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Germination and Regeneration of *Cymbidium findlaysonianum* Lindl. on a Medium Supplemented with Some Organic Sources

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ABSTRACT

Seeds of *Cymbidium findlaysonianum* Lindl. were germinated and regenerated on media supplemented with various additives. After germination for 3 months, solidified Vacin and Went (VW) medium supplemented with, 15 % coconut water, 5 % banana homogenate, 5 % potato homogenate, 0.2 % activated charcoal and 20 g/l sucrose promoted higher seedling germination than the control medium. The number of protocorm cultured in a modified liquid VW medium with Murashige and Skoog (MS) vitamin (VWM) increased 4 times for every month of culture, a significant difference from the VW medium, MS medium and half strength MS medium (½ MS). Moreover, the activated charcoal added to the medium stimulated seedlings growth significantly more than when coconut water, banana homogenate and/or potato homogenate were added to the medium. Healthy plants transferred to a plastic tray containing coconut peat successfully acclimatized (70 %) in the greenhouse. More than 100,000 plantlets may be obtained from a capsule after being cultured for a year. Thus, organic additives and medium components had an effect on the growth and development of asymbiotic seeds in *C. findlaysonianum*.

Keywords: *Cymbidium findlaysonianum*, germination, organic additives, protocorm

INTRODUCTION

Cymbidium is a tropical orchid that belongs to the family Orchidaceae. *Cymbidium*'s genus comprises 44 species, distributed throughout Madagascar, Sri Lanka, India, Japan, Malaysia and Thailand. Among them, 19 species are indigenous to Thailand. *Cymbidium findlaysonianum* Lindl. is a sympodial, pseudobulb orchid. This orchid has long pendulous spikes carrying small yellow and red fragrant flowers [1].

Generally, orchids are propagated through seeds. Orchid seeds are very small, with no endosperm, resulting in a lack of ready germinate in nature. Despite limited food reserves, they can germinate *in vitro* [2]. The introduction of an asymbiotic seed germination method by Kunduson helped in increasing the viability or survival of orchids in nature [3]. This technique has been used in the commercial production of many orchid species such as *Oncidium* [4], *Doritaenopsis* [5], *Dendrobium* [6], *Zygopetalum* [7] and *Ascocenda* [8]. Seeds from immature capsules are suitable for *in vitro* germination as embryos become viable and develop prior to the capsule ripening. They are also easy to surface-sterilize [9]. However, specific nutritional and environmental conditions are needed for *in vitro* germination of orchid seeds. Various organic additives, such as coconut water, banana homogenate, potato homogenate and activated charcoal, alone or in combination, were reported to be successful in inducing growth and development of several orchid species and explants [10]. Thus, this study develops an efficient and simple tissue culture method for obtaining healthy plants via asymbiotic germination of the immature seeds in *C. findlaysonianum* by adding various organic additives into the culture medium.

MATERIALS AND METHODS

Seed Culture and Germination

Seed capsules at 8 weeks after pollination, were collected from a cultivated plant of *C. findlaysonianum*. After sterilization by dipping in 95 % alcohol and passing briefly through the flame, the treated capsules were cut aseptically half longitudinally, scooped out and sown by spreading over the surface of the culture medium. The medium was Vacin and Went (VW) [11] supplemented with 15 % (v/v) coconut water (Medium I) or 5 % banana homogenate or 5 % potato homogenate or 0.2 % activated charcoal (Medium II). Sucrose (20 g/l) was used as a carbon source, and the medium was solidified with 8 g/l agar. Seed weight, 0.1 g, were randomly collected every month to the count germination rate and assess the protocorm on a scale I to III as follows: (I) swollen seed, seed size increasing 3 times showing the green color of chlorophyll; (II) protocorm, globule formation with rhizoid; (III) protocorm, less round with a protrusion of leaves at one end.

