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Comparison of Different Culture Media for the *In Vitro* Culture of *Dendrobium* (Orchidaceae)

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ABSTRACT

The potential of different media, supplements and conditions for reliable *Dendrobium* orchid plantlet regeneration from *in vitro* grown leaf explants were studied. In all three media under investigation, regeneration was *via* the production of protocorm-like bodies (PLBs). Maximum number of PLBs was obtained in Murashige and Skoog (MS) liquid medium agitated at 80 rpm and supplemented with 0.1 mg L^{-1} of BA, 1.0 mg L^{-1} of NAA and 15% (v/v) coconut water. Shoot regeneration was achieved when the PLBs were transferred onto solidified MS medium supplemented with 0.1 mg L^{-1} of BA, 1.0 mg L^{-1} of NAA and 15% (v/v) coconut water and 10 g L^{-1} potato homogenate. The shoots developed roots after four weeks of culture on the same medium. Transferring them onto wood charcoal successfully acclimatized the *in vitro* grown plantlets. The short duration of *in vitro* culture and its high efficiency makes this approach well suited for mass production of *Dendrobium* orchids.

Key Words: Acclimatization; *Dendrobium; In vitro* culture; Plantlets; Protocorm-like bodies

INTRODUCTION

The genus *Dendrobium* was established by Olaf Swartz (1799). Although most *Dendrobiums* have an epiphytic growth habit, some are also found growing on rocks and cliffs and terrestrial in grasslands. They are widely distributed throughout the Asian and South Pacific tropics and subtropics, from lowland warm regions in northern Australia, Papua New Guinea, to Thailand and Himalayan mountains. The truly spectacular genus *Dendrobium* contains the largest diversity of horticulturally interesting specimens. More than 1,000 natural species make *Dendrobium* the second-largest orchid genus (next to *Bulbophyllum*) in the orchid family, which has over 700 genera. Although the color range of *Dendrobiums* is varied, most hybrids are usually lavender, white, golden-yellow, or combinations of these colors. Some of the more unusual species and hybrids can be bluish, ivory colored, brilliant orange or scarlet, or have exotic markings. Most of the evergreen *Dendrobiums* are not fragrant, however the deciduous species such as *superbum, pierardii* and *parishii*, can have fresh citrus scent or smell of raspberries. *Dendrobiums* can often bloom several times a year and the flower sprays make excellent cut flowers for arrangements.

It is widely recognized that world production of potted *Dendrobium* has increased in the last few years. Large scale potted *Dendrobium* production is occurring in the Netherlands, Germany, China, Taiwan, Thailand, Philippines, Unites States, and Japan. However, orchid growing has not fully achieved the transition from a hobby to an industry in Mauritius. The main reason being that conventional methods of orchid propagation are extremely slow and the number of propagules produced by these methods is low. Tissue culture techniques provide a solution for producing large number of propagules within a limited period of time. According to Morel (1974), more than four million plantlets can be obtained in a year from a single explant. Among the explants most commonly used are shoot tips and axillary buds (Intuwong & Sagawa, 1974) although stem, leaf, root and inflorescence can also be used (Homma & Asahira, 1985). Also, a wide range of recipe has been used in the in vitro culture of orchids (Lim-Ho, 1982). In a study carried out by Wu *et al.* (1987), it was observed that several factors influence shoot formation and *in vitro* multiple shoot production in oriental orchids. When growing the orchids in our laboratory, we found that the plantlets of *Dendrobiums* do not grow fast enough in the commonly used Vacin & Went (Vacin & Went, 1949) and Knudson C (KdC) medium (Knudson, 1946) and do not produce roots which are strong enough to withstand the acclimatization conditions. As literature for the commercial propagation of *Dendrobiums* specifically are scarce, due to obvious reasons, a study was conducted to compare the efficiency of different nutrient media, growth regulators and medium supplements for the *in vitro* propagation of orchid *Dendrobium* 'Sonia' suitable for commercial exploitation.

