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# Plant Regeneration from Root Tissue and Establishment of Root Culture of Japanese White Birch (*Betula platyphylla* var. *japonica*)

Lu-Min VAARIO*, Yasuhiro OTOMO*, Ryo SODA* and Yuji IDE**

* *Tsukuba Research Institute, Sumitomo Forestry Co., Ltd.*, Tsukuba 300-26, Japan
** *Research Division of the University Forests, Faculty of Agriculture, The University of Tokyo*, Tokyo 113, Japan

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Protocols for *in vitro* regeneration from root explants and for rapid elongation of root cultures were established on *Betula platyphylla* var. *japonica*. Adventitious shoots were differentiated on IS medium supplemented with BAP and NAA from root explants. The highest differentiation rate (50%) was attained on the medium containing 0.8 mg/*l* BAP and 0.03 mg/*l* NAA. The adventitious shoots successfully regenerated perfect plantlets on the rooting medium. Root culture was established from root segments excised from intact plantlets grown *in vitro* in liquid IS, B5 and WP media containing 1~3% sucrose. B5 medium supported maximum root elongation among the tested media. The use of 100 mg/*l* PVP in B5 medium successfully prevented browning of roots and promoted root elongation. There was no significant difference in the rate of adventitious shoot formation between root explant obtained from root culture and that obtained from intact root from plantlets.

## Introduction

Tissue culture of birch trees has been reported by a numbers of investigators[1]. Plants have been successfully regenerated *in vitro* from various kinds of tissues from various birch species. However, little is known about regeneration from root explants except for *Betula pendula* Roth[2],

Plant regeneration from root tissue is not only useful for rapid propagation of clones, but also useful for plant regeneration from gene-transferred tissues using *Agrobacterium* vectors[3].

Japanese white birch (*Betula platyphylla* Sukatchev var. *japonica* Hara) is one of the promising species for biomass production in Japan[4]. The techniques for *in vitro* plant regeneration and rapid clonal propagation from petiole and stem segments of Japanese white birch have already been established, as reported earlier[5-8].

In this report, we describe the methods for plant regeneration from root tissue and for rapid propagation of root tissue, which will provide more explants for plant regeneration in Japanese white birch.

## Materials and Methods

### 1. *Plant regeneration from root segments excised from intact plantlets*

One cm long root segments were excised from plantlets grown *in vitro*. Root segments both with and without root apices were used as explants. The plantlets were originally obtained from petiole culture of mature birch tree[7] and subcultured for 8 years *in vitro* on MS medium[9] solidified with

0.8% agar and containing 30 g/*l* sucrose, 0.5 mg/*l* IBA and 0.02 mg/*l* NAA. They were kept at 26±2°C under about 3,000 lux fluorescent illumination for a 16 h photoperiod.

Excised root segments were laid on a modified IS medium[7] which was solidified with 0.3% Gelrite and supplemented with a combination of different concentrations of BAP (0.4, 0.8, 1.2, 1.6 mg/*l*) and NAA (0.001, 0.003, 0.01, 0.03, 0.1 mg/*l*). The pH of the media was adjusted to 5.8 before autoclaving. The root segments were cultured at 26±2°C under 3,000 lux fluorescent illumination for a 16 h photoperiod. Thirty explants were cultured for each combination of BAP and NAA.

**2. *Establishment of root culture***

Liquid B5[10], IS[7] and WP[11] media without any growth regulators were used for culture media. All of the media containing 2% sucrose except for the experiment in which the effect of sucrose concentration (0, 0.5, 1.0, 2.0, 3.0 and 5.0%) was tested using B5 medium. Effects of IBA and NAA (0.02 and 0.2 mg/*l*) concentrations were also tested using B5 medium. Antioxidants, polyvinylpyrrolidone (PVP) and ascorbic acid were tested to overcome browning of root tissue at concentrations of 50 and 100 mg/*l*. pH of the media was adjusted to 5.8 in all cases before autoclaving. Each root segment with root apex cut one cm long was cultured in a 250 m*l* Erlenmeyer flask contained 75 m*l* of liquid medium at 26±2°C in the dark with 70 stroke/min. on a reciprocal shaker. Twelve to 14 explants were cultured for each experiment.