Multiplication and Regeneration

The germinated protocorm, 2 mm in diameter, were cultured in 4 liquid media viz. VW, MS [12], a half strength MS medium (½ MS) and modified VW with MS vitamins (VWM). All media were supplemented with 15 % (v/v) coconut water and 20 g/l sucrose. All culture media were replicated 4 times with each replicate consisting of 30 protocorms. The cultured jars were shaken at 110 rpm on a rotary shaker. The number of protocorms was recorded monthly before sub-culturing to a fresh medium of the same components. For plantlet regeneration, three-month-old seedlings (2 mm in diameter) were transferred to a VWM medium supplemented with 20 g/l sucrose and 5 % banana homogenate, 5 % potato homogenate and 0.2 % activated charcoal alone or in combination. Each medium component was replicated 4 times with each replicate having 30 protocorms. The number of plantlets, 3 cm in height with 2 leaves, was recorded monthly for 3 months.

The pH of the media were adjusted to 5.8 and solidified with 8 g/l agar before autoclaving. All cultures were maintained at 25 ± 3 °C for a 16-h photoperiod under cool, white florescent light (ca 1,500 lx).

Hardening of Plantlet

The rooted plantlets with a pair of leaves at 3 - 4 cm in height were removed from the culture vessel, rinsed under tap water to remove the residual medium before culturing in a plastic tray (20 cm wide × 30 cm long × 10 cm high) with or without coconut peat. The plantlets were placed in 75 % shade in a greenhouse at 30 ± 3 ºC. The plants were initially covered with plastic sheets for 1 week to maintain high humidity (above 70 %) and irrigated twice a week with tap water. After 1 week the plants were irrigated once every morning. The percentage of plantlets that survived was counted after culturing for 1 month. Each treatment contained 10 plantlets with 4 replications.

Experimental Design and Data Analysis

Experiments were performed in a complete randomized design (CRD). The data were analyzed by ANOVA using SPSS version 11.5 and the mean values were compared using Duncan’s multiple range test at a 5 % probability level.

RESULTS AND DISCUSSION

Seed Culture and Germination

Tissue culture methods have been successfully used for regeneration and propagation of orchid species. In this study we were interested in the propagation of *C. findlaysonianum*. It is expected that this system would be further established as an efficient genetic transformation scheme for this orchid species. Our results showed that various additives and medium components affected germination, multiplication and regeneration. The effects of additives on morphogenetic response of embryos of *C. findlaysonianum* after culturing for 3 months are shown in **Table 1**. There were

differences in the pattern of germination among additives over the time in culture. VW solidified-medium supplemented with 15 % coconut water, 20 g/l sucrose, 5 % banana homogenate, 5 % potato homogenate, 0.2 % activated charcoal (Medium II) enhanced the development of the embryo. Germination in this medium after 1 month showed a lower percentage of swollen seeds (type I, **Figure 1a**) (79.13 ± 2.34 %) but gave a higher percentage of protocorms (type II, **Figure 1b**), up to 21 %. In contrast, the control medium with 15 % coconut water, 20 g/l sucrose (Medium I) showed a higher germination percentage for type I (93.63 ± 3.21 %), but gave a lower germination percentage for type II (6.37 ± 2.80 %). Further development of protocorms was also rapid in the medium II. The percentage of seedlings with 1 leaf (type III, **Figure 1c**) was 5.40 ± 0.21 and 8.13 ± 0.34 after culturing for 2 and 3 months, respectively. Medium I showed lower rates of germination of type III protocorms, after culturing for 2 and 3 months (2.32 ± 0.19 and 2.66 ± 0.24 %, respectively). Thus, VW solidified-medium supplemented with coconut water and sucrose in combination with banana homogenate, potato homogenate and activated charcoal is more effective in asymbiotic seed germination and protocorm development in *C. findlaysonianum*. Normally, *in vitro* germination of orchid seeds is a long and slow process. In our study, the initiation process was observed only 2 weeks after inoculation. The germinating embryo turned green, swelled to about 3 times its original size and broke through the testa after a month of culturing. Continued culturing for 2 to 3 months, gave a less rounded embryo with a distinctive leaf point at the one end and the presence or absence of rhizoids at the other end. Protocorms transferred to the regeneration medium for 4 - 8 weeks showed slow elongation and proliferation of leaves, but at the end of culturing, protocorms were well developed into normal plantlets. It is known that orchid seeds have neither endosperm nor cotyledons in the embryo as primary storage materials [13]. Thus, nutrients in the culture medium are required for orchid seed germination. In our study, all media were devoid of synthesis plant growth regulators (PGRs). Thus, organic additives had a profound effect on the subsequent germination and regeneration of the protocorms. This could be due to the presence of sufficient endogenous growth regulators required for the initial stages of germination. Similar results were also found in *Dendrobium* that seed germination was successful on a medium without a plant growth regulator [6].

