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RESEARCH ARTICLE

Efficient micropropagation of *Dendrobium aurantiacum* from shoot explant

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ABSTRACT

Embryogenic tissue culture (seed tissue culture) is a common practice in plant industry to speed up and mass production. However, the culture method is not widely adopted in most of the orchid species. Micro-size orchid seeds are difficult to obtain and collect due to ambiguous seed maturation period. Most of orchid seeds have no endosperm and highly dependent on the specific fungi for germination and survival. Micropropagation from shoot culture with meristem tissue is potentially be another alternative for mass propagation of orchid. Therefore, this study examine the potential of micropropagation technique by shoot culture in orchid, *Dendrobium aurantiacum* (F. Muell.) F. Muell. This study reported an effective aseptic technique to develop sterilized *D. aurantiacum* tissue *in vitro*. The callus induction and regeneration from shoot explant by utilization of different plant growth regulator had been examined in this study. Among the treatments, 20% sodium hypochlorite with 15 mins sterilization period showed the highest sterilization efficiency on explants with only 16.7±5.8% of contamination occurred after two weeks and obtained highest survival rate 73.3±5.8% after one month. Callus formed in all combinations of plant hormone treatments. Media treated with 10 mg/L 2,4-D showed the highest callus induction rate but browning condition occur after 3 months of culture. Cell count on callus proliferation showed a significant difference ($p < 0.05$) between control and treatments. In conclusion, micropropagation of *D. aurantiacum* had been shortened almost 9-12 months required for nature germination.

Introduction

Orchids are widely cultured for horticulture and medical purposes. Apart of its ornamental value, orchids has wide application such as raw material for production of medicine, facial beauty products and foods, orchids are thus had high trade market domestically and internationally (1, 2). *Dendrobium* orchids are one of the genus that are popular for its commercial and medical values in cut and potted flower industries (3). *Dendrobium aurantiacum* (F. Muell.) F. Muell. also known as “Shihu” or “Huangcao” in China province and it is widely distributed in temperated and tropical regions such as Southern China, South Asia, Taiwan, Laos and Burma. *Dendrobium aurantiacum* is a growing lithophyte or epiphyte orchid that owned size ranged from a medium to large size (4). *Dendrobium aurantiacum* is a medicinal orchid which play an important role in traditional Chinese medicine for cough relief with lung moisturizing, immunomodulatory effects, antipyretic and anti-aging effects (5). The species contained a variety of secondary metabolites such as phenolic compounds (phenanthrenes, bibenzyls, fluorenones), alkaloids, anthraquinones and sesquiterpenes (6).

However, orchids are often grown in low densities in nature and possess a long life cycle, especially during seed germination. Many orchids

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take up to 5-7 years from seed to flowering stage and some hybrid orchids are about 3 years to complete growing process (7). Seed contained endosperms that serve as important food storage especially for the seed germination and protocorm development before the seed can carried out photosynthesis process. However, majority of the orchid seeds are lacking endosperms. Therefore, they are very much depended on specific fungal associations for germination and food supply. Due to the extremely specific symbiotic relationship, orchids are difficult to germinate and thus having a very low germination rate (8). With the introduction of plant tissue culture technique, the need of mycorrhiza symbiotic is eliminated and hence orchids can be massively propagated *in vitro*. This is advantageous to pharmaceutical industry that use orchids as raw material in bulk quantity.

Plant tissue culture is one of the plant biotechnology techniques used for the mass propagation of plants with the utilization of totipotency and high plasticity properties of plant cell (9). Callus formation and callus regeneration are the special abilities of plants to rebuild themselves after injury. This developmental plasticity is existing only in some animals' and it is remarkable in most of the plants. A complete plant can be constructed starting from a single cell. Micropropagation has several important uses in the production and conservation of rare, endangered, threatened, economically important and disease-free orchid species that are difficult to propagate through traditional horticultural methods (10). As orchids require long maturity time to blossom, micropropagation supports the regeneration from various vegetative parts of mature plants. In fact, by repeated sectioning and subculture, millions of plants can be produced from a single bud within one year (11).

Plant micropropagation is extremely efficient for the synthesis of high-quality plant based pharmaceutical products. The most common explant used in plant tissue culture was embryogenic germination with the use of seed. However, the problem faced by orchid species was the difficulty in obtaining dust-like seed as the seed collection is highly associated with the flowering season and it's also very time-consuming. Therefore, in this study, we focused to examine the use of shoot as explant for micropropagation and further explore its potential to grow and its interaction with plant growth regulators. This study could provide optimum sterilisation method for plant tissue culture and useful data for the effect of plant hormones on callus induction and regeneration of *D. aurantiacum*.