**3. *Plant regeneration from root cultures***

After 6 weeks of culture of root segments in liquid B5 medium containing 2% sucrose and 100 mg/*l* PVP, elongated root cultures were cut into one cm pieces and laid on 0.3% Gelrite-solidified IS medium containing 0.8 mg/*l* BAP, 0.03 mg/*l* NAA and 2% sucrose. One cm long root segments excised from intact plantlets were also cultured on the same medium as a control. They were separated into two groups. One was the segment with root apex and the other was without root apex. Thirty explants were cultured for each type of explants. They were maintained at 26±2°C under 3,000 lux fluorescent illumination for a 16 h photoperiod.

Adventitious shoot regeneration was also examined using 6-month-old root cultures that were subcultured in the same liquid medium as described above by transferring the root apex segments into a fresh medium once a month. Twelve explants were examined for each type of explant. All of culture conditions were the same as described above.

## Results

**1. *Plant regeneration from root segments excised from intact plantlets***

Root segments turned light brown in color during one week of culture. A slight swelling of the root segment occurred after 2 weeks of incubation on IS medium containing 0.5 mg/*l* BAP and 0.02 mg/*l* NAA. There was no obvious callus formation. The swelling gradually developed into a mass of meristematic organ after three weeks of culture (**Fig. 1-a**). They developed into shoots after six weeks of culture (**Fig. 1-b**) In the control cultures on hormone-free IS medium, the swelling occurred in the cut ends of the roots after 2 weeks of culture but meristematic organ was not formed. The responses of root tissues after 7 weeks of culture on the media containing different concentrations of BAP and NAA are shown in **Table 1.** The combination of 0.8 mg/*l* BAP and 0.03 mg/*l* NAA gave the best adventitious shoot formation rate (50%). The hormonal combinations using 0.4 mg/*l* BAP or 0.1 mg/*l* NAA were not effective for adventitious shoot formation. When the adventitious shoots were transferred to MS medium containing 0.02 mg/*l* NAA and 0.5 mg/*l* IBA, rooting occurred within 2 weeks and complete plantlets were obtained.

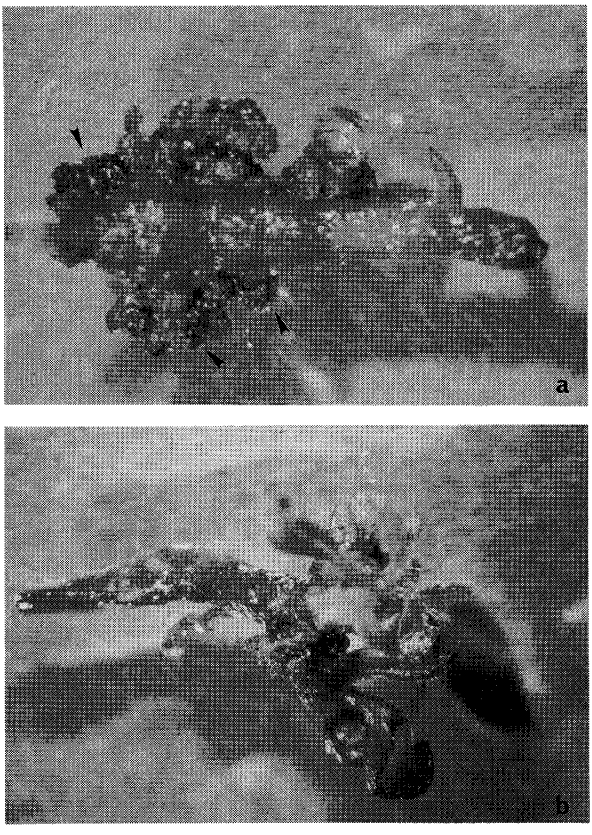


**Fig. 1** Differentiation of adventitious shoot from root explant obtained from intact plantlet of *B. platyphylla* var *japonica*.

a. Meristematic organs appeared on the surface of swelled root explant after 3 weeks of culture. The arrow indicates a mass of a meristematic organ.

b. Adventitious shoots developed from meristematic organs after 6 weeks of culture.

