In vitro Seed Germination, Callus Induction, and Plantlets Growth of *Bulbophyllum auricomum* Lindl. from Artificial Pollination

Win Theingi Aung[1], Myat Myat Moe[2]

Abstract

Bulbophyllum auricomum Lindl. is an endangered orchid and has only ever been found in Myanmar. Therefore, this paper used a tissue culture technique for *in vitro* propagation of the endangered *B. auricomum* Lindl. In this study, mature pods derived from artificial self-pollination were cultured for the best seed germination in MS basal medium. MS+1.0 mg/L of BAP+ 2.0 mg/L of NAA + 1g of AC had a 40% effect on callus induction from sub-cultured pseudobulb explants. Among 11 different concentrations, and combination treatments of natural plant extracts, MS+150 ml/L of coconut water was generally effective in fresh weight of callus proliferation and PLBs induction (1.75±0.08) and (3.01±0.20) at 1 and 2 months, respectively. MS + 30 g/L of banana + 20 g/L of potato extract was followed for 1 month (0.67±0.00), and 2 months (01.87±0.05). Consequently, 2 months old calluses and PLBs (about 0.23 g) were used to compare the effects of natural extracts (CW, BE, and PE) with or without plant growth regulators (BAP and NAA) medium for plant regeneration. Our results showed that MCBN: MS+ 150 ml/L of coconut water+2.0 mg/L of BAP+1.0 mg/L of NAA contained the highest plantlet fresh weight of (3.37±0.17) and (6.41±0.68) after 1 month and 2 months of culturing, respectively. The second highest plantlet growth was MBPBN: MS + 30 g/L of banana extract + 20 g/L of potato extract + 2.0 mg/L of BAP + 1.0 mg/L of NAA. In the present study, the combinations of natural extracts and plant growth regulators are the best for plant regeneration in the findings of a comparative study.

***Keywords*:** artificial self-pollination, *in vitro*, natural extract, and plant growth regulator

Introduction

Bulbophyllum auricomum Lindl., commonly known as Thazin-Pan, is native to Myanmar States of Bago, Tanintharyi, Yangon of Region, Mon and Rakhine (Hundley *et al*., 1987). *B. auricomum* Lindl. is difficult to produce seeds, and pods under natural conditions. If an orchid is not pollinated naturally or is only rarely pollinated, hand pollination has proven to be an effective conservation strategy (Zimmerman and Aide, 1989). Tan, 2009 showed that there are artificial reproductive benefits exist in the *Bulbophyllum* orchid. Orchid seeds have an extremely low germination rate because it difficult to germinate endosperm-less orchids seed in the wild (Dwivedi *et al*., 2020).

Tissue culture research is now established and successfully used in different plant parts as explants for commercial orchid propagation. Among them, *in vitro* germination of some orchid seeds were developed and established in the 1950's (Islam *et al*., 2014). This technic has the potential to save endangered species from extinction in their natural habitat (Sinimbu Neto *et al*., 2011). The tissue culture method can be used for rapid mass multiplication by using appropriate growing media and hormones (Asghar *et al*., 2011). Natural substances and hormones such as auxins and cytokinins are important factors that can be used to improve orchid development from protocorm to plantlet (Parthibhan *et al*., 2015). In addition, organic growth supplements including tomato juice (TJ), coconut water (CW), banana extract (BH), and potato extract (PE) are commonly added to orchid plant tissue culture media

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because they contain natural vitamins, phenols, fiber, hormones, and proteins (Gnasekaran *et al*., 2009). Hormones, natural substances, and carbohydrate sources have different effects in shoot development depending on their types and concentrations (Prasertsirivatna and Koolpluksee, 2011).

The micropropagation of *B. auricomum* Lindl. has been reported (Thet and Aye, 2018; Naing and Lim, 2011; Khin *et al*,. 2008 and Than, 2013). Previous researchers conducted about *in vitro* propagation of *B. auricomum* Lindl. by using MS medium supplemented with various plant growth regulators chosen from any explant parts, or seeds through natural self-pollination. Although the effects of coconut water, potato and banana extract, and plant growth regulators in the addition of MS basal medium were studied *in vitro* for *B. auricomum* Lindl., the combined effect of natural extract and plant growth regulators within or without hormones medium has not yet been reported. Therefore, the purpose of this study was conducted to examine the culture of mature pods using the artificial self-pollination method for *in vitro* seed culture and to compare the effect of natural extracts with or without the plant growth regulators (PGRs) on the *in vitro* large production of plantlets regeneration of *B. auricomum* Lindl.

