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Induction, Regeneration and Artificial Seed Production of Etiolation Stems of *Cymbidium hookerianum*

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Abstract The protocorm-like bodies (PLBs) of *Cymbidium hookerianum* were used as the explants, the effects of different hormones and hormone concentrations on the induction of etiolation stems, the subsequent regeneration of etiolation stems, and artificial seeds production and conservation using etiolation stems were studied. The results showed that the most efficient etiolation stems production induced by PLBs was obtained by adding 0.1 mg/L NAA and 0.1 mg/L 6-BA. The induced etiolation stems cultivated on the medium containing 2.0/0.1 mg/L NAA/6-BA showed the highest induction rate of PLBs, with an average of 80.40%, and the induction number of PLBs was higher, with an average of 1.36. The Induced etiolation shoot tips could regenerate rootless seedlings on the medium, and these seedlings could root and strengthen on the medium of 1/2MS + 20.0 g/L sucrose + 7.5 g/L agar. The highest germination rate of artificial seeds produced by encapsulating etiolation shoot tips was obtained on the MS medium containing 1.0 mg/L 6-BA, with an average of 86.67%. After storing for 6 weeks, the germination rate of artificial seeds preserved at 4°C was the highest, with an average of 48.00%.
Keywords *Cymbidium hookerianum*; Etiolation stem; Artificial seed; Storage

Cymbidium hookerianum is an epiphytic perennial herb of the genus *Cymbidium* in the Orchidaceae family. It is popular for its large flowers, showy color, graceful shape, long flowering period, and elegant fragrance. However, the traditional ramification method of *Cymbidium hookerianum* is of low reproduction rate and long cycle, which is difficult to meet the needs of development, production and resource conservation. Therefore, it is of great significance to study the tissue culture and rapid propagation technology of *Cymbidium hookerianum* and the *in vitro* preservation technology based on it for its development and protection (Cao, 2007). In the past, researchers took *Cymbidium hookerianum* and other orchids as research objects, and the shoot tips (Xiong et al., 2003; He and Wang, 2007), leaves (Cao, 2007) and seeds (Chen et al., 2009) were used as explants to induce PLBs and regenerated plants, and pollen (Zhang et al., 2019) and PLBs (Gogoi et al., 2013) were used as materials for low temperature and cryopreservation. However, these materials are sometimes difficult to obtain due to time constraints, sources, etc., so it is important to find new ways to optimize and enrich the original system. In the past, some studies, such as *Malus domestic* 'Gala' (Liu et al., 2001) and *Anoectochilus roxburghii* (Jiao et al., 2019), established efficient proliferation and regeneration system by inducing adventitious buds or PLBs from etiolation stems. Compared with traditional methods, this method can obtain more etiolation stems, have higher induction efficiency and better quality of PLBs, and improve the utilization rate of initial materials (Jiao et al., 2019), which has a good reference significance. Therefore, in this paper, we used this method to explore the PLBs of *Cymbidium hookerianum*, we first induced etiolation stems, then induced etiolation stems to produce PLBs or plants, and then used the induced etiolation shoot tip and stem segment to make artificial seeds and preserve research, so as to improve the tissue culture propagation and preservation system of this plant.

1 Results and Analysis

1.1 Induction of etiolation stems by PLBs

The average induction rate of etiolation stems of A_4 was 96.40% (Figure 1A), followed by A_5 and B_1, with no significant difference from A_4. In terms of the number of induced strips (Figure 1B), A_5 was the highest, with an

average of 2.48, which was not significantly different from A_4 and A_6. In terms of induction length (Figure 1C), A_4 has the longest average induction length (2.96 cm), which is not significantly different from A_1 and B_3. Since etiolation stem segment was selected as the material for induction of PLBs, the average induction length was longer and more materials were obtained, so A_1, A_4 and B_3 were better choices from the experimental results. Considering the induction rate, the number of etiolation stems and the length of etiolation stems, A_4 was the only one with high induction rate (Figure 1). Therefore, A_4 medium was the best choice, that was, NAA and 6-BA were added 0.1 mg/L respectively (Figure 2A).