* One cm long root explant was cultured on solid IS medium containing 0. 8 mg/*l* BAP and 0. 03 mg/*l* NAA.

** Root apex is on the left in the both photos.

**Table 1.** Effect of growth regulators on adventitious shoot differentiation from root explants of *B. platyphylla* var. *japonica* on IS medium after 7 weeks of culture.

| NAA (mg/*l*) | BAP (mg/*l*) | | | |
|---|---|---|---|---|
| | 0. 4 | 0. 8 | 1. 2 | 1. 6 |
| 0. 001 | 0* | 3 | 0 | 0 |
| 0. 003 | 0 | 17 | 0 | 0 |
| 0. 01 | 0 | 33 | 27 | 10 |
| 0. 03 | 0 | 50 | 10 | 17 |
| 0. 1 | 0 | 0 | 0 | 0 |

* Percentage of explants with adventitious shoot differentiation. 30 explants were cultured for each treatment. Initial length of explants was one cm.

## 2. *Establishment of root culture*

Elongation of root cultures with root apex differed among the basal media tested (**Table 2**). B5 medium yielded the highest root elongation and the IS medium yielded the lowest. There were no

**Table 2.** Effect of different basal media on elongation of excised root tissue of *B. platyphylla* var. *japonica* in liquid media*.

| Medium | Mean root length (cm±S. D.) | | Color of explant after 4-weeks of culture |
|---|---|---|---|
| | 2-week culture | 4-week culture | |
| IS | 1. 42±0. 25 | 1. 97±0. 69 | brown |
| B5 | 3. 17±0. 42 | 5. 58±0. 97 | brown |
| WP | 2. 37±0. 73 | 4. 50±2. 21 | brown |

* Each medium contained 2% sucrose. Twelve explants with root apices were cultured for each treatment. Initial length of explants was one cm.

**Table 3.** Effect of antioxidants on elongation of excised root tissue of *B. platyphylla* var. *japonica*[*1].

| Antioxidant (mg/*l*) | | Mean root length (cm±S. D.) | | Color of explant after 4-weeks of culture |
|---|---|---|---|---|
| | | 2-week culture | 4-week culture | |
| PVP[*2] | 50 | 3. 10±0. 40 | 5. 95±0. 99 | no change in color |
| | 100 | 3. 60±0. 83 | 6. 93±0. 73 | no change in color |
| Ascorbic acid | 50 | 3. 02±0. 44 | 4. 67±0. 52 | no change in color |
| | 100 | 1. 42±0. 38 | 1. 77±0. 59 | brown |
| Control[*3] | | 2. 72±1. 37 | 4. 18±1. 48 | brown |

[*1] Basal medium was B5 medium contained 2% sucrose. Twelve explants with root apices were cultured for each treatment. Initial length of explants was one cm.

[*2] Polyvinylpyrrolidone.

[*3] Without any antioxidants.

**Table 4.** Effect of sucrose concentration on elongation of excised root tissue of *B. platyphylla* var. *japonica*[*1].

| Sucrose concentration (%) | Mean root length (cm±S. D.)[*2] |
|---|---|
| 0 | 1. 00±0. 00 |
| 0. 5 | 1. 46±0. 17 |
| 1. 0 | 3. 92±0. 37 |
| 2. 0 | 3. 44±0. 38 |
| 3. 0 | 3. 52±0. 33 |
| 5. 0 | 1. 28±0. 28 |

[*1] Medium was B5 containing 100 mg/*l* of PVP. Twelve explants with root apices were cultured for each treatment.

[*2] Initial length of explants was one cm. Root length was recorded after 2 weeks of culture.

lateral roots in any cultures. However, most root segments turned brown after 4 weeks of culture.

