

Induction of Adventitious buds from stem explants in *Aoectochilus formosanus*

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Abstract. In order to explore the optimal culture conditions for adventitious bud regeneration of stem explants in *Aoectochilus formosanus*, the stem segments from the sterile seedlings were used as explants, and different concentrations of diethyl aminoethyl hexanoate (DA-6), kinetin (KT), Cu²⁺ and glutamine (Gln) were separately added into MS medium containing 6-benzylaminopurine (6-BA) and naphthaleneacetic acid (NAA), and the induction rate and the induction multiple of adventitious buds were recorded and analyzed. The results showed that the regeneration of adventitious buds could be promoted, when 2 mg/L DA-6, 0.4 mg/L KT and 15 mg/L Gln were added in mediums. However, the effect of Cu²⁺ on the regeneration of adventitious buds in *A. formosanus* was enhanced by low concentration and suppressed by high concentration, and the best concentration of Cu²⁺ was 5 mg/L.

1 Introduction

Anoectochilus formosanus, also known as sapphire orchid, is a perennial herb in *Orchidaceae*. It is mainly distributed in the cool and humid areas at an altitude of 500-1800 m in Taiwan and Fujian, China [1,2]. The overall of plant is small and beautiful, and has a high ornamental value [3].

A. formosanus has high medicinal value, the main chemical components in *A. formosanus* are polysaccharides, flavonoids, glycosides, organic acids, steroids, trienes, alkaloids and nucleosides. These compounds have been shown to be the main biologically active substances, with major pharmacological effects such as anti-diabetes, anti-inflammatory, anti-inflammatory, anti-viral, liver protection, kidney protection, immunomodulation and anti-tumor [4]. *A. formosanus* is considered to be a precious medicinal material in China. It is especially popular in Taiwan province and is known as the “king of medicine” [5].

Due to the growth environment of *A. formosanus* is special, and its seeds have no endosperm and no germination ability. It can promote seed germination in the case of symbiosis with fungi. Because of the low germination rate of *A. formosanus* under natural conditions, it is difficult to breed in large numbers in recent years; furthermore, the deterioration of the natural environment and the excessive collection of humans have made this precious plant threatened with extinction. At present, the rapid propagation of tissue culture

technology can effectively solve this problem, which not only eases the shortage of wild *A. formosanus* resources, but also plays a positive role in the protection of germplasm resources of this species [1, 6]. In recent years, although the tissue culture technology of *A. formosanus* has made some progress, there are still some problems, such as the problem of high seedling variability, low reproductive rate and low uniformity. How to improve the quality of seedlings will become the key to solving actual production problems in the future [7]. In this experiment, the stem segments from sterile seedlings of *A. formosanus* were used as explants, and different concentrations of diethyl aminoethyl hexanoate (DA-6), kinetin (KT), Cu²⁺ and glutamine (Gln) were added into the culture mediums for explore the induction effects of adventitious buds regeneration. Through continuous screening of the mediums to obtain high-quality tissue culture seedlings of *A. formosanus*, it provides a reference for the industrial production of *A. formosanus*.

2 Methods

2.1 Plant Sources

The plantlet of *A. formosanus* adopted were supplied by professor Yue-sheng Yang from College of Life Sciences in South China Agricultural University, in the present study.

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2.2 Preparation of Explants

Stem segments were isolated from the young and healthy tissue culture seedlings of *A. formosanus*, and stem explants were cut from them into 2~3 cm pieces.

2.3 Preparation of Mediums and Condition of Culture

The same conditions of culture were suitable for all experiments. The pH of the medium was adjusted to 5.8-6.0 by 1 mol/L NaOH, and then autoclaved at 1.4 kg • cm⁻² for 20 min. The Culture temperature of the mediums was 25 ± 2 ° C, and light length and light intensity was 12 h and 60-80 μ mol • m⁻² • s⁻¹ with cool white fluorescent tubes.

2.4 Induction of Adventitious Buds Regeneration

For the regeneration of adventitious buds, stem explants were placed on the base medium of Murashige and Skoog (MS) [8] supplemented with 3 mg/L 6-BA and 1 mg/L NAA. The different treatments were conducted by using different concentrations of DA-6 (0, 1, 2 and 4 mg/L), KT (0, 0.2, 0.4 and 0.8 mg/L), CuSO₄ (0, 5, 10 and 15 mg/L) and Gln (0, 5, 10 and 15 mg/L) on the base medium. The adventitious buds regeneration was cultured for 30 days.