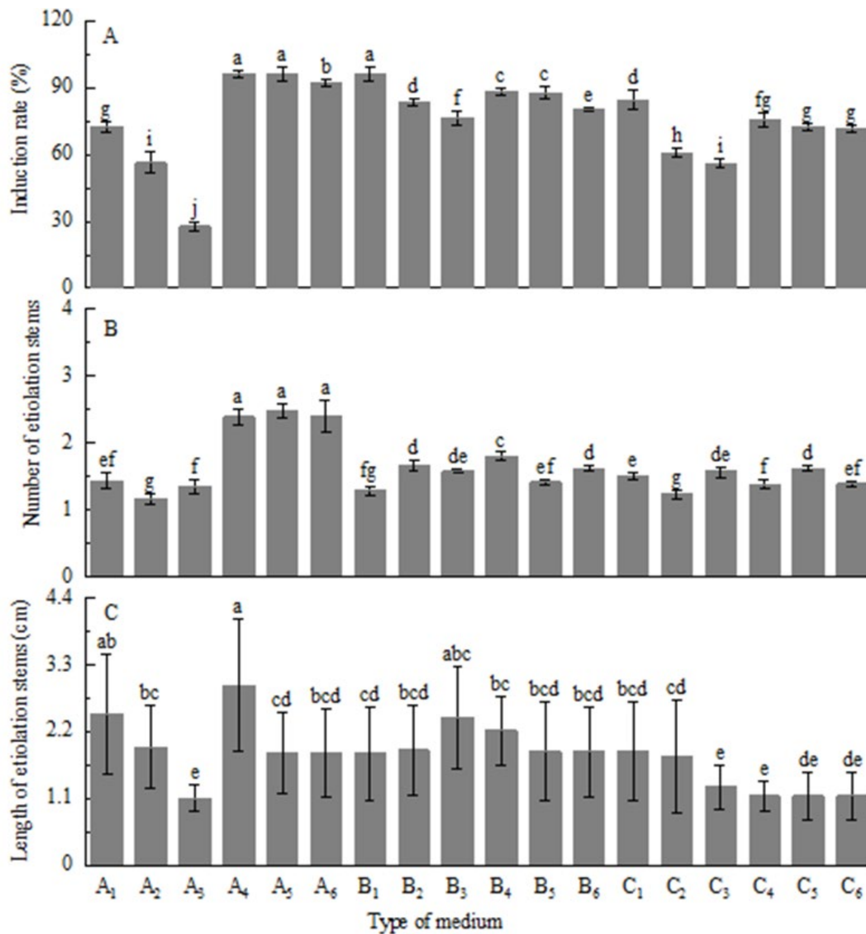

Figure 1 Effect of different compositions of hormone on induction of etiolation stems

Note: A: Induction rate of etiolation stems; B: Number of etiolation stems; C: Length of etiolation stems; Different lowercase letters indicate significant differences at $p < 0.05$ levels

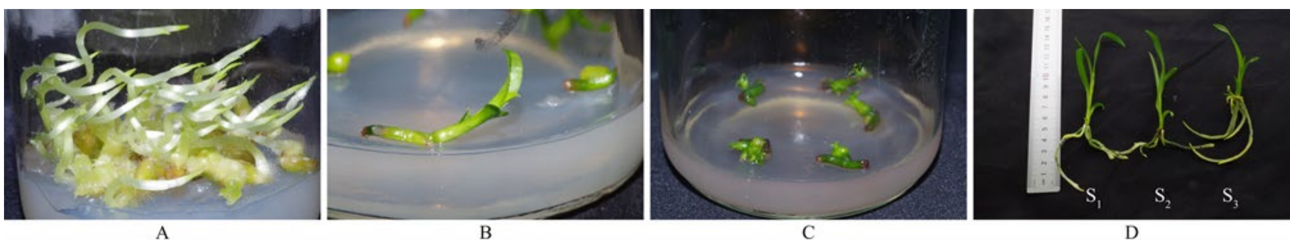

Figure 2 Induction and regeneration of etiolation stems of *Cymbidium hookerianum*

