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The Efficient Tissue Culture System of *Orostachys fimbriata*

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Abstract

This study aimed to investigate the tissue culture and rapid propagation techniques of *Orostachys fimbriata*, a medicinal and ornamental herbaceous perennial herb belonging to the family Crassulaceae. The leaves of *O. fimbriata* were used as explants to investigate the effects of plant growth regulators such as thidiazuron (TDZ), 6-benzylamino purine (6-BA) and 1-naphthaleneacetic acid (NAA), on the induction of callus, differentiation of adventitious buds and rooting of shoots. Our results showed that optimum callus induction medium was MS medium supplemented with 0.5 mg·L^{-1} TDZ and 0.2 mg·L^{-1} NAA. 1.5 mg·L^{-1} 6-BA and 0.2 mg·L^{-1} NAA was the optimum hormone combination for differentiation of adventitious buds. And 0.1 mg·L^{-1} NAA could efficiently promote rooting. The survival rate of transplants reached about 90%. In this study, using leaves of *O. fimbriata* as explants, high efficient tissue culture and regeneration system of *O. fimbriata* were established, and the period from leave to transplant plantlet was about 3 months. The presently developed protocol could be used for large scale clonal propagation and germplasm conservation of *O. fimbriata*. The efficient tissue culture system of *O. fimbriata* would provide technical support for its utilization.

Keywords

***Orostachys Fimbriata*, Differentiation, Callus, Adventitious Buds, Efficient**

1. Introduction

Orostachys fimbriata is a biennial or perennial succulent herb belonging to the Crassulaceae family. The whole

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herb contains a variety of active substances with the effect of cooling blood and hemostasis, detoxication and restraining wound. Therefore, *O. fimbriata* is used for bloody flux, partial blood, hemorrhoids blood and chronic wound which is not cured for a long time [1]. *O. fimbriata* is small and exquisite, with beautiful plant type and panicle with amount of flowers. It has become the "new favorite" of family gardening small fleshy plant and can also be arranged in flower bed, flower border and roof greening [2]. However, due to the great medicinal and ornamental value of *O. fimbriata*, wild resources are subjected to excessively exploitation and thus become threatened. *In vitro* culture techniques represent an excellent option for the study and conservation of rare, threatened or endangered plants [3] [4]. Therefore, the interest in using these techniques for rapid and large-scale propagation of medicinal and ornamental plants has been significantly increased [5] [6]. The present study is undertaken to establish an efficient protocol for rapid large-scale regeneration of plantlets *in vitro* from leaf explants of *O. fimbriata*.

2. Materials and Methods

2.1. Material

O. fimbriata were collected from Wubaoan Mountains of Zoucheng, Shandong in May, 2014, and planted in the greenhouse of School of Life Sciences of Qufu Normal University. Healthy leaves were selected as explants.

2.2. Method

2.2.1. Material Disinfection

Fresh leaves (explants) were washed thoroughly under running tap water with moderate amount of detergent for 30 min. The explants were then surface disinfected with 70% ethanol for 30s followed by immersion in 0.1% (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 min and finally rinsed five times with autoclaved sterile distilled water in a clean bench. The surface sterilized explants were trimmed to about 0.5 - 1.0 cm in diameter prior to inoculation on culture media.

2.2.2. Callus induction

Sterilized explants were inoculated onto callus induction medium with different concentrations of TDZ and NAA (**Table 1**) according to the available research [7]. The induction ratio of callus was recorded as the number of leaves generating callus/the number of leaves for inoculation × 100% after 30 days. There were 15 bottles in each treatment with 3 explants in each bottle.

2.2.3. Differentiation of Adventitious Buds

For adventitious buds differentiation, the callus were excised into small pieces of 1 × 1 cm in size and cultured on MS medium supplemented with various concentrations of 6-BA and NAA (**Table 2**). The induction ratio (the number of differentiated and germination callus/the total number of callus × 100%) of adventitious buds which form 3 - 4 fully expanded leaves were recorded after 30 days. Thirty calluses were randomly selected in each treatment to record the induction rate.

