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High-Efficient Regeneration and Transplanting for Industrialized Seedling Production of Biomedical *Dendrobium officinale*

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Dendrobium officinale was widely used as a rising valuable forest Chinese medicinal herb in recent years in China. By the systematical researches on seed aseptic germination, protocorm induction and proliferation, rooting and hardening-off culture, seedling transplanting and tending, the highly efficient regeneration system and domestication transplanting technology for *D. officinale* were established. The results showed that 1/2 MS without any phytohormone is the optimal medium for seed aseptic germination of *D. officinale*, the medium suitable for protocorm induction and proliferation is MS + 1.0 mg/L 6-BA (6-Benzylaminopurine) +1.0 mg/L NAA (1-Naphthaleneacetic acid) +1.0 mg/L KT (kinetin) according to the orthogonal tests, the optimal medium for rooting and hardening-off culture of *D. officinale* is 1/2 MS+ 0. 2mg /L IBA (3-indolebutyric acid) + 0.5% activated carbon + 40% potato extract, and the corresponding seedling transplanting medium is the 1:1:5 combination of vermiculite, perlite and humus soil. The high-efficient regeneration and transplanting technique obtained will contribute to the industrialized seedling production of Biomedical *D. officinale*.

Key words: Dendrobium officinale; Regeneration system; Seedling transplanting; Medicinal herb, Biomedicine.

As one of perennial epiphytic herb of Orchidaceae family, *Dendrobium officinale* is a perennial valuable forest Chinese medicinal herb[1,2], which was recorded as herbal Dendrobii in the Pharmacopoeia of Peoples Republic of China. The whole plantlet of *D. officinale* has been used as medicine material for two thousand years[2,3]. The earliest record of *D. officinale* was in Shen Nong Materia Medica and Compendium of Materia Medica. Modern pharmacological researches showed that polysaccharides contained in *D. officinale* have the function of antioxidation and antineoplastic, and can relieve fatigue[4,5]. Moreover, many kinds of bioactive components extracted from *D. officinale*, especially including Dendrocandin ingredients, are effectively used to enhance tumor resistance, inhibit cardiovascular discomfort, relieve pain, eliminate evil-heat, moisten lung and relieve coughs[5,6]. The main medicinal part of *D. officinale* plantlets is its stem, which have the practical efficacy for fluid deficiency, dry mouth polydipsia, food retch, deficiency heat after illness and dark eyes[7,8].

The traditional medicinal materials of *D. officinale* relied mainly on its wild resources. Because of the extremely tiny and exalbuminous seeds, the natural germination of *D. officinale* needed very rigor growth environment, this resulted in that the natural propagation capacity of *D.*

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officinale is very low [9]. Moreover, its reproduction rates of ramet and cutting were extremely low, this does not fulfill the production demands[9,10]. In recent years, with the rising price and urged market interests, the uncontrolled over-exploiting of wild source, *D. officinale* is becoming on the verge of extinction in China [11,12]. Especially, more and more drugs were found in plant biosources[13-31]. The above adverse reasons seriously restricted the sustainable development of *D. officinale* industry in China. So, in order to fulfill the market demands, many studies have been carried out on tissue culture, chemiccl components and cultivation technology of *D. officinale* in recent years[11-37].

Therefore, using the *D. officinale* seeds and its protocorms derived from aseptic germination as explants, researches on seed aseptic germination, protocorm induction and proliferation, rooting and hardening-off culture, seedling transplanting and tending were systematically carried out, providing a technology base for industrial seedling production and somatic hybridization breeding of *D. officinale*.

MATERIALS AND METHODS

Material Treatment

Assuming that the ancient pagoda of every layer of the eight points lie on a plane, their connection is rule eight polygon. According to the observation data, we can see that the Z axis of eight observation points of each layer is almost, then we can combine eight points ideal on the same plane, we will draw a two-dimensional eight shape model useing Matlab mathematical software [4] on eight coordinates of each layer (Fig. 1).We use the method of averaging[5], get the average value of each layer eight observation point coordinate, it is the coordinate of the layer of the center point. Then we build a model[6,7]:

The healthy capsules without disease spots and pest wormhole were selected from *D. officinale* individuals, and then the fruit stalks and persistent petals were cut off. Posteriorly, the *D. officinale* capsules were washed thoroughly to remove surface dusts by tap water for 30 minutes.