Material and Methods

Pod materials by artificial self-pollination

B. auricomum Lindl. flower are grown with shade net greenhouse conditions. Mature flowers (5 days after flowering) of were artificial self-pollinated from mother plants. In artificial self-pollination, two pollinia were picked with sterilized tooth sticks sprayed with 70% ethanol solution, and placed inside the stigmatic surface of the same flower by using hand pollination. Pollination rate were determined by counting the fruits formed from the number of flowers per inflorescence. After artificial self-pollination, the mature pods were collected from the Useful Plant Resource Center, Dagon University, Myanmar. For *in vitro* seed culture, the four-month-old mature seed of *B. auricomum* Lindl. was carried out in the tissue culture laboratory, Department of Lifestyle Medicine, Jeonbuk National University, Republic of Korea.

Seed media preparation by aseptic condition

The collected pods were firstly washed under tap water, followed by surface sterilization through soaking in a 25% sodium hypochlorite solution (Cocorex) for 15 minutes. The sterilized seeds were cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with 30 g/L of sucrose and 8 g/L of agar to study seedling growth. The initial pH value was adapted to 5.8 by adding a drop of 1N NaOH or 1N HCL before autoclaving at 121˚C for 15 minutes. Media were cultured in a culture room at 25±2˚C under 16/8 hrs (light/dark light cycle hours) with cool-fluorescent white light intensity of 3000 lux.

Effect of plant growth regulators on plantlet multiplication

The plantlets from three months of seed cultured were sub-cultured on MS basal medium supplemented with different combinations of plant growth regulators (PGRs), such as 2.0 mg/L of 1-napathylacetic acid (NAA), 1.0 mg/L of 6-benzyl aminopurine (BAP) and the addition of 1g/L of activated charcoal (AC) for roots and shoots formation. The percentage of callus induction in each treatment was calculated by dividing the number of explants formed callus by the total number of explants cultured multiplied by one hundred. Out of five culture boxes, only one box was used to grow one plantlet per each treatment.

Effect of different natural extracts on subculture formation from callus culture

After culturing of nine weeks, calli were resulted from the subcultured pseudobulb explants (1.2 cm) of B. auricomum Lindl. in the prior medium. (0.23 g) of calli were sub-cultured using 11 different concentrations of natural extracts such as coconut water (CW), banana extract (BE), and potato extract (PE), which were provided to the MS+1 g/L of activated charcoal to collect a large amount of callus, as shown in Table 1.

Effects of various concentrations of plant growth regulators and natural extracts on multiple plantlets

Consequently, 2 months old of *B. auricomum* Lindl. were carried out to compare the effect of natural extracts with or without the plant growth regulators (PGRs) for *in vitro* plantlets regeneration. PLBs (protocorm-like body) (0.23 g) were grown in MS medium in various concentrations with natural extracts and plant growth regulators. For example, Natural extracts were included in the perfect medium (MS+150 ml/L of coconut water; MS+30 g/L of banana extract + 20 g/L of potato extract) from the above result data. Natural extracts in combination with plant growth regulators medium was MCBN: MS+(150 ml/L) coconut water+(2.0 mg/L) BAP+(1.0 mg/L) NAA; MBPBN: MS+(30 g/L) banana extract+(20 g/L) potato extract+(2.0 mg/L) BAP+(1.0 mg/L) NAA.

Subculture and shoot with pseudobulb multiplication

Consequently, the development of young shoot (0.23 g initial fresh weight/ shoot cluster) formed on the initiation the best medium (MCBN, and MBPBN) were subcultured for further pseudobulbs propagation plantlets experiments.

Statistical analysis

Each experiment consisted of three replicates and ten culture bottles per replicate for the *in vitro* propagation of calluses, PLBs, and shoot regeneration cultures. The data were shown as mean ± standard deviation (SD). The mean data were evaluated statistically by paired t-test and tukey's test. The difference was considered sta-tistically significant at $P<0.05$. All the statistical analyses were performed by using GraphPad Prism (version 9.3.0).