Note: A: Induction of etiolation stems on medium containing 0.1 mg/L NAA and 0.1 mg/L 6-BA (60 d); B: Rootless plants directly induced by etiolation shoot tips; C: PLBs induced by etiolation stems on medium containing 2.0/0.1 mg/L NAA/6-BA; D: Rooting plants on different media

1.2 Induced regeneration of etiolation stems

The isolated etiolation shoot tips and stem segments turned from white to green after about 5 days of culture under light, and most etiolation shoot tips began to differentiate and develop at this time, without the production of PLBs, and eventually developed into rootless plants (Figure 2B). As for the induction rate of PLBs in etiolation stem segment (Figure 3), NAA/6-BA was the highest when it was 2.0/0.1 mg/L, with an average of 80.40%, which was not significantly different from 0.1/0.1 mg/L. In terms of the induction number of PLBs, 0.5/2.0 mg/L was the highest, with an average of 1.38 (Figure 2C), which was not significantly different from 2.0/0.1 mg/L and 2.0/2.0 mg/L. When NAA/6-BA was 2.0/0.1 mg/L, the highest induction rate was obtained, with an average of 80.40%, and the induction number of PLBs was higher, with an average of 1.36.

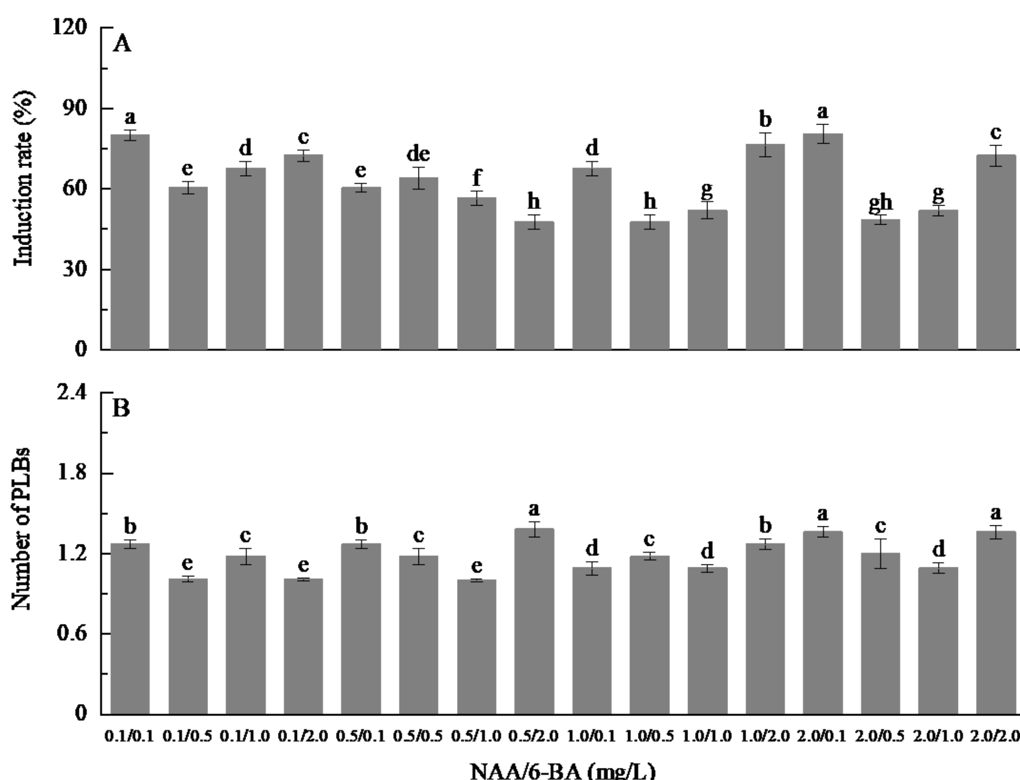

Figure 3 Effect of different compositions of NAA and 6-BA on induction of PLBs

Note: A: Induction rate of PLBs; B: Number of PLBs; Different lowercase letters indicate significant differences at $p < 0.05$ levels