2.5 Data Analysis

Rate of adventitious buds regeneration (%) = (number of stem explants with adventitious buds differentiated/number of stem explants inoculated) × 100%.

Induction of multiple = number of adventitious buds differentiated/number of stem explants inoculated

All of the experiments were established in an absolutely random factorial design and repeated three times. Data analysis was accomplished by using SPSS 17.0 software and the significantly different of data was notarized via Duncan's multiple comparison ($p \leq 5\%$). The results were shown as mean value ± standard error (SE).

3 Results

3.1 Effects of different concentrations of DA-6 on adventitious buds regeneration of *A. formosanus*

The results shown that different concentrations of DA-6 significantly affected the regeneration efficiency of adventitious buds (Table 1 and Figure 1(A-D)). The highest percentage of regeneration was gained at 2 mg/L of DA-6 among the four concentrations, as reflected by a 93.33% of adventitious buds regeneration rate (Table 1 and Figure 1C). Moreover, the maximum induction multiple of buds (2.67) was acquired at 2 mg/L DA-6,

the buds were relatively viable and good quality (Table 1 and Figure 1C).

Table 1 Influencing of DA-6 on adventitious buds induction from stem explants in *A. formosanus*

Number	Concentrations of DA-6 (mg/L)	Rate of adventitious buds regeneration (%)	Induction multiple
0	0	73.33 ± 12.47a	0.87 ± 0.17b
1	1	73.34 ± 6.67a	1.73 ± 0.44ab
2	2	93.33 ± 6.67a	2.67 ± 0.21a
3	4	80.00 ± 13.33a	1.93 ± 0.55ab

Note: Different lowercase in the same column indicated significant difference at ($P \leq 0.05$) level.

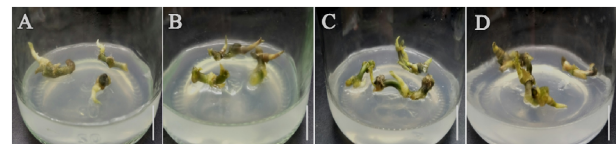


Fig. 1 Effect of DA-6 on adventitious buds induction from stem explants in *A. formosanus*
 A: 0 mg/L; B: 1 mg/L; C: 2 mg/L; D: 4 mg/L.
 (Bars = 1 cm)

3.2 Induction of adventitious buds from stem explants of *A. formosanus* treated with KT

The responses of adventitious buds induction were observably influenced by the concentrations of KT in mediums (Table 2 and Figure 2(A-D)). During the four different concentrations of KT, 0.4 mg/L KT seemed to be most suitable, under which the highest percentage of adventitious buds regeneration (100%) and the most induction multiple of buds (2.40) were gained (Table 2 and Figure 2C). Furthermore, when KT concentration was higher than 0.4 mg/L, the induction multiple of buds was markedly reduced (Table 2).

Table 2 Effect of KT on adventitious buds induction from stem explants in *A. formosanus*

Number	Concentrations of KT (mg/L)	Rate of adventitious buds regeneration (%)	Induction multiple
0	0	80.00 ± 13.33a	1.27 ± 0.24b
1	0.2	86.67 ± 8.16a	1.47 ± 0.20b
2	0.4	100.00 ± 0.00a	2.40 ± 0.19a
3	0.8	86.67 ± 8.16a	1.27 ± 0.12b

Note: Different lowercase in the same column indicated significant difference at ($P \leq 0.05$) level.

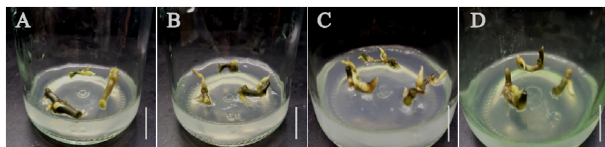


Fig. 2 Effect of KT on adventitious buds induction from stem explants in *A. formosanus*
A: 0 mg/L; B: 0.2 mg/L; C: 0.4 mg/L; D: 0.8 mg/L.
(Bars = 1 cm)

3.3 Results of CuSO₄ on regeneration of adventitious buds from stem explants

Adding different concentrations of CuSO₄ in mediums also significantly affected the regeneration of adventitious buds from stem explants (Table 3 and Figure 3(A-D)). The effects of regeneration would be enhanced with adding high concentrations 5 mg/L of CuSO₄ into the regeneration mediums (Table 3). The highest rate of adventitious buds regeneration (86.67%) and the maximum induction multiple of buds were reflected by 86.67% and 2.20, respectively, when 5 mg/L of CuSO₄ was used into mediums (Table 3 and Figure 3B).

