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# Influence of Organic Supplements on Shoot Multiplication Efficiency of *Phaius tankervilleae* var. *alba*

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***Abstract*—**The influence of organic supplements on growth and multiplication efficiency of *Phaius tankervilleae* var. *alba* seedlings was investigated. 12 week-old seedlings were cultured on half-strength semi-solid Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 8 g/L agar and various concentrations of coconut water (0, 50, 100, 150 and 200 mL/L) combined with potato extract (0, 25 and 50 g/L) and the pH was adjusted to 5.8 prior to autoclaving. The cultures were then kept under constant photoperiod (16 h light: 8 h dark) at 25 ± 2 °C for 12 weeks. The highest number of shoots (3.0 shoots/explant) was obtained when cultured on the medium added with 50 ml/L coconut water and 50 g/L potato extract whereas the highest number of leaves (5.9 leaves/explant) and roots (6.1 roots/explant) could receive on the medium supplemented with 150 ml/L coconut water and 50 g/L potato extract. with 150 ml/L coconut water and 50 g/L potato extract. Additionally, plantlets of *P. tankervilleae* var. *alba* were transferred to grow into seven different substrates i.e. soil, sand, coconut husk chip, soil-sand mix (1: 1), soil-coconut husk chip mix (1: 1), sand-coconut husk chip mix (1: 1) and soil-sand-coconut husk chip mix (1: 1: 1) for four weeks. The results found that acclimatized plants showed 100% of survivals when sand, coconut husk chip and sand-coconut husk chip mix are used as substrates. The number of leaves induced by sand-coconut husk chip mix was significantly higher than that planted in other substrates ($P > 0.05$). Meanwhile, no significant difference in new shoot formation among these substrates was observed ($P < 0.05$). This precursory developing protocol was likely to be applied for more large scale of plant production as well as conservation of germplasm of this orchid species.

***Keywords*—**Acclimatization, coconut water, orchid, *Phaius tankervilleae* var. *alba*., potato extract.

## I. Introduction

THE *Phaius* is a genus of terrestrial orchids belonging to Collabieae tribe of Epidendroideae subfamily. Less than 50 described species were recorded and widely distributed in Africa, Australia, Pacific and Indian Ocean islands and Asia including Thailand [1]. Up to date, six known species of *Phaius* i.e. *P. tankervilleae*, *P. mishmensis*, *P. indigoferus*, *P. takeoi*, *P. flavus* and *P. epiphticus* have been morphologically classified and exhibited throughout Thailand [2]. Among them, *P. tankervilleae* is one of the most ornamentally attractive species. Morphologically, their inflorescences are large, showy and distinctive. They flower easily and have a long duration of blooming which is appropriate to use as potted plants and cut flower [3], [4]. In addition, some countries have applied *P. tankervilleae* as folk medicines to treat rash, freckle, blemish and blister [5]. This orchid species in natural habitat is stated as rare and endangered in Japan [6], India [7] and Australia [8] and is now listed in CITES Appendix II which somehow can be traded under a strict regulation [9]. The extinction is much more concerned with *P. tankervilleae* var. *alba* which is rarely found only in few natural population (Fig. 1 (a)). Therefore, an efficient method for rapid propagation of this variety is needed to study for conservation and sustainable utilization. *In vitro* culture technique is an effective way of propagation and has been successful in propagating many orchid species [10].

The composition of culture medium is one of the key factors affecting the result of *in vitro* orchid culture. Growth and multiplication rate of many orchid species can be enhanced by adding some organic supplements like coconut water (CW) or potato extract (PE) to culture medium [11], [12]. However, the accomplishments are depended on explants type and orchid species [13]. At present, only few publications [14], [15] study about the effect of organic supplements on shoot regeneration capability in the *Phaius* species especially in *P. tankervilleae* var. *alba*. Therefore, this study attempted to observe the influence of organic supplements on shoot multiplications efficiency of *P. tankervilleae* var. *alba.*

