Effects of Natural Additives on Asymbiotic Seed Germination and Growth of Endangered Lady's Slipper Orchid: *Paphiopedilum callosum* var. *sublaeve*

ผลของสารจากธรรมชาติต่อการงอกของเมล็ดและการเจริญเติบโตของกล้วยไม้รองเท้านารีม่วงสงขลา (*Paphiopedilum callosum* var. *sublaeve*)

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บทคัดย่อ

รองเท้านารีม่วงสงขลา (*Paphiopedilum callosum* var. *sublaeve*) เป็นกล้วยไม้รองเท้านารีประจำถิ่นที่อยู่ทางภาคใต้ของไทย ซึ่งเป็นพืชใกล้สูญพันธุ์ จึงถูกจัดให้อยู่ในบัญชีแนบท้ายหมายเลข 1 ตามอนุสัญญาไซเตส การทดลองนี้ศึกษาอิทธิพลของสารจากธรรมชาติ คือ กรดไฮยาลูโรนิค (HA) และไคโตซาน ที่มีต่อการงอกของเมล็ด การเจริญของโพรโทคอร์มและการเพิ่มจำนวนยอดของรองเท้านารีม่วงสงขลา โดยเพาะเลี้ยงเมล็ดในอาหารเหลวสูตร MVW ที่มี HA ความเข้มข้น 0, 0.01, 0.1 และ 1.0 มก/ลิตร หรือไคโตซานความเข้มข้น 0, 1, 5 และ 10 มก/ลิตร พบว่า เมล็ดเริ่มงอกภายใน 45 วัน มีค่าอัตราการงอกสูงสุด 8.34±1.01%, ค่าดัชนีการงอกสูงสุด 0.16±0.02, และอัตราการเกิดโพรโทคอร์มสูงสุด 4.28±0.96% เมื่อเพาะเลี้ยงในอาหารเหลวสูตร MVW ที่มี HA ความเข้มข้น 0.1 มก/ลิตร อย่างไรก็ตาม อาหารวุ้นสูตร MVW ที่มีไคโตซานความเข้มข้น 1.0 มก/ลิตร เป็นอาหารที่มีความเหมาะสมต่อการเจริญของโพรโทคอร์มมากที่สุด เนื่องจากให้ค่าอัตราการงอกในระยะ C สูงสุด 1.93±0.35% และโพรโทคอร์มที่ได้มีสีเขียวและแข็งแรง ส่วนการเพาะเลี้ยงบนอาหารวุ้นสูตร MMS ที่มี HA ความเข้มข้น 1.0 มก/ลิตร พบว่ามีจำนวนการเกิดยอดสูงสุด 3.22±0.36 ยอด/โพรโทคอร์ม และอัตราการเกิดยอดสูงสุด 100% จากนั้นย้ายต้นที่ได้ไปเพาะเลี้ยงในอาหารวุ้นสูตร MMS และย้ายลงปลูกในกระถางที่มีสแฟคนั่มมอสไปอนุบาลในเรือนเพาะชำเป็นระยะเวลา 30 วัน พบว่าต้นรองเท้านารีม่วงสงขลามีการเจริญเติบโตและมีอัตราการรอดชีวิตร้อยละ 90

คำสำคัญ: *Paphiopedilum callosum* var. *sublaeve*, กรดไฮยาลูโรนิค, ไคโตซาน

Abstract

Paphiopedilum callosum var. *sublaeve*, a native lady's slipper orchid of southern Thailand, is under threat of species extinction and listed in Appendix I of CITES. The experiments were conducted to study the influence of natural additives, namely, hyaluronic acid (HA) and chitosan on seed germination, protocorm development, and shoot multiplication of *P. callosum* var. *sublaeve*. Seeds were cultured in modified Vacin and Went (MVW) liquid medium supplemented with various concentrations of HA (0, 0.01, 0.1 and 1 mg/L) or chitosan (0, 1, 5 and 10 mg/L). Swollen seeds exhibited green embryos within 45 days. The highest percentage of seed germination (8.34±1.01%), germination index (0.16±0.02) and

