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Proliferation of Protocorm-*like* Bodies of *Dendrobium mannii* and *Dendrobium mirbelianum* in Chitosan-containing Media *In Vitro*

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Abstract

Dendrobium mannii and *Dendrobium mirbelianum* were rare and endangered orchid species. *In vitro* micro propagation could be used to conserve and proliferate these species. The aims of this study were to determine the effects of chitosan on proliferation of the protocorm-like-bodies (PLBs) of *in vitro* grown *Dendrobium mannii* and *D. mirbelianum.* Chitosan has been reported as an important component to promote orchid growth *in vitro*. In this study chitosan was included in the MS basal media at the concentrations of 0, 1, 2, 3, and 4 mg.L^{-1}. The highest PLBs formation, clump diameter, and fresh weight on *D. mannii* were obtained from explants cultured on medium supplemented with 1 mg.L^{-1} chitosan. After 14 weeks of culture, fresh weight, clump diameter and average number of PLBs of *D. mannii* were 29.52 %, 33.00 % and 35.88% greater at chitosan (1 mg.L^{-1}) compared to control media. *D. mirbelianum* treated with low chitosan concentrations (1 and 2 mg.L^{-1}) had 65.71% more leaves than those without chitosan treatment. Chitosan, however, had and adverse effect on growth of both species at 4 mg.L^{-1}. Medium contain 4 mg.L^{-1} chitosan induced PLBs proliferation of *D. mannii*, but inhibited PLBs proliferation of *D. mirbelianum.* These results suggested that chitosan had important roles in PLB proliferation and could be used for the propagation of *D. mannii* PLBs *in vitro*.

Keywords: rare orchid species, Papua, *in-vitro*

Introduction

Dendrobium mannii Ridl is also known as Mann's Dendrobium, or Ueang Hang Pla (Thailand). The synonym of *Dendrobium mannii* is Aporum manii. Distribution of *D. manii* is centered in Burma, but also found in Malaysia, Indonesian, Papua New Guinea, Vietnam, Thailand and northeast and southern China. In nature *D. mannii* grow on elevations of 300 to 900 m dpl. As *Dendrobium* in general, *D. mannii* is epiphytic; it has thin basal, expanding and flattened above stem. *D. mannii* species has 10 to 12 distichous, equitant, fleshy, triangular, deciduous leaves; the flowers are triangular and have white petals of about 1 cm diameter which bloom between November to January (Anonym, 2014; Rao, 1990; Seidenfaden et al., 1992).

Native orchid species are facing extinction due to extensive natural habitat conversion and destruction due to illegal logging and orchid trades. *D. mannii* is a rare orchid species (Lokho, 2013) and CITES (the Convention on International Trade in Endangered species of wild fauna and flora) has included *D. mannii* in appendix II that were protected from trading to avoid extinction. The inclusion of *D. mannii* in appendix II was due to their adaptability to certain habitats, and unlikely to be survived when moved to a new location. Appendix II lists species that are not necessarily threatened with extinction but that may become so unless trade is closely controlled. It also includes so-called "look-alike species", i.e. species whose specimens in trade look like those of species listed for conservation reasons (CITES ENVIS 2015).

Dendrobium mirbelianum is a dwarf *Ceratobium* from the Moluccas and Papua New Guinea with canes 20-60 cm long. *D. mirbelianum* inflorescence is 30 cm long with 12-15 flowers of ± 5 cm across with attractive flower shapes. The sepals and petals are a light yellow-green spotted with violent brown, and lip has five keels, all green and veined with purplish brown. *D. mirbelianum* has attractive flowers; unfortunately it is a cleistogamous or self-pollinating. Distribution of *D.mirbelianum* are in the north of Australia, Indonesia (Papua, Maluku island), and New Guinea (Teoh, 2005; Tirta, 2013). *D.mirbelianum* was listed as endangered species under the Environment Protection and Biodiversity Conservation Act (EPBC Act) (Australian Departement of the Environment, 2008).

