



## Non-involvement of Activated Charcoal and Plant Growth Regulators in PLB Proliferation of *Rhynchostylis coelestis* Rchb.f.

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### บทคัดย่อ

เอื้องเขาแกะเป็นหนึ่งในกล้วยไม้ป่าซึ่งมีความเสี่ยงต่อการสูญพันธุ์ของประเทศไทย อย่างไรก็ตาม การขยายพันธุ์ด้วยวิธีการเพาะเลี้ยงเนื้อเยื่อสามารถนำมาช่วยเพิ่มปริมาณของกล้วยไม้หลายชนิดได้เป็นอย่างดี งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการชักนำให้เกิดและเพิ่มจำนวนโปรโตคอร์มไลค์บอดี (Protocorm-like Bodies: PLBs) ของเอื้องเขาแกะโดยวิธีการเพาะเมล็ดซึ่งเจริญเต็มวัย เมื่อเพาะเลี้ยงเมล็ดเจริญเต็มวัยของเอื้องเขาแกะบนอาหารดัดแปลงสูตร VW และอาหารสูตร MS ที่เติมและไม่เติมน้ำมะพร้าว 15 เปอร์เซ็นต์ ผลการศึกษาพบว่าเมล็ดแห้งของเอื้องเขาแกะสามารถเจริญไปเป็นโปรโตคอร์มไลค์บอดีได้มากที่สุดเมื่อเพาะบนอาหารดัดแปลงสูตร VW ที่เติมน้ำมะพร้าว 15 เปอร์เซ็นต์ และอัตราการเกิดโปรโตคอร์มไลค์บอดีสูงสุดมีค่าประมาณ 84.52 เปอร์เซ็นต์ จำนวนโปรโตคอร์มไลค์บอดีของเอื้องเขาแกะเพิ่มมากกว่า 30 เท่าบนอาหารสูตรนี้ การเติมผงถ่านและสารควบคุมการเติบโตในอาหารเพาะเลี้ยงไม่มีผลต่อการเพิ่มจำนวนโปรโตคอร์ม

ไลค์บอดีของเอื้องเขาแกะ เมื่อย้ายโปรโตคอร์มไลค์บอดีลงบนอาหารดัดแปลงสูตร VW และอาหารสูตร MS ที่มีและไม่มี NAA 0.5 มิลลิกรัมต่อลิตร พบว่าพืชสามารถเจริญได้ดีบนอาหารสูตรพื้นฐาน MS

**คำสำคัญ:** เอื้องเขาแกะ โปรโตคอร์มไลค์บอดี การเพาะเมล็ด การเพิ่มจำนวนโปรโตคอร์มไลค์บอดี

### Abstract

*Rhynchostylis coelestis* Rchb.f. is one of the nation's endangered wild orchids facing a high risk of becoming extinct. However, the propagation through plant tissue culture could splendidly provide the increasing number of various orchids. The objectives of this research were to study the initiation and proliferation of *R. coelestis* protocorm-like bodies (PLBs) via mature seed germination. Mature seeds of *R. coelestis* were cultured on modified VW medium

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and MS medium with or without 15% coconut water. The results revealed that dry seeds of *R. coelestis* could maximally develop into PLBs after being germinated on modified VW medium supplemented with 15% coconut water. The highest percentage of PLB formation was 84.52 percent approximately. PLB proliferation increased more than 30 times on this medium. Adding activated charcoal and plant growth regulators into the medium had no effect on *R. coelestis* PLB proliferation. When PLBs were transferred to modified VW medium and MS medium containing or lacking 0.5 mg/l NAA, it was found that plantlets could develop well on MS basal medium.

**Keywords:** *Rhynchostylis coelestis*, Protocorm-like Bodies, Seed Germination, PLB Proliferation

## 1. Introduction

Thailand is one of the centers for wild orchids since there are a huge number of these species found in Thai forest throughout various parts of the country. Because of the suitable ecosystem, Thai wild orchids could grow as terrestrials, epiphytes and lithophytes in both monopodial and sympodial forms. *Rhynchostylis* is a monopodial genus of the orchid family (Orchidaceae). This genus is closely related to vandas and easily recognized by the thick, leathery leaves with striate venation. The flowers are fragrant and borne in dense, pendent inflorescences on short-stemmed plants. They are quite large, with a backward-pointing, flattened spur. These plants need warm, humid and shady conditions for healthy development [1], [2].