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percentage of protocorm formation (4.28±0.96%) were obtained from 0.1 mg/L HA. However, MVW agar medium supplemented with 1.0 mg/L chitosan was the most suitable medium for protocorm development because it gave the highest seed germination percentage at stage C (1.93±0.35) and gave the vigorous protocorms. The highest number of shoots (3.22±0.36) and the maximum shoot formation rate (100%) were recorded in the modified Murashige and Skoog (MMS) agar medium containing 1.0 mg/L HA. To promote growth, the obtained plantlets were transferred to MMS agar medium and then planted them in pots filled with sphagnum moss. The acclimatized plantlets in the greenhouse grew well with 90% survival rate.

Keywords: *Paphiopedilum callosum* var. *sublaeve*, Hyaluronic acid, Chitosan

Abbreviations: HA, hyaluronic acid; MMS, modified Murashige and Skoog medium; MVW, modified Vacin and Went medium; TTC, 2,3,5-Triphenyltetrazolium chloride

Introduction

Many orchid populations are under extinction menace as a result of over-collection and habitat destruction (Zeng, 2012). *Paphiopedilum callosum* var. *sublaeve*, a lady's slipper orchid native to the southern Thailand, is protected under the appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013). This orchid species has sensationally beautiful flowers, marbled and evergreen foliage which make it delightful and distinctive (Ng and Saleh, 2011). *Paphiopedilum* orchid is generally propagated through the division of axillary bud of the mother plant which is a very time-consuming and its seed germination is also slow growth (Nhut et al., 2006; Long, 2010; Ng et al., 2010). Accordingly, technique of plant tissue culture is being used for large-scale plantlet production within a short period and has become an optimal solution for conservation of genus extinction (Nhut et al., 2006). Especially, the supplementation of natural substances to the medium can enhance in vitro seed germination, promote growth and prevent somaclonal variation, (Pornpienpakdee et al., 2010). Chitosan and hyaluronic acid (HA) are polysaccharides which act as plant growth regulators and as safe materials for humans and the environment (Nge et al., 2006; Nahar et al., 2012). These substances were added to germination media to improve seed germination and to retain somaclonal variation (Pornpienpakdee, et al., 2010). Chitosan is a biodegradable polymer, which comprises of a copolymer of *N*-acetyl-D-glucosamine residues, β-1, 4 glycosidic bonds (Croisier and Jerome, 2013). The structure of chitosan relates to glycosaminoglycans (GAG) which is significant structural element of extracellular matrix of many tissues (Enrione et al., 2010). This chitosan was used to promote seed germination and protocorm development in many orchids such as *Dendrobium phalaenopsis* (Nge et al., 2006), *D. bigibbum* var. *compactum* and *D. formosum* (Kananont et al., 2010), *Grammatophyllum speciosum* (Sopalun et al., 2010) and *Cymbidium insigne* (Nahar et al., 2012). Hyaluronic acid (HA) is a natural polysaccharide which composed of alternating (1→4) - β linked D-glucoronic acid and (1→3) - β linked *N*-acetyl-D-glucosamine residues (Kogan et al, 2007). The application of HA in plant tissue culture has also been shown to enhance the micropropagation in many orchid species,

for instance, *C. dayanum* (Nahar et al., 2011), *Cymbidium* Waltz 'Idol' (Kaewjampa et al., 2012), and *D. kingianum* (Habiba et al., 2014). Therefore, present study aimed to examine the natural substances (HA and chitosan) affecting on seed germination, protocorm development and multiple shoot induction of *P. callosum* var. *sublaeve*.

