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Tissue Culture of *Betula microphylla* Bunge var. *paludosa*

Aihua CHEN, Gang LIU, Wei HU*

Forest By-product and Speciality Institute in Heilongjiang Province, Key Laboratory of Non-wood Forest Products in Heilongjiang Province, Mudanjiang 157011, China

Abstract [**Objectives**] This study aimed to study the tissue culture technique of *Betula microphylla* Bunge var. *paludosa*. [**Methods**] Taking seeds as explant, seedlings of *B. microphylla* were cultured. Then, stem sections with leaves were cut off and subjected to induction of clustered shoots. Finally, the rooting and transplanting of adventitious shoots were completed. Thus, the tissue culture system of *B. microphylla* was established. [**Results**] The natural seed germination induction medium was Murashige and Skoog (MS), and the germination rate was 31%. The most suitable shoot induction medium was WPM medium added with 1.0 mg/L of 6-benzylaminopurine (BA), and the multiplication coefficient was 13.7. The rooting medium was Woody Plant medium (WPM) added with 1.0 mg/L of indole-3-butytric acid (IBA), and the rooting rate was 100%. The transplanting substrate for tissue-cultured seedlings was composed of humus soil, vermiculite and perlite (*V*:*V*:*V* = 4:3:3), and the survival rate reached 88.75%. [**Conclusions**] The experimental materials are easy to obtain and preserve, and the proliferation is rapid. This study provides technical support for the rapid acquisition of the tissue-cultured seedlings of *B. microphylla*.

Key words *Betula microphylla* Bunge var. *paludosa*, Tissue culture, Shoot induction, Transplanting

1 Introduction

Betula microphylla Bunge var. *paludosa* is a new variety in the subgroup of *Betula* L. discovered and named by Professor Yang Changyou in 2006[1]. It is an extremely rare and endangered temperate deciduous broad-leaved tree species. It has non-strict requirements on the soil, and has strong waterlogging resistance, salt-alkali resistance and cold resistance. The tree shape is beautiful. It can be used as a soil and water conservation tree species for saline-alkali land, lakes and shoals, especially for afforestration in alpine regions[2], with very high ecological and economic value. Therefore, the research on the tissue culture of *B. microphylla* has a very important significance for its popularization and application, germplasm preservation and protection of the ecological environment.

In the different climatic zones of Shanghai, Hebei, Tianjin, *etc.*, the promotion and application of *B. microphylla* has been carried out. However, there are no reports on the introduction and cultivation in the three provinces. In terms of tissue culture, Zhang Qun from Shanghai Institute of Landscape Gardening Science conducted shoot induction and rooting culture with shoot tips as explants, and the multiplication ratio reached 5.7[3]. At the same time, *B. microphylla* is able to grow normally under the environment with salinity less than 4 ‰[4-5], and it is resistant to high temperature and high humidity and other stresses[6]. These studies provide technical support for large-scale seedling rearing. However, the current research is still in its infancy. Axillary buds or top buds of the hydroponic overwintering branches are often used as explants for tissue culture, and sampling is limited by time. In this experiment, the seeds stored at room temperature were used as explants to conduct a tissue culture system of *B. microphylla*. The sampling is convenient, not limited to the season. This study will lay a foundation for further research on *B. microphylla* and provide technical support for the preservation and promotion of germplasm resources.

2 Materials and methods

2.1 Material The seeds of *B. microphylla* were used as explant. They were harvested from the artificially constructed protected forests in the Altay region of Xinjiang in early September, with 1 000-seed weight of (0.155 1 ± 0.003 6) g (the average of five measurements).

2.2 Pretreatment and disinfection of seeds The seeds were selected randomly. After removing the seed wings, the seeds were rinsed with running water for 12 h. Subsequently, they were immersed in 75% alcohol for 30 - 60 s and disinfected with 1% - 5% sodium hypochlorite for 10 - 20 min. Then, they were soaked in sterile water for 1 h and re-disinfected with 1% sodium hypochlorite for 15 min. Finally, they were washed five times in sterile water. After drying the surface moisture, the seeds were placed in Murashige and Skoog (MS) medium and cultured in dark. There were 10 seeds in each flask, and total 10 flasks were arranged for each time. The infection rate was analyzed.

Infection rate(%) = (Number of infected seeds/Total number of seed inoculated) × 100 (1)

2.3 Seed germination The disinfected seeds were inoculated in MS, Schenk and Hildebrandt medium (SH) and WPM, respectively and cultured in dark. There were 10 seeds in each flask

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* Corresponding author. E-mail: 258265658@qq.com

and 10 flasks were arranged for each time. After one week, the seed germination rate was counted.

