

Full Length Research Paper

Effects of plant growth regulators on *in vitro* propagation of *Cymbidium faberi* Rolfe

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Cymbidium faberi Rolfe is one of the oldest cultivated orchids in China, which is often sold as a precious potted specimen because of its beautiful and fragrant flowers in eastern Asia. However, the propagation of *C. faberi* is greatly restricted by the low seed germination rate due to immature embryo and no endosperm under natural conditions. In this study, the effects of plant growth regulators on *in vitro* propagation of *C. faberi* were investigated to establish *in vitro* propagation protocol. The results demonstrated that seeds began to form into white spots on 2.0 mg/L N6-benzyladenine (BA) + 1.5 mg/L α -naphthaleneacetic acid (NAA) and the highest number was on 2.0 mg/L BA + 2.0 mg/L NAA. And 1/2 Murashige and Skoog (MS) medium + 0.5 mg/L NAA was the optimal medium for protocorm-like bodies (PLB) turning green. In the subculture of PLB, 1/2 MS supplemented with NAA was more effective, its optimal concentration for growth and multiplication of PLB was 2.0 and 1.0 mg/L, respectively. In adventitious shoots induction, the optimal medium was 1/2 MS + 1.0 mg/L thidiazuron (TDZ) + 0.5 mg/L NAA whose induction rate was up to 56.8%. Additionally, 1/2 MS supplemented with 0.5 mg/L indole-3-butyric acid (IBA) was the best culture medium for rooting. These results may provide available technique for cultivar breeding and large-scale propagation of *C. faberi*.

Key words: Orchids, *Cymbidium faberi* Rolfe, propagation, plant growth regulators.

INTRODUCTION

Orchids are valued ornamentals which have become the second largest cut flowers and potted floricultural crop (Hossain et al. 2010) all over the world, and they include three groups, epiphyte orchids, terrestrial orchids and saprophytic orchids. However, the orchids are usually divided into two categories from cultivation in China, Chinese orchids and tropical orchids. Chinese orchids are one of ten traditional famous flowers in China, which have been favored by people because of their humanistic

and ornamental values from time immemorial.

As one of the oldest cultivated and most popular Chinese orchids in China, *Cymbidium faberi* Rolfe is often sold as a precious potted specimen because of its beautiful and fragrant flowers in eastern Asia (Chen et al. 2005). Meanwhile, breeding new cultivars of *C. faberi* including novel flower colors, color patterns, flower size, and other high commercial values are mainly through hybridization (Vendrame et al. 2007). The demands for *C. faberi* plants are increasing with the improvement of people's living standards. However, low propagation rate due to restricted seeds germination in the natural reproduction and lagged scale production make it difficult to meet market demands (Knudson 1946; Yang 2008).

Plant tissue culture refers to *in vitro* culture of plant cell, tissue and organ, and induction of whole-plant generation in the culture medium, which is an important tool in both basic and applied studies as well as in commercial production (Thorpe 2007). It is an effective means for studying the rapid propagation of plants, like orchids,

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Abbreviations: AC, Activated charcoal; BA, N6-benzylaminopurine; d, diameter; GA, gibberellin; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; NAA, α -naphthaleneacetic acid; PLB, protocorm-like bodies; TDZ, thidiazuron.

which grow and multiply slowly, because of its advantages, such as large propagation coefficient, short reproductive cycle, whole-year production and supply, etc (Nge et al. 2006). In a previous study, it was found that orchids seeds could germinate with high frequency and form plantlets in artificial nutrient medium (Knudson 1946). Subsequently, many researches on orchids rapid propagation protocols were reported, however, they were mainly focused on tropical orchids (Shimasaki and Uemoto 1991; Lu et al. 2001; Huang and Okubo 2005; Vendrame et al. 2007; Hossain et al. 2009 and 2010). Plant regeneration of national orchids was difficult to obtain by seed, or tissue and organ culture *in vitro* in comparison with tropical orchids (Shimasaki and Uemoto 1991). Concerning *C. faberi*, few reports established an *in vitro* plant regeneration protocol so far (Chen et al. 2005). In this paper, we describe the effects of plant growth regulators on *in vitro* propagation of *C. faberi*, including seeds germination, protocorm-like bodies (PLB) formation, turning green and multiplication, shoots and roots induction. The results may provide an available technique for new varieties breeding and large-scale propagation of *C. faberi*.