Research Methodology

Plant material

P. callosum var. *sublaeve* (figure 1a) were maintained in the greenhouse of Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. The capsules at 180 days after pollination (DAP) were collected from hand self-pollinated flowers (figure 1b). The seed viability was evaluated by 1% 2,3,5-Triphenyltetrazolium chloride (TTC) (Vellupillai, 1997). The viable seeds exhibiting dark red color (figure 1c) were counted. Seed viability (SV) was reported as a percentage which was calculated as follows:

% SV= [Red embryos/(numbers of red embryos + colorless embryos)]*100

Natural additives affecting on seed germination and protocorm development

Seed preculture

The capsules were surface-sterilized with 1.2% (v/v) sodium hypochlorite (NaOCl) containing 1-2 drops of Tween-20 for 20 min and rinse 2-3 times with sterile distilled water (DW). They were cut longitudinally and the seeds were scooped out and placed into a 125 ml Erlenmeyer flask containing 40 ml of sterile DW. For preculture, the seed suspension (5 ml) was pipetted into a 125 ml Erlenmeyer flask containing 20 ml of sterile DW supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). These cultures were maintained on a shaker at 50 rpm in the culture room (25±2°C) under the dark condition for 2 weeks.

Figure 1 *Paphiopedilum callosum* var. *sublaeve* plant showing (a) single flower and (b) capsule (180 DAP) (c) dark red embryo of viable seed using TTC staining. Scale bar: (a) and (b) 1 cm, (c) 5 μm.

Seed germination and protocorm development

After preculture for 2 weeks, 1-ml seed suspension (approximately 294 seeds/ml) was placed into each 125 ml Erlenmeyer flask containing 20 ml of modified Vacin and Went (MVW) medium (Vacin and Went, 1949) supplemented with either HA (0, 0.01, 0.1, and 1.0 mg/L) or chitosan (0, 1, 5, and 10 mg/L). Each treatment was conducted with 15 replicates (culture flask). Visual observation was observed at a 15-day interval. The subculture was carried out in the same fresh medium for every 2 months. The percentages of seed germination including germination index and protocorm formation were examined after 2 month of culture.

Evaluation of seed germination and growth

The seed germination percentage and the germination index were calculated by formulae proposed in Pierik et al. (1988) as follows:

$$\% \text{ Seed germination} = \frac{[100(B+C+D)]}{(A+B+C+D)}$$

$$\text{Germination index} = \frac{[10(1B+2C+3D)]}{(A+B+C+D)}$$

Where A is seeds containing an embryo but not germination (swollen), swollen seeds.
B is germinating, but not yet rupturing the seed coat.
C is seeds with embryos just rupturing the seed coat.
D is seeds with embryos completely out of the seed coat.

The percentage of seed/protocorms formation at each developmental stage was calculated by dividing the number of seed/protocorms in each stage by the total number of cultured seeds in each flask.

Shoot multiplication

Three-month-old seedlings (2-3 cm in height) were used as materials for shoot multiplication. They were cultured on modified Murashige and Skoog (MMS) agar medium (Kaewubon et al., 2010) added with 2 g/L peptone, 2 g/L activated charcoal and various concentrations of HA (0, 0.01, 0.1 and 1 mg/L) or chitosan (0, 1, 5 and 10 mg/L) for 2 months. All experiments consisted of 9 culture flasks and each flask contained 1 seedling. Number of shoots and multiplication rate were examined after 2 months of culture.

Shoot development and rooting

Single shoot (3-4 cm. height derived from shoot multiplication) was transferred to the regeneration medium (Kaewubon et al., 2010) for 2 months to induce root growth. These plantlets were then transferred to pots containing sphagnum moss and maintained in the shaded greenhouse. The survival rate of these plantlets was recorded after transplanting for one month.

Culture conditions

The pH of the media was adjusted to 5.2±0.1 with 1N NaOH or HCl prior to autoclaving at 121°C for 20 min. All treatments/experiments cultures were cultured at 25±2°C under a 16-h photoperiod at irradiance of 23 μmol/m^2s provided by cool white fluorescent tubes (Philips).

Statistical analysis

Each experiment was arranged in a completely randomized design (CRD). Fifteen replicates (for seed germination) and 9 replicates (for shoot multiplication) were performed for each treatment. Data were analyzed using SPSS 17.0 program for Windows (SPSS Inc. Chicago, IL, USA). Data of percentage of seedling in each developmental stage, percentage of seed germination, seed germination index, number of shoots and multiple shoot formation rates were subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range tests. Data of percentage of protocorm formation were analyzed by using Kruskal-Wallis test.