Seed germination rate(%) = (Number of germinated seeds/Total number of seeds inoculated) × 100 (2)

2.4 Effects of medium type, combined concentration of 6-BA and NAA on induction of clustered shoots After the germination, rootless seedlings of *B. microphylla* grew out. Stem sections with leaves, in the length of about 1.5 cm were cut off from well grown *B. microphylla* seedlings. They were then inoculated into the shoot induction medium.

(i) LS, 1/2 MS, MS, WPM and SH media were added with 2.0 mg/L of BA and 0.1 mg/L of NAA, respectively. A total of 5 treatments were designed.

(ii) WPM was used as the basic medium, which was added with 1.0 mg/L of BA, 2.0 mg/L of BA + 0.1 mg/L of NAA, and 2.0 mg/L of BA + 0.1 mg/L of NAA, respectively.

A total of 10 flasks were arranged for each treatment. There were 2 – 4 stem sections in each flask. After eight weeks, three flasks were selected randomly from each treatment to investigate the effects of different treatments on the proliferation of adventitious buds.

Multiplication coefficient of adventitious buds = Total number of adventitious buds/Total number of adventitious buds inoculated. (3)

2.5 Rooting culture Adventitious buds were inoculated into rooting medium, with 1 – 4 adventitious buds in each flask. After four weeks, five flasks were selected randomly to observe the rooting situation.

Rooting rate(%) = (Number of rooted plants/Number of inoculated plants) × 100 (4)

2.6 Hardening and transplanting of rooted seedlings When the root length of the tissue-cultured seedlings was more than 3 cm, the seedlings were taken out carefully. The medium on the surface of the roots was rinsed off with running water. The seedlings were transplanted into sterilized substrate composed of humus soil, vermiculite and perlite (V: V: V = 4 : 3 : 3). The substrate was watered and covered with light-transparent plastic bottles to moisturize. The seedlings were placed under natural light at room temperature, and watered once every three days to make them adapt to the temperature and humidity of the environment. After two weeks, the bottles were removed, and the seedlings were transplanted in a weak wind shed, in which the temperature was 20 – 30℃ and the shading rate was 70%. After growing more than two new leaves, the survival rate of transplanting was counted.

Survival rate(%) = (Number of live tissue-cultured seedlings/Total number of seedlings transplanted) × 100 (5)

2.7 Culture conditions and statistical analysis The pH of the used media in the experiment was adjusted to 5.8. They were added with 2.0% sucrose and 5.5 g/L agar. The used media were all sterilized at (121 ± 1)℃ for 20 min. In each flask, 40 mL of medium was poured, which was replaced once every four weeks. The incubation was carried out in a culture chamber at (25 ± 1)℃ with an illumination intensity of 36 lux. All experiments were repeated three times. The collected data were analyzed ANOVA with SPSS16.0 software, and multiple comparisons of means were performed by Tukey's HSD method.

3 Results and analysis

3.1 Treatment and disinfection of seeds A total of nine disinfection treatments were designed for the seeds of *B. microphylla* (Table 1). The seeds were then soaked in sterile water for 1 h and disinfected with 1% sodium hypochlorite for 15 min. As shown in Table 1, when the 300 seeds were soaked in 75% alcohol for 30 s and disinfected with 5% sodium hypochlorite for 20 min, none of them was infected with bacterial, with infection rate of zero, indicating good treatment effect.

Table 1 Disinfection situation of seeds of *Betula microphylla* Bunge var. *paludosa*

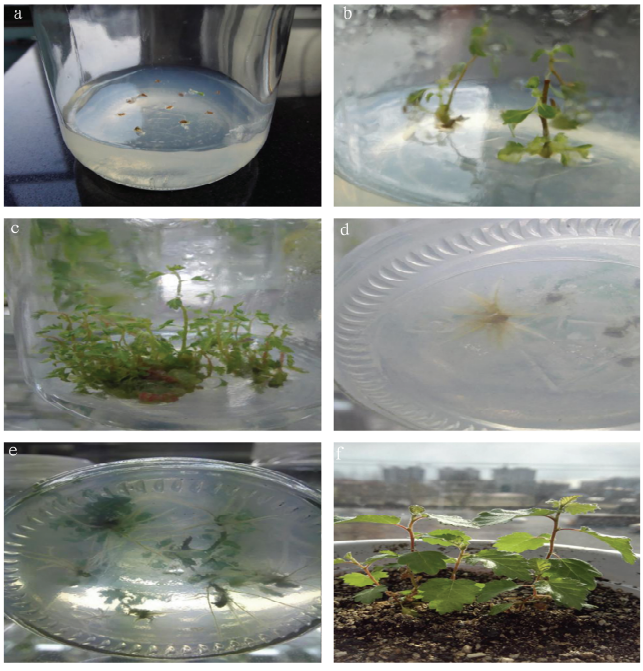
Treatments	75% alcohol Immersion time// s	Sodium hypochlorite concentration// %	Sodium hypochlorite disinfection time// min	Total number of seeds	Number of seeds infected with bacteria	Pollution rate //%
1	10	1	10	300	300	100.0
2	10	2	15	300	300	100.0
3	10	5	20	300	289	96.3
4	20	1	15	300	299	99.7
5	20	2	20	300	172	57.3
6	20	5	10	300	283	94.3
7	30	1	15	300	297	99.0
8	30	2	10	300	300	100.0
9	30	5	20	300	0	0