MATERIALS AND METHODS

Plant materials and tissue culture conditions

C. faberi was cultivated in the greenhouse of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32°30'N, 119°25'E). The immature seeds in green capsules used as explants were sampled in September 2007, and then stored at 4°C for one month. The capsules were immersed in detergent solution for 20 min and washed in running tap water, followed by sterilization in 70% alcohol and 0.1% mercuric chloride solution (plus 1 to 2 drops of Tween®) for 2 to 20 min, respectively. After rinsing three times with sterile distilled water, the seeds were placed on medium individually. Without special instructions in this paper, the medium was 1/2 Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, 6.8 g/L agar, 2 g/L AC and 0.5 g/L casein hydrolysate, with a pH 5.6. After preparation, it was sterilized at 121°C for 20 min. The culture room was exposed to artificial light with light intensity of 1500 to 2000 Lux. The photoperiod was 12 h a day and the temperature was 25 ± 2°C.

Aseptic germination of seeds

The seeds were inoculated on 1/2 MS supplemented with AC (1.0 g/L), cross-group designed BA (0.5, 1.0, 2.0 mg/L) and NAA (0.5, 1.0, 1.5, 2.0 mg/L). There were 20 inoculating flasks per treatment, and each inoculating flask contained about 100 seeds which were cultured in darkness.

Turning green of PLB

The white PLB with similar size were transferred to 1/2 MS randomly supplemented with 0.5 mg/L BA, 0.5 mg/L NAA, and 0.5 mg/L NAA + 0.5 mg/L BA. There were 10 inoculating flasks per treatment, and each inoculating flask contained 10 white PLB.

Subculture of green PLB

Green PLB with consistent size from the 1/2 MS supplemented with 0.5 mg/L NAA were selected, and then transferred to 1/2 MS containing different concentrations of NAA (0.0, 0.5, 1.0, 2.0 mg/L), BA (0.0, 2.0, 4.0, 8.0 mg/L) and gibberellin (GA) (0.0, 0.5, 1.0, 2.0 mg/L). There were 12 inoculating flasks per treatment, and each inoculating flask contained four green PLB.

Induction of adventitious shoots

The green PLB that were cultured on 1/2 MS supplemented with 1.0 mg/L NAA were transferred to 1/2 MS containing different concentrations of BA (1.0, 3.0 mg/L), TDZ (1.0, 3.0 mg/L), 1.0 mg/L TDZ + 0.5 mg/L NAA and 3.0 mg/L TDZ + 0.5 mg/L NAA. The aim was to compare the impact of BA and TDZ on adventitious shoots induction from green PLB and study whether NAA could accelerate the adventitious shoots induction. There were 22 inoculating flasks per treatment, and each inoculating flask contained four green PLB.

Rooting of shoot seedlings

Shoot seedlings with 1 to 2 cm height were cultured on the MS without any plant growth regulators for one month, and then transferred to 1/2 MS supplemented with different concentration of indole-3-butyric acid (IBA) (0.0, 0.5, 1.0, 1.5, 2.0 mg/L). There were seven inoculating flasks per treatment, and each inoculating flask contained four shoot seedlings.

Statistical analysis

All data were means of three replicates at least with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA). The difference between the means was tested by least significant difference at $P_{0.05}$ (LSD_{0.05}).

RESULTS

Aseptic germination of seeds

After sowing for 30 days, the seeds of *C. faberi* began to swell. At 90 days, white spots appeared firstly on the 2.0 mg/L BA + 1.5 mg/L NAA medium, and then on the 2.0 mg/L BA + 1.0 mg/L NAA medium. At 120 days, the seeds treated with 12 treatments germinated one after the other. It was found that the seeds germination got a long duration, the period of germination was different between treatments, and the phenomenon of seeds germination still occurred after one year. With the growth and development of white spots, they gradually formed into white PLB which did not directly develop into shoots, but could constantly extend or branch (Figure 1b).