Results and discussion

Natural additives affecting on seed germination and protocorm formation

The viable seeds with dark red color due to the formation of red formazan were presented at 27.19%. Process of seed germination and protocorm development is presented in table 1 and figure 2. Seeds started to germinate after culture for 60 days. The highest seed germination percentage (8.34±1.01), germination index (0.16±0.02) and protocorm formation percentage (4.28±0.96) were observed on MVW liquid medium added with 0.1 mg/L HA (table 1, figure 3a, 3b). This result conformed to Nahar et al. (2011) who reported that the application of 1 mg/L HA in MMS medium promoted the formation rate of protocorm like bodies (PLBs) and shoots of *Cymbidium dayanum*. In addition, 0.1 mg/L HA-supplemented MMS medium gave the highest number of PLBs (12.4) of Cymbidium Walts 'Idol' (Kaewjampa et al., 2012). Although HA, abiotic elicitor, was reported to enhance the production of secondary metabolite in medicinal plant tissue culture (Zhou and Wu, 2006), it plays important roles in living organism including control of tissue hydration, water transport, tissue repair and various receptor-mediated functions in cell detachment (Kogan et al., 2008). Moreover, Lee and Spicer (2000) claimed that HA is essential for normal development in higher eukaryotes namely regulation of cell proliferation, migration and tissue architecture at multiple levels. However, visual observation of protocorm which cultured in MVW medium added with chitosan was green and more vigorous than those in HA. Unfortunately, it was possible that the HA application was not suitable for seed germination of *Paphiopedilum* species due to orchid seed coat presenting the impermeable testa which may act as a barrier to water and nutrients (Zhang et al., 2015) including HA permeation.

MVW medium supplemented with 1.0 mg/L chitosan was the most suitable medium for subsequent protocorm development of *P. callosum* var. *sublaeve*. This treatment gave seed germination percentage and protocorm formation at 4.89±0.54% and 2.47±0.46%, respectively. This treatment also provided the highest seed germination percentage (1.93±0.35) at stage C (seeds with embryos just rupturing the seed coat) and exhibited vigorous green protocorms (figure 3c). In addition, Nge et al. (2006) and Nahar et al. (2011) revealed that chitosan presents a unique combination of properties and was widely available, inexpensive, environmentally friendly and as well as non-toxic to human. Chitosan may be involved in some signaling pathway related to auxin biosynthesis via a tryptophan-independent pathway which supported growth and development in plant (Uthairatanakij et al., 2007). It has been reported that chitosan may act as a plant growth stimulator in some plant species including orchids (Sopalun et al., 2010). Chitosan was effective for producing seed germination and protocorm formation in *Dendrobium phalaenopsis* (Nge et al., 2006) and *Grammatophyllum speciosum* (Sopalun et al., 2010). In the latter case, the application of 15 mg/L chitosan could enhance the PLBs growth rate. Therefore, chitosan might be suitable substrate for seed germination and protocorm formation of *P. callosum* due to the requirement for safety and reducing cost.

The process of seed germination of *P. callosum* var. *sublaeve* (table 2, figure 2) was divided into the following six categories according to developmental stages of embryos (Miyoshi and Mii, 1995).

Table 1 Hyaluronic acid (HA) and chitosan affecting seed germination, germination index and protocorm formation of *Paphiopedilum* callosum var. *sublaeve*.