Table 3 Results of Cu²⁺ on adventitious buds induction from stem explants in *A. formosanus*

Number	Concentrations of Cu ²⁺ (mg/L)	Rate of adventitious buds regeneration (%)	Induction multiple
0	0	60.00 ± 6.33c	1.33 ± 0.42b
1	5	86.67 ± 8.16a	2.20 ± 0.25a
2	10	72.14 ± 2.47b	0.93 ± 0.22b
3	15	60.25 ± 4.23c	0.67 ± 0.21b

Note: Different lowercase in the same column indicated significant difference at ($P \leq 0.05$) level.

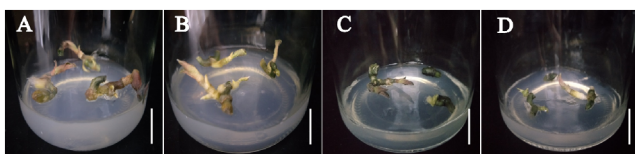


Fig. 3 Effect of Cu²⁺ on adventitious buds induction from stem explants in *A. formosanus*
A: 0 mg/L; B: 5 mg/L; C: 10 mg/L; D: 15 mg/L.
(Bars = 1 cm)

3.4 Influences of glutamine (Gln) on regeneration of adventitious buds from stem explants

As shown in Table 4 and Figure 4(A-D), the concentrations of Gln affected the regeneration of adventitious buds significantly. From the results summarized in table 4, it's explicit clear that increased

the concentration of Gln could improve the rate of adventitious buds regeneration and the induction multiple. When the concentration of Gln increased to 15 mg/L, the highest regeneration rate of adventitious buds (86.67%) and the best induction multiple of buds (2.93) were obtained (Table 4 and Figure 4D).

Table 4 Results of Gln on adventitious buds induction from stem explants in *A. formosanus*

Number	Concentrations of Gln (mg/L)	Rate of adventitious buds regeneration (%)	Induction multiple
0	0	40.00 ± 12.47b	0.47 ± 0.13b
1	5	53.33 ± 8.17ab	1.40 ± 0.40b
2	10	60.00 ± 16.33ab	1.87 ± 0.62ab
3	15	86.67 ± 13.33a	2.93 ± 0.59a

Note: Different lowercase in the same column indicated significant difference at ($P \leq 0.05$) level.

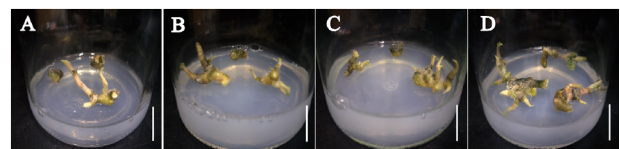


Fig. 4 Effect of Gln on adventitious buds induction from stem explants in *A. formosanus*
A: 0 mg/L; B: 5 mg/L; C: 10 mg/L; D: 15 mg/L.
(Bars = 1 cm)

4 Discussion

As a new plant growth regulator, DA-6 is suitable for a variety of crops [9,10]. However, DA-6 is fewer used to explore the regeneration of adventitious buds from stem explants in *A. formosanus*. The results found that the addition of appropriate concentration of DA-6 can increase the rate of adventitious buds regeneration and induction multiple of buds. This might be due to the fact that DA-6 could promote the synthesis of chlorophyll and nutrient absorption of plants [9,10].

KT is one type of cytokinins. When the concentration of KT is appropriate, it could promote cell division and differentiation [11]. It's beneficial for inducing of adventitious buds regeneration from stem explants in *A. formosanus*.

Cu²⁺ is one of the essential trace elements for plant growth, but high concentration will cause toxicity to plants. Our results shown that the low concentration of Cu²⁺ can promote the regeneration of adventitious buds of *A. formosanus*. A study reported that when Cu²⁺ was used at the suitable concentration, the differentiation of plant organs would be promote observably [12].