Seed Aseptic Germination

The surface sterilization of cleaned *D. officinale* capsules was carried out in super-clean bench by the following treatment program. Firstly soaked in 75% alcohol for 55s, followed by washing with sterile water for 4 times, After sterilized in 0.1% HgCl2 for 8 minutes, the *D. officinale* capsules were washed again with sterile water for 6 times, and then its surface water were absorbed by putting on sterile filter papers. The *D. officinale* capsules were split, and were flicked to deliver seeds to the surface of 1/2 MS medium. The seeds of each *D. officinale* capsule should be sowed for 50-100 tissue-cultured bottles. During the culture, the intensity of illumination in culture room was maintained between 1000-1500lx, the illumination period was 12h per day, and the temperature was maintained at (25±1)°C.

Protocorm Induction and Proliferation

After successful aseptic germination of the *D. officinale* seeds, the strong clusters were selected out as explants for protocorm induction and proliferation in different MS medium, which were added 3 kinds of hormones including 0.6-1.2 mg/L 6-BA (6-Benzylaminopurine), 0.6-1.2 mg/L NAA (1-Naphthaleneacetic acid) and 0-1.5 mg/L KT (kinetin). Each hormone has 4 levels, and the orthogonal test L16 (43) was designed to explore the effect of protocorm induction and proliferation of *D. officinale* (Table 1). 5 tissue-cultured bottles were used for each treatment group, and 6 protocorms were inoculated in each bottle. Observation and record about the protocorm growth of *D. officinale* were carried out periodically, then its growth status and proliferation coefficient were calculated among a period of 30d. During the culture period, the illumination intensity in culture room was maintained between 1000-1500lx, the illumination time was 14h per day, and the temperature was maintained at (25±1) °C.

Rooting and Hardening-off Culture

In order to analyze the effect of different organic extracts on rooting and hardening-off culture of *D. officinale*, the strong adventitious buds (2.0 cm in height) differentiated from *D. officinale* protocorms were inoculated in basic medium: 1/2 MS + 0.2mg/L IBA (3-indolebutyric acid) + 0.5% activated carbon, which were added kind of organic extracts including potato extract (40%), banana extract (40%) and apple extract (40%) (Table 2). 30 tissue-cultured bottles were used for each treatment group, and 6 adventitious buds were inoculated in each bottle. The root length, root number and relative indicators of plantlet were

calculated after 60 days culturing period. During the culture period, the intensity of illumination in culture room was maintained between 1000-1500lx, the illumination time was 12h per day, and the temperature was maintained at (25±1) °C.

Domesticating and Transplanting of Seedlings

When the tissue-cultured seedlings of *D. officinale* grew up to the status in which the plantlets are 5-7 cm in height, have 4-5 leaves in dark green, and have 3-4 white roots in the length above 2cm[18], these strong plantlets were selected to be domesticated and transplanted. The plantlets were domesticated periodically to enhance the adaption to the natural light and temperature of culture rooms. All plantlets in bottles were divided into 3 treatment groups by hardening time (3d, 5d and 7d) to observe the influence of different hardening time on domestication and posterior transplanting.

During the transplanting, the roots and stems of tissue-culture plantlets should not be damaged when removed out of the bottles. After washing the attached medium with flowing water, all plantlets were put on the shady place to airing, until their roots become a little white. Finally, these plantlets were transplanted into matrix with the ratio 1:1:5 of vermiculite, perlite and humus soil. After transplanting, the surfaces of seedlings leaves should be maintained moist by spraying, and foliar fertilization should be performed every week.

RESULTS AND ANALYSIS

High-efficient aseptic germination of D.officinale seeds

Among the 50 bottles for aseptic germination of *D. officinale* seeds, only 3 bottles were contaminated by microbes and failed to

Table 1. Orthogonal Design Table for Protocorm Induction and Proliferation of *D. officinale*

Factor level	6-BA / (mg/L)	NAA / (mg/L)	KT/ (mg/L)
1	0.6	0.6	0.0
2	0.8	0.8	0.5
3	1.0	1.0	1.0
4	1.2	1.2	1.5

Table 2. Treatments of Different Organic Extracts on Rooting and Hardening-off Culture of *D. officinale*

Treatment No.	IBA / (mg/L)	Activated carbon / (%)	Extracts / (%)
1	0.2	0.5	40% potato extract
2	0.2	0.5	40% apple extract
3	0.2	0.5	40% banana extract