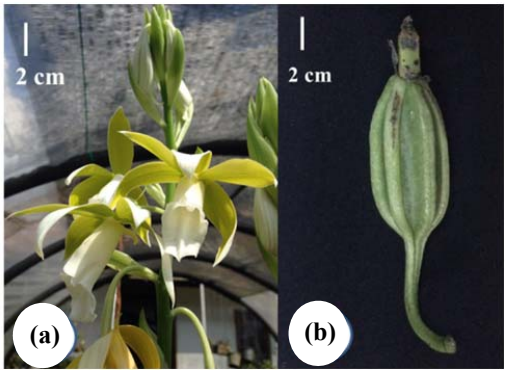


Fig. 1 Flowers (a) and pod (b) of *P. tankervilleae* var. *alba*

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## II. Materials and Methods

### A. In vitro Culture of *P. tankervilleae* var. *alba*

The pod of *P. tankervilleae* var. *alba* (Fig. 1 (b)) obtained from Romklao Botanical Garden under the Royal Initiative, Phitsanulok, Thailand was sterilized with 15% (v/v) Clorox® solution for 20 minutes. The seeds were collected from sterilized pod and cultured on modified VW [16] medium supplemented with 150 mL/L CW and 50 g/L PE. The media was solidified by 8 g/L agar and pH was adjusted to 5.2. After 12 weeks of seed culture, the seedlings were cultured on half-strength MS medium [17] supplemented with different concentrations of CW and PE. The media were added with 15 g/L sucrose, 8 g/L agar and adjusted pH to 5.8. The seedlings were cultivated under cool-white LED lamp (20 µmol/m$^2$/s) for 16 h/day at 25 ± 2 °C. Growth and development of seedlings were measured and recorded after 12 weeks of culture. Each treatment was performed in 10 replicates and repeated 3 times. The statistical differences of data were analyzed by One-way ANOVA and Duncan's multiple range tests (DMRT).

### B. Acclimatization of *P. tankervilleae* var. *alba* Plantlets

The *in vitro* rooted plantlets were washed with sterile water to remove the agar medium and immersed in fungicide solution for 20 min. The treated plantlets were then transplanted to plastic pot (5 cm wide and 5 cm height) containing different planting substrates and cultured under greenhouse (28–34 °C air temperature, relative 50–60% humidity and natural sunlight with 70% shade nets). After 4 weeks of transplantation, survival and growth of transplanted plants were recorded. The experiment was conducted with 3 replications of 10 plantlets per replication

## III. Results and Discussion

### A. In vitro Growth and Multiplication Efficiency

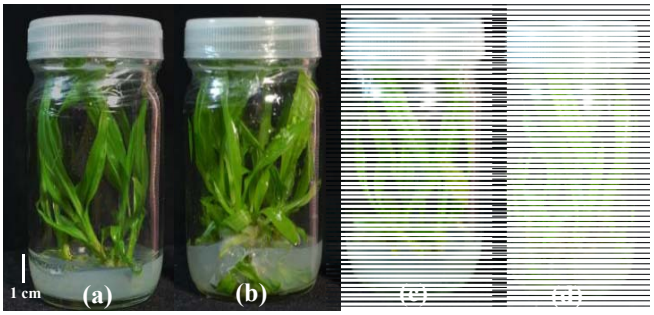


Fig. 2 *In vitro* plantlets of *P. tankervilleae* var. *alba* 12 weeks after cultured on (a) ½MS medium, (b) ½ MS supplemented with 150 mL/L CW, (c) 50 g/L PE and (d) 50 mL/L CW combined with 50 g/L PE

For *in vitro* orchid culture, CW and PE are extensively used because they effectively promote growth and development of orchids [18], [19]. Therefore, CW and PE were applied in this study. The seedlings were cultured on half-strength semi-solid MS medium supplemented with various concentrations of CW (0, 50, 100, 150 and 200 mL/L) combined with PE (0, 25 and 50 g/L).

After 12 weeks of culture, the seedlings were all survived in every tested media. However, diverse growth performances were found among the different PE and CW supplementations (Fig. 2).