Natural additive (mg/L)		Percentage of seedlings in each development stage (mean±SE)				Total seed germination percentage	Seed germination index	%Protocorm formation*	visual observation of protocorm
		A	B	C	D				
Control	0	95.36±1.18^{a}	2.85±0.97^{ns}	0.71±0.46^{b}	0.36±0.36^{b}	4.65±1.18^{b}	0.06±0.02^{ab}	3.08±0.97^{ns}	Pale yellow
HA	0.01	95.15±1.57^{a}	3.59±1.21^{ns}	0.47±0.33^{b}	0.78±0.52^{b}	4.84±1.58^{b}	0.07±0.03^{ab}	1.51±0.66^{ns}	Pale and green
	0.1	91.67±1.01^{b}	3.75±0.77^{ns}	1.35±0.39^{ab}	3.23±0.64^{a}	8.34±1.01^{a}	0.16±0.02^{a}	4.28±0.96^{ns}	Pale and green
	1.0	96.25±0.45^{a}	2.23±0.46^{ns}	0.71±0.21^{b}	0.80±0.28^{b}	3.75±0.45^{b}	0.06±0.01^{b}	1.83±0.53^{ns}	Pale and green
Chitosan	1.0	95.11±0.54^{a}	2.16±0.56^{ns}	1.93±0.35^{a}	0.79±0.35^{b}	4.89±0.54^{b}	0.08±0.01^{ab}	2.47±0.46^{ns}	Green, ,healthy
	5.0	96.92±0.50^{a}	1.83±0.33^{ns}	0.77±0.27^{b}	0.48±0.22^{b}	3.34±0.47^{b}	0.05±0.01^{b}	1.23±0.29^{ns}	Green, Healthy
	10.0	94.90±0.88^{a}	2.79±0.67^{ns}	1.06±0.49^{ab}	1.25±0.44^{b}	5.10±0.89^{b}	0.08±0.02^{ab}	2.56±0.71^{ns}	Green, Healthy

Data were taken after culture on MVW medium supplemented with various additives for 3 months.

Means in column followed by the same letters were not significantly different at $P \leq 0.05$ as determined by DMRT.

*The Kruskal-Wallis test was used.

ns: non-significant

A: swollen seeds, no embryo germination; B: swollen seeds, embryo germination but not yet rupturing the seed coat; C: seeds with embryos just rupturing the seed coat; and D: seeds with embryos completely out of the seed coat

Table 2 Developmental stages of *Paphiopedilum callosum* var. *sublaeve* protocorms.

Stage	Days after culture (d)	Description
0, No germination	0-40	No growth of embryo occurs.
1, Pre-germination	40-45	Embryo swells to fill the seed coat.
2, Germination	40-50	Embryo emerges from the seed coat.
3, Protocorm	60-65	Embryo is completely discharged from the seed coat.
4, Rhizoid	60-75	Rhizoids are formed on the protocorm surface.
5, Shoot	70-85	Shoot is differentiated from the protocorm.

Figure 2 In vitro seed germination and seedling development of *Paphiopedilum callosum* var. *sublaeve*. (a) stage 0; swelling and ungerminated seeds, (b) stage 1; testa ruptured, (c) stage 2; appearance of the shoot, (d) stage 3; appearance of the shoot and rhizoids, (e) stage 4; emergence and elongation of first leaf, (f) stage 5; presence of two or more leaves, (g) seedling with multiple leaves and root. Scale bars: (a) 100 μm, (b) 200 μm, (c-f) 1.0 mm, (g) 5.0 mm. S: seed coat. E: embryo.

Effects of natural additives on shoot multiplication

The effects of natural additives on number of shoots and shoot formation rate were shown in table 3 and figure 3. The highest number of shoot (3.22±0.36) and the maximum shoot formation rate (100%) were derived from the explants which were cultured on MMS medium containing 1.0 mg/L HA. In present experiment, HA at low concentration could induce shoot formation of *P. callocum*. This result was conformed to Nahar et al. (2011) who reported that the optimum concentration for promoting the highest number of shoot (3.0±0.4) and shoot formation rate (9.3%) of *Cymbidium dayanum* tissue was 1.0 mg/L HA. Furthermore, Kaewjampa et al. (2012) reported that HA at 1.0 mg/L gave the highest number of shoots (2.7±0.5)

in *Cymbidium* Waltz 'Idol'. Moreover, HA at the same concentration also gave the highest shoot formation rate (53.3% and 66.7%) in *Cymbidium kanran* Makino (Kamal et al., 2014) and *D. kingianum* (Habiba et al., 2014). However, Sultana et al. (2015) reported that 0.1 mg/L HA application was the optimal concentration for the highest shoot number in *Phalaenopsis* 'Fmk 02010'. Therefore, a function of HA was entirely different from orchid species to species (Sultana et al., 2015).

