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Optimizing conditions for shoot multiplication of *Anoectochilus formosanus* Hayata through protocorm-like body in *in vitro* condition

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Anoectochilus formosanus Hayata is a valuable medicinal herb used in traditional remedies of Vietnam based on its high medicinal properties. Due to over-exploitation, this plant is disappeared rapidly in wild. The traditional propagation of this plant is not effective because of the low multiplication efficiency. Furthermore, the quality plantlets produced through traditional method such as stem cutting or plant separation are highly variable resulting inconsistency in herbal products. In this study, several parameters for *in vitro* multiplication of *A. formosans* through protocorm-like body (PLB) were examined with the aim of contributing to the conservation and economic development of the plant. The obtained results showed that the PLB formation is most suitable in Murashige Skoog medium (MS) supplemented with 0.5 mg/L kinetin in shaken condition whereas the Schenk Hildebrandt medium (SH) is suitable for the PLB development. SH medium added with 0.5 mg/L BA is found to be the most suitable for shoot multiplication from PLB. This study could be useful for rapid propagation of conservation programs or mass production to meet the market demand of this herbal plant.

Keywords: *Anoectochilus formosanus,* jewel orchid, micro propagation, protocorm-like bodies

INTRODUCTION

Anoectochilus formosanus Hayata is a rare species of orchid with high medicinal and economic value, which widely distributes in tropical regions of Asia such as China, India, Laos, Indonesia and Vietnam. This plant is commonly called as “jewel orchid” due to its beautiful venation on the leaves. According to traditional medicine, fresh or dried *A. formosanus* were used to treat several diseases such as pleurodynia, cardiovascular diseases, hypertension, fever, nephristis, bruises and poisonous snake bites. Plant products are applied as dehumidification and detoxification which show effects in treating hyperactivity cough, tuberculosis vomiting blood, hematuria, hypertension, pediatric shock, tetanus, and nephritis edema (Ho et al. 2018). Extracts of *A. formosanus* have also been reported to prevent CT26 colon cancer in mice (Yang et al. 2014). The extract of this plant possesses different biological activities including antioxidant, antidiabetic, antitumour and immunostimulatory, anti-inflammatory, hepatoprotective, renal protection and anti-hyperlipidemia effects (Tang et al. 2018). Several medicinal compounds were also characterized from this plant such as pharmaceutical constituents including flavonoid, polysaccharides, glycoside derivative kinsenoside, and steroids (Yang et al. 2022).

The existence of *A. formosanus* is currently being threatened by its low seed germination rate, habitat loss and degradation, animal consumption, indiscriminate collection, and increasing demand by humans (Zhang et al.2013). At present, *A. formosanus* is included in the list of endangered species in group IA of Decree 32/2006/CP of Vietnam government for being prohibited from commercial exploitation and classified as endangered forest plants (EN A) 1 a, c, d) in the Red Book of Vietnam. Realizing the potential, economic value of this plant, numerous studies on *in vitro* propagation have been carried out by several research groups in order produce large quantities for conservation and to supply raw materials continuously and stably for pharmaceutical industry. Since *in vitro* propagation allows the regeneration of huge number of shoots or organs from limited starting tissues such as leaves, stems, flowers or roots. This technique overcomes the limitations of conventional propagation methods such as production of homogenous, disease-free seedlings, good growth, high yield, and a high yield in short time, meeting the needs of large-scale planting. Therefore, several studies to optimize

the *in vitro* protocols for propagating *A. formosanus*. In 2002, Shiau and colleagues developed method to conserve this plant through artificial cross-pollination and in vitro culture of seeds (Shiau et al. 2002), Winarto and Samijan developed mass propagation *in vitro* using shoot tips (Winarto and Samijan, 2018).