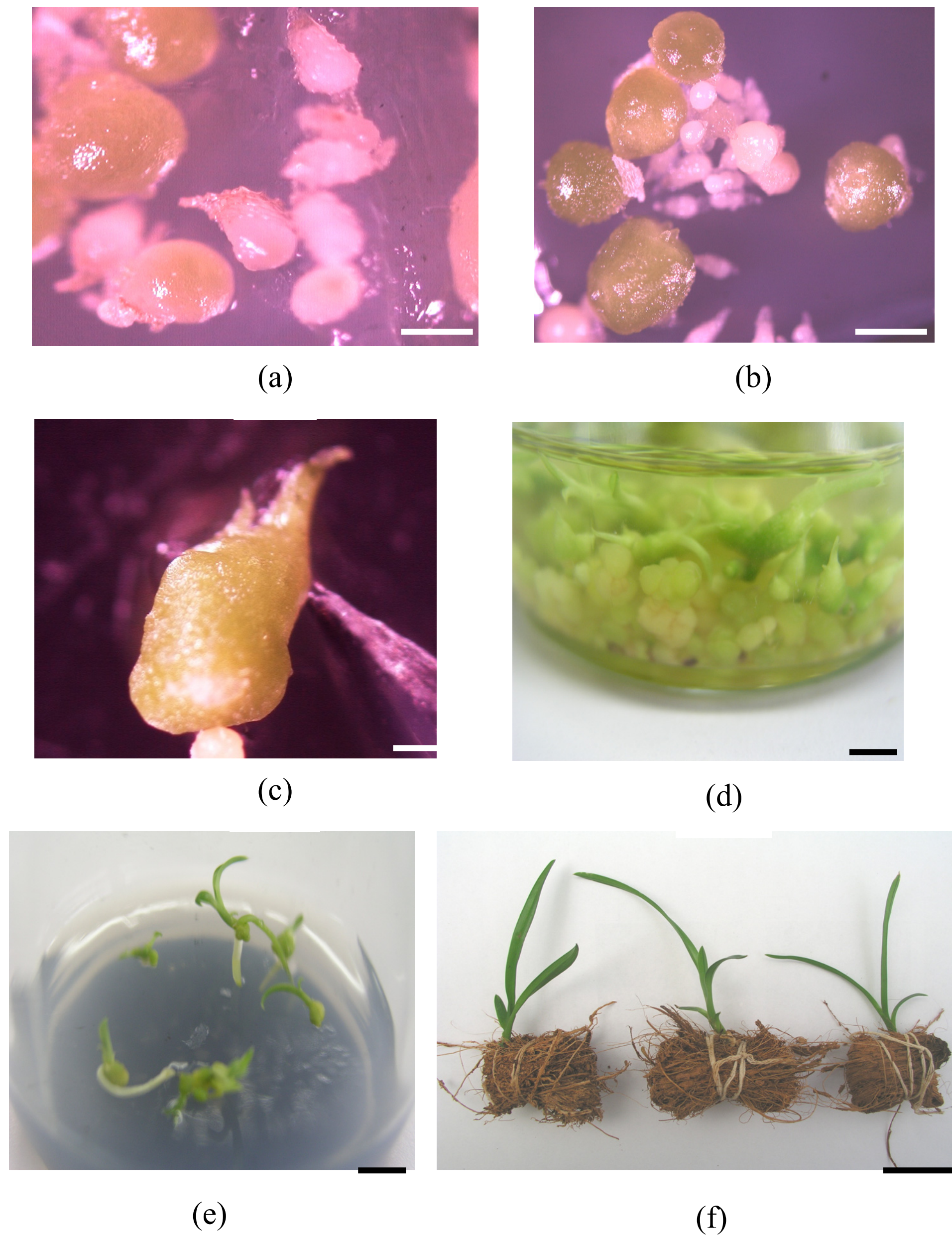

Figure 1 *In vitro* germination of *Cymbidium findlaysonianum* on VW solidified-medium supplemented with 15 % coconut water, 5 % banana homogenate, 5 % potato homogenate, 0.2 % activated charcoal and 20 g/l sucrose for 3 months. (a) Type I, swollen seed; seed size 3 times greater with the green color of chlorophyll (*Bar = 1 mm*). (b) Type II, Protocorm; globule formation with rhizoid (*Bar = 1 mm*). (c) Type III, Protocorm; less round with a protrusion of leaves at one end (*Bar = 1 mm*). (d) Multiplication of protocorms in a VWM liquid medium supplemented with 15 % coconut water and 20 g/l sucrose after culturing for 1 month (*Bar = 1 mm*). (e) Plantlet regeneration on VWM medium supplemented with all additives for 3 months (*Bar = 5 mm*). (f) 1 month plantlets hardening on coconut peat. (*Bar = 1 cm*).

Table 1 Effect of organic additives on *in vitro* germination of *C. findlaysonianum* after culturing for 3 months.

Months	Medium I*			Medium II		
	Germination rate (%)					
	Type I**	Type II	Type III	Type I	Type II	Type III
1	93.63 ± 23.21	6.37 ± 2.89	0	79.13 ± 12.34	20.87 ± 7.34	0
2	88.03 ± 18.03	9.66 ± 4.66	2.32 ± 0.19	78.01 ± 10.98	16.59 ± 5.56	5.40 ± 0.21
3	87.77 ± 14.67	9.57 ± 3.08	2.66 ± 0.24	74.76 ± 9.11	17.12 ± 3.14	8.13 ± 0.32

*Medium I = VW with 15 % coconut water, 20 g/l sucrose + 8 g/l agar
Medium II = VW with 15 % coconut water + 5 % banana homogenate + 5 % potato homogenate + 0.2 % activated charcoal + 20 g/l sucrose + 8 g/l agar
**Type I = swollen seed; seed size 3 times that of the original with the green color of chlorophyll
Type II = Protocorm; globule formation with rhizoid
Type III = Protocorm less round with a protrusion of leaves at one end

