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Mass propagation of *Dendrobium* 'Zahra FR 62', a new hybrid used for cut flowers, using bioreactor culture

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ABSTRACT

A mass propagation protocol for *Dendrobium* 'Zahra FR 62', the result of hybridization between *Dendrobium* 'Sonia Deep Pink' × *Dendrobium* 1265, was successfully established. Half-strength Murashige and Skoog (MS) medium containing 1 mg/L thidiazuron (TDZ) and 0.5 mg/L N^6-benzyladenine (BA) resulted in high protocorm-like body (PLB) formation and initial proliferation of *Dendrobium* using shoots as the donor explant *via* repeated subcultures. High proliferation and optimal growth of PLBs was possible by culturing PLBs in a 3-L bioreactor with 10 vessel volumes per minute (vvm) (CS-9) containing half-strength MS medium with 0.05 mg/L BA (M-2). A total of 807.7 PLBs formed with an average of 247.5 new PLBs per subculture. The culture system was improved by adding 15 g of explant to 5 vvm of dissolved oxygen, although good quality PLBs formed when 10 g of explant was combined with 10 vvm of dissolved oxygen. PLBs converted into healthy plantlets by culturing them on half-strength MS containing 0.05 mg/L BA. Thereafter, germinated PLBs were subcultured on half-strength MS medium with full vitamins and free of plant growth regulators (RM-1). Rooted plantlets were successfully acclimatized *ex vitro* with 90–100% survival using *Cycas rumphii* bulk and grew well after repotting individually into a mixture of *C. rumphii* bulk and wood charcoal (1:1, v/v).

1. Introduction

Dendrobium 'Zahra FR 62' is a new and promising ornamental cut flower derived from breeding activities of the Indonesian Ornamental Crop Research Institute (IOCRI). The cultivar was the result of hybridization between *Dendrobium* 'Sonia Deep Pink' × *Dendrobium* accession 1265. *D.* 'Zahra FR 62' was released and registered by IOCRI as a new superior hybrid in October 2010 under the Indonesian Center for Plant Variety Protection and Agricultural Permit. This *Dendrobium* has a flower stalk length of 27–30.3 cm, 5–8.6 cm flower diameter, 8–13 flower buds per stalk with a vase life of three months (Catalog on Superior Varieties of Ornamental Plants, 2012). Moreover, based on a consumer preference test conducted by IOCRI with 100 respondents (consumers, florists and growers), *D.* 'Zahra FR 62' flowers have high potential for substituting *D.* 'Sonia' as a cut flower product, especially for the Indonesian orchid cut flower market. However, ever since the development of this new hybrid in 2010, there has been insufficient quality material for nation-wide distribution to farmers for commercial production. Therefore, the development of an *in vitro* clonal propagation protocol that would deliver high-quality planting material in sufficient quantities to satisfy current demand was an important consideration when embarking on this study.

Dendrobium species are generally propagated asexually by the division of offshoots, but the multiplication rate is extremely low with only 2–4 plants being produced per year (Nasiruddin et al., 2003; Martin and Madassery, 2006). Thus, conventional methods are not suitable for preparing a large amount of high-quality planting material. Many orchids can be propagated sexually by seeds, but *Dendrobium* seeds lack an endosperm and nutritive substances and are generally difficult to grow into complete plants in nature because seed germination requires a symbiotic fungus (Anjum et al., 2006). Thus, an *in vitro* propagation method for *D.* 'Zahra FR 62' would be particularly useful for commercialization of the variety.

Several *in vitro* propagation protocols for *Dendrobium* spp. and varieties have been successfully established. Nayak et al. (2002) used thin cross sections (equivalent to thin cell layers) of *Dendrobium nobile* protocorm-like bodies (PLBs) cultured on modified

Abbreviations: BA, N^6-benzyladenine (BA has been used to represent BA or BAP, N^6-benzylaminopurine, according to Teixeira da Silva, 2012); CS, culture system; CW, coconut water; DO, dissolved oxygen; FW, fresh weight; GA_3, gibberellic acid; NP, Ichihashi New *Phalaenopsis* (1998) medium; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kin, kinetin; MS, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; PLB, protocorm-like body; SDW, sterile distilled water; TDZ, thidiazuron; vvm, vessel volumes per minute; VW, Vacin and Went (1949) medium.