In Vietnam, there are various studies to develop the *in vitro* propagation programs for different jewel orchid species belonging to *Anoectochilus* genus such as *A. setaceus* Blume (Nguyen and Phi, 2012), *A. lylei* Rolfe ex Downnies (Phan et al. 2016) and *A. roxburghii* (Wall.) Lindl. (Nguyen and Mai, 2019). Presently, orchid micropropagation based on protocorm-like bodies (PLBs) got great attention as the PLB-produced plants resemble somatic embryos in formation and development (Lee et al. 2013). Diverse work on applying PLB to multiply different orchids have been published such as *Dendrobium aqueum* (Parthibhanet al. 2015), *Rhynchostylis gigantea* (Prasongsomet al. 2016), *Dendrobium chrysotoxum* (Kaur, 2017), *A. roxburghii* (Wang et al. 2022). Nevertheless, research on rapid multiplication of *A. formosanus* through induction generation is still scant. Giap and colleagues surveyed the effect of different organic extracts on quality and quantity of *A. formosanus* biomass (Giap et al. 2018). This plant was also *in vitro* propagated directly from shoot (Phan and Nguyen, 2017; Nguyen et al. 2020). Therefore, this study was carried out with the aim of establishing a rapid multiplication process of *A. formosanus* through induction of PLBs generation in order to increase the shoot multiplication capacity. The obtained results would provide scientific and practical bases to preserve and develop this rare medicinal plant.

MATERIALS AND METHODS

Materials

Thirty-day old *A. formosanus* plantlets grown in *in vitro* condition provided by the Laboratory of Faculty of Biology and Environment – Ho Chi Minh City University of Food Industry (Vietnam) was utilized for experiments. Three culture media consisted of Murashige Skoog (MS), Schenk Hildebrandt medium (SH), and Knudson C media were supplemented with 10% coconut water and 30 g/L sucrose. Depending of experiments, different concentrations of plant hormones and nutrition were supplemented namely 6-Benzyl Adenin (BA), kinetin, and agar. The pH values of media were adjusted to 5.8 before autoclaving at 121°C for 15 min.

Investigate the effect of kinetin and medium condition on PLB formation

The young shoots were cut into 4 mm fragments and inoculated into MS medium containing 30 g/L sucrose supplemented with kinetin at different concentrations: 0; 0.25; 0.50; 0.75; 1.00 mg/L to induce PLB generation. In this experiment, shoot fragments were cultured in three different medium systems: solid medium with agar added at 8 g/L, stationary liquid, and shaken liquid using orbital shaker at 120 rpm. PLB formation rate (%) and number of PLBs per bottle were counted after 30 days of culture.

Investigate the effect of mineral media on PLB development

PLB clusters (weighing approximately 0.15 g per bottle) obtained from best condition of previous experiment (shaken liquid and 0.5 mg/L kinetin) were sub-cultured to different mineral media namely MS, SH and Knudson C. Fresh weight of PLB (g) and PLB regeneration coefficient were measured after 30 days of culture.

Investigate the effect of BA concentration on shoot formation from PLB

PLB clusters from SH medium of previous experiment (weighing approximately 0.15 g per bottle) were continued sub-culturing in SH medium with different BA concentrations: 0; 0.5; 1.0; 1.5 and 2.0 mg/L to induce shoot formation. Number of shoots per bottle and fresh weight of shoots (g) were recorded after 45 days of culture.

Investigate the effect of the medium condition on shoot development from PLB

Shoots were inoculated on SH medium supplemented with optimum BA concentration from previous experiment. Amount of 1.0 g of initial shoot weight were cultured in each bottle. Samples were then cultured in four different medium conditions: solid medium with agar concentration at 8 g/L, stationary liquid, shaken liquid using orbital shaker at 120 rpm, and liquid medium with the addition of cotton wool. Fresh weight of shoots (g) and shoot regeneration rate (times) were recorded after 30 days of culture.

Culture conditions and data analysis

Culture conditions of all treatments were maintained at 23 ± 2^{o}C under 12-hour photoperiod with 2500 ± 200 lux light intensity using fluorescent light. Culture room humidity was maintained at 55-60%. The experiments were arranged in a completely randomized design with three replications in 500 ml glass bottles containing 100 ml medium. The collected data were subjected to analysis of variance (ANOVA), the mean values were further separated through Duncan's multiple range test at the confidence level of $p < 0.05$ using the STATGRAPHICS Centurion XV statistical software.