In Thai forest, there are 3 species of *Rhynchostylis* which had been recorded: *Rhynchostylis gigantea* (Ldl) Ridl., *R. retusa* Bl. and *R. coelestis* Rchb.f.. Though

most species in the genus *Rhynchostylis* are indigenous in mainland Southeast Asia, Malaysia and the Philippines, *R. coelestis* is native to Thailand, Cambodia and Vietnam. It is flowering in the summer with upright inflorescences with waxy white flowers tipped in blue-violet [2]. Recently, *Rhynchostylis* was reported as one of the wild orchids that were threatened with the danger of extinction through deforestation and indiscriminate collection [3].

Plant tissue culture is one of the important biotechnological tools which could be applied in various aspects [4]. In the past decade, there were some publications on shoot regeneration of *Rhynchostylis gigantea* [5] and *R. retusa* [6]. However, effects of activated charcoal and plant growth regulators on protocorm-like bodies (PLBs) proliferation of *R. coelestis* had never been studied. Thus herewith we report our study on the initiation of *R. coelestis* protocorm-like bodies. In addition, activated charcoal and plant growth regulators were also used to investigate PLBs proliferation on this plant.

## 2. Materials and Methods

### 2.1 Dry Seeds Sterilization and Protocorm-like Bodies (PLBs) Initiation

The 10-month-old brown capsules of *Rhynchostylis coelestis* Rchb.f. (Figure 1 left) were purchased from a local orchid farm in Warin Chamrab district, Ubon Ratchathani, Thailand. Surface-sterilization of dry seeds (Figure 1 right) began with removing the mature seeds from the seed pod and then immersing in 15% (v/v) Clorox (a commercial bleach solution containing 5.25%, w/w, sodium hypochlorite as available chlorine) for 1 min. Afterward, they were rinsed with sterile distilled water 3 times each for 1 min



**Figure 1** A seed pod of *Rhynchosstylis coelestis* Rchb.f. (left) and mature seeds of *R. coelestis* Rchb.f. (right, scale bar = 0.1 mm).

before placing on 4 types of media: modified VW medium [7]; modified VW medium supplemented with 15% CW (coconut water); MS medium [8]; MS medium supplemented with 15% CW. The data on PLB initiation were collected after 10 weeks.

## 2.2 Protocorm-like Bodies (PLBs) Proliferation

After PLBs had been initiated for 7 weeks, some of them were transferred on various culture media: modified VW medium supplemented with 15% CW; modified VW medium containing 15% CW and 0.2% activated charcoal (AC); modified VW medium comprised 15% CW, 0.1 mg/l NAA and 1 mg/l BA; modified VW medium consisted of 15% CW, 0.1 mg/l NAA, 1 mg/l BA and 0.2% AC. The data on PLB proliferation were also observed for 10 weeks.

## 2.3 Plantlet Developed from Protocorm-like Bodies (PLBs)

The 7 weeks old of PLBs at initial stage were placed on 4 kinds of media: modified VW medium; modified VW medium filled with 0.5 mg/l NAA; MS medium; MS medium mixing with 0.5 mg/l NAA. Root number, root length, leaf number, leaf length and plantlet height were recorded for 6 weeks.

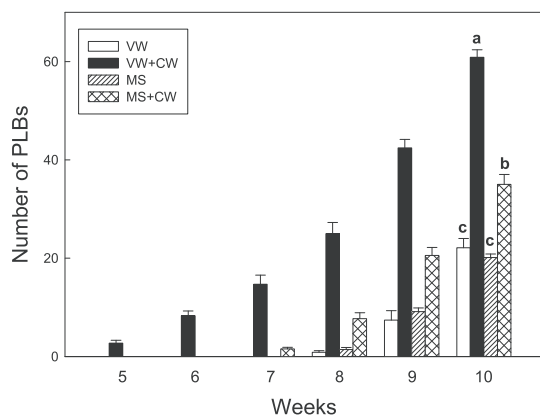
## 2.4 Media and Growth Room Conditions

Modified VW and MS media were adjusted to pH 4.8 and 5.7, respectively. All media in this research were gelled with 0.9% (w/v) agar and autoclaved at 121°C and 15 psi for 20 minutes. All cultures were kept in a growth room under 16 h illumination from white fluorescent lamps ( $12.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 8 h darkness at  $25 \pm 2^\circ\text{C}$ .