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Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2.5 mg/L N^6-benzyladenine (BA) (Teixeira da Silva, 2012) for initiation and proliferation, and 2.0 mg/L indole-3-butyric acid (IBA) for root formation. *D. nobile* shoot tips were cultured on Mitra et al. (1976) medium supplemented with 4.0 mg/L triacontanol (TRIA) for shoot induction and proliferation and 2.0 mg/L TRIA for plantlet development (Malabadi et al., 2005). *Dendrobium* 'Sonia' flower stalk nodes were cultured on MS medium supplemented with 10 mg/L of BA for induction of multiple shoots, subculturing these shoots in the same medium for induction of PLBs and rapid PLB proliferation on half-strength MS containing 2 g/L activated charcoal for plantlet development (Martin and Madassery, 2006). In *Dendrobium candidum*, protocorm segments cultured on MS medium with half-strength macronutrients and full-strength micronutrients, 2.0 mg/L BA and 0.5 mg/L α-naphthaleneacetic acid (NAA) successfully induced callus, which was subcultured in the same medium without any plant growth regulators (PGRs) to produce PLBs and the basal medium containing 0.5 mg/L for plantlet development (Zhao et al., 2007). *Dendrobium* 'Serdang Beauty' PLBs could be induced on MS medium containing 1.5 mg/L IBA and 1 mg/L kinetin (Kin) or MS medium supplemented with 1 mg/L Kin for high plantlet formation (Khosravi et al., 2008). *Dendrobium densiflorum* PLBs were successfully regenerated by culturing stem segments on MS medium supplemented with 5.0 mg/L BA, proliferated in the same medium, completely converted into shoots on MS medium containing 2.0 mg/L BA and rooted on MS medium supplemented with 2.0 mg/L neodymium nitrate (Luo et al., 2008). In *Dendrobium transparens*, multiple shoot induction was carried out by culturing axenic nodal segments on half-strength MS medium supplemented with 1 mg/L IAA, IBA or NAA while shoot multiplication was possible using 1 mg/L NAA and 2 mg/L BA and plants could be rooted with 1 mg/L IAA (Sunitibala and Kishor, 2009). Shoots of *Dendrobium nobile* var. 'Emma white' could be induced from axillary buds on Phytotechnology medium (O753) supplemented with 2 mg/L BA, 1.5 mg/L Kin and 2 mg/L IBA while the application of 2 mg/L IBA rooted plantlets more efficiently than NAA (Asghar et al., 2011). No *Dendrobium in vitro* study in the literature has used VW medium. Each *Dendrobium* thus requires specific *in vitro* conditions, including the correct choice of explant source and medium components at each stage, including initiation, proliferation or multiplication and root formation right through to the acclimatization stage. Despite a variety of explant sources, basal media and PGRs having been described for initiation, proliferation and root formation in *Dendrobium*, no report on *D.* 'Zahra FR 62' exists.

In this study, a mass propagation protocol for *D.* 'Zahra FR 62' was successfully developed, including PLB initiation, proliferation, germination, plantlet preparation and acclimatization. The protocol has now been successfully applied to the production of clonal plant material for commercial purposes. Commercially, from 1 g of PLBs (61 PLBs) in the initial culture, 100,000 or more PLBs/year can be produced with a multiplication rate of 4 PLBs/month and approximately only 3.5% of PLBs browning from initial PLBs until a proliferated state. The establishment of a mass propagation protocol using a bioreactor-based culture system is a new and as yet unpublished achievement in *Dendrobium* (and rare for orchids; Hossain et al., 2013) biotechnology.