The number of seeds germination on different medium was different (Table 1); however, the one that germinated most was on the 2.0 mg/L BA + 2.0mg/L NAA medium, and the one that germinated least was on the 0.5 mg/L BA + 0.5 mg/L NAA medium. When the concentration of BA was 2.0 mg/L, total number of *C. faberi* seeds germi-

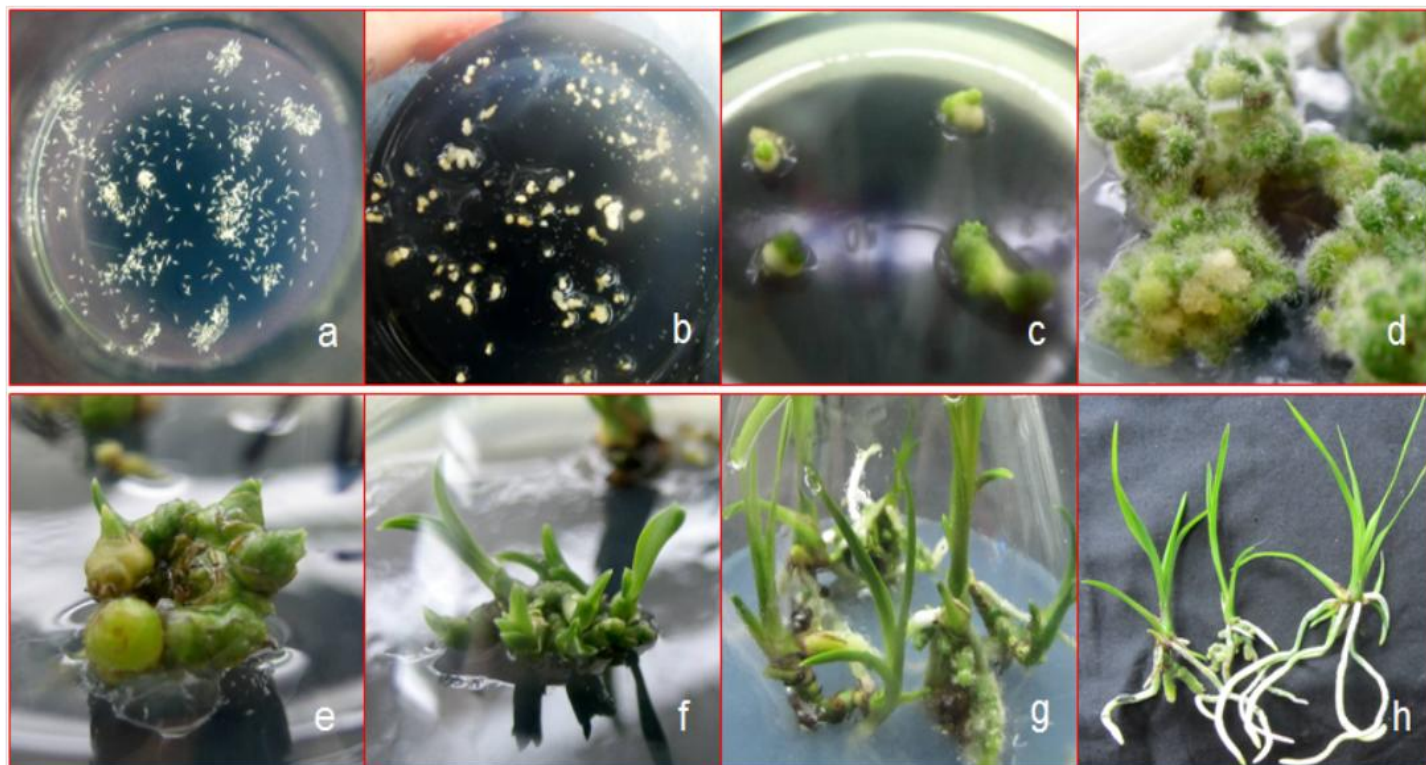


Figure 1. Effects of plant growth regulators on *in vitro* propagation of *C. faberi* Rolfe. a, Seed sowing; b, seed germination and PLB formation; c, green PLB formation; d, PLB multiplication; e, differentiation of adventitious shoots from PLB; f, multiply shoots from PLB; g, adventitious roots regenerating; h, seedlings.

Table 1 Effects of BA and NAA on the germination of *C. faberi* seeds after 120 days culture.