Note: The seeds in the treatment groups were further soaked in sterile water for 1 h and disinfected with sodium hypochlorite for 15 min after treated as described in the table.

3.2 Seed germination As shown in Table 2, the overall germination rate of the natural seeds of *B. microphylla* was low. In the three kinds of medium, the seeds sprouted as early as 2 d later, no longer than 6 d. The highest germination rate was 31%, and the lowest germination rate was 21.67% (Fig.1 a). In the MS medium, the seeds sprouted fastest and showed the highest germination rate (31%).

Table 2 Germination induction of seeds of *Betula microphylla* Bunge var. *paludosa*

Treatments	Medium type	Number of explants	Number of seeds germinated	Time of germination // d	Germination rate // %
1	MS	300	93	2 – 5	31.00
2	SH	300	65	4 – 6	21.67
3	WPM	300	84	4 – 6	28.00



Note: a, seeds germinating in MS medium; b, leafy stem sections subjected to shoot induction; c, clustered buds after induced in WPM medium added with 1.0 mg/L of BA for four weeks; d, roots cultured in WPM medium added with 1.0 mg/L of IBA for two weeks; e, roots cultured in 1/2 MS for four weeks; f, tissue-cultured seedlings transplanted in flowerpot.

Fig. 1 Tissue culture of *Betula microphylla* Bunge var. *paludosa*

3.3 Effects of medium type and combined concentration of 6-BA and NAA on induction of clustered shoots The leafy stem sections and the new axillary buds (Fig. 1 b) were placed in the re-differentiation culture process. After eight weeks, the data were analyzed. As shown in Table 3, under the condition of same hormone concentrations (BA 2.0 mg/L, NAA 0.1 mg/L), different types of medium had a significant effect on the multiplication coefficient of the shoots ($P < 0.001$). In the WPM medium, the multiplication coefficient was highest (11.5), After one subculture, adventitious buds in WPM medium grew in large clusters, the stems were hard (Fig. 1 c), the degree of lignification was slightly higher, and white adventitious roots grew out at the base of some adventitious buds. In the MS medium, the multiplication coefficient of adventitious buds was 10.6. According to Tukey's multiple comparisons, there was no significant difference in the induction of shoots between MS and WPM medium ($P = 0.238$). However, they showed significant differences compared with LS, 1/2 MS and SH. The adventitious shoots induced by MS medium were very young, and were easily broken by tweezers. There were no calluses at the base of the adventitious shoots induced by the two kinds of medium. Among different types of medium, the induction of adventitious shoots was weakest in the LS medium, with multiplication coefficient of only 1.3. A large callus was formed at the base of each explant, accompanied by several hairy roots. Adventitious shoots were formed occasionally on the calluses.

As shown in Table 4, when WPM was used as the basic medium, different combined concentrations of BA and NAA had a significant effect on the induction of adventitious shoots. The differences between treatments 1, 2 and 3 were significant ($P = 0.004$, $P < 0.001$, $P = 0.001$). The WPM medium added with 1.0 mg/L of 6-BA showed the best effect for induction of adventitious shoots, with multiplication coefficient up to 13.7. Moreover, the buds were green and grew into large clusters. The axillary buds were easy to form, and no calluses appeared.

Table 3 Effects of different types of medium on the induction of adventitious shoots of *Betula microphylla* Bunge var. *paludosa*

Basic medium	Number of explants	Number of adventitious buds induced	Multiplication coefficient
LS	12.0	15.3	1.3 d
1/2 MS	12.0	38.3	3.2 c
MS	12.0	127.7	10.6 ab
WPM	11.7	133.7	11.5 a
SH	9.0	23.0	2.6 cd

Note: Different lowercase letters in the same column indicate significant differences at the 0.05 level.