Materials and Methods

Chemical List

The sterilization agents used 70% ethanol (diluted from 95% ethanol, Universal Science Trading), Clorox household bleaching agent (6.0% sodium hypochlorite) and commercial detergent (Decons 90). The chemical used for media preparation including KNO_3, NH_4NO_3, $MgSO_4.7H_2O$, $CaCl_2.\ 2H_2O$, $NaHPO_4.2H_2O$, H_3BO_4, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, KI, FeEDTA, myo-inositol, Thiamine-HCl, Pyrodoxine, Nicotinic Acid, Sucrose, and Phytagel. The plant growth regulator (PGR) used is 2,4-Di-chlorophenoxy-acetic acid (2,4-D) and Kinetin (6-furfuryl aminopurine, KIN). All the media preparation chemical and PGR used are purchased from Sigma- Aldrich (M) Sdn Bhd.

Orchid Sources

Orchid *Dendrobium aurantiacum* was selected in this study. *D. aurantiacum* was also known as *D. denneanum*, had been listed in Taxonomy ID 181002 in the NCBI Taxonomy (12). *D. aurantiacum* is widely distributed in Southern China, South Asia, Laos and Burma (5). The plants were collected from Nurseri Harapan, Terengganu, Malaysia with a GPS coordinate of 5°16'03.6N, 103°10'35.2'E (Fig. 1). The plants were kept in the greenhouse prior to shoot cutting for tissue culture.

Fig. 1. *Dendrobium aurantiacum* orchid. **1a**: the whole plant. **1b:** flower.

Optimization of Sterilization Technique

Orchid shoot was used as explant for tissue culture. The shoots of explant were excised cut at region between nodes. The white external layer of dead cells around the shoot explants were rubbed with hand and immersed in commercial detergent (Decons 90) for 30 mins. The explants were then rinsed with running tap water for another 15 mins and subsequently washed with double sterilization agent (70% ethanol and Clorox bleaching agent). The experimental design was set as in Fig. 2. There are 30 replicate for each treatment in order to test the optimal sterilization effect in orchid shoot explant.

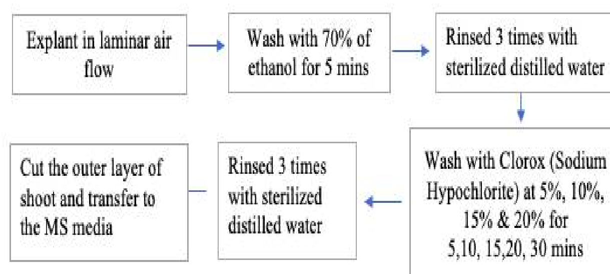

Fig. 2. The flowchart showing overall experimental design for the optimization of sterilization process.

The optimization of sterilization was observed based on the contamination rate after 2 weeks, survival rate after one month and browning rate of the shoot explant.

Media Preparation

Murashige and Skoog medium (MS medium) was were prepared based on the specific formulation composition for callus induction and proliferation (13). M3S mixture was then added with 2.5 g/L of phytagel to solidify the medium. The pH of the medium was then adjusted to pH 5.7± 0.2 and well mixed with hot plate stirred. The MS media is then sterilized at 121 ^{0}C for 15 min in an autoclave (Hirayama HVE-50 series, Amerex Instruments, Inc). Plant hormone (Table 1) was added into the media once the temperature had been cooled down to 50-60 ^{o}C. The media were poured into petri dish in the laminar air flow chamber BBS-V1300/1800 (Biobase Biodustry Co., Ltd).

Table 1. Media treatment for callus induction and callus proliferation

Treatment	Callus induction	References
A	MS (Control)	(8)
B	MS + 1.0 mg/L 2,4-D + 0.5 mg/L KIN	(26)
C	MS + 10.0 mg/L 2,4-D	(19)

Note: 2,4-D represent 2,4-Dichlorophenoxyacetic acid and KIN represent kinetin.