Attempts to reduce browning of excised root segments, which probably resulted from phenolic oxidation in the tissue, were made using two antioxidants, PVP and ascorbic acid at various concentrations. The highest root elongation was attained on the medium with 100 mg/*l* PVP after 4 weeks of culture (**Table 3**). Ascorbic acid was not effective for root elongation and had a detrimental effect at 100 mg/*l*.

Sucrose concentration also affected the elongation of root. As shown in **Table 4**, 1-3% sucrose resulted in better root elongation with maximum root length at 1%, whereas 0. 5 and 5% resulted in less elongation.

It was found that auxin was effective for root elongation and branching of lateral roots. Both IBA and NAA stimulated root elongation at 0. 02 mg/*l*, and promoted the formation of lateral roots

**Table 5.** Effect of IBA and NAA concentrations on root growth of *B. platyphylla* var. *japonica*[*1].

| Auxins (mg/*l*) | | Mean root length[*2] (cm±S. D.) | | Mean number of lateral roots (no±S. D.) | |
|---|---|---|---|---|---|
| | | 2-week culture | 4-week culture | 2-week culture | 4-week culture |
| IBA | 0. 02 | 3. 55±0. 43 | 6. 50±0. 70 | 2. 91±1. 04 | 3. 36±1. 29 |
| | 0. 2 | 1. 64±0. 21 | 2. 10±0. 76 | 1. 09±0. 94 | 3. 73±2. 37 |
| NAA | 0. 02 | 2. 96±0. 81 | 5. 21±1. 78 | 0 | 0. 27±0. 47 |
| | 0. 2 | 1. 85±0. 25 | 2. 44±0. 74 | 3. 09±2. 12 | 4. 73±2. 33 |
| Control | | 2. 75±1. 31 | 4. 22±1. 73 | 0 | 0 |

[*1] Medium was B5 with 2% sucrose and 100 mg/*l* PVP. Fourteen explants each one cm long with root apices were cultured for each treatment.

[*2] Main root length.

**Table 6.** Adventitious shoot formation from root cultures after 7 weeks of culture on shoot differentiation medium[*1].

| Source of explant | Type of explant | |
|---|---|---|
| | Without root apex | With root apex |
| Plantlet[*2] | 33. 3% | 40% |
| Root culture[*3] | 30% | 33. 3% |

[*1] Explants were cultured on solid IS medium contained 0. 8 mg/*l* BAP, 0. 03 mg/*l* NAA and 2% sucrose. Thirty explants, each 1 cm long, were cultured for each treatment.

[*2] Intact roots were excised from *in vitro*-cultured plantlets.

[*3] Root segments were prepared from the root cultures 6 weeks after inoculation of the root segment with apex in liquid B5 medium with 2% sucrose and 100 mg/*l* PVP.

at 0. 2 mg/*l* (**Table 5**). IBA at 0. 02 mg/*l*, however, gave the best stimulation of root elongation and a good formation of lateral roots compared with the other auxin and still concentrations used.

### 3. *Plant regeneration from root cultures*

Root segments obtained from the root cultures showed the same response as those obtained from intact plantlets after 2 weeks of culture. They turned light brown in color and showed slight swelling.

Adventitious shoots were directly obtained from root culture-derived segments after 7 weeks of culture, whereas those from intact plants took a little longer. The rate of plant regeneration from root cultures was almost the same as that from the root segments obtained from intact plantlets (**Table 6**). The root segments with root apex resulted in a slightly higher adventitious shoot regeneration rate than those without apex.

Adventitious shoot formation also occurred on 6-month-old root cultures. On root cultures with apex, the regeneration rate was 41% (5 out of 12), whereas 33. 3% (4 out of 12) on those without apex.

## Discussion

The protocols of *in vitro* regeneration of plants from root explants has previously been reported in some woody species[2,12]. In this study, we succeeded in the establishment of fast growing root cultures, the regeneration of shoots, and the production of plantlets of Japanese white birch.