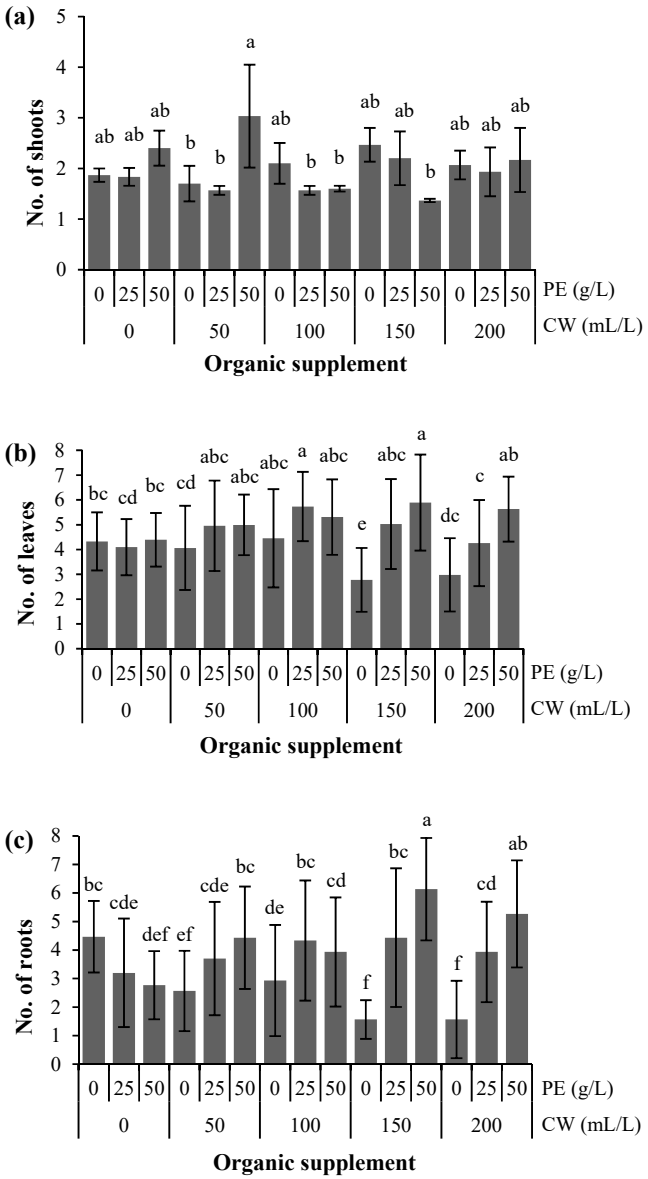


Fig. 3 Effect of CW and PE supplementation on number of shoot (a), leaves (b) and root (c) per explant of *P. tankervilleae* var. *alba* after culture for 12 weeks. The bars and error bars represent means and SE of 3 replicates (each with 10 plants), respectively. Different letters above the bar show significant differences analyzed by DMRT at $p \leq 0.05$

Different concentrations of CW affected shoot production of seedling randomly (Fig. 3 (a)). At low concentration of CW supplementation (0 and 50 mL/L), shoot number had a tendency to increase with the concentration of PE supplementation. On the other hand, shoot numbers tend to decrease with increasing PE content at higher CW

supplementation (100 and 150 mL/L). Nevertheless, different PE supplementation did not affect shoot number at the highest CW addition (200 mL/L). In summary, the most effective combination for shoot multiplication of *P. tankervilleae* var. *alba* seedling was 50 mL/L CW with 50 g/L PE (Figs. 2 (d) and 3 (a)). Although PE and CW have been used in orchid culture media, combination effect has been observed in only few orchid species [11]. Previously, enhancement growth of *Calanthe* hybrid plantlet by CW supplement has been reported [20]. The optimum concentration was 50 mL/L which was consistent with this study. However, the higher CW concentration promotes better growth and shoot multiplication in some orchid species [11], [21]. So, the effect of CW may be depending on species and explants.