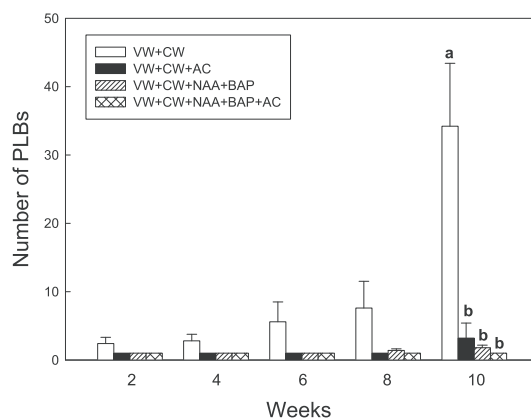
## 3. Results and Discussion

In all habitats of Thailand, the Orchidaceae has the largest number of vascular plants compared with other families. It consisted of 176 genera and 1,157 species [9]. Among these, *Rhynchosstylis coelestis* Rchb.f. is a native orchid of Thailand that currently need to be conserved and propagated since it is vulnerable for the extinction. As a consequence, plant tissue culture, a biotechnological tool, could be possible to apply for solving this problem. The orchid tissue culture in Thailand has presently been an essential means for the commercial production of elite selections owing to low cost, uniformity, fast propagation and high yield in a short period of time [10], [11]. This research also reports a simple and efficient method to increase the number of *R. coelestis*.

Asymbiotic seed germination of *R. coelestis* was successfully done after surface-sterilization. The approximate 72 mature dry seeds in each jar (4.5 cm diameter and 8.5 cm height) were developed into protocorm like-bodies (PLBs) after culturing for 5 weeks on modified VW medium supplemented with 15% CW while other media induced PLBs after 7 weeks (Figure 2). When compared the PLB formation at the end of experiment, modified VW medium comprised 15% CW gave the highest number of PLB forming and had the significantly different from other PLB initiation



**Figure 2** PLBs initiated from mature seeds of *Rhynchosstylis coelestis* Rchb.f. during 5 to 10 weeks on different media under light condition. Values are means of 7 replications±SE. Results that share the same letters are not significantly different at P<0.05.



**Figure 3** PLB proliferation starting from one PLB of *Rhynchosstylis coelestis* Rchb.f. during 2 to 10 weeks on different media under light condition. Values are means of 5 replications±SE. Results that share the same letters are not significantly different at P<0.05.

media (Figure 2). The best percentage of PLB forming was about 84.52% on modified VW medium consisted of 15% CW. Media adding coconut water seemed to have the potential to induce PLBs better than the basal media (VW and MS). Thus, coconut water could be one of the important factors for the rapid initiation of *R. coelestis* protocorm-like bodies. This result was similar to the in vitro culture of immature seeds of *R. retusa* which seeds were germinated on ½ MS medium supplemented with various concentrations of CW and 15% CW could provide the efficacious response [6].

After mature seeds were initiated into PLB state, the PLBs at week 7 had been selected to conduct the PLB proliferation on different media. At this week PLBs were still undifferentiated into plantlets. One PLB was basically placed on modified VW medium containing 15% CW and on this medium in combination with activated charcoal (AC) and plant growth regulators

(NAA and BAP). It was found that PLB multiplication could take place on semi-solid medium. The single PLB fabulously multiply on modified VW medium containing 15% CW (Figure 3). PLB proliferation occurred about 34 times on this medium after 10 weeks. Nonetheless, AC and plant growth regulators in the medium obviously showed no effect on *R. coelestis* PLB proliferation (Figure 3). This finding was opposing to the proliferation of *Phalaenopsis gigantea* PLBs [12] since their media (New Dogashima Medium) containing low concentrations of BAP (0.5 to 3.5 mg/l) promoted multiplication and development of this orchid. Thus, at this stage, we can conclude that proliferation of mature seed-derived PLBs in our experiment was not affected by AC, NAA and BAP. It might occur from inappropriate growth factors or concentrations as well as the amount of AC. To increase PLB proliferation, therefore, others plant growth regulators and AC in

**Table 1** Different parameters from *Rhynchosytilis coelestis* Rchb.f. plantlets which occurred on each medium after cultured for 6 weeks under light condition. Values are means of 4 replications±SE. Data marked by same letter in a column are not significantly different (ANOVA, P < 0.05).