Multiplication and Regeneration

We found that the culture media had a positive effect on the multiplication rate of protocorms. Upon culturing for 3 months, multiplication of protocorms responded variably in 4 different liquid media viz., VW, MS, ½ MS and VWM (**Table 2**). VWM medium resulted in the best response in terms of the number of protocorms produced at all period of times. This medium increased the number of protocorms nearly 4 times compared with the non modified VW medium in each month of culturing and was significantly better than the other media. This might be due to its rich microelement regime [8] or due to the synergistic effect and interacting influences of MS vitamins on cell division and elongation [14]. Also, the morphology and color of protocorms varied in each medium. A single green protocorm when cultured in a VWM medium for a month showed a yellow-green color. Continuous culturing of these protocorms in a fresh medium for an additional 2 - 3 months showed the protrusion of leaves on the apical region of the yellow or pale yellow protocorm or well-developed small plantlets with a yellow-green color (**Figure 1d**). Whereas protocorms cultured in a ½ MS medium and MS medium for 2 - 3 months showed a yellow-green and light-green color, respectively. In contrast, protocorms cultured in a VW medium showed browning or necrosis without further growth. This is a common phenomenon in orchids [14] that may be due to inappropriate culture conditions, such as improper balance of nutrients or the lack of required growth-stimulating components [15].

Table 2 Effect of media on multiplication of protocorms after culturing for 3 months.