MATERIALS AND METHODS

Plant materials. Leaves obtained from *in vitro* grown protocorm-like bodies (PLBs) of *Dendobrium* 'Sonia' were used as starting material. The PLBs were cultured in liquid MS medium (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} sucrose on an orbital shaker at 80 rpm and

Fig. 1. Effects of BA and NAA on the initiation of *Dendrobium* 'Sonia' Protocorm-like bodies on three different liquid basal media

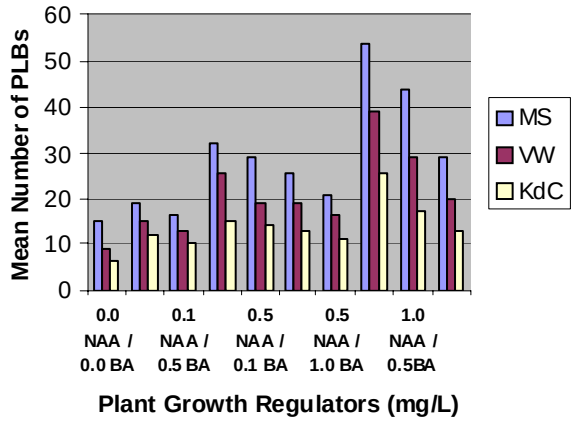


Fig. 2. Effects of BA and NAA on the initiation of *Dendrobium* 'Sonia' Protocorm-like bodies on three different solid basal media

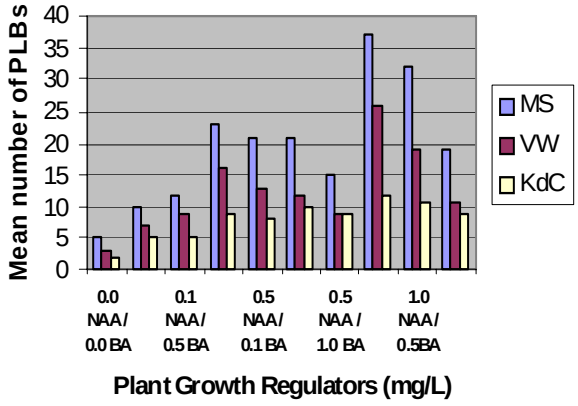


Fig. 3 Effects of the speed of agitation on the propagation of *Dendrobium* 'Sonia'. The medium consisted of MS medium supplemented with 0.1 mgL^{-1} of BA and 1.0 mgL^{-1} of NAA. Values are means of five replicates and five explants per treatment.

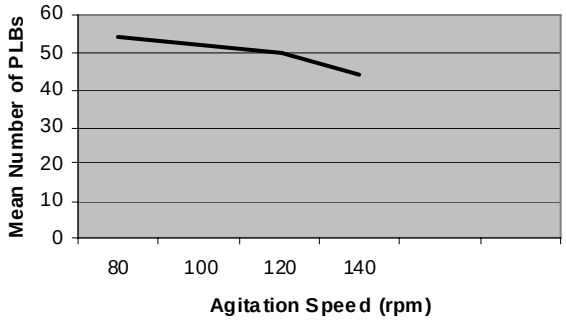
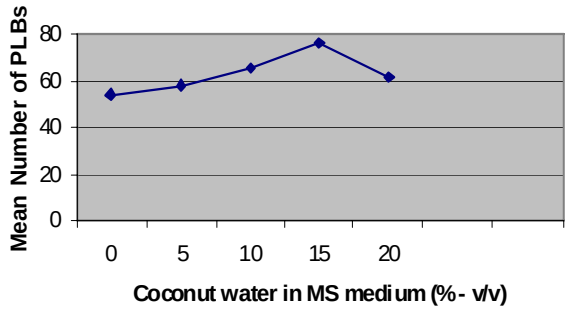


Fig. 4 Effects of adding coconut water on the propagation of *Dendrobium* 'Sonia' PLBs. The medium consisted of MS medium supplemented with 0.1 mgL^{-1} of BA and 1.0 mgL^{-1} of NAA. Values are means of five replicates and five explants per treatment.



under conditions of 26±2^0 C and 16 h of light daily provided by cool-white fluorescent tubes (50 µmol m^{-2} s^{-1}). Sub-culturing was performed at 2-weeks intervals by the transfer of terminal cuttings to the same medium and under the same conditions.