1.3 Rooting and strengthening of regenerable plants

Etiolation shoot tips can directly differentiate into plants when cultured on different media (Figure 2B), but most of these plants have no roots, and it is difficult to develop into complete plants when cultured on the original media, so they need to be transferred to the rooting medium for rooting and strengthening. Rootless plants were able to root in all three rooting media, even in hormone-free media, and grew well (Table 1; Figure 2D). In terms of the number of leaves, roots and root length, there was no significant difference between the number of roots and root length except the number of leaves S_1 was better (Table 1). Therefore, considering the cost and hormone effects, using S_3:1/2MS+20.0 g/L sucrose +7.5 g/L agar without plant hormones can make rootless plants take root and grow well.

Table 1 Effect of different media on rooting of regenerable plants

Number of medium	Rooting rate	Number of leaves	Number of roots	Length of roots
S_1	100.00±0.00	4.35±0.18 a	4.67±0.58 a	4.67±2.52 a
S_2	100.00±0.00	3.98±0.03 b	4.00±0.00 a	3.00±1.00 a
S_3	100.00±0.00	3.90±0.13 b	4.33±0.58 a	4.67±1.53 a

Note: Different lowercase letters indicate significant differences at $p < 0.05$ levels

1.4 Artificial seed production and germination of etiolation stem

Using the ionic reaction of 3.0% SA and 100.0 mmol/L $CaCl_2 \cdot 2H_2O$, etiolation shoot tips and stem segments were mostly embedded into equi-diameter, uniform artificial seeds (Figure 4A). The artificial seeds made from etiolation shoot tips were cultured on different media (Figure 5), and more than 70.00% of them could germinate, and the leaves directly grew from the embedded beads (Figure 4B), which could take root and become seedlings at later stage. Among them, the germination rate of artificial seeds on MS+1.0 mg/L 6-BA medium was the highest, with an average of 86.67%. There was no significant difference between MS and MS, 1/2MS+1.0 mg/L 6-BA, MS+2.0 mg/L 6-BA. However, the germination rate of artificial seeds made from etiolation stem segments was lower on different media, significantly different from that of etiolation shoot tips, and PLBs were mainly generated first (Figure 4C). The germination rate was the highest in MS+1.0 mg/L 6-BA, with an average of 33.33%. There was no significant difference between MS and MS, 1/2MS+1.0 mg/L 6-BA, MS+2.0 mg/L 6-BA.

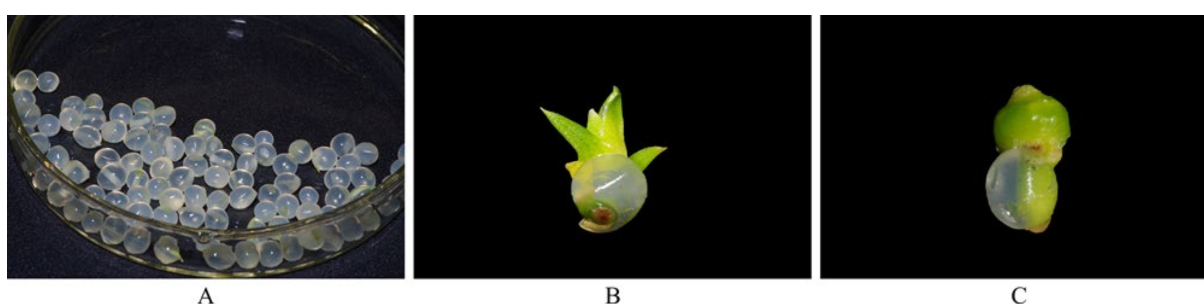

Figure 4 Production and germination of artificial seeds

Note: A: Artificial seeds produced by etiolation stems; B: Germination of artificial seeds produced by etiolation shoot tips; C: Germination of artificial seeds produced by etiolation stem segments