Table 3. Effect of Different Phytohormone Combinations on Protocorm Induction and Multiplication of *D. officinale*

Treatment	Number of flowering			Number of protocorms			protocorm
No.	15d	30d	average	15d	30d	average	proliferation rate (%)
A1	12	7	9.5	16.80	18.40	17.60	293
A2	13	7	10	15.60	18.80	17.20	287
A3	12	14	13	15.20	18.00	16.60	277
A4	6	7	6.5	14.60	16.60	15.60	260
A5	9	3	6	17.00	19.00	18.00	300
A6	12	12	12	13.00	16.40	14.70	245
A7	3	7	5	13.60	17.00	15.30	255
A8	3	4	3.5	12.40	16.60	14.50	242
A9	2	5	3.5	14.40	18.80	16.60	277
A10	5	7	6	11.60	16.20	13.90	232
A11	4	8	6	15.80	20.60	18.20	303
A12	1	0	0.5	14.75	15.60	15.18	253
A13	2	3	2.5	14.00	17.00	15.50	258
A14	1	3	2	14.20	17.80	16.00	267
A15	4	1	2.5	12.80	15.60	14.20	237
A16	3	2	2.5	15.00	19.20	17.10	285

germinate, while the seeds in other 47 bottles remained normal aseptic germination. After cultured for 15 days, these seeds began to germinate and became markedly swollen in light green. Until to 30 days culture, the seeds initiated to grow light green leaf primordiums and shoot tips on the apex, while the body began to present greenish or yellowish-white. After 45 days culture, the seedlings began to grow in appearance of protocorm clusters; during the next 15 days culture, the leaf primordiums were slowly turn into dark green, and the seedlings grew to 0.5-1.5 cm in height with blackish green leaves, which implied a healthy growth status.

Influence of Phytohormone Combinations on Protocorm Induction and Proliferation

During the plant tissue culture, regulatory effects of different phytohormone combinations on organ differentiation and development were extremely significant. By analyzing the influence of different combinations of 6-BA, NAA and KT on the protocorm induction and proliferation of *D. officinale*, the results showed that the average number of flowering presents a trend of anterior increase and posterior decrease, whose peak is reached when the KT concentration is up to 1.0 mg/L in medium. When keep concentrations of 6-BA and NAA unchanged, the protocorm proliferation rate gradually decreases with increasing KT concentration. According to the analytical result, the optimal phytohormone combination in MS medium (treatment A11) is: 1.0 mg/L 6-BA + 1.0 mg/L NAA + 1.0 mg/L KT, which is most suitable for protocorm induction and proliferation of *D. officinale* (Table 3).

Table 4. The effect of different extracts on rooting and hardening-off culture of *D. officinale*

Additives	Plantlet number of all bottles	Average Plantlet number per bottle	Individual number of cluster	Plantlet height (cm)	Root number	Root length (cm)
potato extract	6/8/13/10/9	9.2	1.891	5.086	9.978	3.946
apple extract	6/7/8/8/13	8.4	2.317	3.495	15.22	2.92
banana extract	5/8/7/6/7	6.6	2.091	3.339	9.273	4.033

Fig. 1. Procedure for High-efficient Regeneration and Seedling Transplanting of *D. officinale*

Effect of Plant Extracts on Rooting and Hardening-off Culture of *D. officinale*

The plantlet samples were shifted out from the bottles, and then the multiplication rate, plant height, root number, root length and individual number per cluster were calculated. As shown in table (Table 4), the effect of different organic extracts on rooting and hardening-off culture of *D. officinale* plantlets is significantly different. In multiplication rate, the average plantlet number of *D. officinale* per bottle in medium with potato extract was the highest at 9.2, followed by apple extract at 8.4, and the least in banana extract at 6.6, respectively. The difference in individual number per cluster among three organic extracts

Table 5. The Growth Status of *D. officinale* Seedlings after Hardening and Transplanting

Days	Growth status of seedlings
5	Plantlets were a little wilting, and timely sprayed over the plantlets to keep leaf surface humid every day.
10	The color of leaves changed from light green to dark green gradually, and the plantlets grew well.
15	A small number of leaf apex turned yellow, but its leaves became thicker and wider.
20	The color of roots recovered from white to dark green, and green root-shoot grew from the root-tip.
25	1-2 new leaves grew, and the seedlings continued to grow tall.