2.2.4. Rooting of Shoots

For root induction, excised plantlets (2 - 3 cm in length) from *in vitro* callus were transferred to MS basal

Table 1. Effects of TDZ and NAA on callus induction of *O. fimbriata.*

TDZ concentration ($mg \cdot L^{-1}$)	NAA concentration ($mg \cdot L^{-1}$)	Induction rate of callus (%) ± SD	Character of callus
0.3	0.2	86.3 ± 3.40^{ab}	Compact, green, with slight brownness
0.5	0.2	93.3 ± 3.35^{a}	Compact, green, with slight brownness
1.0	0.2	$81.1 \pm 6.96b^{c}$	Loose, green
0.3	0.5	$80 \pm 3.30b^{c}$	Compact, light green
0.5	0.5	76.7 ± 6.65^{c}	Compact, light green
1.0	0.5	74.1 ± 4.50^{c}	Loose, light green

Note: Different letters following the data in the same column indicated significant differences ($p < 0.05$). The same below.

Table 2. Effects of different plant regulators on differentiation shoots of callus.

6-BA concentration (mg·L^{-1})	NAA concentration (mg·L^{-1})	Induction rate of adventitious buds (%)	Buds no. per callus (piece)
1.0	0.2	15.6 ± 1.15^{b}	8.4 ± 2.36^{b}
1.5	0.2	32.6 ± 1.68^{a}	12.4 ± 2.58^{a}
2.0	0.2	9.3 ± 1.70^{d}	6.3 ± 1.84^{c}
1.0	0.5	6.7 ± 1.11^{d}	4.6 ± 1.50^{d}
1.5	0.5	13.7 ± 1.73^{bc}	5.2 ± 1.73^{cd}
2.0	0.5	15.2 ± 2.35^{bc}	8.5 ± 1.80^{b}

medium supplemented with different concentrations of NAA at the range of 0.1 - 0.3 mg·L^{-1} (**Table 3**). The percentage of rooting, the mean number of roots per shoot and the length of root were calculated after 30 days of induction with randomly selected 30 shoots in each treatment.

2.2.5. Culture Media and Conditions

This study regards MS medium as basic medium, which supplemented with 3% (w/v) sucrose and gelled 0.6% (w/v) agar. The pH of the medium was adjusted to 5.8 - 6.0 before autoclaving at 121˚C for 20 min. All the cultures were incubated in the culture room at 25˚C ± 1˚C under a 12 h photoperiod with illumination intensity of 1500 - 2000 lx supplied by cool white fluorescent light.

2.2.6. Acclimatization and Transplanting

The culture bottle was opened for 4 - 5 days for acclimatization the *in vitro* plantlets before transplant [8]. The plantlets with developed well roots were removed from the culture medium and the roots were washed gently under running tap water to remove agar. The plantlets were transplanted to the flower cultivation soil and humus mixing vermiculite (1:1) under controlled growth chamber conditions (temperature 25˚C ± 1˚C, 12 h photoperiod, 80% relative humidity) and the effects of the two different matrixes on the survival rate of the *in vitro* plants were compared.

2.2.7. Statistical Analysis

The treatments were repeated three times. The data were analyzed statistically using SPSS 19.0 Statistical Software. The significance of differences among means was carried out using Duncan's multiple range test at $p < 0.05$. The results are expressed as the means ± SD of three experiments.

3. Result and Analysis

3.1. Effects of TDZ and NAA on the Induction of Callus

The leaves were inoculated on MS medium with different combination of TDZ (0.3, 0.5, 1.0 mg·L^{-1}) and NAA (0.2 and 0.5 mg·L^{-1}) for callus induction (**Table 1**). The results showed that all of the 6 kinds of medium can induce the production of callus after about 30 days of culture (**Figure 1(A)**). Among these phytohormones tested, the best induction rate (93.3%) was obtained in the presence of MS medium with 0.5 mg·L^{-1} TDZ and 0.2 mg·L^{-1} NAA in the present study. The callus was compact or loose block, with color of green to light green. About 50% of callus had a large number of small white particles structure on the surface. The anatomical observation indicated that they were parenchymatous tissue (data not shown). About 20% of callus surface was relatively dry, with brown spots, the adventitious bud differentiation ability from such callus was stronger than green or light green callus (**Figure 1(B)**).