Medium[1]/Months	Number of protocorms		
	1	**2**	**3**
VW	31.00 ± 4.47 d**	58.40 ± 6.91 d	92.20 ± 47.02 c
MS	75.20 ± 5.50 c	111.60 ± 6.50 c	278.60 ± 32.96 b
Half-strength MS (½MS)	91.00 ± 3.16 b	153.80 ± 5.54 b	378.60 ± 55.89 ab
VW+MS vitamin (VWM)	121.40 ± 5.73 a	237.00 ± 6.78 a	465.80 ± 31.74 a
CV (%)	**8.32**	**4.61**	**25.37**

**Means followed by the same letter within a column were not significantly different at 5 % probability level by Duncan's multiple range test.
[1]All of the media were supplemented with 15 % coconut water and 20 g/l sucrose.

Moreover, we were able to successfully induce plantlets on the medium consisting of organic additives, such as coconut water, banana homogenate, potato homogenate and activated charcoal. During the first month of culturing, protocorms grew very slowly and no plantlet was developed in any culture media. Additional culturing, for 2 and 3 months, in an activated charcoal medium tended to promote the best growth of the protocorms and was significantly different to other media (**Table 3**). Activated charcoal added-VWM medium resulted in the highest percentage of plantlet regeneration at 50 and 70 % after culturing for 2 and 3 months, respectively. At the end of 3 months, 1 - 2 complete plantlets per protocorm were developed (**Figure 1e**).

Table 3 Effect of organic additives on plantlet regeneration after culturing for 3 months.

Media	Plantlets induction (%)*	
	2 Months	**3 Months**
VW + 15 % coconut water	2.5 b*	7.5 b
VW + 15 % coconut water + 0.2 % activated charcoal	40.0 a	65.0 a
VW + 15 % coconut water + 5 % banana homogenate	7.5 b	17.5 b
VW + 15 % coconut water + 5 % potato homogenate	2.5 b	12.5 b
VW + 15 % coconut water + 5 % banana homogenate + 5 % potato homogenate	2.5 b	10.0 b
VW + 15 % coconut water + 5 % banana homogenate + 5 % potato homogenate + 0.2 % activated charcoal	50.0 a	70.0 a
CV (%)	**33.10**	**58.79**

*Means followed by the same letter within a column were not significantly different at 5 % probability level by Duncan's multiple range test.

We added coconut water in the medium because of its beneficial effects on seed germination, which have been reported in culturing endangered orchids like *Rhynchostylis retisa* and *Vanda coerlea* [16]. Coconut water is commonly used in the tissue culture of orchids. It is known to contain zeatin and zeatin ribosides as a major cytokinin which supports cell division [17]. Coconut water at 10 - 30 % stimulated growth of Cymbidium's protocorm [17]. On the contrary, coconut water had no significant effect on the growth or protocorm regeneration capability of *D. fimbriatum* callus [18], *Oncidium varicosum* [19] and *Aeride*s *crispum* [20]. Thus, the effect of coconut water on the growth of protocorms might be dependent on the species. We also found that a medium supplemented with activated charcoal stimulated germination and induced plantlet regeneration. Activated charcoal was originally used in tissue culture to darken the medium in order to simulate soil conditions. Activated charcoal not only showed a positive stimulating effect in many developmental processes, but also had detrimental effects on others. This may be due to its ability to adsorb various media components [21]. In this study, the medium that consisted of activated charcoal at 0.2 to 3 g/l enhanced growth and development of the seedlings and plantlets of *Cymbidium* [13,17]. This agrees with the results reported by Ichihashi and Islam which showed that potato homogenate enhanced seedling growth of *Phalaenopsis* and banana homogenate enhanced seedling growth of *Doritaenopsis* [22]. Potato and banana homogenate contain carbohydrate, fat, vitamins, phenolic compounds, amino acids, and organic acids. The use of these homogenates alone or in combination might enhance plantlet regeneration in orchids compared to a non-additive medium. So, supplementation of organic additives to an orchid culture medium is simple, practical, beneficial and convenient to improve culture media used for commercial production.