Media and culture conditions. The basal media used were Vacin and Went (VW) medium (Vacin & Went, 1949), Knudson C (KdC) medium (Knudson, 1946) and Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). Five leaf explants (~1 cm^2) per flask were cultured in each of these media supplemented with various amounts of BA (0–1.0 mg L^{-1}) and NAA (0–1.0 mg L^{-1}) both in liquid and Phytagel solidified media (2 g L^{-1}) for the determination of the optimum plant growth regulator combination. Similar experiments designed to assess the effects of the speed of agitation (80–140 rpm) and the effects of coconut water (0–20% v/v) on the propagation of the PLBs. The coconut water was prepared from selected coconuts obtained from a local market and processed to remove most of the proteins. Any precipitate formed was removed by further filtration. The pH of the media was adjusted to 5.4 with 1 *N* KOH or 1 *N* HCl and 25-mL aliquots of medium were dispensed into 125-mL bottles with subsequent autoclaving for 15 min at 121^{0}C. For the solidified media, Phytagel (2 g L^{-1}) was added to the liquid media followed by warming. The Phytagel dissolved media were then dispensed into 125 mL bottles and autoclaved. The bottles were cultured in a culture room at 26±2^{0}C and 16 h of light daily provided by cool-white fluorescent tubes (50 µmol m^{-2} s^{-1}). Bottles containing the liquid media were also cultured in the same culture room but on an orbital shaker (Edmund Bühler®, Swip SM 25 DIGI, Germany) at 80 rpm unless otherwise stated. After six weeks in culture, explants were evaluated in terms of the number of PLBs formation (>1mm in diameter) per treatment.

Plantlet regeneration. In order to obtain plantlets, the PLBs were transferred to the same medium as that for PLBs initiation but solidified with 2 g L^{-1} Phytagel. The effect of

including potato homogenate (10 mg L^{-1}) to the medium was also investigated. The potato homogenate was prepared, immediately before use, by grating 100 g of fresh potatoes followed by blending with 500 mL of liquid medium for 2 min. The pH of the medium was adjusted to 5.4 before autoclaving. The cultures were maintained in a culture room at 26±2^{0}C and 16 h of light daily provided by cool-white fluorescent tubes (50 µmol m^{-2} s^{-1}).

Acclimatization. Plantlets were taken out from bottles and gently washed with distilled water. They were then separated, transplanted onto wet wood charcoal in a basket at a temperature of 26±2^{0}C and 16 h light: 8 h dark photoperiod provided by white fluorescent tubes (500 µmol $m^{-2}s^{-1}$). The basket was covered with polyvinylidenechloride film to avoid desiccation. Between 2–4 weeks after the start of acclimatization, the film was gradually removed.

Experimental design. A completely randomized design was used for all experiments with the only recognizable differences between the explants being the treatments applied to them. All treatments consisted of five replicates and each replicate contained five explants. The number of PLBs formed (>1mm in diameter) after six weeks of culture as well as the number of plantlets regenerated per PLB after a further six weeks of culture was recorded. The mean for each treatment was calculated.

RESULTS

Effects of plant growth regulators and type of media. The mean number of PLBs (>1mm) obtained with the three combinations of BA with NAA in liquid and solidified media is presented in Fig. 1 and 2, respectively. All explants cultured in the liquid media started swelling after about one week in culture. However, those cultured onto solidified media took longer to swell. Similarly, PLBs formation were visible in all the liquid media after a further two weeks in culture while in solidified media they were visible only after six weeks in culture. Explants cultured in media devoid of plant growth regulators also responded, however, many of them died after eight weeks in culture. The maximum number of PLBs was obtained with liquid MS medium that contained 0.1 mg L^{-1} of BA and 1.0 mg L^{-1} of NAA (54 PLBs). In all cases, it was found that cultures grown in liquid medium gave better response than their counterpart solidified medium. For example, the solidified MS medium supplemented with 0.1 mg L^{-1} of BA and 1.0 mg L^{-1} of NAA gave only 37 PLBs compared to 54 in the liquid medium. MS medium was superior at all plant growth regulator combinations followed by Vacin and Went medium. Least PLBs development was obtained on un-supplemented solidified Knudson medium (only 2). Even when supplemented with plant growth regulators and using liquid medium, the response obtained with Knudson C medium was lowest.

Speed of agitation. The influence of the speed of agitation of the shaker was examined, from 80–140 rpm, using MS medium that contained 0.1 mg L^{-1} of BA and 1.0 mg L^{-1} of NAA (Fig. 3). Although insignificant, a general decline in the number of PLBs was noted with increase in agitation speed.

Effects of adding coconut water to MS medium. Addition of coconut water to the MS medium supplemented with 0.1 mg L^{-1} of BA and 1.0 mg L^{-1} of NAA proved to be an important factor in the propagation of *Dendrobium* 'Sonia' PLBs (Fig. 4). In medium that contained 15% (v/v) of coconut water, the highest number of PLBs was obtained.