RESULTS AND DISCUSSION

Effect of kinetin and medium condition PLB formation

PLBs play an important role in the propagation of orchids in general. In this experiment, stems of *A. formosanus* plantlets were fragmented into 4 mm and then cultured in three different culture conditions (shaken liquid,

stationary liquid, solid) with kinetin added at four concentrations of 0.0; 0.25; 0.5 and 1.0 mg/L to determine the best culture conditions and kinetin concentrations on PLB formation. After culturing 30 days, the PLB formation rate and PLB number per stem fragment were variable among treatments and detail data are shown in Table 1 and Figure 1.

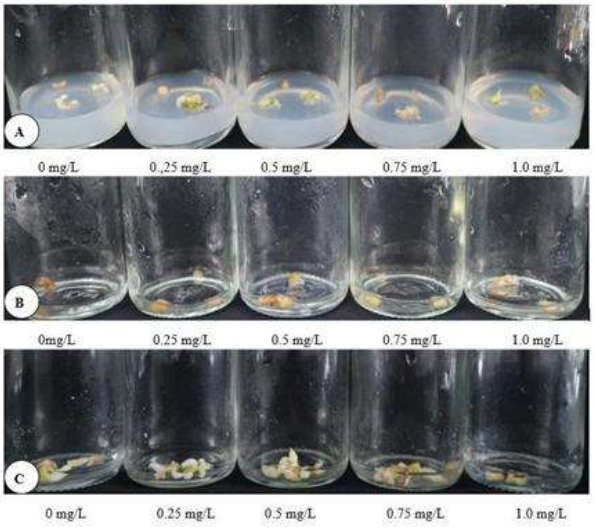

Figure 1: Effect of kinetin and culture system on the induction of PLB formation of *A. formosanus* after 30 days (A: Solid; B: Stationary liquid; C: Shaken liquid).

In stationary liquid media, shoots were submerged in the medium resulting in death of shoots and no PLB formation regardless the addition of kinetin (Figure 1B). On the other hand, on solid and shaken media, the number of PLBs were formed and increased correspondingly with kinetin addition. Furthermore, PLB morphology is healthy, with plenty of shoots (Figure 1A and Figure 1C). Nevertheless, most of monitored parameters increased and got peak at 0.5 mg/L of kinetin and no significant difference were found between these two medium conditions except the PLB formation rate in solid medium is highest at 0.75 mg/L. When kinetin concentrations were higher, PLB formation and number PLB per sample decreased. Thus, when kinetin concentration is higher than 0.5 mg/L, PLB formation could be inhibited. The results of this study are in line with previous study of Tran et al. (2015), when kinetin supplementation with a concentration of 0.5 mg/L in the culture medium was showed as the best ability to induce PLB generation from *A. setaceus* shoots. Nevertheless, the formation of *A. roxburghii* required much higher concentration of kinetin at 1.5 mg/L (Zhang et al. 2015).

In more detail observation, at 0.5 mg/L kinetin, the shaken liquid culture medium was the most optimal (Figure 2), the number of PLBs formed is highest (4.8 PLB/sample), PLBs show good quality, strong, and white-green colour (Figure 2B). It could be explained that in a shaken liquid medium, the samples were exposed to the culture medium on all sides at the same time. This condition promotes gas exchange, enhances the ability to absorb nutrients and hormones of the culture. When cultured in a stationary liquid medium (Figure 2A), the samples were submerged in the medium resulting in oxygen deficiency which could cause mass death of the explants. Although the generated PLB per sample was also high (3.7 PLBs/sample) in solid medium (Figure 2C), this value is still lower than that in the shaken liquid medium. The solid medium can serve as a support for the tissue in the meanwhile it could reduce the exposure of the culture to nutrient uptake.