Callus Induction and Callus Proliferation

Plant hormone were used to initiation the formation of mass of differentiate cells called callus by using different concentration or types of hormones. In this study, three treatments had been tested for shoot induction *of Dendrobium aurantiacum* (Table 1). The orchid explants was sub-cultured every one month with the same hormone treatment for the callus proliferation. The observation parameters such as callus formation period, callus induction rate, colour of callus formation, callus type and browning condition had been observed and recorded after one month period of the callus.

Cell count

For better understanding on the effect of hormone to the callus proliferation rate, cell count in liquid suspension culture had been performed. The callus obtained in the agar plate were transferred into liquid MS media with the same hormone treatment as in Table 1 in 100 ml conical flask and allow evenly agitated with the orbital shaker for one week period. The concentration of callus cell in each treatment were measured using automated cell counter (Thermofisher) on Day 1 and Day 7.

Somatic Embryogenesis

The somatic embryo of *Dendrobium aurantiacum* at different growth stage was observed in the light microscope. A 5 ml volume of cells from liquid suspension culture were pipetted and transferred into a glass slide and then covered with cover slip. The glass slide was then observed under light microscope. The observation was carried out every week to check the somatic embryo process.

Data Analysis

The sterilization and callus proliferation data were subjected to analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) Version 20. Significant difference between the treatments were analysed by ANOVA and Post Hocs Tukey test for comparison by mean values of treatment $p<0.05$. The homogenous subset group of each treatment have determined from SPSS and labelled with small letter of alphabets.

Result and Discussion

Optimization of Sterilization Effect of Clorox Sterilization Agent

The contamination rate of explants after two weeks and the survival rate of explants after 1 month were recorded (Table 2). During cultural transfers, dust particles containing microorganisms are continuously contact with cultural media, which act as enrich resources that promote their flourishing growth (14). Hence, endogenous microbial contamination may occur easily if proper sterilization techniques does not adopted.

The optimal sterilization requirements of shoot explant was ethanol 70% for 5min following to 20% clorox for 15 mins, with approximately 83.3% and no browning condition observed on the explants. Commercial clorox contained sodium hypochlorite (NaOCl) had been widely known as cheap and efficient universal disinfectant in plant tissue culture. NaOCl can dissolved in water to produce HOCl and OCl^{-} ions. The molecular form of HOCl can destroy the microorganisms and pathogens by penetrating their cell walls, breaking their slimy and protective layers, and finally resulting in reproductive failure (15, 16). Therefore, direct contact of the explant with the disinfectant during sterilization will increases the percentage of sterile culture (17). Low contamination rate of explants were observed with high dose of sodium hypochlorite. Due to rapid degradation, small dose of sodium hypochlorite does not cause toxicity to shoot explants (18). Nevertheless, the concentration of Clorox should be kept in the lowest possible rate because our result also show that high concentration of Clorox even provide higher sterilization efficiency but have negatively causing browning on plant cells that lead to poor survival rate.

Effect of Hormone on Callus Induction and Proliferation of *Dendrobium aurantiacum*

Since auxin could benefit to the callus induction, the effect of applying high amount of 2,4-D, and the combination of 2,4-D with KIN were examined (Table 3). Callus were 100% successfully induced from all the media combination (Fig. 3). The results showed that treatment C required the shortest time for callus formation where tiny callus started to develop at 3 days under stereomicroscope observation, followed by treatment B and A, which took 5 and 7 days respectively (Fig. 3). White, compact and hard-textured callus was found in all the control and treatments. After three months observation, there were no

Table 2. Sterilization effect of chlorox on the shoot explant of *D. aurantiacum*

