention on International Trade in Endangered Species (CITES). This species is usually propagated through the formation of new buds at the bases of mature plants. However, this method of propagation is very inefficient as the number of new buds produced by a plant is very low.

Tissue culture has been used widely for mass propagation of superior varieties of *Phalaenopsis* (Tokuhara and Mii, 1993). This technique can be used not only for rapid and large-scale propagation of the species but also for *ex situ* conservation. Many protocols have been developed for large-scale propagations of a number of orchid species (including *Cymbidium*, *Vanda*, *Phaphiopedilum* and *Phalaenopsis*) through *in vitro* culture of various plant parts (Arditti and Ernst, 1993). Protocols for *Phalaenopsis in vitro* micropropagation utilize flower stalk buds (Arditti, 1977; Tanaka and Sakanishi, 1978; Tokuhara and Mii, 1993, 2001; Košir et al., 2004) entire shoots, shoot tips, stem nodes (Griesbach, 1983), leaf tissues/segment (Tanaka and Sakanishi, 1980; Park et al., 2002) or root tips culture (Tanaka et al., 1976; Park et al., 2003). Unfortunately, these methods are very difficult and inefficient. Propagation through protocorms derived from seedling proliferation has been studied by Yam et al. (1991) and Chen et al. (2000).

The protocorm is a structure unique to orchids, including *Phalaenopsis*. It is the earliest structure formed during embryo

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development during orchid seed germination (Ishii et al., 1998). Proliferation of protocorms- and protocorm-like bodies (PLB) is often the only means of increasing the number of orchids, which produce few seeds or may not germinate well. In an effort to increase and/or accelerate proliferation, propagators have resorted to media which contain very high hormone levels (Arditti, 1977). Vajrabhaya (1997) reported that some media containing hormones may accelerate the rate of increase, but they can also bring about undesirable mutations. These undesirable side effects may be reduced or eliminated through the use of media, which induce proliferation of protocorms or PLB but contain low concentrations of hormones (Gu et al., 1987) or by using hormone-free media supplemented with complex additives. Based on research by previous investigators we determined the optimal conditions required for *Phlaenopsis in vitro* culture and micropropagation. Thus far, we found that medium choice, concentrations of mineral nutrients (Hinnen et al., 1989; Tokuhara and Mii, 1993; Košir et al., 2004), addition of complex additives such as banana homogenate (Yam et al., 1991) and coconut water (Yam et al., 1991; Ishii et al., 1998), explants source (Ishii et al., 1998), maturity (Chen et al., 2000), and size (Park et al., 2002) profoundly affect the success culture procedures.

Therefore, this study was carried out to determine the effects of different concentration of coconut water and activated charcoal on the multiplication *P. gigantea* protocorms through the use of protocorms with trimmed and untrimmed bases. These protocorms were used as a model system because they are easier to obtain than protocorm-like bodies produced through tissue culture and their use does not damage the mother plants. As a rule protocorms and PLB respond similarly to media and culture conditions.

2. Material and methods

2.1. Plant material and inoculation

A capsule of *P. gigantea* was harvested 167 days post-pollination and seeds from the disinfected capsule were cultured on XER medium (Ernst, 1994) supplemented with 10% (v/v) coconut water (CW) and 0.2% (w/v) activated charcoal (AC) (charcoal, wood powder, BDH chemicals, LTD Poole, England). Protocorms were used 150 days after the seeds germinated. Trimmed bases protocorms were prepared by hand cutting of the protocorm base (approximately 1 mm) with razor (Albion surgical Limited, Sheffield, England).

The experiments were carried out in sterile plastic Petri dishes containing 25 ml medium. Cultures were maintained at $25 \pm 2\,^{\circ}C$ under continuous illumination (20–50 μmol $m^{-2}\,s^{-1}$) provided by cool white florescent tubes (Philips, Malaysia).

2.2. Culture medium

The protocorms with trimmed and untrimmed bases (Fig. 1A and B) were cultured on XER (Ernst, 1994) medium containing 20 g l^{-1} fructose. The medium was further supplemented with 0, 10, 15 and 20% (v/v) CW and 0, 1, 2 and 2.5 g AC l^{-1} either individually or in combination. The pH of the medium was adjusted to 5.7 ± 0.03 before gelling with 1% (w/v) agar (Sigma). Media were autoclaved at 104 kPa and 121 °C for 20 min. CW was obtained by draining the liquid endosperm of ripe nuts.

The new protocorms formed from trimmed protocorms were transferred into the growing medium (XER medium supplemented with 20 g l^{-1} fructose, 2 g AC l^{-1}, 10 g l^{-1} agar pH 5.7 ± 0.03).

2.3. Experimental design and data analysis

Experiments were carried out in a completely randomized design (CRD). Media were replicated ten times with 20 protocorms cultured per Petri dish. Results were evaluated 112 days after cultures were initiated. The parameters recorded were the percentages of proliferating protocorms and the number of new protocorms produced by a protocorm. The data were subjected to analysis of variance. Significant differences among the experimental treatments were tested using two-way ANOVA and F-test followed by Tukey's multiple comparison at $p = 0.05$.

3. Results

Initial response of protocorms to different media was observed only 14 days after cultures were initiated. It consisted of with emerging pappilae and/or swelling of protocorms

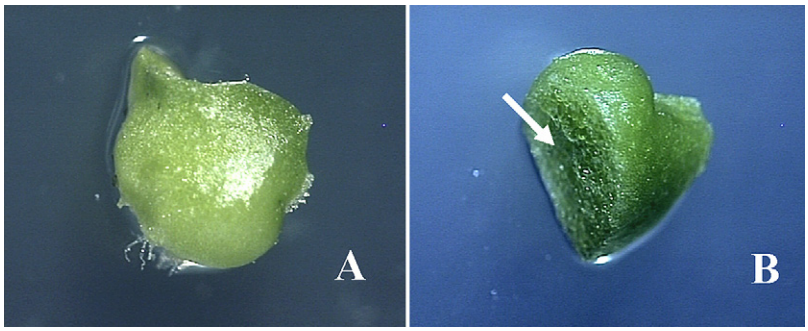

Fig. 1. *Phalaenopsis gigantea* protocorms (150 days) used as explants: (A) protocorm with untrimmed base; (B) protocorm with trimmed base. The arrow points to the surface after the base of a protocorm has been trimmed.