Results and Discussion

Morphology of flower and pod collection by artificial self-pollination

The inflorescence is a raceme flower (about 20-28) with peduncle that is slightly arched from the base of pseudobulbs. In this study, the fertilized flower petals turned from white to yellow color after one week. The petals gradually became dry and the flowers successfully turned into mature pods after artificial self-pollination for four months. The mature pods measured 1.0-1.5 x 7.0-9.0 cm, ovate in shape, dark green in color, and ridges appeared. The pods of *B. auricomum* Lindl. contained thousands of tiny white seeds as shown in Figure 1. The pollination rate is 50%, because 10 flowers per inflorescence were artificially pollinated.

Thakur *et al*., 2012 who reported that artificial self-pollination increased the success of capsule productions. In previous studies, pods of *B. auricomum* Lindl. from natural self-pollination were used as explants (Thet and Aye, 2018; Naing and Lim,

2011; Than, 2013). In the present study, these results indicated that artificial self-pollination is required to quickly mature pods.

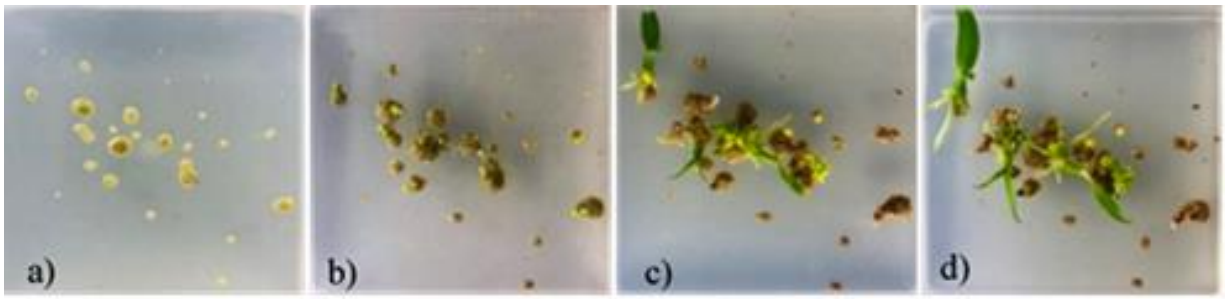

Figure 1. Developmental stages from flower to mature pod by artificial self-pollination of Bulbophylllum auricomum Lindl. a) flowering in the greenhouse; b) artificial self-pollination; c and d) fully mature pod after artificial self-pollination

Effect of germination and protocrom to plantlets formation on culture medium

Orchid seeds lack functional endosperm and thus cannot grow on their own (Rasmussen *et al.*, 2015). Hence, seeds from the four-month-old mature pod of *B. auricomum* Lindl. collected after artificial self-pollination, and were cultured in MS basal medium supplement without plant growth regulators (PGRs) for seed germination. Figure 2 shows that the formation of well-developed protocorms that eventually become plantlets with shoot and root differentiation occurred within two to three months of seed culture. Data on seed germinations were recorded after one month of cultur when the majority of viable seeds had germinated. The germination rate of seeds on the MS medium was found nearly 90%, but the development of plantlets was very slow in our study. Our results showed that *in vitro* seed germinate was stimulated on MS basal medium. Then, the growth of protocorms and ddifferentiation of roots and shoots were enhanced, but their growth was very slow.

Previous researchers (Thet and Aye, 2018; Than, 2013) who used MS medium for seed germination and subsequent differentiation of *B. auricomum* Lindl. Furthermore, MS medium is used in the seed germination of several *Coelogyne* spp., (Kaur and Bhutani, 2014). The findings of this results are in agreement with previous research.

Figure 2. Seed germination and protocorm to plantlets development of B.auricomum Lindl; a) swelling of seed after one week of culture; b) seed germination followed protocorm formation after 1 month of culturing; c, and d) shoots appeared differentiation from protocorm after 2 to 3 months of culturing.