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Micro propagation *in vitro* could be used to conserve and propagate *D. mannii* and *D. mirbelianum.* Previous studies have showed that the optimization of medium composition played important roles to improve the success of orchid micro propagation using PLBs, and that optimum media is species-specific (Shimura and Koda 2004; Luo et al., 2009). Organic materials have been used in the media for propagation *in vitro*, e.g. coconut water (Kanika and Vij, 2004; Mitra et al., 1976) and chitosan (Uthairatanakij et al., 2007; Nge et al., 2005). These organic components are reported to be effective, easy to get and cheaper than plant growth hormones.

Chitosan is an N-deacetylated product of chitin which is natural carbohydrate polymer found in crab and shrimp shells, which are waste products from food-processing (Devlieghere et al., 2004). For industrial uses chitin is extracted from Crustaceans by acid treatment to dissolve their calcium carbonate content, followed by alkaline extraction to solubilize proteins. When the degree of deacetylation of chitin reaches about 50% (depending the origin of the polymer), it is called chitosan. Chitosan is water soluble in acidic media (Rinaudo, 2006). Shrimp shells could be converted into chitosan through demineralization, deproteination and deacetylation process (Hargono and Djaeni, 2003).

In agriculture chitosan has been primarily used to enhance plant growth and to boost the ability of plant in defending against fungal infections (Barka et al., 2004). Chitosan improved multiplication of PLBs, enhanced formation of juvenile leaves, and increased fresh weight of *Phalaenopsis gigantean* (Samarfard et al., 2013). Chitosan-treated *Dendrobium* 'Sensational Purple' had more flower stems and better quality flowers per stem (Chandrkrachang et al., 2005). In addition, chitosan at 10 ppm increased the number of *Dendrobium* 'Woxinia' pseudobulbs (Suningsih, 2012).

In this work we studied the effects of chitosan on proliferation of the protocorm-like-bodies of *in vitro* cultured *D. mannii* and D. *mirbelianum* to determine whether or not chitosan can be used to stimulate plant growth *in vitro*.

Materials and Methods

Plant Materials

This study consists of two separate experiments, each with one species of *Dendrobium*. The first experiment used PLB clumps of *D. mannii*; the second experiment used a single PLB of *D. mirbelianum.* PLBs of both species were proliferated from 2-month-old PLBs after subcultured in the modified Murashige and Skoog medium (Nurhasanah and Wiendi, 2009). Green PLBs of ± 3 mm diameter were used.

Culture Methods

This study used basal modified Murashige and Skoog solid medium (Nurhasanah and Armini, 2009). This medium consist of 1.5 $mg.L^{-1}$ of 2-ip (2-isopentenyl adenine), 0.1 $mg.L^{-1}$ IAA (Indole 3-acetic acid), 4 $mg.L^{-1}$ calcium panthotenate, 10% (v/v) coconut water, 30 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ agar. The pH was adjusted to 5.9 before autoclaving.

Chitosan used for this study was derived from processed shrimp shells. Chitosan powder was used to prepare chitosan stock solution by grinding the powder to get softer and smaller size of chitosan. Stock solution of 1 $mg.mL^{-1}$ chitosan was prepared by adding two to three drops of 1 N HCl into 100 mg chitosan powder and stirred until the chitosan powder was fully dissolved. Distilled water was then added to make 100 mL of 1 $mg.mL^{-1}$ chitosan stock solution.

Chitosan at concentration 0, 1, 2, 3, and 4 $mg.L^{-1}$ were added to basal culture medium before autoclaved at 121°C and 17.5 psi for 20 mins. Five explants were placed in each flask, and four flasks were used for each treatment as replication. The explants were grown in a controlled environment with a light intensity of 640 lux for 16 h at 19-22° C for 14 weeks.

Data Analysis

Data were collected by counting the number of PLB, number of shoots and roots for both species. Secondary PLBs of *D. mirbelianum* were collected after 14 weeks, whereas secondary PLBs of *D. mannii* until 8 weeks, because their clumps had become so large and the number of PLBs was difficult to count. Clump diameter, number of PLBs and PLB fresh weight were measured to estimate proliferation of *D. mannii* PLBs. The data were analyzed by calculating standard deviation of the means (n=20) using MS Excel 2010.