Hardening of Plantlet

The regenerated plantlets (3 - 4 cm in height) with a pair of leaves and 2 - 3 roots, hardening in coconut peat before putting in a plastic tray for 4 weeks showed higher rates of survival, up to 70 % with healthy and greenish leaves (**Figure 1f**). Whereas culturing in a plastic tray without coconut peat gave no survival of plantlets. The leaves turned yellowish brown when the plants were drying. In another study, hardening of *in vitro* seedlings in the greenhouse under high humidity conditions was found to be highly beneficial for successful acclimatization. A higher number of plants survived in the coconut peat rather than in non coconut peat. Supporting materials with maximum water holding capacity, porosity and drainage is essential for proper growth and development of *in vitro* raised seedlings of orchids [8]. The high humidity conditions prevented the wilting of plants. Similar results were also found in *Dendrobium* plantlets hardened in moss and tree fern in the greenhouse [6].

CONCLUSIONS

A simple protocol for rapid production of *C. findlaysonianum* from seeds has been summarized. Maximum seed germination was achieved on a VW solidified-medium supplemented with 15 % coconut water, 5 % banana homogenate, 5 % potato homogenate, 0.2 % activated charcoal and 20 g/l sucrose. The number of protocorms increased 4 times when cultured in a VWM medium supplemented with 15 % coconut water and 20 g/l sucrose. The seedlings were further grown on a VWM medium with added organic additives (15 % coconut water, 5 % banana homogenate and 5 % potato homogenate) for 3 months. Plantlets could be hardened under greenhouse conditions on coconut peat. By using this protocol, over 100,000 healthy orchid plants could be produced from a capsule in a year. This technique has also proven to be valuable for breeding of the desired characters of *C. findlaysonianum*.

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บทคัดย่อ

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การงอกของเมล็ดและการพัฒนาเป็นต้นของกล้วยไม้กะเร่กะร่อนปากเป็ด (*Cymbidium findlaysonianum* Lindl.) บนอาหารเติมสารอินทรีย์บางชนิด

เมล็ดกล้วยไม้กะเร่กะร่อนปากเป็ด (*Cymbidium findlaysonianum* Lindl.) นำมาเพาะให้งอกและชักนำให้เป็นต้นบนอาหารสูตรวาชีนและเวนท์ (VW) เติมสารต่างๆ หลังจากเพาะเมล็ดเป็นเวลา 3 เดือน อาหารที่เติม น้ำ-มะพร้าว 15 เปอร์เซ็นต์ กล้วยหอมบด 5 เปอร์เซ็นต์ มันฝรั่งบด 5 เปอร์เซ็นต์ ผงถ่าน 0.2 เปอร์เซ็นต์ และน้ำตาลซูโครส 20 กรัมต่อลิตร ส่งเสริมการงอกของเมล็ดสูงกว่าอาหารสูตรควบคุม โปรโตคอร์มอายุ 3 เดือน ที่เลี้ยงในอาหารเหลวสูตรวาชีนและเวนท์ ดัดแปลงด้วยการเติมวิตามินของอาหารสูตรมูราชิเกะและสกูค (MS) (VWM) เพิ่มปริมาณ 4 เท่า ในแต่ละเดือนของการเลี้ยง แตกต่างอย่างมีนัยสำคัญทางสถิติจากอาหารสูตร VW MS และ MS ที่ลดองค์ประกอบของธาตุอาหารลงครึ่งหนึ่ง (1/2 MS) นอกจากนี้อาหารที่มีผงถ่านเป็นองค์ประกอบกระตุ้นการเจริญเติบโตของต้นกล้า แตกต่างอย่างมีนัยสำคัญกับอาหารที่มีน้ำมะพร้าว กล้วยหอมบด และ/หรือมันฝรั่งบด ต้น-กล้าที่แข็งแรงเมื่อย้ายไปปลูกในถาดพลาสติกที่มีขุยมะพร้าวสับสามารถมีชีวิตรอดได้ 70 เปอร์เซ็นต์ การเพาะฝักกล้วยไม้กะเร่กะร่อนจำนวน 1 ฝัก อาจชักนำต้นกล้าได้มากกว่า 100,000 ต้น ในระยะเวลา 1 ปี ดังนั้นอินทรีย์สารและองค์ประกอบของอาหารจึงมีผลต่อการเจริญเติบโตและพัฒนาการของเมล็ดกล้วยไม้กะเร่กะร่อนปากเป็ดที่เพาะเลี้ยงในสภาพปลอดเชื้อ

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