Plant growth regulator (mg/L)		Number of inoculating flask	Rate of seed germination (%)
BA	NAA		
0.5	0.5	20.0	3.9±0.9 ⁱ
	1.0	20.0	4.3±0.9 ^{hi}
	1.5	20.0	4.6±1.0 ^{hi}
	2.0	20.0	4.8±1.0 ^{fgh}
1.0	0.5	20.0	5.3±1.1 ^{efg}
	1.0	20.0	5.6±1.5 ^{def}
	1.5	20.0	5.8±1.2 ^{cde}
	2.0	20.0	6.4±1.2 ^{bcd}
2.0	0.5	20.0	6.4±2.0 ^{bcd}
	1.0	20.0	6.6±1.5 ^{bc}
	1.5	20.0	7.1±1.4 ^b
	2.0	20.0	8.8±1.8 ^a

Rate of seed germination (%) = (number of germinated seeds / number of inoculating seeds) × 100%.

nation was the most regardless of NAA concentration; while the concentration of BA was fixed, total number of *C. faberi* seeds germination gradually increased with elevated NAA concentration.

Turning green of PLB

The effects of plant growth regulators on the PLB turning green were shown in Figure 2. After 10 days light culture,

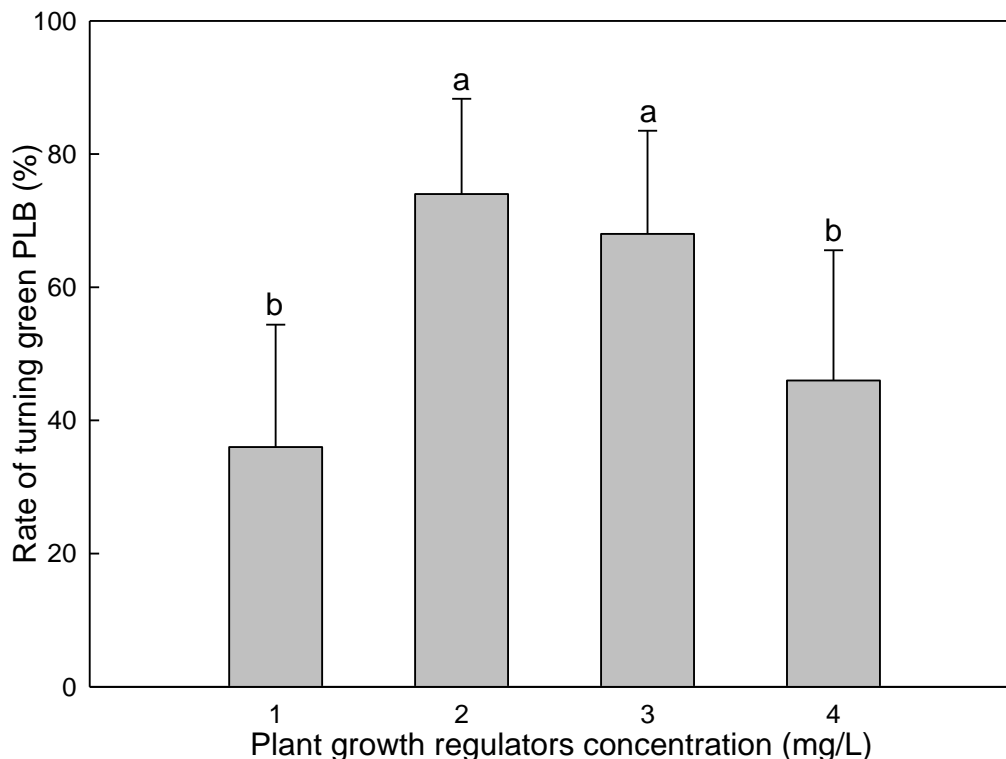


Figure 2. Effects of plant growth regulators on the formation of green PLB after 30 days culture. 1, CK; 2, NAA 0.5 mg/L; 3, 6-BA 0.5 mg/L; 4, NAA 0.5 mg/L + 6-BA 0.5 mg/L. Rate of turning green (%) = (number of turning green PLB / number of inoculating PLB) × 100%.

The PLB which began to turn green were firstly discovered in the medium supplemented with 0.5 mg/L NAA (Figure 1c). The effect of the medium supplemented with 0.5 mg/L NAA on turning green was the best after 30 days, reaching to the highest rate of 74% which was twice of that in the control followed by the 0.5 mg/L BA medium with 68% rate of turning green. However, the turning green rate of PBL in the medium supplemented with 0.5 mg/L NAA+ 0.5 mg/L BA was less than that with NAA or BA alone, and had no significant difference with the control.