Table 1. Effect of chitosan on clump diameter, number of secondary PLBs, and fresh weight of *D. mannii* at 4, 8 and 14 weeks*

Chitosan (mg.L $^{-1}$)	Diameter of clump (mm)			Number of secondary PLBs		Fresh weight (mg)
	4	8	14	4	8	14
0	0.61 ± 0.15	1.09 ± 0.37	2.03 ± 0.62	5.65 ± 7.38	102.26 ± 33.50	2095.10 ± 928.35
1	0.75 ± 0.21	1.42 ± 0.42	2.70 ± 0.41	4.55 ± 4.22	132.45 ± 40.99	2846.80 ± 357.79
2	0.79 ± 0.27	1.13 ± 0.37	1.80 ± 0.61	3.50 ± 4.06	119.60 ± 39.24	1935.20 ± 741.97
3	0.66 ± 0.14	1.08 ± 0.36	1.84 ± 0.57	4.80 ± 3.55	109.45 ± 35.12	2089.10 ± 833.02
4	0.79 ± 0.27	1.23 ± 0.40	1.86 ± 0.73	7.11 ± 4.37	118.58 ± 49.59	1820.25 ± 689.26

*Values represent means ± standard deviation; n = 20.

Table 2. Effect of chitosan on number of leaves, shoots and roots per explant of *D. mannii* at week 4, 8 and 14*.

Chitosan	Number of shoots per explant			Number of leaves per explant			Number of roots per explant		
(mg.L $^{-1}$)	4	8	14	4	8	14	8	10	14
0	0	2.63 ± 4.37	12.90 ± 15.27	0	1.37 ± 2.67	8.80 ± 11.21	0	0.43 ± 0.75	1.80 ± 2.70
1	0	0.40 ± 1.19	5.80 ± 8.56	0	0.10 ± 0.45	3.40 ± 5.64	0	0.05 ± 0.22	1.00 ± 1.41
2	0.05 ± 0.22	1.53 ± 2.72	6.60 ± 9.04	0	0.47 ± 1.13	3.80 ± 5.31	0	0.27 ± 0.59	0.40 ± 0.89
3	0	0.35 ± 1.18	6.89 ± 9.28	0	0.10 ± 0.45	4.33 ± 6.16	0	0	0.33 ± 1.00
4	0	0.37 ± 0.83	0.75 ± 1.50	0	0 ± 0	0.25 ± 0.50	0	0	0

*Values represent means ± standard deviation; n = 20.

Results and Discussion

Effect of Chitosan on D. mannii Growth

All chitosan-treated and untreated PLBs in this study proliferated (Table 1). Chitosan in *D. mannii* media enhanced PLB formation, increased clump diameter and fresh weight, but inhibited shoot, leaf and root growth compared to control (without chitosan) (Table 1). After 14 weeks of culture, PLBs treated with 1 mg.L^{-1} chitosan had the highest number of PLBs, larger clump diameter, and greater fresh weight than control (Table 1, Figure 1). The number of secondary PLBs treated with 1 mg.L^{-1} increased exponentially (Figure 1). However, *D. mannii* grown in the control media had more shoots, leaves and roots per explant than those with chitosan treatment (Table 2).

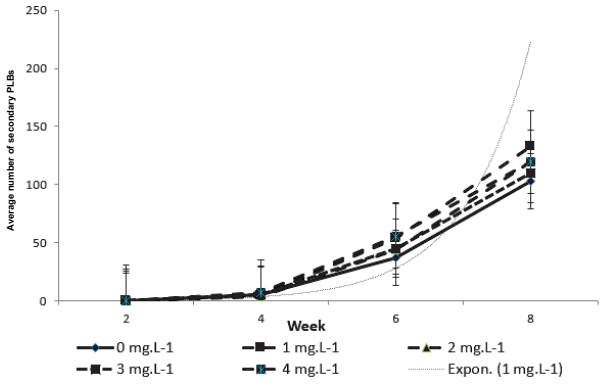

Figure 1. PLB growth of Dendrobium mannii at two to eight week of culture. Chitosan at 1 mg.L-1 increased PLB growth exponentially.