Callus induction from subcultured pseudobulb explants

Subsequently, three-month-old plantlets of *B. auricomum* Lindl. were transferred to the MS +1.0 mg/L of BAP+ 2.0 mg/L of NAA and 1g/L of AC were also added for the developed pseudobulb plantlets. These plantlets develop into pseudobulb explants after 2-3 months of subculture. The callus is friable, and yellowish green on sub-cultured pseudobulb explants. The unorganized mass or a callus that grows in the suitable nutrient medium from the original explant. The results showed that the callus was extremely slow and difficult from the pseudobulb explants and the percentage of callus induction was 40% (Figure 3).

Previous studies have revealed that the callus culture of leaf segments of *B. auricomum* Lindl. (Naing and Lim, 2011). Callus induced on a MS basal medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) alone or in combination with different concentrations of thidiazuron (TDZ) from the bisected PLB segment of *B. auricomum* Lindl. (Than, 2013). This finding is in agreement with Sharma *et al*., 2015 who observed callus proliferation in nodal segments on NAA (2.0 mg/L)+ BAP (1.0 mg/L). The callus induction and PLBs proliferation in MS+ NAA (2.0 mg/L)+ BAP (1.0 mg/L) culture media in this study agree with references.

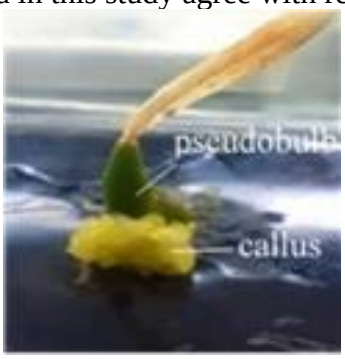

Figure 3. Callus induction from subcultured pseudobulb explants after 2 months of culturing

Effects of different natural extracts on subcultured callus and PLBs formation from callus culture

These callus masses (about 0.23g) were subcultured in a MS basal medium supplemented with 11 different natural extract treatments. Among them, MS+150 ml/L of coconut water and MS+30 g/L of banana extract+20 g/L of potato extract observed in a large amount of callus induction and PLBs formation. The results presented in Figure 4 and Table 1 show the fresh weight of callus and PLBs measured 1 month and 2 months in grams. PLBs formation were occurred on MS+150 ml/L of coconut water and yielded the highest fresh weight of (1.75±0.08) and (3.01±0.20) at 1 and 2 months, respectively (Figure 4: 2a and 2b). The second highest fresh weights at 1-month (0.67±0.09) and 2-months (1.87±0.05) of callus and PLBs were obtained by MS + 30 g/L of banana extract+20 g/L of potato extract (Figure 4: 9a and 9b). An orchid callus is thought to be extremely difficult to grow from a callus subculture. For example, subculture callus broken into small pieces, and was not achieved (Chang and Chang, 1998). However, We successfully observed numerous callus and PLBS formations in my research study.

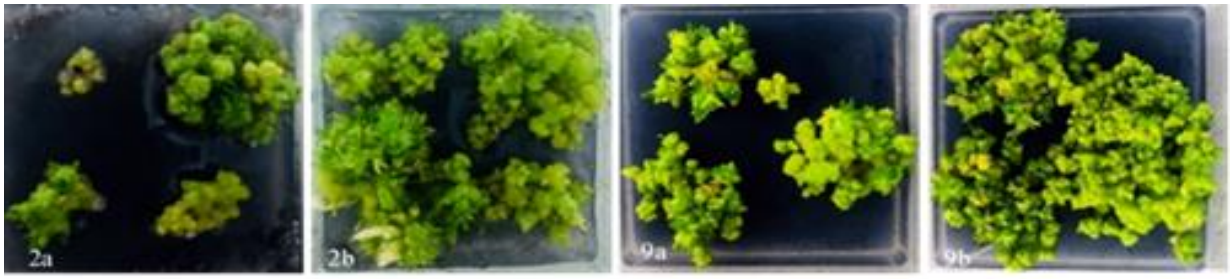

Figure 4. Effects of various combinations of natural extracts on callus induction and PLB formation in fresh weight of Bulbophyllum auricomum *Lindl. after 1 and 2 months of culture. 2a, 2b) MS+150 ml/L of coconut water at 1 and 2 months of culture; 9a, 9b) MS+30 g/L of banana extract+20 g/L of potato extract, respectively.*

Table 1. Effects of various combinations of natural extracts on callus induction and PLBs formation in fresh weights of *B. auricomum* Lindl. after 1 month and 2 months of culture.