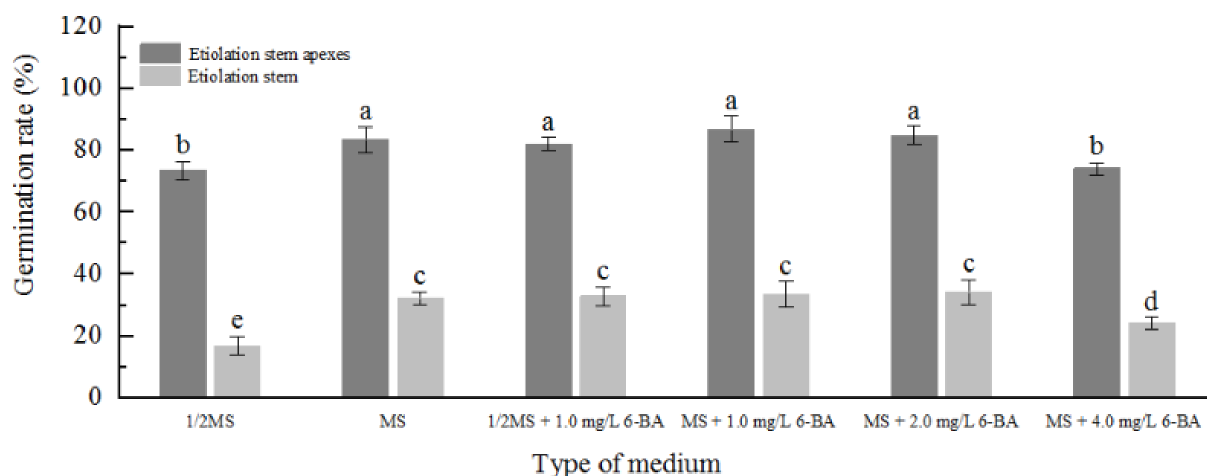

Figure 5 Effect of different media on germination of artificial seeds

Note: Different lowercase letters indicate significant differences at $p < 0.05$ levels

1.5 Artificial seed preservation

When stored at 4°C and 25°C (Figure 6), the germination rate of artificial seeds decreased gradually with the extension of storage time, until the germination rate decreased to less than 50.00% after 6 weeks. In contrast, the unembedded etiolation shoot tips all died after 3 weeks. Compared with artificial seeds stored at different temperatures, the germination rate of artificial seeds stored at 25°C was higher after 1 week, but there was no significant difference between them. In the following 2 to 6 weeks, the germination rate of artificial seeds stored at 4°C was higher than that of artificial seeds stored at 25°C, but the difference was not significant. After 6 weeks of storage, the results showed that the germination rate of artificial seeds made from etiolation shoot tips was the highest at 4°C, with an average of 48.00%.