3.2. Effects of 6-BA and NAA on Adventitious Buds Differentiation from Callus

In order to obtain multiple tissue culture seedlings of *O. fimbriata*, the interactions of various concentrations of 6-BA (1.0, 1.5, 2.0 mg·L^{-1}) and NAA (0.2 and 0.5 mg·L^{-1}) were evaluated on the rate of adventitious bud induction (**Table 2**). The result showed that the highest rate of adventitious bud induction (32.6%) was recorded when 1.5 mg·L^{-1} 6-BA and 0.2 mg·L^{-1} NAA were added into MS medium. Under this condition, about 12.4 ± 2.58

Table 3. Effects of different NAA concentration on rooting of *O. fimbriata*.

NAA concentration (mg·L^{-1})	Rooting rate (%)	Average root no. per plantlet	Average root length (cm)
0.1	92.2 ± 2.20^{a}	28.1 ± 6.39^{a}	4.7 ± 1.11^{a}
0.2	84.1 ± 2.81^{b}	24.3 ± 5.63^{b}	4.2 ± 0.99^{b}
0.3	78.9 ± 3.30^{b}	22.2 ± 6.39^{b}	3.4 ± 0.82^{c}

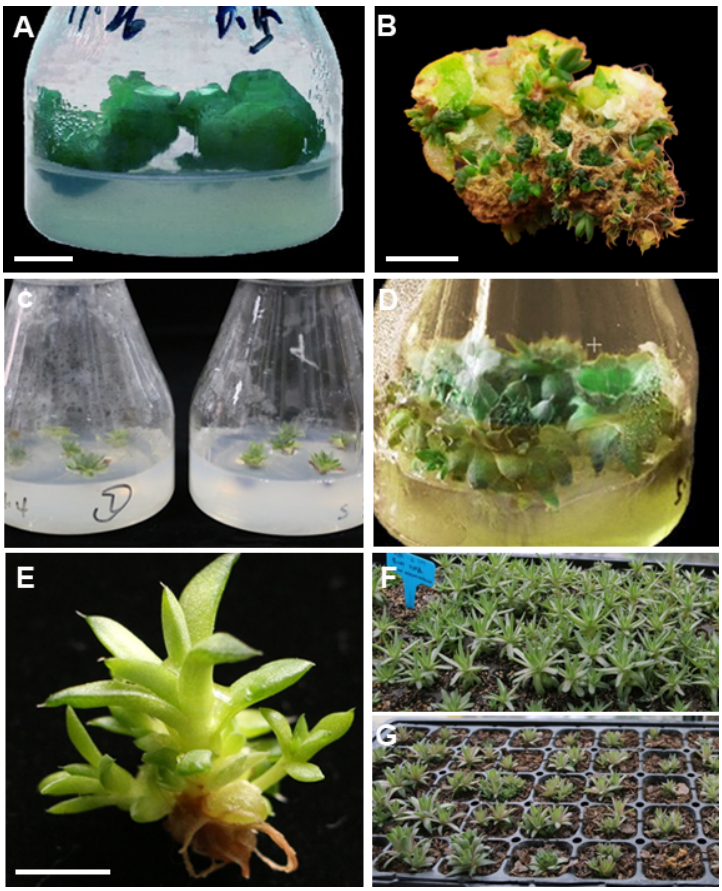

Figure 1. Tissue culture of *O. fimbriata* (A: Callus; B. Differentiation of adventitious buds from callus; C. Adventitious buds; D: Rooting of adventitious buds; E: Plantlet before transplant; F: Plants grown in composite soil of humus and vermiculite; G: Plants grown in flowers nutritional soil Bar: 1 cm).

adventitious buds were formed from per callus. The results also showed that callus with light brownness on the surface could differentiate more adventitious buds, and the quality of buds was better (**Figure 1(B)**).

