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Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan

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ABSTRACT

The appropriate chitosan types and concentrations for enhancing the *in vitro* micropropagation of *Dendrobium* 'Eiskul' were studied using 70, 80 and 90% N-deacetylated polymeric (P-70, P-80 and P-90) and oligomeric (O-70, O-80 and O-90) forms of crab (*Portunus pelagicus*) chitosan. For the initial protocorm-like body (PLB) multiplication, the application of 10 mg/L of P-70 or 20 mg/L of P-90 was optimal, although 10 mg/L of P-80 and O-70 were also effective, and attained maximal PLB replication rates without increasing the detectable levels of somaclonal variation. However, during PLB-shoot induction, 10 or 20 mg/L of O-80 was the most appropriate chitosan and also induced further PLB formation. For plantlet regeneration, the addition of 10 mg/L of O-80 or P-80 gave the best quantity and quality, respectively, of plantlets. Finally, 20 mg/L of P-70 chitosan as a supplement during exflasking enhanced both the survival rate and the growth of the plantlets at one month after exflasking. Together, these data reveal a potentially beneficial and applicable protocol for commercial orchid micropropagation.

1. Introduction

Orchids are one of the most popular ornamental crops worldwide. The orchid industry of Thailand is well known for both cut tropical orchid flowers and the supply of different growth stages from *in vitro* and exflasked plantlets to preblooming sized plants. The increasing demand for, and export values of, orchids each year (KRC, 2008) is driving the expansion of orchid growing areas. Coupled with the replanting of disease-free clones in place of old plantations and the replacement of old cultivars with new hybrids, this is leading to an increasing demand for the large scale production of orchid plantlets. The development of more efficient micropropagation methods will be advantageous for the industry, especially for Thailand since most *Dendrobium* hybrids are micropropagated and they are the major export cut flower of Thailand. Moreover, such techniques could help repopulation and translocation projects for biodiversity conservation.

Since the initial work of Morel (1960), plant tissue culture techniques have been applied to obtain the relatively rapid mass clonal propagation of orchids and now play an important role in the industry. The general protocol of *Dendrobium* micropropagation by shoot tip culture is well known (Sagawa and Shoji, 1967). In commercial production, the acceleration of the multiplication rate and total yield of protocorm-like bodies (PLB) and their subsequent development into orchid plantlets without any enhanced mutation rates are highly desirable traits to reduce investment costs.

Phytohormones, both from natural substances, such as coconut water, which contains cytokinin (Arditt and Ernst, 1993) and synthetic ones, such as 6-benzyl amino purine (BAP), thidiazuron (TDZ), naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA), have been carefully applied to stimulate PLB and plantlet development (Nayak et al., 1997; Roy and Banerjee, 2003; Saiprasad et al., 2004). The disadvantages of using synthetic hormones are the additional costs, the inhibitory effect on shoot regeneration (Nayak et al., 1997), and the increased mutation rate amongst the plantlets produced (Arditt and Ernst, 1993).

Chitosan, poly [β-(1 → 4)-2-amino-2-deoxy-D-glucopyranose], is a biopolymer derivative of chitin, is the main component found in the exoskeleton of arthropods and crustaceans. The shells of prawns and crabs are an abundant, somewhat sustainable and readily available natural waste product from the seafood industry in Thailand. Chitosan is becoming more wildly used in agriculture due to its beneficial effects on plant growth and development and its environmental friendliness. Thus, for example, chitosan is

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reported to stimulate plant defense mechanisms (Vander et al., 1998) and to restrict fungal growth (Ait Barka et al., 2004).

Chitosan has been reported to reduce the stomatal aperture in tomatoes and the Asiatic dayflower, *Commelina communis*, (Lee et al., 1999) which may be advantageous for the plant's self-defense against pathogen infection. Likewise, transpiration rates were found to decrease in pepper plants after chitosan application resulting in a 26–43% water conservation without any significant change in biomass production and yield (Bittelli et al., 2001).

When added to the soil mix, chitosan remarkably enhanced the growth of Lisianthis, *Eustoma grandiflorum* (Raf.) Shinn, with flowering occurring 15 days earlier than in the control plants (Ohta et al., 1999). The growth promotion and early flowering stimulation effect of chitosan have also been found in seedlings of some other ornamental plants (Ohta et al., 2004). Within orchids, in the cut flower hybrid orchid, *Dendrobium* 'Eiskul', chitosan of 45 kDa molecular weight and a >90% degree of deacetylation (DD) applied at a concentration of 1–100 mg/L significantly increased the inflorescence yield, both quantitatively and qualitatively (Limpanavech et al., 2008). Moreover, an increased number of vascular bundles containing silica bodies was also reported, which might relate to the plant stress tolerance (Limpanavech et al., 2008).

Chitosan, in the form of chitogel at 1.75% (v/v) concentration, enhanced the *in vitro* grapevine root and shoot growth (Ait Barka et al., 2004), whilst in liquid culture it also enhanced the shoot growth in *Lippia dulcis* Trev. (Sauerwein et al., 1991). However, there have been only a limited number of reports on the *in vitro* effects of chitosan on orchids and orchid propagation.