For the elongation of roots in excised root cultures, B5 medium containing 1% of sucrose and 100 mg/*l* of PVP was the most appropriate among tested media. The highest adventitious shoot formation rate was attained on IS medium containing 0.8 mg/*l* BAP and 0.03 mg/*l* NAA. This result coincided with those reported by Ide[5] who showed that the combination of 0.8 mg/*l* BAP and 0.02 mg/*l* NAA was the best for formation of adventitious shoots is stem culture, and that differentiation of adventitious shoots was inhibited by 0.2 mg/*l* NAA.

One key point for the successful elongation of root tissue of *B. platyphylla* var. *japonica* in liquid media was the selection of a suitable basal medium. In this case, B5 without any auxin was enough to induce elongation of excised root tissues.

Sucrose in the medium has metabolic and osmotic functions as well[13]. In the present study, lower concentrations (1-3%) of sucrose promoted root elongation, whereas higher concentration (5%) was rather inhibitory to root elongation. Ide[5] also reported that 2% sucrose was appropriate for elongation of roots of *B. platyphylla* var. *japonica*. In our present study, 1% sucrose showed the best root elongation.

The second important point for elongation of root tissues is the addition of antioxidants ot the basal media to prevent the browning, which probably resulted from phenolic oxidation of the tissue[14,15]. Many woody plants are rich in phenolic compounds in general. These products are well known to inhibit the establishment of primary cultures[16]. In the present study, PVP was found to be an effective antioxidant for *B. platyphylla* var. *japonica* when used at 100 mg/*l* (0.01%).

These results are consistent with the findings of Hohtola[17] and Gupta[18] in mature woody plant tissue cultures. Hohtola found 1% PVP gave the best result against browning in mature Scot pine culture. Gupta reported that blackening was reduced by 0.7% PVP treatment in culture of tissues from 100-year-old teak trees.

Rumary and Thorpe[19] reported that ascorbic acid in the medium prevented browning during secondary shoot formation in black spruce culture and that the optimum concentration of ascorbic acid was $10^{-4}$ M (17.6 mg/*l*). However, in the present study ascorbic acid was not effective in preventing cultured tissues from browning. Especially, a high concentration (100 mg/*l*) of ascorbic acid was detrimental to root elongation. It is not clear whether our negative result with ascorbic acid was because the concentration was too high, or the explant used in our study was different from that used by Rumary and Thorpe.

Although auxin stimulated lateral root formation and promoted root elongation, adventitious shoot formation rate from root tissues cultured in media containing auxin was comparatively lower than in auxin free media (data was not shown). We therefore concluded that auxin was not effective for rapid clonal propagation through root cultures.

As only small differences were found in adventitious shoot formation rate between the root segments derived from root culture and those from intact root and between the explants with root apex and those without root apex, any type of root explant may potentially have the ability to regenerate adventitious shoots.

Plant regeneration from root culture of *B. pendula* through callus formation on the MS mediuum containing 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 2iP (5-(3-methyl-2-butenyl-amino) purine) has previously been reported[2]. In this report, the authors pointed out that the use of callus caused genetic instability of propagated plantlets. According to Bhat *et al.*[20], Chaturvedi & Sharma[21] found no variation among the plants regenerated from 9-year-old root cultures of *Solanum khasianum* and 5 to 6-year-old root cultures of *Atropa belladonna*. Although there was slight callusing on the explant, shoot regeneration occurred almost directly from the explant in our

present study. Therefore, our regeneration system may have higher genetic stability than that of *B. pendula* and is expected to be used not only for mass propagation of elite clones but also for genetic transformation studies of Japanese white birch.

We needed intact plant tissue such as stems and petioles for *in vitro* plant regeneration of Japanese white birch before establishing the plant regeneration system reported in this paper. As the root cultures retained the potential for continuous *in vitro* growth for long periods as well as shoot regeneration ability, we may not need to repeat the initiation of tissue culture from the intact plants after establishment of root culture for clonal propagation of individual genotypes of Japanese white birch.