2. Materials and methods

2.1. Plant material, explant preparation, media and culture conditions

D. 'Zahra FR 62' donor plants (18 months old) were obtained from an IOCRI orchid breeder. The plants were cultivated in small pots (15 cm in diameter) containing wood charcoal and *Cycas rumphii* bulk (1:1, v/v) and placed in a glasshouse at 30–37 °C during the day and at 15–21 °C at night (temperature assessed by a thermo-hygrometer, Haar-Synth-Hygro, Germany), 60–90% relative humidity (RH) during the day and 75–90% at night, also assessed with a Haar-Synth-Hygro, and a 12-h photoperiod with 175–350 μmol/m^2/s light intensity during the dry season (April–October) and 30–100 μmol/m^2/s in the rainy season (November to March). Light intensity was measured using a Digital Lux Meter, Lutron LX 101 (Lutron Electronic Enterprise Co., Ltd., Taiwan). Measurement data from the Lutron LX 101 which was in lux was then converted to μmol/m^2/s by multiplying each data point with a conversion factor for sunlight, *i.e.*, 0.0185 (Thimijan Richard and Heins, 1982).

Plants were watered every morning at 7.00–8.00 am. Liquid fertilizer (2 g/L of N:P:K, 20:20:20; Nusa Tani, Ltd., Jakarta) containing 2 ml/L BioSugih Tani, a liquid fertilizer (PT. Sugih Cipta Santosa, Jakarta, Indonesia) was applied twice a week to maintain the vigorous vegetative growth of donor plants and to accelerate the development of new pseudobulbs. Pesticides (fungicides or insecticides) were used as little as possible to reduce the potential of environmental contamination. Initially, *D.* 'Zahra FR 62' was multiplied several times through conventional propagation, *i.e.*, splitting of pseudobulbs, to increase the number of stock plants. A total of 20 stock plants were used in this study. The lateral shoots (0.5–1.0 cm in length) and terminal shoots that emerged from active growth of the donor plants were harvested and used as donor explants.

The shoots were washed by placing them under tap water for 30–60 min then immersed in 1% Tween 20 (Sigma–Aldrich Ltd.) solution for 30 min and rinsed with sterile distilled water (SDW) 5 times (5 min each rinse). After pretreatment, the shoots were surface sterilized by immersing them in 0.05% mercury chloride ($HgCl_2$, Merck, Darmstadt, Germany) plus a few drops of Tween-20 for 10 min, followed by 5–6 rinses in SDW (5 min each rinse). After sterilization, the damaged surface of explants caused by chemicals was sliced off with a tissue culture blade (BB510, Aesculap AG & Co. KG AM, Tutlingen, Germany) and shoot size was reduced by removing several leaves. Finally, the size of the donor explant used as the *in vitro* explant was approximately 0.4 cm in length (Fig. 1A). The explants were then cultured in PLB initiation medium.

Three basal media were tested in this study: MS, Vacin and Went (VW; Vacin and Went, 1949) and Ichihashi New *Phalaenopsis* (NP; Islam et al., 1998). MS medium was used for PLB initiation and proliferation, while VW and NP media were used and tested for PLB germination. All media components used were chemicals of analytical grade supplied by Merck. Thidiazuron (TDZ), BA and NAA were purchased from Sigma-Aldrich (St. Gallen, Germany). All media used in these studies contained 2% sucrose (Merck) and 2.0 g/L Gelrite (Duchefa-Biochemie, RV Haarlem, The Netherlands) to create a semi-solid medium. The pH of media was adjusted to 5.8 (Model 420A pH meter, Thermo Orion, Beverley Hills, USA) and sterilized for 20 min at 121 °C and 15 kPa atm (Pressure Sterilizer Model No. 1941X, Foundry Co. Inc., WI, USA).

In this study, two types of media were used, semi-solid and liquid. Semi-solid medium was utilized in the initial stage, proliferation and conversion of PLBs, while liquid medium was only applied for the proliferation of PLBs. All liquid cultures were placed on an orbital shaker (GFL 3017, Geselischaft fűr Labortechnik mbH, Burgwedel, Germany) on incubation racks under fluorescent lamps (SL-Shinyoku, PT. Ningbo Global Lamp, Jakarta, Indonesia). Cultures were shaken at 80–100 rpm under ~13.5 μmol/m^2/s light intensity in a 12-h photoperiod, at 23.5 ± 1.1 °C, and at 60.6 ± 3.8% RH. All semi-solid cultures were placed on incubation racks under fluorescent lamps under the same light intensity.