The results of this study demonstrated that chitosan had improved proliferation of secondary PLBs, increased fresh weight and diameter of clump of *D. mannii* (Table 1, Figure 1). These results were similar to those reported by Hasegawa et al. (2005) in *Arisaema ternatipartum*. *A ternatipartum* corms grown in chitosan-containing media had larger diameter and taller shoots (Hasegawa et al., 2005). Dewanty (2011) reported that chitosan at 5, 10, 15, and 20 ppm increased *Phalaenopsis* PLBs proliferation and fresh weight; whereas Limpanavech (2003) reported that chitosan at 10 mg.L^{-1} enhanced the growth and the number of PLBs of *Dendrobium formosum* Roxb. ex Lindl, and *Paphiopedilum sanderianum* Rchb. f. Nahar et al. (2012) reported chitosan (Chitosan H) at concentrations of 0.1 mg.L^{-1} significantly enhanced the average number of PLBs per explant of *Cymbidium insigne*. These reports suggested that the responses of orchid to chitosan treatment vary with species.

Previous studies have reported that chitosan can be used as a plant-growth enhancer for tissue culture orchid multiplication. Chitosan increased stalk length of Dendrobium “Missteen” and it was speculated that chitosan's effects were related to the actions of gibberellins (Uthairatanakij et al., 2007). Changdrkrachang (2006) also reported that chitosan induced signaling to synthesize giberellin in *Phalaenopsis* sp. L., whereas Zhou et al (2002) reported that chitosan increased gibberelic acid and indole-acetic acid levels in germinating peanut

seeds. Souter and Lindsay (2000) reported the role of gibberelin (GAs) are primarily nutritive for plant response in tissue culture. Auxin plays a major role in establishing the embryonic body plant via effects on apical-basal polarity/pattern formation and vascular development (Vogler and Kuhlemeier, 2003), whereas Novak et al. (2014) reported auxin promoted formation and development of protocorm-like bodies in orchids. Addition of chitosan to the culture media in our study inhibited shoot and leaf growth, and root initiation. High chitosan concentrations have been reported to inhibit cell growth (Chang and Chang, 2000), whereas Suningsih (2012) reported that chitosan at 10 and 20 ppm inhibited leaf growth of Dendrobium "Woxinia". Similar results were reported in *Phalaenopsis amabilis* (Sulistiana and Sukma, 2014), and *Phalaenopsis cornu-cervi* (Breda). Blume & Rchb.f. (Prasertsongskun and Chaipakdee, 2011).

Effect of Chitosan on D. mirbelianum Growth

Addition of chitosan to the culture media inhibited proliferation of secondary PLBs (Figure 2), growth of shoot and root of *D. mirbelianum* (Table 3 and 4) PLBs of the control treatment increased exponentially (Figure 2). Increased concentration of chitosan generally decreased proliferation of *D. mirbelianum* (Table 3 and 4). The highest chitosan concentration in this study (4 mg. L^{-1}) completely inhibited proliferation of secondary PLBs and root growth. PLBs of *D. mirbelianum* treated with chitosan at 4 $mg.L^{-1}$ did not proliferate, even turned brown and died.

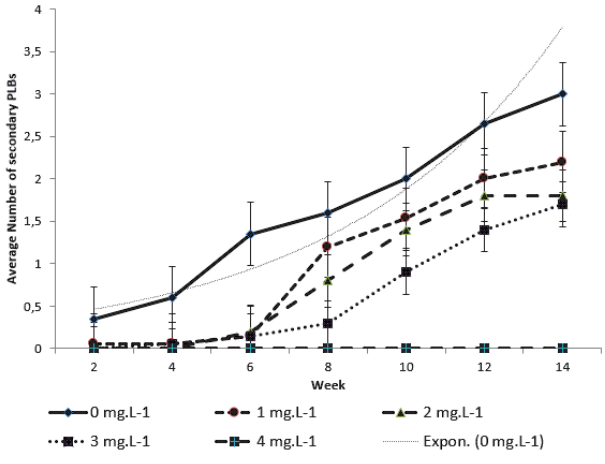

Figure 2. PLB growth of Dendrobium mirbellianum at 2 to 14 week of culture. Chitosan treatments inhibited the number of secondary PLBs, whereas control PLBs grew exponentially during 12 weeks of culture.

Table 3. Effect of chitosan on number of secondary PLBs of *D. mirbelianum* at 4, 8 and 14 weeks after culture

Chitosan ($mg.L^{-1}$)	Number of secondary PLBs		
	4	8	14
0	0.60 ± 1.50	1.60 ± 2.72	3.00 ± 5.16
1	0.05 ± 0.22	1.20 ± 1.66	2.20 ± 3.61
2	0	0.80 ± 0.92	1.80 ± 0.84
3	0.05 ± 0.22	0.30 ± 0.67	1.70 ± 1.89
4	0	0	0

*Values represent means ± standard deviation; n = 20.