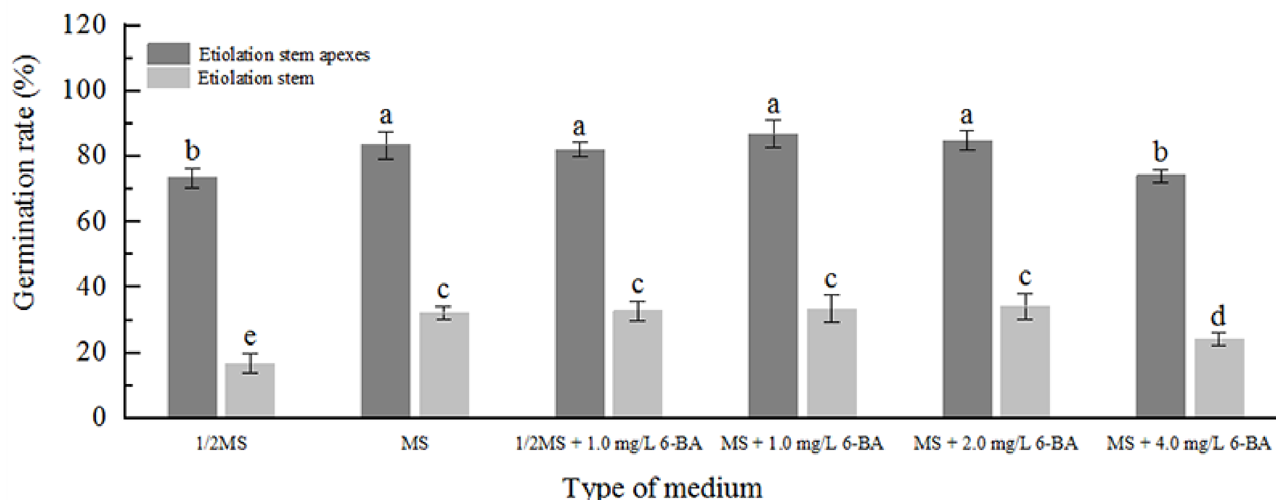

Figure 6 Effect of different temperatures and different conservation times on germination of artificial seeds
Note: Different lowercase letters indicate significant differences at $p < 0.05$ levels, NA not applicable due to non-viable capsule

2 Discussion

Dark culture is the decisive factor for the induction of PLBs of *Cymbidium hookerianum*. The inoculated PLBs produced green adventitious buds in different media under light condition, and then produced normal plants. Etiolation stems were only produced under dark conditions, and under different experimental treatments, etiolation stems were induced from inoculated PLBs (Figure 1A), with large number and slender form (Figure 2A). These materials could recover green after re-illumination cultivation, and their leaves also extended quickly, but they could not return to the same state as normal plants. These results suggest that dark culture may have an irreversible effect on the differentiation and development of PLBs.

The method of separate culture of etiolation stem tip and etiolation stem segment was adopted to induce PLBs from etiolation stem, in which etiolation stem tip showed strong differentiation and regeneration ability and directly developed into plants. However, although the induction rate of PLBs from etiolation stem segment was high (60.00%~80.00%), the amount of PLBs obtained was all low (Figure 3B). This was quite different from the yield of PLBs induced by Cao (2007) from the stem segment of test-tube plantlets, which may be related to different material maturity or hormone concentration (the concentration in this paper is relatively low). Although the overall efficiency is low (more material and less PLBs yield), these induced large numbers of etiolation stems provide material for other studies, such as artificial seed production.

Endosperm and germination media for artificial seed seeding are the nutrient sources of embedded tissues, so the germination rate of artificial seeds can be improved by adjusting the contents of inorganic salts and hormones in artificial endosperm or germination media (Chen et al., 1995). For example, artificial seeds made from PLBs of hybrid species *Aranda* and *Vanda* had the highest germination rate in 1/2MS medium without hormones (Gantait et al., 2012). Artificial seeds made from some stem tips had the highest germination rate on full MS salt and hormone-added medium (Nower, 2014; Bhattacharyya et al., 2018). In this article, the germination rate of artificial seeds made from etiolation stem tips on medium containing only 1/2MS salt was significantly different from that on medium containing only MS salt and 1/2MS salt supplemented with 1.0 mg/L 6-BA, which not only indicated the effect of full MS salt. It also indicated that the addition of appropriate hormones could promote germination to a certain extent (Figure 4). However, different from etiolation stem tips, artificial seeds made from etiolation stem segments had low germination rate in the experiment and were not suitable for embedding seeds. Similarly, in many studies, materials with strong differentiation and regeneration ability, such as regenerative somatic embryos (Nirala et al., 2010) and PLBs (Gantait et al., 2012), are often selected to produce artificial seeds.