Gln plays an important role in plant metabolism, which is widely involved in several biosynthesis pathways, such as amino acids, nucleic acids and other N-compounds. As one of the main endogenous amino

acid, Gln is frequently chosen as an organic nitrogen source applied in plant tissue culture medium [13,14]. It has been reported that exogenous application of Gln could increase the regeneration frequency and explant biomass in plant tissue culture [15,16]. Our study found that Gln can promote the regeneration of adventitious buds, moreover, with the increase of concentration of Gln, the induction rate of adventitious buds was also enhanced.

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6 Conclusion

For exploring the best culture conditions for adventitious buds regeneration of stem explants in *A. formosanus*. The results showed that the regeneration of adventitious buds could be promoted, when 2 mg/L DA-6, 0.4 mg/L KT and 15 mg/L Gln were added in mediums. However, the effect of Cu²⁺ on the regeneration of adventitious buds in *A. formosanus* was enhanced by low concentration and suppressed by high concentration, and the best concentration of Cu²⁺ was 5 mg/L.

References

1. Liu F, Wei P X, Cen X F, et al. Effects of explants and basic medium on the induction of cluster buds of clematis chinensis from Taiwan [J]. Northern horticulture, 2009, (4): 103-104.
2. Shiau Y J, Abhay P S, Chen U C, et al. Conservation of *Anoectochilus formosanus* Hayata by artificial cross-pollination and in vitro culture of seeds[J]. Botanical Bulletin - Academia Sinica Taipei, 2002, 43(2): 123-130.
3. Jiang A M, Zhou X Y. Tissue culture and rapid propagation of *Anoectochilus formosanus* [J]. Journal of hanzhong normal university, 2017, 37(3): 17-21.
4. Ye S, Shao Q, Zhang A. *Anoectochilus roxburghii*: A review of its phytochemistry, pharmacology, and clinical applications[J]. Journal of Ethnopharmacology, 2017, 209: 184-202.
5. Tan X J, Su C X, Yu X G, et al. Application value of lotus seed and rapid propagation of seedlings [J]. Pharmaceutical biotechnology, 2017, 24(1): 88-91.
6. Li F H. Research progress of *Anoectochilus formosanus* [J]. Yunnan journal of traditional Chinese medicine, 2016, 37(11)
7. Jiao Z, An S J, Shao T M. "two plus two" method for the establishment of a new system for the cultivation of protocorm of *Anoectochilus formosanus* [J]. Journal of tropical crops, 2019, 40(2): 314-322.
8. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 1962;15:473-479.
9. Peng Z P, Yu J H, Hu J R, et al. Effects of DA-6 on nutrient uptake and yield of cauliflower root [J]. Guangdong agricultural sciences, 2012, 39(22): 80-81.
10. Yang X Y, Li S H, Mei Y C, et al. Effects of da-6 on growth and physiological characteristics of hydroponic lettuce [J]. Agricultural environmental science, 2017, 36(1): 32-38.
11. Luo Q G, Ye W, Jiang J L, et al. Study on tissue culture and rapid propagation of clematis chinensis [J]. Southern agriculture, 2011, 5(5): 43-44.
12. Zhao F C. In vitro culture and plant regeneration of *Echinacea purpurea* [D]. Master's thesis of south China agricultural university, 2006.
13. Coruzzi G, Last R. Amino acids. In: Buchanan B B, Groissem W, Jones R, editors. *Biochemistry and molecular biology of plants*. Rockville (MD): American Society of Plant Physiology Press; 2000. p. 358-410.
14. Franklin C I, Dixon R A. Initiation and maintenance of callus and cell suspension cultures. In: Dixon RA, Gonzales RA, editors. *Plant cell culture a practical approach*, 2nd ed. New York (NY): Oxford University Press; 1994. p. 1-29.
15. Franklin C I, Trieu T N, Gonzales R A, et al. Plant regeneration from seedling explants of green bean (*Phaseolus vulgaris* L.) via organogenesis. *Plant Cell Tissue Organ Culture*. 1991;24:199-206.
16. Vasudevan A, Selvaraj N, Ganapathi A, et al. Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. *Biologia Plantarum*. 2004;48:125-128.