Subculture of PLB

Effects of plant growth regulators on the growth of PLB

1/2 MS supplemented with different concentrations of NAA, BA and GA was used to study the effects of plant growth regulators on the growth of PLB. Statistic analysis of the PLB diameter (d) at 30 days and 60 days indicated that the PLB grew gradually large with time increase, and most of the PLB diameters were between 3 and 5 mm (Figure 3).

For NAA 2.0 mg/L, the PLB growth rate was the fastest, and the number of PLB (d > 5 mm) was 12 at 30

days, and 34 at 60 days, respectively. At 60 days, the number of PLB (d > 5mm) increased with the enhancement of NAA level, reaching to the maximum in 2.0 mg/L. These revealed that NAA could obviously accelerate the growth of *C. faberi* PLB.

When PLB were treated with BA, the number of PLB (d > 5 mm) had only a slight increase as the culture days increased. With the enhancement of BA level, the number of PLB (d < 3 mm) gradually increased, whereas the number of PLB (d > 5 mm) was in a balanced state at 30 days; but the number of PLB (d < 3 mm) showed little difference and the number of PLB (d > 5 mm) was gradually reduced at 60 days. It is suggested that high concentration of BA may inhibit the growth of *C. faberi* PLB.

When PLB were treated with GA for 60 days, the number of PLB (d > 5 mm) was six times of that for 30 days, but the overall trend was that the number of PLB (d > 5 mm) decreased with the enhancement of GA level. These results demonstrate that GA was not beneficial to the growth of *C. faberi* PLB.