Treatment	Chlorox concentration (%)	Time (min)	Contamination Rate (%)	Survival Rate after one month (%)	Browning rate (%)
T1		5	100.00[a]	0.0 [a]	0.0 [a]
T2		10	86.7± 11.5 [a,b]	10.0± 0.1 [a,b]	0.0 [a]
T3	5	15	46.7±20.8 [b,c,d]	43.3±15.2 [b,c,d]	0.0 [a]
T4		20	53.7±11.5[a,b,c,d]	26.7±25.2 [a, b,c]	0.0 [a]
T5		30	46.7±5.8[c,d]	53.3±20.8 [c,d]	0.0 [a]
T6		5	73.3±25.2[a,b,c]	26.7±25.2 [a,b,c]	0.0 [a]
T7		10	56.7±15.3[b,c,d]	43.3±15.3 [b,c,d]	0.0 [a]
T8	10	15	66.7±5.8[a,b,c,d]	26.7±11.5 [a,b,c]	0.0 [a]
T9		20	56.7± 11.5 [b,c,d]	33.3±11.5 [a,b,c,d]	0.0 [a]
T10		30	46.7±15.3 [c,d]	46.7±5.8 [c,d]	3.3±3.3[a]
T11		5	63.3±15.3 [a,b,c,d]	33.3±11.5 [a,b,c,d]	0.0 [a]
T12		10	53.3±5.8 [b,c,d]	46.7±5.8[b,c,d]	0.0 [a]
T13	15	15	60.0±10.0 [a,b,c,d]	40.0±10.0 [a,b,c,d]	0.0 [a]
T14		20	50.0±10.0 [b,c,d]	50.0±10.0 [b,c,d]	10.0±5.8 [a,b]
T15		30	46.7± 11.5 [b,c,d]	53.3± 11.5 [c,d]	23.3±8.8 [b,c]
T16		5	60.0±10.0 [a,b,c]	30.0±10.0 [a,b,c]	0.0 [a]
T17		10	63.3±11.5 [a,b,c,d]	36.7±11.5 [a,b,c,d]	0.0 [a]
T18	20	15	16.7±5.8 [d]	73.3±5.8[d]	3.3±3.3[a]
T19		20	23.3±5.8 [c,d]	56.7±5.8 [c,d]	13.3±3.3[a,b]
T20		30	13.3±5.8 [c,d]	86.7±5.8 [c,d]	33.3±8.8[c]

Data recorded in the mean with n=30 of the frequency of contamination and survival of explants after two weeks of culture. Data expressed in different upper case letters showed significantly different at $P < 0.05$ by Tukey's HSD test. (+) represents browning and (-) not browning.

Table 3. Callus induction of *Dendrobium aurantiacum* under different hormone treatment

Treatment	2,4-D (mg/L)	KIN (mg/L)	Callus formation time (days)	Callus induction rate (%)	Callus colour	Callus type	Browning condition
A	-	-	7	100	white	compact hard	0.00 [a]
B	1.0	0.5	5	100	white	compact hard	0.00 [a]
C	10.0	0.0	3	100	white	compact hard	13.33 [b]

Data is presented in the mean with n=5 for callus browning condition. Data expressed in different upper case letters showed significantly different at $P < 0.05$ by Tukey's HSD test.

browning condition occurred in callus supplemented with treatment A and B but about 13.33% of callus on treatment C showed slight browning. Explant browning is mainly caused by the oxidation of phenolic compounds (19). However, the callus on treatment C with totipotency properties can continuous propagated in the agar media with multiple subculture in both solid and liquid MS culture media with appropriate hormone as designed in the experiment.

Plant hormone 2,4-Dichlorophenoxyacetic acid was invented in 1967 as the first synthetic herbicide that commonly used at high dose to control annual and perennial weeds (20). Since then, 2,4-D were also found to be useful in promoting cell elongation and division (21) and also plant responses for regeneration (22). Our result is in agreement that 10 mg/L 2.4-D is the best auxin for enhancing callus induction, callus proliferation and subculture of grass (23). Research on *in vitro* regeneration of *Dendrobium* sp. (24) also reported that the maximum protocorm-like bodies (PLBs) formation (90%) was observed in 10 mg/L 2, 4-D after 60 days of culture.

The combination of 2,4-D and kinetin is more effective for fresh weight and dry weight of shoot-derived callus, but 2,4-D alone is more effective in inducing callus growth (25). Results showed that callus induction occurred in both MS media with and without hormones, but the process is very slow in the MS media without hormone.

Cell Count on Callus Proliferation

To determine the callus cell proliferation, the changes in number of callus cell in the control and treatments were measured using cell counter. Callus cell concentration of on day 0 was 2.35×10^4 live cells per ml. The orchid callus in treatment C media has the highest cell concentration with 43.73×10^6 live cells per ml, showed about 14-fold compared to treatment A media showed only 2.98×10^6 live cells per mL of callus cell concentration (Fig. 4).

Somatic Embryogenesis

Proliferated callus was transferred to MS medium with the supplement of 0.5 mg/L BAP and 0.5 mg/L NAA hormone for regeneration. Fig. 5 showed the somatic embryogenesis of *Dendrobium aurantiacum*.