3.3. Effects of NAA on Rooting of Plantlets for *O. Fimbriata*

The adventitious buds (2 - 3 cm in length) were excised and inoculated on MS medium with different concentrations of NAA (0.1, 0.2 and 0.5 mg·L^{-1}) (**Table 3**) for induction of roots. The effects of NAA on root induction as well as the length and number of roots were examined after 15 days of culture. In this experiment, roots formed at a high frequency (92.2% ± 2.20%) on MS medium containing 0.1 mg·L^{-1} NAA. In this medium, a maximum number of roots (28.1 ± 6.39) attaining a length of (4.7 ± 1.11) cm were obtained (**Figure 1(D)**, **Figure 1(E)**). Forming good roots system with low concentration of NAA proved that *O. fimbriata* is a plant which can produce roots easily.

3.4. Acclimatization and Transplant

The growth was observed after transplanting for 30 days and the survival rate was counted. The result showed that the survival rate in the substrate mixing humus with vermiculite was 89.8% with good growth vigor (**Figure 1(F)**), and the survival rate in cultivatable soil of flowers was 91.7% but the growth vigor was worse than that of the former (**Figure 1(G)**). This result indicated that the substrate mixing humus with vermiculite was more applicable for the cultivation of *O. fimbriata*.

4. Discussion

The dedifferentiation and re-differentiation capacities of plants were mainly regulated by the growth substance in plant, such as auxin NAA and cytokinin 6-BA, TDZ, etc. As a high efficient growth regulator, TDZ was widely used in rapid propagation system for tissue culture of plants [9]-[12]. In tissue culture, TDZ worked on plant cells separately or jointly with other growth regulatory substances. A great deal of scientific researches proved that adding TDZ in various plant cultivation systems could induce the forming of callus with a higher cell proliferation rate than other plant growth regulatory substances [13] [14]. Furthermore, the study of Murthy suggested that low concentration of TDZ could promote the forming of compact-type green modular callus [15]. Through combining TDZ and NAA at different concentrations in this research, the callus of *O. fimbriata* was obtained and the best induction medium was MS medium with 0.5 $mg \cdot L^{-1}$ TDZ and 0.2 $mg \cdot L^{-1}$ NAA and the induction rate was up to 93.3%, forming massive compact or loose callus with the color from light green to green.

In tissue culture for plant, differentiation of adventitious buds from callus was determined by the ration of cytokinin and auxin, that is, relatively higher 6-BA and lower NAA led to high shoot induction rate otherwise led to root induction [16] [17]. In this study, the highest induction rate of adventitious bud was obtained when the ration of 6-BA and NAA was 7.5, that is, MS medium with 1.5 $mg \cdot L^{-1}$ 6-BA and 0.2 $mg \cdot L^{-1}$ NAA was the best medium for differentiation of adventitious bud. The induction of adventitious bud was closely related to the growth of callus. When the callus color of *O. fimbriata* turned from light green to brown, the induction rate of adventitious buds would be higher while that of adventitious bud always maintaining light green would be lower, which was similar to that the callus in *Hevea brasiliensis* anther culture maintaining light green without turning brown after 50 days was adverse to the induction of somatic embryo [7]. The difference for differentiation capacity of adventitious buds of the two different calluses needed to be studied further. During the culture of *O. fimbriata*, the callus induction rate of explants from the leaf bases was higher than the distal portions of the leaf, which was similar to the previous research result [18]. This result supported the notion that growth and organizational process occurred in basal portions of the leaf while many important energy-related metabolic process occurred at the distal end [19] [20], and provided suggestion for selection of explants for succulent plants tissue culture. In a word, this research reported a high frequency protocol for *in vitro* propagation of *O. fimbriata* which could provide a large quantity of tissue culture seedlings in a period as short as 3 months, thus providing a reliable technical support for resource protection and usage of *O. fimbriata*.

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