No.	Medium concentration	callus & PLBs fresh weight (g)	
		1 month	2 months
1.	MS+ (100 ml/L) CW*	$0.12\pm0.05^{d\dagger}$	0.17 ± 0.01^{e}
2.	MS+ (150 ml/L) CW	1.75 ± 0.08^{a}	3.01 ± 0.20^{a}
3.	MS+ (20 g/L) BE**	0.08 ± 0.01^{e}	0.12 ± 0.03^{f}
4.	MS+ (30 g/L) BE	0.37 ± 0.07^{c}	0.74 ± 0.11^{c}
5.	MS+ (20 g/L) PE	0.13 ± 0.02^{d}	0.23 ± 0.02^{d}
6.	MS+ (30 g/L) PE***	0.12 ± 0.02^{d}	0.26 ± 0.02^{d}
7.	MS+ (20 g/L) BE+ (20 g/L) PE	0.13 ± 0.04^{d}	0.21 ± 0.02^{d}
8.	MS+ (20 g/L) BE+ (30 g/L) PE	0.57 ± 0.11^{b}	0.95 ± 0.19^{c}
9.	MS+ (30 g/L) BE+ (20 g/L) PE	0.67 ± 0.00^{b}	1.87 ± 0.05^{b}
10.	MS+ (30 g/L) BE+ (30g/L) PE	0.37 ± 0.04^{c}	0.78 ± 0.11^{c}
11.	MS**** Control	0.08 ± 0.028^{e}	0.28 ± 0.09^{d}

† Tukey's test results are mean ± SD and super-script letters in each column indicate statistically significant different at $P<0.05$

CW*: Coconut water; BE**: Banana extract; PE***: Potato extract

Comparative effects of various combinations of natural extracts and plant growth regulators on shoot regeneration of *B. auricomum* Lindl.

Consequently, 2 months old callus and PLBs (about 0.23 g) were used to compare the effects of natural extracts with or without the plant growth regulators (PGRs) on regeneration. Our results in Figure 5, and Table 2 showed that the highest fresh weight was observed in MS+150 ml/L of coconut water+2.0 mg/L of BAP+1.0 mg/L of NAA by (3.37±0.17) and (6.41±0.68) in 1 month and 2 months, respectively. The number of developed plantlets was 35 plantlets multiplication with per box was 10 roots long (1.6 cm). The second media was MS+30 g/L of banana extract+20 g/L of potato extract+2.0 mg/L of BAP+1.0 mg/L of NAA by (2.33±0.12) and (4.21±0.21) in 1 month and 2 months respectively. The intermediate growth was observed from a natural extract only. The MS basal medium (control) produced the lowest and slowest shoot regeneration. However, natural extract combined with plant

growth regulators (PGRs) was more effective than natural extract alone in producing a high quantity of plantlets of *B. auricomum* Lindl.

Nayak *et al*. (1998) and Sheelavantmath *et al*. (2000) who found that using BAP and NAA improved plantlet growth. For example, high concentrations of BAP and low concentrations of NAA were more efficient for shoot multiplication of *Dendrobium* orchid (Talukder *et al*., 2003) and *Dendrobium primulinum* (Pant and Thapa, 2012). In epiphytic orchids, adding 15% CW to the basal medium improves growth performance in a variety of parameters, including stimulating new shoots (Baque *et al*., 2011; Paris *et al*., 2019). Similarly, enhanced shoot development using CW has been observed in a variety of *Dendrobium* and *Cymbidium* (Parthibhan *et al*., 2015). The present study discovered that the above characteristics agree with previous research.