Numbers of leaves of seedling are obviously affected by the supplementation of CW and PE (Fig. 3 (b)). Without PE, adding high concentration (more than 150 mL/L) of CW inhibited leaves production. However, the leave production was obviously improved along with the increasing concentration of PE augmentation in high CW containing media. On the contrary, PE combination did not significantly increase the number of leave at CW supplementation lower than 150 mL/L. The highest average leaves number per explant was achieved from the medium containing 150 mL/L CW and 50 g/L PE. The combination effect of CW and PE on number of leaves was also formerly reported in *Bulbophyllum nipondhii* [11]; however the number of leave did not decrease in high concentration of CW supplementation.

*In vitro* plantlet with root is preferred before transfer to ex vitro environment. The plants without root or poor root quality hardly survive under ex vitro condition [22]. In this study, roots were successfully produced in every plantlet. Like leaves production, without PE, root was reduced with the increasing of CW concentration (Fig. 3 (c)). Similarly, root was also decreased with the increasing of PE concentration in media without CW. However, PE combinations were significantly improved root production in media holding CW, especially at high CW concentration (more than 150 mL/L). Therefore, root productions appear to be induced by the combination effect of CW and PE. This effect was aforementioned in *Bulbophyllum nipondhii* [11].

The main composition of CW is sugars, amino acids, minerals, vitamins and particularly plant hormones like auxin and cytokinin [23] which has been proposed as the key ingredient affecting explant organogenesis, growth and development. For PE, although the main composition is carbohydrate, protein, and vitamin [25], the important composition influenced plant growth and development was still doubtful. Previously, it was proposed that organic nitrogen was easier to process by orchid protocorms in comparison to inorganic nitrogen because it may by pass some step in nitrogen metabolic pathways [25]. Thus, the improvement of shoot, leaves and root number of *P. tankervilleae* var. *alba* probably due to the action of some phytohormones, organic nitrogen compounds, phosphorus, or vitamins from both CW and PE. In summary, the most suitable culture medium for *in vitro* shoot multiplication of *P. tankervilleae* var. *alba* was half-strength MS supplemented with 50 mL/L CW and 50 g/L PE. This medium provides the highest shoot number with fair leaves and roots number.

*B. Survival and Growth of Acclimatized Plant*

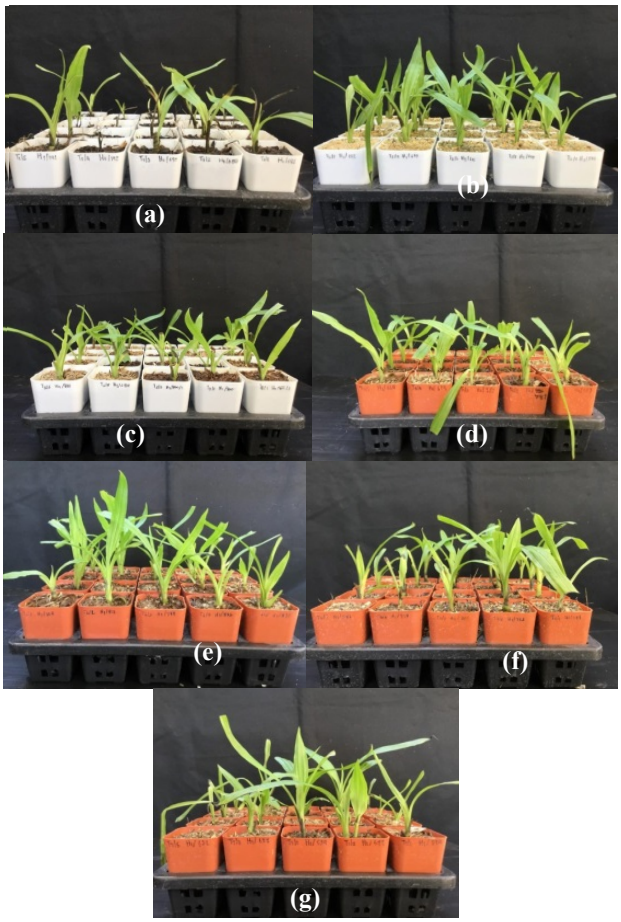


Fig. 6 Growth of *P. tankervilleae* var. *alba* planting with soil (a), sand (b), coconut husk chip (c), soil-sand mix, 1: 1 (d), soil-coconut husk chip mix, 1: 1 (e), sand-coconut husk chip mix, 1: 1 (f), coconut husk chip mix, 1: 1: 1 (g). The pictures were taken after 4 weeks of greenhouse acclimatization