Table 3 Effect of hyaluronic acid (HA) and chitosan on number of shoots and multiple shoot formation rate of *Paphiopedilum callosum* var. *sublaeve*.

Natural additive (mg/L)		Number of shoots (shoots) (mean±SE)	Multiple shoot formation rate (%)
Control	0	1.00±0.37^{b}	55.56±17.57^{ab}
HA	0.01	1.33±0.76^{b}	44.44±17.57^{b}
	0.1	1.11±0.26^{b}	77.78±14.67^{ab}
	1.0	3.22±0.36^{a}	100.00±0.00^{a}
Chitosan	1.0	1.33±0.41^{b}	77.78±14.67^{ab}
	5.0	1.33±0.47^{b}	88.89±11.11^{ab}
	10.0	1.67±0.58^{b}	66.67±16.67^{ab}

Data were taken after culture on MMS medium supplemented with various additives for 2 months. Means in column followed by the same letters were not significantly different at $P \leq 0.05$ as determined by DMRT.

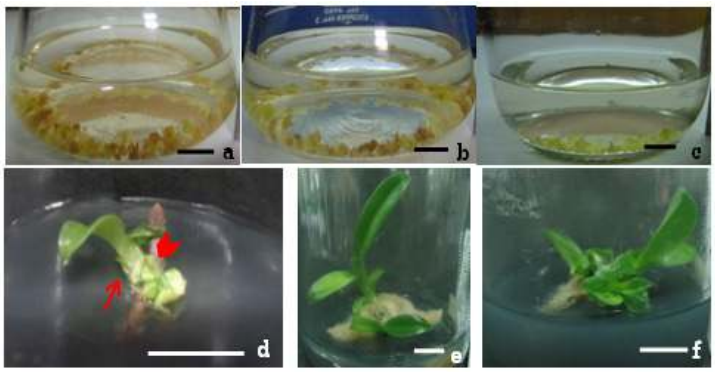

Figure 3 Protocorm developments and shoot multiplication of *Paphiopedilum callosum* var. *sublaeve*. Sixty-day-old protocorms exhibiting (a) pale yellow in MVW medium (control), (b) pale and green and (c) vigorous green protocorms in MVW medium containing HA (0.1 mg/L) and chitosan (1.0 mg/L), respectively. (d) seedling (arrow) presents a new shoot (arrow head) on MMS supplemented with 0.1 mg/L HA for 4 weeks. Multiple shoots presenting vigorous growth on MMS supplemented with chitosan at (e) 1.0 mg/L and (f) HA at 1.0 mg/L. Photographs were taken after seedlings were cultured for (d) 4 weeks and (e-f) 10 weeks. Scale bar: (a)-(f) 1 cm.

Shoot development and rooting

Four-month-old plantlets (figure 4a) which cultured on MMS agar medium supplemented with 50 g/L banana homogenate (Kaewubon et al., 2010) were transferred to sphagnum moss filled pots (figure 4b). These plants presented at 90% survival rate after being transferred to the greenhouse for one month.

Figure 4 Multiple shoot-derived plantlet exhibits (a) vigorous shoot with root after cultured on MMS agar medium supplemented with 50 g/L banana homogenate for 2 months. (b) a representative plantlet after being transferred to the greenhouse for one month.

Conclusion

In conclusion, the most appropriate procedure for seed germination and protocorm formation was performed by the pre-culturing in sterile DW added with 1.0 mg/L chitosan for the first 2 weeks in the dark followed by the culture in MVW medium supplemented with 1.0 mg/L chitosan for the next 3 months. This procedure provided healthy protocorms and seedlings. Moreover, MMS medium supplemented with HA (1.0 mg/L) was suitable for shoot multiplication. Plantlets grew well with 90% survival rate in a shaded greenhouse. In the near future, the experiment to elucidate the effective protocol for seed germination and protocorm development of *P. callosum* var. *sublaeve* will be gained. HA can be included in culture medium to promote number of seed germination (quantity) and shoot multiplication followed by chitosan application to encourage quality of protocorm and seedling.

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