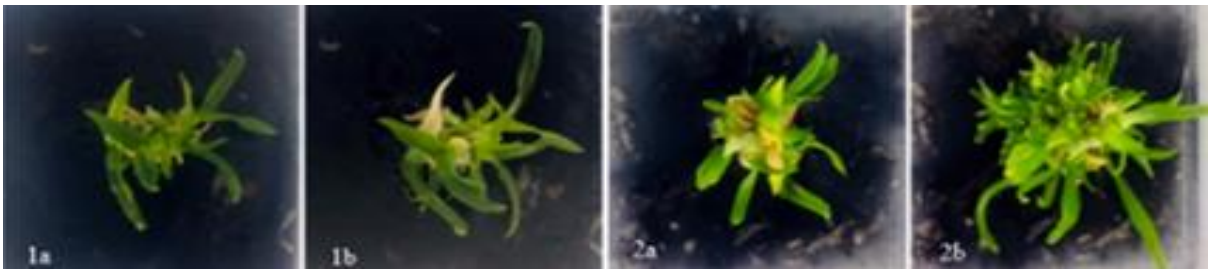

Figure 5. Comparative of various concentrations of plant growth regulators and natural extracts on shoot regeneration of B. auricomum Lindl.. 1a, 1b) MS+150 ml/L of CW; 2a, 2b) MS +150 ml/L of CW+2.0 mg/L of BAP+1.0 mg/L of NAA after 1 and 2 months of culturing respectively.

Table 2. Comparative effects of various combinations of natural extracts and plant growth regulators on shoot regeneration of *B. auricomum* Lindl. after 1 month and 2 months of culture.

No.	Medium composition	shoot fresh weight (g)	
		1 month	2 months
1.	MS+(150 ml/L) CW*	2.79±0.13^{b†}	3.79±0.25^{b}
2.	MS+(150 ml/L) CW+ (2.0 mg /L) BAP****+ (1.0 mg/L) NAA	3.37±0.17^{a}	6.41±0.68^{a}
3.	MS+(30 g/L) BE**+ (20 g/L) PE***	2.59±0.16^{b}	3.39±0.43^{c}
4.	MS+(30 g/L) BE+ (20 g/L) PE+ (2.0 mg /L) BAP + (1.0 mg/L) NAA*****	2.33±0.12^{c}	4.21±0.21^{b}
5.	MS+(2.0 mg/L) BAP +(1.0 mg/L) NAA	2.46±0.10^{c}	2.78±0.18^{c}
6.	MS****** Control	1.37±0.05^{d}	1.60±0.07^{d}

The mean ± SD of three replicate experiments was comparison between 1 and 2 months fresh weight data.

† Tukey's test results are mean ± SD and super-script letters in each column indicate statistically significant different p- value at P<0.0.

*CW: Coconut water; **BE: Banana extract; ***PE: Potato extract; ****BAP: 6-benzyl aminopurine; *****NAA: 1-napathyl acetic acid; ******MS: Murashige and Skoog

Combined effect of natural extract and plant growth regulators on subculture shoot with pseudobulb formation

In this study, MS supplemented with 150 ml/L of coconut water+ 2.0 mg/L of BAP+ 1.0 mg/L of NAA and MS supplemented with 30 g/L of banana extract+ 20 /L

of potato extract+ 2.0 mg/L of BAP + 1.0 mg/L of NAA was chosen from the above experiment. The best of different two media were subcultured on the same medium to development pseudobulb from shoot plantlets. We found that table 6 on MS supplemented with 150 ml/L of coconut water+ 2.0 mg/L of BAP+ 1.0 mg/L of NAA promote the multiple number of new pseudobulbs (5-7 pseudobulbs),pseudobulb size with mean and standard deviation (0.16±0.21in cm), the number of roots (27.33±2.52), and root length (2.11±0.38) per box; whereas MS supplemented with 30 g/L of banana extract+ 20 /L of potato extract+ 2.0 mg/L of BAP + 1.0 mg/L of NAA observed 4–6 number of pseudobulb, (0.07±0.1cm) in the size, the number of roots (25.00±2.00), and root length (1.45±0.55) after subcultured for 3 months. MS supplemented with 150 ml/L of coconut water+ 2.0 mg/L of BAP+ 1.0 mg/L of NAA promotes elongated root length compared to MS supplemented with 30 g/L of banana extract+ 20 g/L of potato extract+ 2.0 mg/L of BAP + 1.0 mg/L of NAA. Production of pseudobulbs was not significantly influenced by the application of natural extracts. On the other hand, plants treated with coconut water had slight increase in the number of pseudobulbs (Ranawake *et al*., 2016). As a result, the current study agrees with previous research.