## References


1) Meier-Dinkel, A., 1992. In "Biotechnology in agriculture and forestry 18" (ed. by Bajaj, Y. P. S.), p. 40-80, Springer-Verlag, Berlin, Heidelberg.
2) Srivastava, P. S., A. Steinhauer, H. Glock, 1985. Plant Science, **42**: 209-214.
3) Torrey, J. G., 1988. In "Genetic manipulation of woody plants" (ed. by Hanover, J. W.), p. 1-21, Plenum Press, New York.
4) Sasaki, S., 1989. J. Jpn. Wood Res. Soc., **35**(10): 865-874. (in Japanese).
5) Ide, Y., 1987. Bulletin of the Shizuoka Prefecture Forestry Experiment Station, No. 16.
6) Saito, A., Y. Ide, 1985. J. Jpn. For. Soc., **67**: 282-284.
7) Saito, A., Y. Ide, 1985. J. Jpn. For. Soc., **67**: 373-375.
8) Sato, T., Y. Ide, A. Saito, 1986. J. Jpn. For. Soc., **68**: 343-346.
9) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15**: 473-497.
10) Gamborg, O. L., R. A. Miller, K. Ojima, 1968. Exp. Cell. Res., **50**: 151-158.
11) Lloyd, G., B. McCown, 1981. Proc. Int. Plant. Prop. Soc., **30**: 421-427.
12) Son, S. H., R. B. Hall, 1990. Plant Cell Tissue and Organ Culture, **20**: 53-57.
13) Bonga, J. M., P. von Aderkas,1992. In "*In vitro* culture of trees", p. 45-48, Kluwer Academic Publishers, Dordrecht, Boston & London.
14) Bar-Nun, N. A. M. Mayer, 1983. Phytochemistry, **22**(6): 1329-1333.
15) Preece, J. E., M. E. Compton, 1991. In "Biotechnology in agriculture and forestry 17" (ed. by Bajaj, Y. P. S.), p. 168-189, Springer-Verlag, Berlin, Heidelberg.
16) Roy, S. C., A. Sarkar, 1991. Scientia Horiculturae, **47**: 107-113.
17) Hohtola, A., 1988. Plant Cell, Tissue and Organ Culture, **15**: 211-222.
18) Gupta, P. K., A. L. Nadgir, A. F. Mascarenhas, V. Jagannathan, 1980. Plant Science Letters, **17**: 259-268.
19) Rumary, C., T. A. Thorpe, 1984. Can. J. For. Res., **14**: 10-16.
20) Bhat, S. R., P. Chitralekha, K. P. S. Candel, 1992. Plant Cell Tissue and Organ Culture, **29**: 19-25.
21) Chaturvadi, H. C., M. Sharma, 1987. In "Plant Cell and Tissue Culture of Economically Important Plants" (ed. by Reddy, G. M.), p. 175-179, Osmania University, Hyderabad.

《和文要約》

# シラカンバの根の組織からの植物体再生と根培養の確立

Lu-Min VAARIO*・大友泰弘*・曽田　良*・井出雄二**

* 住友林業筑波研究所
** 東京大学農学部附属演習林研究部

シラカンバの根の組織からの植物体再生法と培養根の急速な成長のための手法を確立した．BAP と NAA を添加した IS 培地で培養した根の組織からシュートが分化した．シュートの分化率は，BAP を 0.8 mg/*l*，NAA を 0.03 mg/*l* 含んだ培地で 50% と最も高かった．分化したシュートは発根培地上で発根し完全な植物体を再生した．培養植物の根を切り取って，ショ糖を 1～3% 含んだ B5, IS, WP の 3 種の液体培地中で振とう培養することで，根の培養系を確立した．PVP 100 mg/*l* を B5 培地に添加することにより，培養根の褐変を抑制し，伸長を促進することができた．培養根と培養中の幼植物体から得られたインタクトな根の間には，シュートの分化率に大きな差はなかった．