Effects of plant growth regulators on the multiplication of PLB

The effects of plant growth regulators on PLB multipli-

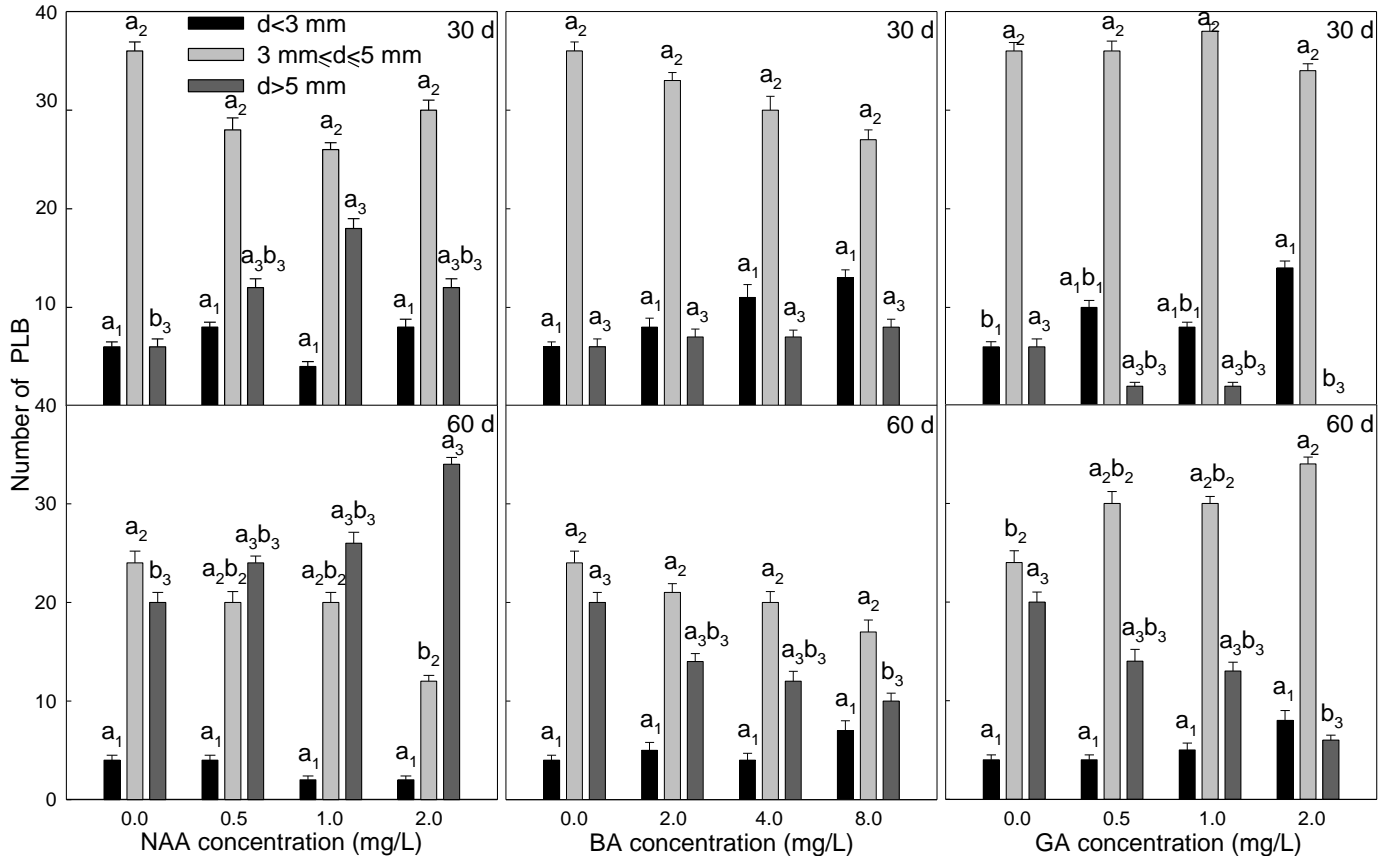


Figure 3. Effects of plant growth regulators on PLB growth after 30 and 60 days culture. d stands for the diameter of PLB.

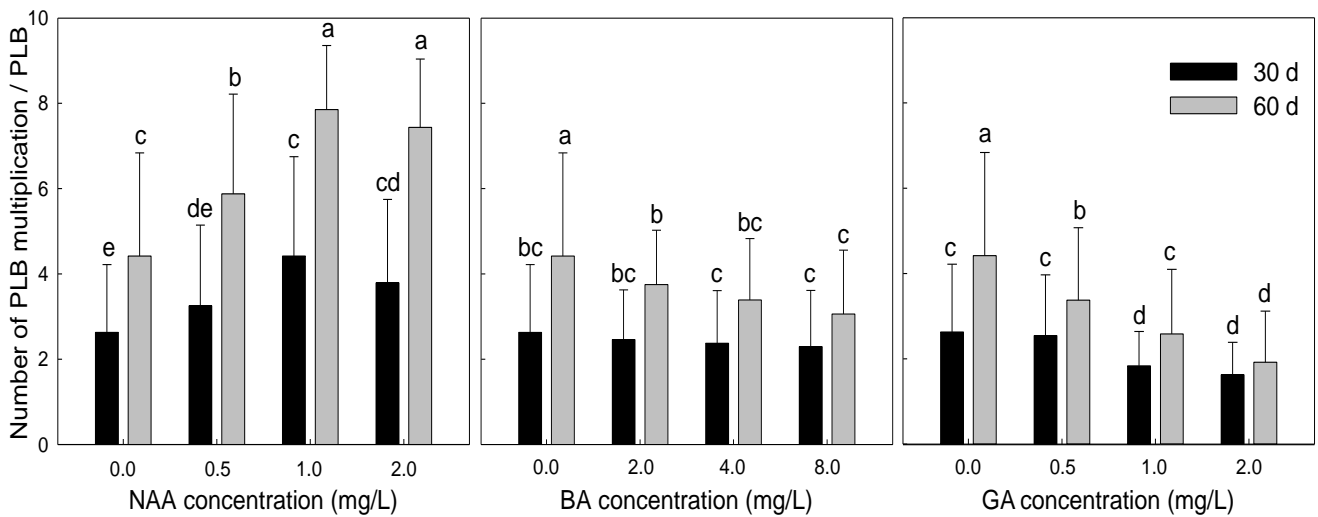


Figure 4. Effects of plant growth regulators on PLB multiplication after 30 and 60 days culture.

cation after 30 and 60 days culture were shown in Figure 4. Whether they were cultured for 30 or 60 days, the number of PLB multiplication increased with the elevated NAA level at first, and reached the maximum (7.9) in 1.0

mg/L. When NAA level arrived at 2.0 mg/L, the number of PLB multiplication was 7.4 which began to show a downward trend.