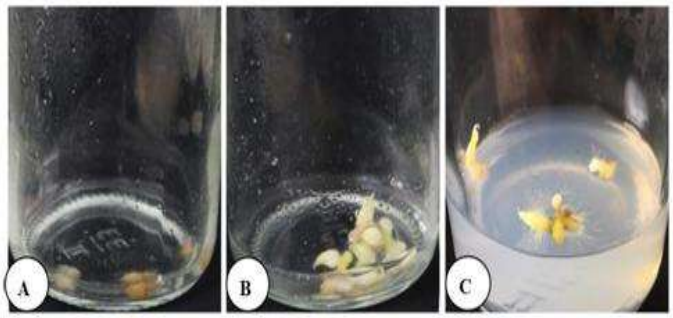

Figure 2: Effect of culture system supplemented with 0.5 mg/L kinetin on the induction of PLB formation *A. formosanus* after 30 days of culture (A: Stationary liquid; B: Shaken liquid; C: Solid media).

Effect of mineral media on PLB development

Mineral composition is an important chemical factor for plant growth under natural conditions which play an important role in the growth of *in vitro* cultured plants. The types of culture media contain most of the nutrients necessary for plant growth including macronutrients, trace minerals, vitamins and mineral content in each type of medium. However, previous publications on plant tissue culture of jewel orchids showed the inconsistency results when different mineral medium were used for various species in *Anoectochilus* genus. For examples, Zhang and colleagues reported that half-strength MS medium is most suitable for *in vitro* propagation of *A. roxburghii* (Zhang et al. 2015), full strength MS medium was reported as the most suitable for PLB induction and proliferation, shoot formation and rooting of *A. elatus* (Sherifet al. 2017) or *A. roxburghii* (Wang et al. 2022). In this experiment, the suitability of three mineral media namely SH, MS and Knudson C for *A. formosanus* PLB development were tested, the results obtained after 30 days of culture are presented in Table 2.

Table 1: Effect of kinetin concentration and culture system on PLB formation of *A. formosanus* after 30 days.

Medium condition	Parameters	Kinetin concentration (mg/L)				
		0	0.25	0.50	0.75	1.0
Solid	PLB formation rate (%)	61.3^{d}	84.0^{c}	96.0^{ab}	97.3^{a}	90.7^{b}
	PLB number per stem fragment	1.3^{c}	2.8^{b}	3.7^{a}	2.8^{b}	2.7^{b}
Stationary liquid	PLB formation rate (%)	0	0	0	0	0
	PLB number per stem fragment	0	0	0	0	0
Shaken liquid	PLB formation rate (%)	76.0^{c}	97.3^{a}	100^{a}	88.0^{b}	21.3^{d}
	PLB number per stem fragment	1.9^{d}	3.8^{b}	4.8^{a}	2.5^{c}	1.4^{e}

* The letters a, b... in the same row indicate the significant difference among the treatments with $p < 0.05$ in Duncan's test.

Table 2: Effect of mineral medium on the PLB proliferation of *A. formosanus* after 30 days.

Medium	Weight after 30 days (g)	Regeneration coefficient (times)
SH	0.64^{a}	4.27
MS	0.48^{b}	3.20
Knudson C	0.32^{c}	2.13

* The letters a, b... in a same row indicate the significant difference among treatments with $p < 0.05$ in Duncan's test.