was not significant, while that of potato extract is 1.891, which was less than those of other two extracts. In plantlet height, the average plantlet height derived from potato extract is 5.086 cm, and is higher than those of the other two extracts, which are 3.495 cm derived from apple extract and 3.339 cm derived from banana extract, respectively. In root number and root length, the average number of roots derived from apple extract is higher than those of the other two extracts, however, the roots derived from potato extract are stronger and structurally uniform, and have leaves in blackish green, this will provide better effect to improve posterior transplanting survival rate of *D. officinale* tissue-cultured seedlings. Therefore, in view of the production cost, growth cycle and rooting status, the optimal culture medium is confirmed as: 40 potato extract added to basic medium (1/2 MS +0.2mg/L IBA + 0.5% activated carbon), which can effectively promote rooting and hardening-off culture of *D. officinale* tissue-cultured seedlings.

Effect of Domesticating and Transplanting of *D. officinale* Seedlings

When the *D. officinale* seedlings in bottles grew up to reach about 7 cm in height and developed into 3-4 individuals per cluster, then were hardened for five days in culture room by removing the lids of bottles. Until the leaves turned into dark green and the roots turned into white, the seedlings were transferred out from the bottles by tweezers. Wash these seedlings carefully to remove the attached medium thoroughly. Before transplanting, carbendazim solution was used to sterilize the seedlings for 8-10 mins, and then spread out these seedlings to dry until the roots became a little white. Finally, the seedlings were transplanted into the medium combined with vermiculite: perlite: humus soil = 1:1:5. Each cave was planted one cluster with 3-4 individuals of *D. officinale* seedlings.

After transplanting, the leaf surfaces of seedlings were kept moist by spraying each day, and foliar fertilization was performed every week. In the later-stage management of nutrient and water, the changes of seedlings were observed every 5 days (Table 5).

CONCLUSION AND DISCUSSION

In the industrialization of seedling production of *D. officinale*, the vital technical processes are the regeneration system and the seedlings transplanting. Therefore, using the extremely tiny seeds of *D. officinale* as materials, the techniques of high-efficient regeneration and seedlings transplanting for industrialized seedling production of *D. officinale* were established successfully by optimizing the seed aseptic germination, protocorm induction and proliferation, rooting and hardening-off culture, seedling transplanting and posterior tending. The result will also provide a vital theoretical base for industrial seedling production and somatic hybridization breeding of *D. officinale*.

The results showed that 1/2 MS without any plant growth hormone is the optimal medium for seed aseptic germination of *D. officinale*, in which the seed germination rate is higher and protocorm grows well. The optimal medium suitable for protocorm induction and proliferation of *D. officinale* is MS + 1.0 mg/L 6-BA+1.0 mg/L NAA+1.0 mg/L KT according to the orthogonal tests, in which the proliferation rate of protocorm is higher and the differentiation is better to bring high-quality seedlings. The strong plantlets of *D.*

officinale can be obtained in optimal medium (1/2 MS+ 0.2 mg/L IBA + 0.5% activated carbon + 40% potato extract) for rooting and hardening-off culture, in which the seedlings of *D. officinale* grow strongly and the rooting status is well. Moreover, this optimal medium may be recommended to reduce costs for the large-scaled industrialized production of *D. officinale* seedlings. The tissue-cultured seedlings of *D. officinale* display a survival rate of up to 90% and better growing performance when hardened for 5 days. The optimal transplanting medium of *D. officinale* seedlings is the 1:1:5 combination of vermiculite, perlite and humus soil. During the later-stage management, a suitable environment similar to laboratory conditions should be provided to shorten the adaptation period of *D. officinale* seedlings to the industrialized production conditions when these seedlings were transferred out to the field environment.

Generally, researches on the tissue regeneration system and the tissue-cultured seedlings transplanting for *D. officinale* will provide a vital technology base for the enough supply of artificial seedlings. However, *D. officinale* grows naturally in the extremely rigor environment, which limits the planting industry development of *D. officinale*. Therefore, basing on the established technique of high-efficient regeneration and seedlings transplanting for industrialized seedling production of *D. officinale*, more researches on the suitable microclimate condition and later-stage management for the posterior growth of *D. officinale* in field environment should be carried out to simultaneously improve its output and quality in future. In addition, the storage room should be treated with formaldehyde free board[38-44].

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