No obvious impact was found on the PLB multiplication

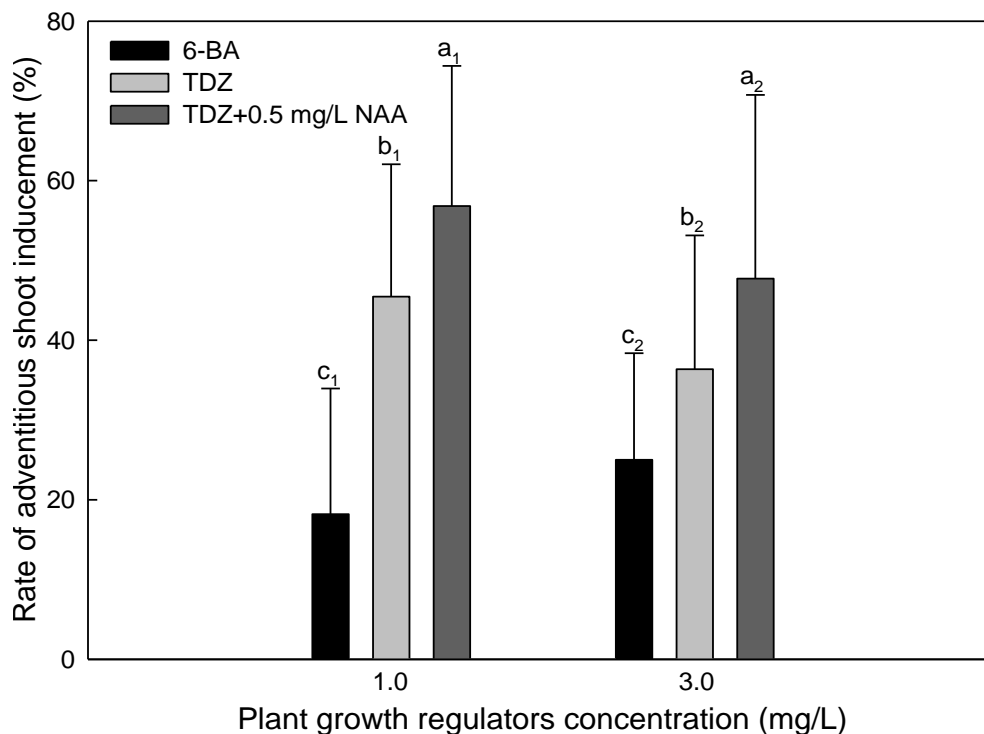


Figure 5. Effects of plant growth regulators on adventitious shoot induction from PLB after 60 days culture. Rate of adventitious shoot induction (%) = (number of adventitious shoot induction from PLB / number of inoculating PLB) × 100%.

at 30 days between the different BA levels. However, the number of PLB multiplication reduced correspondingly, and the size of multiplied PLB became smaller and prone to browning at 60 days along with the enhancement of BA level. Meanwhile, the number of PLB multiplication decreased with the increased GA level, and the new PLB of multiplication presented long slender strips which were detrimental to the differentiation of late shoots. In a word, supplementation with BA and GA in the medium was adverse for PLB multiplication.

Induction of adventitious shoots

BA and TDZ had been extensively used in plant tissue culture, which could enhance adventitious shoots initiation in orchids (Chen and Piluek 1995). In order to induce the adventitious shoots of *C. faberi*, uniform PLB were selected for inoculation separately on the medium supplemented with BA, TDZ and TDZ + NAA, and the effects of these plant growth regulators on the induction of adventitious shoots were compared (Figure 1e, f). As shown in Figure 5, when the concentrations of BA and TDZ were 1.0 mg/L, the adventitious shoots induction rate of PLB in the culture medium supplemented with TDZ was up to 45.5% which was 2.5 times of that treated with BA. However, when TDZ concentration increased to 3.0 mg/L, shoots differentiation from PLB was inhibited,

the rate of adventitious shoots induction was reduced by 9%, the young shoots were slim and fragile, as well as the leaves were thin and curving which grew slowly. The medium with proper TDZ and NAA could improve the shoots differentiation of *C. faberi* effectively, and helped to obtain healthy and strong shoot seedlings. TDZ + 0.5 mg/L NAA in the differentiation medium could improve the rate of adventitious shoots induction significantly which was about 11.4%.