Table 4 Effects of BA and NAA on induction of adventitious shoots of *Betula microphylla* Bunge var. *paludosa*

Treatments	BA concentration // mg/L	NAA Concentration // mg/L	Multiplication coefficient	Description of growth
1	1.0	–	13.7 a	Large clusters, many axillary buds, no callus, young
2	2.0	0.1	11.5 b	Large clusters, some axillary buds, no callus
3	3.0	0.3	3.4 c	Small clusters, some calluses, white hairy roots

Note: Different lowercase letters in the same column indicate significant differences at the 0.05 level.

3.4 Rooting of tissue-cultured seedlings Two types of medium, 1/2 MS and WPM + IBA 1.0 mg/L were used for rooting culture. As shown in Table 5, in the two types of medium, the rooting rate all reached 100%. They had their own advantages. The WPM medium added with IBA showed the fastest rooting, taking about 6 d; and the root system was many and short (Fig. 1 d), with an average of 5 roots. In the 1/2 MS medium, the average length of the roots was 4.54 cm (Fig. 1 e); there were many fibrous roots; and the main roots were scarce and slender.

Table 5 Rooting situation of *Betula microphylla* Bunge var. *paludosa* after four weeks

Basic medium	IBA//mg/L	Average length of roots//cm	Rooting rate// %	Number of roots	Rooting time//d
1/2 MS	–	4.54	100	3	9
WPM	1.00	3.10	100	6	6

3.5 Transplanting of rooted seedlings When a large number of tissue-cultured seedlings are transplanted outdoors, due to uneven growth of the seedlings, during the transplant management process, the tissue-cultured seedlings will inevitably wilt and even die, so the hardening process is particularly important. Hardening can greatly improve the survival rate of tissue-cultured seedlings and facilitate management. Under the conditions of room temperature and natural light, bottle inverting is often used for seedling hardening, and it is quick, simple, and easy to handle and not need to slow down. New leaves begin to grow after 12 – 15 d (Fig. 1 f). It also showed that the substrate composed of humus soil, vermiculite and perlite ($V:V:V=4:3:3$) is suitable for the growth of *B. microphylla*, and the total transplant survival rate reached 88.75%.

4 Conclusions and discussions

The seeds of *B. microphylla* have seed wings, which makes bacterial not easy to remove. Disinfection of explants with alcohol and sodium hypochlorite is an effective method. The best disinfection method for the seeds of *B. microphylla* is as follows: 75% alcohol 30 s, 2% sodium hypochlorite 2 min, sterile water 1 h, repeated once. Huang Gang *et al.* found that healthy seeds accounted for a small promotion in natural needs of *B. microphylla*[7-8], there were seeds with bacteria, residual embryos and insects. Therefore, the overall germination rate of *B. microphylla* seeds is low. This is consistent with the result of this study that the seed germination rate of *B. microphylla* was only 31%. This is also one of the reasons for the gradual reduction of wild *B. microphylla* resources under natural conditions.

Cytokinin has a strong ability of induce bud formation. The role of auxin is to induce callus formation and rooting[9]. In general, high ratio of auxin to cytokinin is beneficial to root differentiation, inhibiting the formation of buds; and low ratio is conducive to bud differentiation, inhibiting root formation; and moderate ratio is conducive to callus growth[10-11]. When BA (0.5 mg/L) and NAA (0.4 mg/L) were added, a large amount of green calluses are formed. It suggests that the concentrations of BA and NAA used in this experiment were suitable for the growth of calluses. The best medium for shoot induction of *B. microphylla* was WPM medium added with 1.0 mg/L of BA.

The rooting induction of adventitious buds of woody plants naturally does not require the addition of hormonal substances[12-13]. However, the addition of rooting hormone can accelerate root formation and increase rooting coefficient[4]. When the root length is greater than 2 cm, the seedlings can be transplanted. The substrate used is generally a mixed substrate composed of peat soil or humus soil, vermiculite and perlite[14-15]. The tissue-cultured seedlings need to be constantly hardened after transplanting. In the growing environment, the light intensity should be best from weak to strong, the humidity should be best from high to low, and the temperature should be best from low to high[12]. From the perspective of survival rate, in the WPM medium added with 1.0 mg/L of IBA, the degree of lignification was higher, and the survival rate of transplanting was also higher. This study not only provides a method for asexual reproduction of *B. microphylla* but also provides the basis for its genetic research.

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