Acclimatization is an important step for *in vitro* plant propagation. This process enhances the survival of *in vitro* plantlet after transfer to ex vitro environment [26]. The *in vitro* plantlets of *P. tankervilleae* var. *alba* were successfully transplanted to greenhouse and grew healthily (Fig. 6). Up to 90% of them were survived in all tested substrates, however there were no statistically significant different among the tested substrates (Table I) although survival variant between different substrates have been previously observed in some orchids [27], [28]. This may be due to all substrates were able to keep the minimum requirement of ground moisture for *P. tankervilleae* var. *alba* plants. Nonetheless, different growth of acclimatized plant was observed among the tested substrates. Highest leaves number was obtained from the plants culturing with the sand-coconut husk chip mix (Fig. 6 (f)). The longest leaf length was gained from the plants growing with the soil-sand mix (Fig. 6 (f)) while the broadest leaf width was obtain

form soil-grown plants (Fig. 6 (a)). However, regarding the overall performances, sand-coconut husk chip mix seem to be the suitable substrate for *P. tankervilleae* var. *alba* acclimatization.

## IV. Conclusions

This research presents the first protocol for *in vitro* shoot multiplication of *P. tankervilleae* var. *alba*. The most suitable culture medium was half-strength MS supplemented with 50 mL/L CW and 50 g/L PE. The *in vitro* plantlets were successfully acclimatized and survived under the greenhouse. The mixture of sand and coconut husk ship (1: 1) was the most appropriate substrate for acclimatization. This study provides the useful information applicable for commercial propagation and conservation.

TABLE I
Effect of Planting Substrates on Acclimatization of *P. tankervilleae* var. *alba* in Greenhouse for 4 Weeks

| Substrates | Survival (%) | No. of leaves per plant | Leaf size (cm)[1] | | New shoot Formation (%) |
|---|---|---|---|---|---|
| | | | Length | Width | |
| Soil | 92.5 ± 15.0 ns | 2.6 ± 1.0 d | 5.5 ± 2.4 abc | 1.8 ± 3.0 a | 2.4 ± 4.0 ns |
| Sand | 100.0 ± 0.0 | 3.6 ± 1.1 b | 5.6 ± 1.9 abc | 0.7 ± 0.2 b | 1.7 ± 2.8 |
| Coconut husk chip | 100.0 ± 0.0 | 3.3 ± 0.8 bc | 5.5 ± 2.0 bc | 0.9 ± 0.2 b | 1.9 ± 3.2 |
| Soil + Sand (1: 1) | 87.5 ± 5.0 | 3.3 ± 0.9 bc | 6.8 ± 2.4 a | 0.8 ± 0.2 b | 2.4 ± 4.0 |
| Soil + Coconut husk chip (1: 1) | 92.5 ± 15.0 | 2.7 ± 0.5 d | 6.6 ± 1.9 ab | 1.0 ± 1.0 b | 0.0 ± 0.0 |
| Sand + Coconut husk chip (1: 1) | 100.0 ± 0.0 | 4.2 ± 1.0 a | 4.9 ± 1.8 c | 0.8 ± 0.3 b | 0.0 ± 0.0 |
| Soil + Sand + Coconut husk chip (1: 1: 1) | 95.5 ± 2.8 | 3.0 ± 1.0 cd | 6.6 ± 2.6 ab | 0.8 ± 0.2 b | 6.6 ± 7.2 |

Values are means ± SE of 3 replications (10 plants per replication). Different letters within the same column show significant differences analyzed by DMRT at $p \leq 0.05$. ns = not significant. [1] Data were obtained from the 3rd leaf of each plant.

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