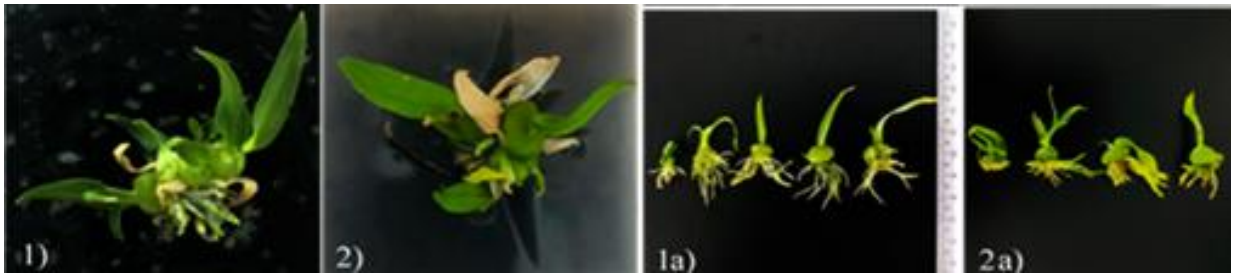

Figure 4. Effects of different concentrations of natural extracts and plant growth regulators on pseudobulb formation of fresh weight of B. auricomum Lindl. after 3 months of culture. 1, 1a) MS+ 150 ml/L of coconut water+ 2.0 mg/L of BAP+ 1.0 mg/L of NAA; 2, 2a) MS+ 30 g/L of banana extract+ 20 g/L of potato extract+ 2.0 mg/L of BAP + 1.0 mg/L of NAA.

Table 3. Effects of various combinations of natural extracts and plant growth regulators on pseudobulb regeneration of *B. auricomum* Lindl.

No.	Number of shoots/ plantlets	Size (width) of pseudobulb (cm)	Length of pseudobulb (cm)	Root length (cm)	Number of root
1.	7.00 ± 2.00^{a}†	0.16 ± 0.21^{a}	0.16 ± 0.21^{a}	2.11 ± 0.38^{a}	27.33 ± 2.52^{a}
2.	5.33 ± 1.53^{b}	0.07 ± 0.10^{b}	0.07 ± 0.10^{b}	1.45 ± 0.55^{b}	25.00 ± 2.00^{b}

† Tukey's test results are mean ± SD and super-script letters in each column indicate statistically significant different at $P<0.05$.

1. MS+(150ml/L) CW* +(2.0 mg/L) BAP****+ (1.0 mg/L) NAA*****

2. MS+(30 g/L) BE**+ (20 g/L) PE***+(2.0 mg/L) BAP+(1.0 mg/L) NAA of culture medium composition

*CW: Coconut water; **BE: Banana extract; ***PE: Potato extract; ****BAP: 6-benzyl aminopurine; *****NAA: 1-napathyl acetic acid

Conclusion

In this research, it is concluded that four months old of maturity pods were collected as explant after artificial self-pollination. These pods can successfully have large numbers of seeds germination of *B. auricomum* Lindl. orchid by MS basal medium. And then, calli were observed from subcultured pseudobulbs on MS + 1.0 mg/L of BAP + 2.0 mg/L of NAA. The addition of 150 ml/L of coconut water, and/or a combination of 30 g/L of banana extract and 20 g/L of potato extract produced a maximum of callus-induced callus. The replacement of plant growth regulators is made possible by this natural extract. The combination of natural extracts and plant growth regulators can efficiently support shoot induction, multiplication, and multiple root formation in our result. In addition, the growth effects of pseudobulb were obtained in MS medium supplemented with coconut water and plant growth regulators and/or in MS medium supplemented with the combination of banana extract and potato extract with plant growth regulators. This result suggested that the system could be used for micropropagation as a substitute for the conventional method of plant regeneration from lateral shoot culture of the pseudobulb. This formation can be further used for mass multiplication and large-scale pseudobulb size of *Bulbophyllum* species and other orchid species.

Acknowledgments

Firstly, I would like to extend my gratitude to my supervisors Professor Bang Keuk Soo for my research. I sincerely thank Professor Myat Myat Moe, Professor and Head Tin Moe Aye, and Professor Yee Yee Thu for their support and mentorship to build my professional career.

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