In orchids, somatic embryogenesis is part of the early steps of PLB regeneration (10) and can be divided into three stages: initial somatic embryo cell generation, globular somatic embryo formation and

	A	B	C
Diagram	2.5mm	2.5mm	2.5mm
Cross section of explant	callus 2.5mm	callus 2.5mm	callus 2.5mm
Callus proliferation	3.0mm	3.0mm	3.0mm

Fig. 3. Callus induction of *Dendrobium aurantiacum* treated with different hormone treatment. **A** is MS media without hormone supplement, **B** is MS media supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L KIN, and **C** is Ms media supplemented with 10.0 mg/L 2,4-D.

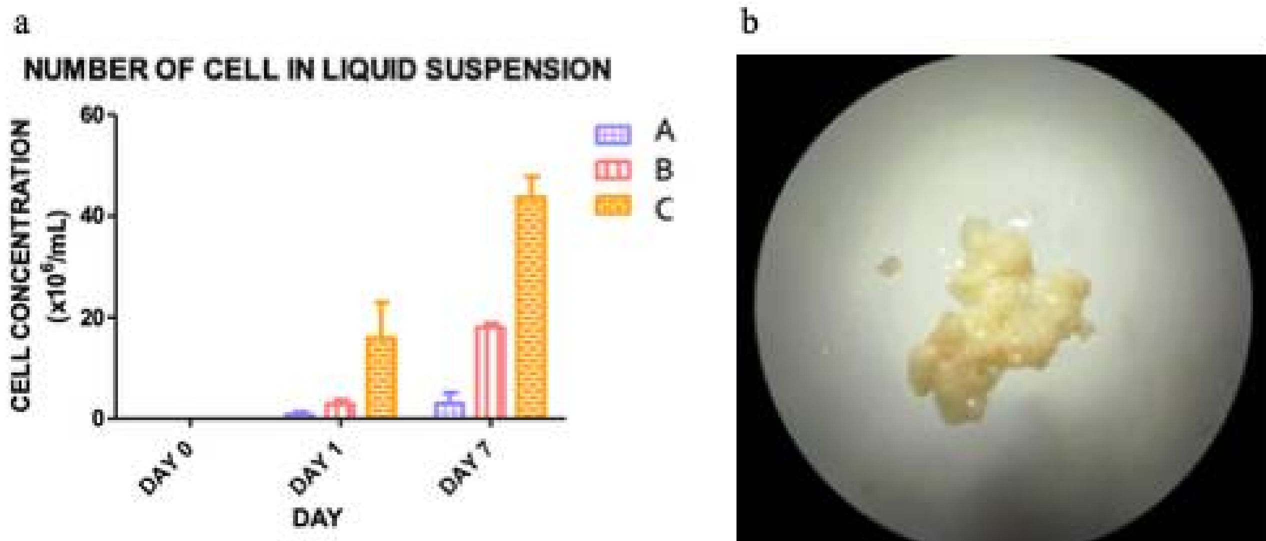

Fig. 4. Callus proliferation in liquid suspension culture. **4a** represents the cell count of callus proliferation at Day 0,1 and 7; **b** represents the structure of callus proliferated in the treatment of 10 mg/L 2,4-D hormone medium.

somatic embryo differentiation (26). There are two pathways in somatic embryogenesis. First, single-cell-derived somatic embryo is generated, develops into a globular somatic embryo similar to zygotic embryogenesis (27) which later become PLBs with shoot tip, and eventually develops into plantlets (10). The other is the production of multiple-cell-derived somatic embryos that directly form globular or multicellular somatic embryos, lacking the typical early stages of embryogenesis (27).

During the transition process, condensation and devacuolation occur continuously in the cytoplasm of embryogenic callus cells, and the cell fate changes (26), thereby forming an initial somatic embryo cell