Rooting of shoot seedlings

The differentiated shoot seedlings of *C. faberi* were transplanted to MS without plant growth regulators, and after 30 days, they were transferred to 1/2 MS supplemented with IBA. After 60 days culture, we obtained rooted plantlets (Figure 1g), and the effects of IBA on root induction from the shoot seedlings were listed in Table 2. The results showed that shoot seedlings of *C. faberi* on 1/2 MS without plant growth regulators started to grow roots after culturing for 44 days, and after 60 days culture, the rooting rate was up to $46.4 \pm 1.3\%$. Compared with the control, the rooting time was ahead, the average number of root per plantlet and the rooting rate all increased after adding different concentration of IBA to 1/2 MS. However, the rooting time was delayed, the average number of root per plantlet and the rooting

Table 2 Effects of IBA on root induction from the shoot seedlings after 60 days culture.

IBA concentration (mg/L)	Rooting rate (%)	Average number of root per plantlet	Time of rooting (day)
0.0	46.4±1.3 ^c	1.3±0.1 ^c	44.0
0.5	96.4±1.3 ^a	6.6±0.1 ^a	35.0
1.0	92.9±1.7 ^{ab}	5.6±0.1 ^{ab}	37.0
1.5	85.7±3.4 ^{ab}	5.6±0.2 ^b	37.0
2.0	78.6±3.2 ^b	5.2±0.2 ^b	39.0

Rooting rate (%) = (number of plantlets with root / number of inoculating plantlets) × 100%; average number of root per plantlet = total rooting number / number of plantlets.

rate all decreased with the enhancement of IBA level. 1/2 MS supplemented with 0.5 mg/L IBA was the best culture medium for root induction of *C. faberi* shoot seedlings, which reached the highest rooting rate of 96.4±1.3%.

DISCUSSION

BA was known to increase seed germination frequency (De pauw et al. 1995), and NAA also possesses this function, but it would result in gradual large dead number of seeds during growth and development after germination; if NAA was used together with BA, the effects were better (Tan 1991). A certain concentration ratio of NAA and BA could accelerate germination of *C. faberi* seeds to some extent (Sun 2010). We also found the germination rate of *C. faberi* seeds rose on 1/2 MS supplemented with both BA and NAA, which validated the previous conclusion.

Chen et al. (2005) also reported that NAA benefited the multiplication of PLB, and the multiplication of orchids rhizome increased five times within 40 days at 1.0 mg/L NAA. In this study, we also found that NAA was beneficial to the propagation of *C. faberi* PLB, and the optimal concentration was 1.0 mg/L which was consistent with previous studies (Chen et al. 2005). Additional BA or GA had a negative effect on the multiplication of PLB, and its number constantly decreased with their elevated concentration, but a clear trend emerged in shoots differentiation. These could be related to different kinds of plant growth regulators.

Plant growth regulators play an essential role in *in vitro* culture, and their type and ratio not only regulated cell dedifferentiation, multiplication and growth, but also manipulated cell differentiation and morphogenesis (Skoog and Miller 1957; Zheng et al. 1999). In *C. faberi* tissue culture, the induction of adventitious shoots almost needed the involvement of cytokinin, especially for BA (Hasegawa 1985). In this study, BA could accelerate the growth and differentiation of PLB with increased concentration. However, the higher the concentration was, the higher the degree of browning was, and the results are consistent that of with previous study (Fu et al. 1997).

Additionally, there were few reports about adventitious shoots induction from Chinese orchids PLB with TDZ, a cytokinin-like plant growth regulator, which had only been reported in the study of *C. sinense* Willd until now (Chang and Chang, 2000). Studies on many plants all showed that the effect of TDZ on adventitious shoots induction was better than that of BA under the same conditions (Fan et al. 2010). Our experiment demonstrates that the effects of different growth regulators on adventitious shoots induction from *C. faberi* PLB were completely different. The action of TDZ was extremely remarkable while BA was less effective. Meanwhile, TDZ used in combination with NAA could improve the shoots germination rate of *C. faberi* PLB, and the shoot seedlings were healthy and strong.

When PLB were cultured in the differentiation medium, they could also differentiate shoots and roots, form plantlets, but the number of plantlets and roots was very few. Therefore, screening out suitable rooting medium was essential for plant tissue culture. Shoot seedlings which were transferred from MS to 1/2 MS supplemented with IBA, and with 0.2% AC benefited the adventitious roots regeneration in this study. These were in agreement with existing reports (Zeng et al. 2000; Xiang et al. 2003), as well as clarified that the additional of AC and reduced macro-elements play important roles in rooting of orchids and were beneficial for root differentiation. All the results obtained in this study would provide the basis for cultivars breeding and large-scale factory production of *C. faberi*.

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