The obtained results showed that PLBs formed shoots in all three types of mineral media (Figure 3). Among which, the sample weight after culture 30 days increased highest in SH media, reaching the top value of 0.64 g with multiplication coefficient up to 4.27 times. In this medium, PLBs generated vigorous shoots and green leaves (Figure 3A). In MS medium, the sample weight after inoculation was lower than that of SH mineral medium, only 0.48 g with regeneration coefficient of 3.2 times. The PLB cluster also induced shoot formation, the shoots were healthy but slower growing (Figure 3B). On Knudson C medium, the lowest sample weight was observed at only 0.32 g with regeneration coefficient of 2.13 times. Although in this medium PLBs also showed shoot formation, the shoots look weak and blackened (Figure 3C). Although in plant micropropagation, MS mineral medium is commonly used for micropropagating of different jewel orchid species such as *A. roxburghii* (Zhang et al. 2015), *A. elatus* (Sherif et al. 2017), *A. formosanus* (Winarto and Samijan, 2018) because of its richness in nitrogen both nitrate and ammonium. However, this medium possesses the nitrogen-rich composition which is not suitable for the growth of *A. formosanus*, therefore the weight of *A. formosanus* PLB obtained in MS medium of our study was lower than in SH medium. Knudson C is also not suitable for the growth of this orchids which could be due to the nutrient-poor environment properties of this medium.

Figure 3: Effect of mineral medium on the proliferation of *A. formosanus* PLB after 30 days in different medium SH (A); MS (B) and Knudson C (C).

Do et al. (2015) also reported that SH medium is suitable for shoot induction and shoot growth of *A. setaceus* Blume.

Effect of BA on shooting formation from PLB

Cytokinin is well known to be a group of growth regulators stimulating cell division, the formation and growth of shoots *in vitro*. To increase the shoot multiplication factor, the cytokinin was supplemented into the culture medium to enhance the multiplication rate of shoot. Several types of cytokinin are commonly used in shooting stage such as BA, kinetin, TDZ. In this experiment, the shoot formation from PLBs under BA added conditions is shown in Table 3 and Figure 4.

Table 3: Effect of BA on shoot formation from PLB of *A. formosanus* after 45 days

BA concentration (mg/L)	Shoots per bottle	Fresh biomass of shoot (g)
0	11.80^{c}	4.18^{b}
0.5	21.80^{a}	5.90^{a}
1.0	20.60^{ab}	5.89^{a}
1.5	20.40^{ab}	5.82^{a}
2.0	19.40^{b}	5.62^{a}

* The letters a, b... in the same column indicate the significant difference between the treatments with $p < 0.05$ in Duncan's test.

After 45 days of culture, PLB clusters on all media induced shoot formation. In the medium without BA addition, the number of shoots formed and fresh shoot weight are lowest with 11.8 shoots per bottle and 4.18 g per shoot, respectively. New shoots were also not observed in this treatment (Figure 4A). When BA was added to the medium, the number of shoots and the fresh

weight of shoots increased significantly. Thus, BA could have a markable effect on the formation of *A. formosanus* shoots. When 0.5 mg/L BA was added the culture medium, the growth of *A. formosanus* shoots increased up to 21.8 shoots per sample and average of 5.9 g per shoot. New formed shoots look healthy with dark green leaves (Figure 4B). Continuing to increase the concentration of BA at 1.0 - 1.5 mg/L, the number of shoots and the fresh weight of the shoots obtained were not statistically different compared with the additional treatment at 0.5 mg/L BA (Figure 4C and Figure 4D). When concentrations of BA were increased up to 2.0 mg/L, the growth and development of *A. formosanus* shoot did not rise correspondingly. Even the too much BA could cause the weakness of shoots (Figure 4E). The shoot development in this experiment is also similar to the results of Nguyen and Phi (2012) when using 0.5 mg/L BA in research on propagation of *A. setaceus* Blume *in vitro* condition.

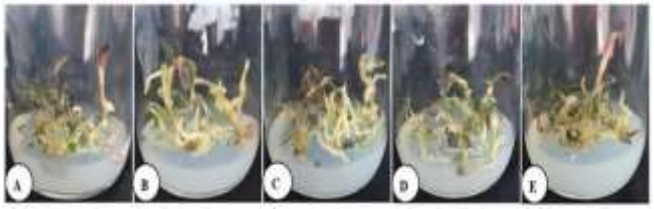

Figure 4: Effect of BA on shoot regeneration from *A. formosanus* PLB after 45 days of culture (A: 0.0 mg/L; B: 0.5 mg/L; C: 1.0 mg/L; D: 1 ,5 mg/L; E: 2.0 mg/L).