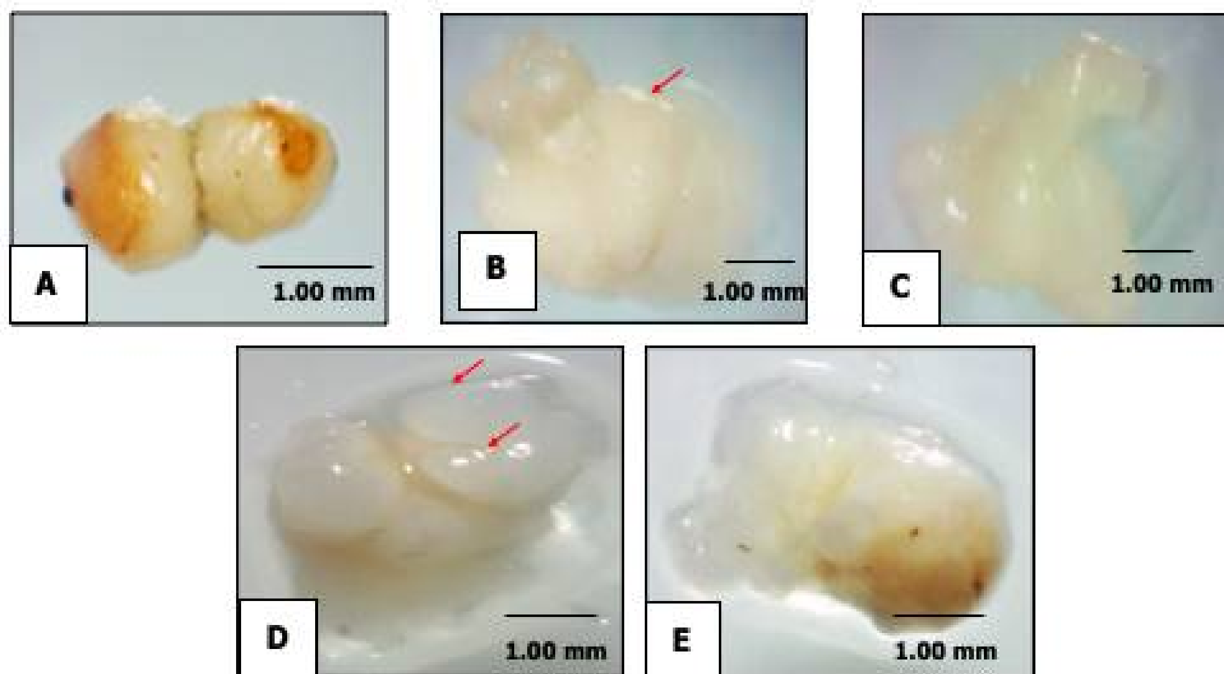

Fig. 5. The transformation of somatic embryos at different growth development stages of *Dendrobium aurantiacum*, where (**A** & **B**) Globular-shaped somatic embryo; (**C**) Heart-shaped somatic embryo; (**D**) Early torpedo-shaped embryo; (**E**) Torpedo-shaped of somatic embryo. Scale of magnification: (**A**) 50x, (**B** and **C**) 25x, (**D** and **E**) 32x.

composed of dense cytoplasm, a clear nucleus and small vacuoles (27). This process continues at various stages of differentiation and development, such as pro-embryo, globular, heart- shaped and torpedo embryos (28).

The orchid embryo is small with a reduced number of cells compared to other flowering plants (29). Therefore, their rate of cell division is slow, and the cell cycle time is long. The absence of a cotyledon is a common characteristic of orchid species (29). Several factors such as medium composition, genotypic properties, and explants used also affect processes associated with callus induction, embryonic differentiation and plant regeneration (30).

Conclusion

The development of orchid industry is required to meet up the annual rising demand for high quality and medicinally important materials. Therefore, the application of modern micropropagation technique and tissue culture laboratories can help to promote the development of orchid industry. This study provides a protocol for the establishment of *D. aurantiacum*. The sterilization efficiency of sodium hypochlorite has been tested on *D. aurantiacum* by using different concentration. Results showed that 20% sodium hypochlorite has the optimal sterilization effect with 83.33±5.8% of survival rate after 1 month and no browning condition. Callus induction were obtained in all plant hormones. Highest callus induction and proliferation was achieved in medium supplemented with 10 mg/L 2,4-D. High callus productions with effective micropropagation technique can create mass orchid market with uniform propagated plants. Further exploitation and conservation efforts on the valuable, endangered or rare species are essential for conservation and commercialization purposes. More efforts including standardized the protocols based on orchid species, phenology and habitat nature are required for the development of laboratory procedures and there by making the techniques more accessible and applicable to the local farmers and orchid developers.

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Authors' Contributions

MYL constructed the experimental design and initial of the overall project, completed and finalized the whole manuscript. KSC carried out the optimization of sterilization technique in orchid, *Dendrobium aurantiacum* and drafted the manuscript. LJX performed the callus induction and proliferation data analysis. SCF and NAAS conceived the overall experimental design and study. All authors read and approved the final manuscript.

Conflict of Interests

There is no competing interest in this project.

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