Fig. 5 Leaves protruding from PLBs

Fig. 6 Shoot and root formation on regeneration medium

Fig. 7 Hardened *Dendrobium* plants

At higher (20% v/v) and lower (10% v/v) levels, the propagation of PLBs was inferior.

Plantlet regeneration and acclimatisation. When the PLBs were sub-cultured onto MS medium supplemented with 1.0 mg L^{-1} NAA, 0.1 mg L^{-1} BA, 10 g L^{-1} potato homogenate, 150 mL L^{-1} coconut water, 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel, they grew in size. After two weeks leaves were seen protruding from the PLBs (Fig. 5). After six weeks in culture, the medium supplemented with the potato homogenate gave an average of ten shoots per PLBs cultured compared to an average of only six in the medium devoid of potato homogenate. Upon sub-culturing onto fresh medium, plantlets with well-formed leaves and roots were obtained (Fig. 6). These were potted into baskets containing wood charcoal and acclimatized under the conditions mentioned in the materials and methods section to obtain normal *Dendrobium* plants (Fig. 7). Hardened plantlets showed significantly high survival rate (84%) after eight weeks**.**

DISCUSSION

This study deals with the mass propagation of *Dendrobium* 'Sonia' orchid. Among the three different media and the different combinations of the two plant growth regulators used in this study, liquid MS medium (Murashige & Skoog, 1962) agitated at 80 rpm and supplemented with 1.0 mg L^{-1} NAA, 0.1 mg L^{-1} BA, 150 mL L^{-1} coconut water and 30 gL^{-1}sucrose was the most efficient in inducing protocorm-like bodies (PLBs). This is due to the fact that liquid medium provides better aeration and optimum conditions for nutrient uptake. In orchids, PLB regeneration has been suggested to be comparable to somatic embryogenesis pathway (Morel, 1974). In other orchid species, a callus stage has proved to be a necessary condition for in vitro regeneration (Stewart & Button, 1978; Kerbauy, 1984; Chen & Chang, 2001).

For the regeneration of plantlets, addition of potato homogenate as well as coconut water proved to be beneficial. Addition of naturally occurring supplements in plant tissue culture has been investigated by a number of workers. One of the earliest reports is that of Overbeek *et al.* (1941), who succeeded in growing immature *Datura* embryos in culture by including the liquid endosperm of *Cocos nucifera* (coconut milk) in their culture medium. Coconut milk was also shown to stimulate cell division in other cultured tissues due to the presence of cytokinins and its use as a supplement was adopted in many laboratories (Duhamet & Gautheret, 1950; Morel, 1950; Nickell, 1950; Duhamet, 1951; Henderson *et al.,* 1952; De Ropp *et al*., 1952; Archibald, 1954; Wiggans, 1954; Dix & van Staden, 1982). Other complex plant juices and liquid endosperms have been shown to possess stimulatory properties more or less similar to those of coconut milk. These include liquid endosperm from immature corn (Nétien *et al.*, 1951), tomato juice (Nitsch, 1951; Straus & La Rue, 1954), immature fruits and seeds (Steward & Caplin, 1952; Steward & Shantz, 1959), orange juice, malt extract, yeast extract, casein hydrolysate, leaf extracts, sap from a number of plants and tumour extracts. Park *et al*. (2003) have used potato homogenate in the regeneration of *Doritaenopsis.*

For the hardening of the orchid plantlets, it was essential to maintain a high humidity (about 90%) around the plantlets for two weeks. Also, wood charcoal was used as substrate as it provides good drainage and adequate aeration to the roots, which is of primordial importance in the culture of orchids. Substrates, which have the tendency to retain too much moisture, should be avoided in all cases for success in the hardening of orchid plantlets.

This study has shown that the orchid *Dendrobium* 'Sonia' can be regenerated on a large scale using a liquid medium for the efficient initiation of PLBs and their eventual regeneration into plantlets using a number of supplements. The active ingredients of these supplements and their role in promoting regeneration in *Dendrobium* 'Sonia' needs to be investigated so as the use of other supplements such as bananas. Other substrates in the hardening of the plantlets such as wood chips, sand, rocksand, coconut coir, vermiculite or a mixture of these could also be investigated.

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