Types of media	Root number	Root length (mm)	Leaf number	Leaf length (mm)	Plantlet height (mm)
VW	2.250±0.250b	3.954±0.421b	3.250±0.250b	2.696±0.254b	3.625±0.125b
VW+NAA	0±0a	0±0a	0.5±0.5a	0.5±0.5a	2.875±0.125a
MS	2.500±0.289b	5.000±0.906c	3.500±0.289b	3.756±0.245b	4.750±0.144c
MS+NAA	0.500±0.289a	1.750±1.181a	3.250±0.250b	3.688±0.361b	3.750±0.250b

different concentrations could be considered for further investigation.

When the 7-week-old PLB had been subcultured on various media, young leaves apparently started developing on the second week in all media while root had not been found on modified VW medium containing 0.5 mg/l NAA (Table 1). Adding NAA did not stimulate root formation at this step. This may occurred because the concentration of this plant growth regulator was inappropriate to enhance root regeneration. Hence, it would be better to examine the appropriate amount of artificial hormone or add some organic additives to the medium for encouraging root development of *R. coelestis*. However, the number of leaf formation on modified VW medium containing 0.5 mg/l NAA was also less than other media whereas the best plantlet height was achieved after the axenically grown PLB was placed on plant growth regulator-free MS medium. This outcome was dissimilar to *R. retusa* which plantlet growth was maximum on MS medium consisted of 1.35 mg/l BAP, 0.037 mg/l NAA and 0.1% AC [6].

## References

- [1] J. Stewart, *Orchids*, Portland, Oregon: Timber press, 2000, pp. 109.
- [2] Ortho Books, *Complete guide to orchids*, Meredith Corporation, 2005, pp. 207.
- [3] S. Chugh, S. Guha, and I.U. Rao, "Micropropagation of orchids: A review on the potential of different explants," *Scientia Horticulturae*, vol. 122, pp. 507–520, 2009.
- [4] K. Bodhipadma, S. Noichinda, S. Udomrati, G. Nathalang, B. Kijwijan, and D.W.M. Leung, "Anthocyanin accumulation in the hypocotyl and petal of red agati (*Sesbania grandiflora*), an ornamental legume," *Journal of Applied Horticulture*, vol. 8, no. 2, pp. 143–146, 2006.
- [5] B. van Le, N.T. Hang Phuong, L.T. Anh Hong, and K. Tran Thanh Van, "High frequency shoot regeneration from *Rhynchosytilis gigantea* (orchidaceae) using thin cell layers," *Plant Growth Regulation*, vol. 28, pp. 179–185, 1999.
- [6] T.D. Thomas and A. Michael, "High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchosytilis retusa* Blume., an exquisite orchid," *Plant Biotechnology Reports*, vol. 1, no. 4, 243–249, 2007.
- [7] E. Vacin and F.W. Went, "Some pH changes in nutrient solutions," *Botanical Gazette*, vol. 110, pp. 605–613, 1949.
- [8] T. Murashige and F. Skoog, "A revised medium for rapid growth and bioassays with tobacco tissue culture," *Physiologia Plantarum*, vol. 15, pp. 473–497, 1962.



- [9] Queen Sirikit Botanic Garden, *Thai Native Orchids I*. The Botanical Garden Organization, Ministry of Natural Resources and Environment of Thailand, Chiang Mai, 2008, pp. 25.
- [10] K. Thammasiri, *Orchids in Thailand: A success story*, APAARI, FAO, New Delhi: Angkor Publishers (P.) Ltd., 1997, pp. 19.
- [11] K. Sopalun, K. Thammasiri, and K. Ishikawa, "Micropropagation of the Thai orchid *Grammatophyllum speciosum* blume," *Plant Cell Tissue and Organ Culture*, vol. 101, pp. 143–150, 2010.
- [12] M.A. Latip, R. Murdad, Z.A. Aziz, L.H. Ting, L.M. Govindasamy, and R. Ripin, "Effects of N<sup>6</sup>-benzyladenine and thidiazuron on proliferation of *Phalaenopsis gigantea* protocorms," *Asia-Pacific Journal of Molecular Biology and Biotechnology*, vol. 18, no. 1, pp. 217–220, 2010.