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

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**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-stemed 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.
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 μmol m\(^{-2}\) s\(^{-1}\)) and 8 h darkness at 25±2°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, *Rhynchostylis 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.
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
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