Nge et al. (2006) found that both the size and the origin of the chitosan (although that was related to chitosan size), as well as the concentration applied at, all affected the number of *Dendrobium phalaenopsis* plantlets that were regenerated from PLB. Spraying with chitosan was reported to significantly reduce the severity of leaf spot disease in orchids, to act as a plant growth promotor in some orchids, and to increase the size and length of the *Dendrobium* florets and inflorescences, respectively (reviewed in Uthairatanakij et al., 2007). However, across these limited studies, the degree of N-deacetylation and the concentration of applied chitosan appear to have varying but poorly discriminated effects on the growth and development of orchid cultures *in vitro*.

The aim of this research was to determine the optimal type and concentration of blue swimming crab (*Portunus pelagicus*) chitosan required to improve the micropropagation efficiency of *Dendrobium* orchids, using the hybrid *Dendrobium* 'Eiskul', one of the most popular cut flower orchids in the market, as the experimental plant material. To this end, the effects of chitosan were evaluated on four distinct cultivation stages of orchid micropropagation: (i) PLB multiplication in liquid medium, (ii) stimulation of PLB differentiation into shoots, (iii) the growth and development of shoots and roots to become plantlets and (iv) the transplanting of plantlets to the greenhouse.

2. Materials and methods

2.1. Plant materials and culture conditions

A flask of *Dendrobium* 'Eiskul' PLB, derived from a shoot tip culture containing not less than 300 PLB, was used as the starting material for the first two stages of culture, the multiplication of PLB in liquid medium and the stimulation of PLB differentiation into shoots on agar medium. For the development of shoots to plantlets (stage 3), the shoots derived from PLB that had been precultured conventionally without chitosan supplementation were used as the starting material. Finally, for the transplanting of the plantlets to the greenhouse (stage 4), *in vitro* plantlets obtained from non-chitosan treated, conventional micropropagation grown plantlets were used as the experimental material. Thus, in this study, for any given plantlet, only one stage of its developmental (culturing) pathway, as outlined above, was exposed to chitosan treatment.

All *in vitro* cultures were maintained at 25 ± 2 °C. For PLB multiplication (culture stage 1), the cultures were shaken (platform shaker) at 120 rpm under 24 h cool white fluorescence light at an intensity of 20 μmol m^{-2} s^{-1}. The cultures on agar medium (stages 2 and 3) were stationarily incubated under 16 h daily cool white inflorescence light at an intensity of 35 μmol m^{-2} s^{-1} in the same temperature controlled room.

2.2. Media and chitosan preparation

Standard Vacin and Went (1949) media was modified by replacing ferric tartrate with Na_2EDTA 37.3 mg/L and $FeSO_4 \cdot 7H_2O$ 27.8 mg/L as the iron source, together with the addition of 2% (w/v) sucrose and 15% (v/v) coconut water for the liquid medium used in the first culture stage (multiplication of PLB). Orchid PLBs were cultured in a 50 ml Erlenmeyer flask containing 20 ml of medium. For the second and third cultivation stages the same modified VW medium constituents were used only they were supplemented with 10% (w/v) banana pulp and 0.8% (w/v) agar in glass culture bottles containing 25 ml of medium (Intuwong and Sagawa, 1973).

Six types of chitosan molecules, that is the 70%, 80% and 90% DD polymeric (P-70, P-80 and P-90) and oligomeric (O-70, O-80 and O-90) forms, were prepared from blue swimming crab (*P. pelagicus*) shells as described in Limpanavech et al. (2008), and used in these experiments. Briefly, 600 g of shells were soaked in 10 L of 1.5 M hydrochloric acid (HCl) for 24 h. The acid solution was replaced with a freshly prepared solution every 8 h. Then it was transferred to 10 L of 1.5 M sodium hydroxide (NaOH) for 24 h. The sodium hydroxide solution was replaced with freshly prepared solution every 8 h. The resulted chitin product was then deacetylated in 50% (w/w) sodium hydroxide (NaOH) for 48 h, changing the NaOH solution after the first 24 h. The chitosan product was washed in distilled water until the pH was neutral. The average chitosan molecular mass was determined by gel permeation chromatography (GPC) and the % DD was measured by UV spectrophotometry, as previously described (Limpanavech et al., 2008).

2.3. Chitosan effects on PLB multiplication, shoot differentiation and plantlet development

The six types of chitosan were separately added to the medium for each of the four culture stages of the plant development at one of four (10, 20, 40 and 80 mg/L) concentrations. Therefore, including the no chitosan control, the experiment was performed in a total of 25 treatments.

For evaluating the multiplication of PLB (stage 1), three pieces of PLB were cultured in 20 ml of modified VW liquid medium in a 50 ml flask. The PLB produced in each flask were transferred to freshly prepared medium every 10 days for three months. The number of PLB and their fresh and dry weights were determined at the end of the experiment, which was performed with four replicates.

The effect of chitosan on the differentiation of PLB into shoots (stage 2) was evaluated by selecting equal-sized 0.5 cm clumps of PLB as the starting material and culturing them as above. The experiment was performed with eight replicates. The number of shoots was determined monthly and the number of shoots with roots and the number of PLB were determined after three months.

The development of the rooted shoots or plantlets (stage 3) was evaluated using the shoots regenerated from PLB on the modified VW agar medium without chitosan as the starting material. Six shoots without roots were subcultured in each culture bottle with modified VW media supplemented with one of the six types of