Low temperature and darkness can reduce the metabolic activities of plant tissues to achieve the purpose of preservation (Zhou and Xiang, 2008). However, directly exposing materials to such environment without treatment will often cause the materials to be affected by low temperature and water loss, resulting in their death. The artificial seeds produced by the ion reaction embedding of alginate and calcium chloride can not only protect plant tissues from mechanical damage and prevent drying and dehydration during storage, but also reduce the influence of these adverse factors to a certain extent, thus prolongating their preservation time (Gantait et al., 2012). This method is simple and has been used in many orchids. For example, *Aranda* and *Vanda* hybrids (Gantait et al., 2012) and *Dendrobium* (Saiprasad and Polisetty, 2003). The survival rate of the embedded etiolation stem tips in this paper was 44.00%~48.00% after 6 w, and the germinated artificial seeds could regenerate into plants (data not shown), regardless of whether the embedded etiolation stem tips were stored in the dark at 4°C refrigerator or 25°C room temperature. However, all the unembedded etiolation stems died within 3 w. This is consistent with the preserved results of *Coelogyne breviscapa* (Mohanraj et al., 2009).

In this study, the PLBs of *Cymbidium hookerianum* were used as the explants, the effects of the induction of etiolation stems, the subsequent regeneration of etiolation stems, and artificial seeds production and conservation using etiolation stems were studied. The results showed that except for etiolation shoot tips, the efficiency of etiolation stem segment induction regeneration and artificial seed germination was not high. But this method enriches the tissue culture propagation system of *Cymbidium hookerianum*, and a large number of etiolation stems produced in the first step provided a large number of materials for other studies, such as callus induction, genetic transformation, cryopreservation and so on.

3 Materials and Methods

3.1 Experimental material

The test tube plantlets of *Cymbidium hookerianumand* and their induced PLBs were used as materials. The test tube plantlets were seeded aseptically, while the PLBs were induced by test tube plantlets, which had been proliferated and subcultured for 2 years.

3.2 Induction of etiolation stem by protocorm-like stem

The basic medium was MS, supplemented with 20.0 g/L sucrose and 7.5 g/L agar, to obtain hormone types and combinations (Table 2). After pH was adjusted to 5.8, sterilization was performed. At the time of inoculation, the PLBs of the same size and state were taken and cut into a single medium with the same size and state. Each treatment was inoculated in 5 bottles, and 5 in each bottle, a total of 25, and repeated 5 times, cultured at 25°C in the dark. After 60 d, the induction of etiolation stem in each treatment was observed and counted, including the induction rate, the number of etiolation stems, the length of etiolation stems.

Table 2 Hormone compositions of etiolation stem induction

No.	Type and concentration of hormone (mg/L)		No.	Type and concentration of hormone (mg/L)		No.	Type and concentration of hormone (mg/L)	
	NAA	6-BA		IBA	6-BA		NAA	IBA
A_1	0.1	0.0	B_1	0.1	0	C_1	0.1	0.1
A_2	0.5	0.0	B_2	0.5	0	C_2	0.5	0.1
A_3	1.0	0.0	B_3	1.0	0	C_3	1.0	0.1
A_4	0.1	0.1	B_4	0.1	0.1	C_4	0.1	0.5
A_5	0.5	0.1	B_5	0.5	0.1	C_5	0.5	0.5
A_6	1.0	0.1	B_6	1.0	0.1	C_6	1.0	0.5

Induction rate of etiolation stem (%)=Number of PLBs induced by etiolation stem/Number of inoculated stem ×100%
Average number of induced etiolation stems =Total number of induced etiolation stems/Number of PLBs
Average induced length of etiolation stems (cm)=Total length of all induced etiolation stems/Number of induced stems

3.3 Induction of etiolation stem regeneration

The induced etiolation stem segments were cut into sterile petri dishes in the clean bench, each segment was about 1.0 cm, and all the other segments were inoculated as stem segments except the shoot tip alone. The basic medium was MS, supplemented with 20.0 g/L sucrose and 7.5 g/L agar, and the hormones were NAA and 6-BA at 0.1 mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L, respectively, in a completely random 4×4 design. Each treatment was inoculated with 5 bottles. A total of 25 were inoculated 5 times in each vial and repeated 5 times. The culture was conducted at 25°C and 12 h/d of light. After 30 days, the induction of PLBs in each treatment was observed and counted, including the induction rate and the number of PLBs.