Previous studies have reported similar negative responses as a result of high concentration of chitosan in the culture media. Chang and Chang (2000) reported that accumulation of high concentration of chitosan inhibited cells division in *Cymbidium sinensis* Wild. Pornpeanpakdee et al. (2006) reported that chitosan concentration of 80 $mg.L^{-1}$ inhibited growth of PLBs *Dendrobium* Sonia Jo 'Eiskul'; PLBs were bleached and died when treated with 160 $mg.L^{-1}$ of any chitosan type. Supplementation with 100 $mg.L^{-1}$ chitosan on *Grammatophyllum speciosum* media caused PLB necrosis and releases browning compounds into medium (Sopalun et al., 2010). Young and Kauss (1983) reported that chitosan caused the release of Ca^{+2} from *Glycine max* cells and/or plasma membrane, resulting in leakage of intracellular electrolytes, and cell death.

Low concentrations of chitosan in the culture media (1 and 2 $mg.L^{-1}$) promoted leaf growth of *D. mirbelianum* compared to control (Table 4). The number of leaves per explant of *D. mirbelianum* treated with 1 and 2 $mg.L^{-1}$ chitosan was 8.0 ± 6.93 and 5.80 ± 1.92, respectively, whereas the control plants had 3.50 ± 2.35 (Table 4). Previous studies reported that low concentrations of chitosan enhanced leaf growth, but at higher concentrations chitosan inhibited leaf growth. Dewanty (2011) reported that Phalaenopsis treated with 5 ppm chitosan had more leaves than control, but leaf growth was inhibited at chitosan concentration of 10 to 25 ppm. Prasertsongskun and Chaipakdee (2011) reported that chitosan at 15 $mg.L^{-1}$ induced leaf growth of *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb.f. compared to control, but leaf growth was inhibited at 20 $mg.L^{-1}$ chitosan.

Conclusion

Low chitosan concentration (1 $mg.L^{-1}$) in the MS basal media had shown promising results on PLB proliferation of *D. mannii*; it increased the number of

secondary PLBs, diameter of clump and fresh weight by 29.52 %, 33.00 % and 35.88 %, respectively, compared to control. *D. mirbelianum* treated with chitosan at 1 and 2 mg.L^{-1} had 65% more leaves than control. Chitosan at 4 mg.L^{-1} inhibited growth of *D. mirbelianum* PLBs, increased PLB browning, and inhibited shoots and roots formation of both *D. mannii* and *D. mirbelianum*.

Table 4. Effect of chitosan on number of leaves, shoots and roots per explant of *D.mirbelianum* at week 4, 8 and 14 after treatment*

Chitosan	Number of shoots per explant			Number of leaves per explant			Number of roots per explant		
(mg.L^{-1})	4	8	14	4	8	14	8	10	14
0	1.00 ± 0.32	1.55 ± 1.15	2.30 ± 2.94	1.05 ± 0.83	2.00 ± 1.38	3.50 ± 2.35	0.40 ± 0.75	0.60 ± 0.99	1.00 ± 1.72
1	1.00 ± 0.32	1.40 ± 1.18	1.93 ± 2.49	0.85 ± 0.81	2.40 ± 2.13	5.80 ± 6.39	0.07 ± 0.26	0.20 ± 0.56	0.33 ± 0.90
2	1.00 ± 0.00	1.30 ± 0.48	1.80 ± 0.84	0.55 ± 0.69	1.80 ± 1.14	5.80 ± 1.92	0	0.20 ± 0.45	0.20 ± 0.45
3	0.85 ± 0.37	0.80 ± 0.63	1.20 ± 1.03	0.70 ± 0.57	1.50 ± 1.18	2.70 ± 2.06	0	0	0.10 ± 0.32
4	0.33 ± 0.49	0.50 ± 0.53	0.50 ± 0.53	0.33 ± 0.49	0.50 ± 0.53	0.50 ± 0.53	0	0	0

*Values represent means ± standard deviation; n= 20.

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