A. formosanus is jewel orchid species with a relatively slow growth rate, current researches mainly conduct rapid multiplication of shoots through shoot culture (Giap *et al.* 2018). The shoot multiplication directly from shoot is usually lower than that of the culture from PLBs. In this study, from an initial PLB cluster containing about 4 - 5 PLBs, approximately 20 shoots were formed while if using directly stem, only 1-2 shoots per sample could be produced. The obtained results show the potential of rapid multiplication of *A. formosanus* shoots through PLB culture.

Effect of the medium condition on shoot development from PLB

In plant micro propagation, medium conditions play crucial roles in the growth and development of regenerated explants. In this experiment, *A. formosanus* were cultured in SH medium supplemented with 0.5 mg/L BA under four culture conditions consisting of shaken liquid, stationary liquid, liquid with cotton wool and solid with agar in order to find out the appropriate culture conditions on the ability to rapidly multiply shoots of *A. formosanus.*

Table 4: Effect of culture system on shoot multiplication of *A. formosanus* after 30 days.

Medium condition	Harvested weight (g)	Multiplication coefficient (times)
Shaken liquid	6.88[a]	6.88
Stationary liquid	6.12[b]	6.12
Liquid with cotton wool	6.26[b]	6.25
Solid with agar	1.45[c]	1.44

* The letters a, b... in the same column with each shoot type indicate the significant difference between the treatments with $p < 0.05$ in Duncan's test.

After 30 days of culture, the obtained results showed that the shaken liquid was the most suitable for the rapid multiplication of *A. formosanus* shoots (Table 4).

When cultured in solid medium, the shoot biomass obtained after culture is lowest with an average of 1.45 g and multiplication coefficient of 1.44 times, respectively. In addition, the shoot morphologies are stunted, small thin, yellowish leaves (Figure 5A).

Figure 5:Effect of culture medium system on the shoot proliferation of *A. formosanus* after 30 days of culture.

Although agar solid medium helps to support plants, it could limit the contact of the explants to nutrients, so the explants can only use nutrients beside them resulting in stunted plants. Three remaining media showed the significantly increase biomass and new shoot formation, nevertheless, shaken liquid revealed the highest value in both parameters. *A. formosanus* is a moisture-preferring plant so that the shoot multiplication in liquid media is faster than that of solid medium. The results of this experiment are similar to the study of Phan and colleagues (2017) who also studied the effects of liquid medium in micropropagation of another species in *A. annamensis* which is also a jewel orchid genus. The results showed that the growth ability of the shoots cultured in shaken liquid is two times higher than in stationary liquid and semi-solid media with the same medium composition. Nevertheless, Do and colleagues reported that liquid medium with cotton wool is most suitable for development of *A. setaceus* Blume (*Do et al.* 2015). Thus, depending on jewel orchid species, the culture conditions have different effects on the growth and development and investigation the most suitable medium condition for each species is necessary.

CONCLUSION

In conclusion, investigating the optimum condition for micropropagation of *A. formosanus* is important step for conservation and development of this herbal plant. The obtained results show that the kinetin concentration at 0.5 mg/L is suitable for the formation of PLB of *A. formosanus* with culture in shaken condition and SH medium is suitable for the proliferation of PLB. To enhance the formation and rapid multiplication of shoots, adding BA at

a concentration of 0.5 mg/L to the medium is suitable and use shaken liquid media will promote the shoot development. This study provides useful information for *in vitro* propagation of *A. formosanus*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

THT: Conceptualization, Methodology, Laboratory work, Writing and editing the manuscript. TTTN: Sample collection, Data analysis, Laboratory work. TTT: Laboratory work, Writing - original draft. DGD: Laboratory work, Writing - original draft. TKAN: Laboratory work, Writing - original draft. MPN: Laboratory work, Writing - original draft. VTH: Conceptualization, Methodology, Supervision, Writing and editing the manuscript.

All authors read and approved the final manuscript.

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