PLBs induction rate (%)=Number of induced PLBs segments/Number of inoculation×100%
Number of PLBs induction =Number of PLBs induction/Number of inoculation×100%

3.4 Rooting and strengthening of regenerable plants

3 rooting media were set up: S_1: 1/2MS+0.1 mg/L NAA+0.1 mg/L 6-BA+1.0 g/L activated carbon+20.0 g/L sucrose +10.0% (W/V) mashed potato+7.5 g/L agar; S_2: Huabao No.1 3.0 g/L+2.0 mg/L NAA+1.0 mg/L 6-BA+1.0 g/L activated carbon+20.0 g/L sucrose+10.0% (V/V) coconut juice+7.5 g/L agar; S3:1/2 ms+20.0 g/L sucrose+7.5 g/L agar, rootless plants induced by etiolation stem tips were inserted into the medium with tweezers at the bottom, and 5 bottles were inoculated in each treatment, 4 plants were inoculated in each bottle, a total of 20 plants were inoculated and repeated 3 times. After 30 days, they were taken out from the bottle, and the rooting rate, leaf number, rooting number and root length were counted.

Rooting rate (%)=Number of plants with induced root/Number of inoculated ×100%
Number of roots=Total number of roots/Total number of seedlings

3.5 Artificial seed preparation and germination of etiolation stem

The induced etiolation stem segments were cut into a sterile petri dish in the clean bench, each segment was about 0.3 cm, and the stem tip and stem segment were separated. Then the cut stem tip and stem segment were mixed with MS (excluding $CaCl_2 \cdot 2H_2O$)+2.0 mg/L NAA+0.1 mg/L 6-BA+30.0 g/L sucrose and 3.0% (W/V) sodium alginate (SA). Then, a single sample was dropped into 100.0 mmol/L $CaCl_2 \cdot 2H_2O$ solution with tweezers and reacted for 20 min. The embedded beads produced by the above steps were removed and cleaned with sterile distilled water for 3 times and dried with sterile filter paper before being connected to different media. Different media refer to six media including 1/2MS without hormones, MS without hormones, 1/2MS+1.0 mg /L 6-BA and MS+1.0, 2.0 and 4.0 mg/L 6-BA. 30.0 g/L sucrose +7.5 g/L AGAR were added to the above medium. 5 vials were inoculated in each treatment and 10 vials were inoculated for 3 times. Germination rate was calculated 30 days later.

Germination rate(%)=Number of germinated artificial seeds/Number of inoculated artificial seeds×100%

3.6 Artificial seed preservation

The artificial seeds made from etiolation stem tips were dried with filter paper and placed into 1.8 mL frozen tubes with no bacteria, 5 seeds were placed in each tube. The frozen tubes were sealed with sealing film and stored in the dark at 4°C refrigerator and 25°C of room temperature for 6 w. At the same time, unembedded etiolation stem tips were used as control. A total of 25 seeds were taken out of 5 tubes of frozen tubes per week and transferred into the germination medium. The experiment was repeated 3 times, and the germination rate was calculated after 30 days.

3.7 Data analysis

All values were expressed by means and standard deviation. OriginPro 2020 (Learning Edition, Origin Lab, USA) was used for drawing. SPSS 26.0 was used for analysis of variance for data. Duncan was used for multiple comparison (P<0.05).

Authors' contributions

FSB was the experimental designer and executor of this study, completed data analysis and written the first draft of the paper. YYP participated in experimental design and analysis of experimental results. ZZ was the architect and principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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