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IN VITRO MULTIPLE SHOOT INDUCTION AND PLANT REGENERATION IN *BETULA PENDULA*

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Shoot development and plant regeneration through axillary bud culture of the European birch (*Betula pendula* Roth.) were successfully achieved. The modified ACM medium supplemented with 2.2 μM BA was used as initial medium. Both adventitious and axillary shoot development was induced in 2-year-old genotype explants, while in 4--year-old genotype cultures only axillary shoots developed. The establishment of multiple shoot culture was genotype depending. It was found that ACM nutrition medium, supplemented with 2.2 μM BA, was more effective for shoot multiplication than WPM and BTM. Microcuttings were easily rooted on ACM medium supplemented with 1.0 μM IBA. The complete plants obtained were successfully transferred to soil.

Introduction

The European birch (*Betula pendula* Roth.) has been identified as potential woody bioenergy crop and a species with positive influence on soil pH. It is known that vegetative propagation of species in the genus *Betula* by cuttings is of limited succes (S u z s k a 1979) which is the main problem for foresters in mass propagation of selected trees. The application of tissue culture methods offers new prospects for their rapid multiplication. In recent years numerous studies on *in vitro* propagation of woody plants have shown that these techniques may be a solution for rapid propagation of selected forest trees (C h a l u p a 1987, M c C o w n and M c C o w n 1987, B o u l a y 1987). Moreover, the microshoots ob-

tained by these methds could be the material source for studying the morphogentic capacity of different organs and tissues, appearance of somaclonal variants (Besendorfe et all. 1989) and resistance to pests or diseases. *In vitro* regenerated plants of some species in the family *Betulaceae* were used for studying the micorrhizal formation *in vitro* (Perinet and Lalonde 1983, Grellier et all. 1984, Tremblay and Lalonde 1984).

The investigations on tissue culture in the genus *Betula* has been reported by Chalupa (1981, 1983), McCown and Amos (1979) and Welander (1988) but the genetic effect on the morphogenetic capacity was not discussed. It is well known that the genotype as well as the physiological condition of the explants is a very important factor determining the possibility of vegetative propagation *in vitro* as well as *in vivo*.

In the present investigation we have tried to work out not only an *in vitro* method, but also to establish how the genotype and the age of donor plants affected shoot multiplication and plant regeneration of *Betula pendula* Roth.

Material and Methods

Plant material

Axillary buds of 2-year-old (genotypes JV_1, JV_2, JV_3) and 4-year-old (genotypes M_1, M_3, M_4) plants of *Betula pendula* Roth. were used as primary explants. The buds were excised from twigs collected in February to March. Dormant buds were surface sterilized by being agitated in 3% chlorine product Izosan-G for 20 minutes and then in 6% hydrogen peroxide for 5 minutes. After sterilization the buds were washed in sterile distilled water.

Media composition

Modified Aspen Culture medium, ACM (Ahuja 1983), without adenin sulfate, with 342 μM lysine and 2.2 μM 6-benzylaminopurine (BA) was used as induction medium.

The folowing three media: mod. ACM, Woody Plant Medium, WPM (McCown and Lloyd 1981) with 200 μM Na_2EDTA and $FeSO_4 \cdot 7H_2O$, and Broadleaved Tree Medium, BTM (Chalupa 1983), supplemented with 2.2 μM BA, were used for shoot multiplication.

Microcuttings were rooted on ACM medium supplemented with 1.0 μM indole-3-butyric acid (IBA).

Sucrose (2%) and Bacto-agar (0.9%) were added to all the media. The pH was adjusted to 5.7—5.8 before autoclaving.

Culture conditions

Cultures were kept in an air-conditioned chamber at temperature of 25±1°C under the illumination of fluorescent lamps (800—1500 lx) and during a light-dark cycle of 16—8 hours.

As a potting substrate the horticultural mixture of peat/perlite was used.

Results and Discussion

Culture initiation and establishment

After removal of the scales, single explants were inoculated on the induction medium, mod. ACM supplemented with 2.2 μM BA. The development of axillary shoot was induced in about 60% of the bud cultures. Within 4 weeks the elongated shoots were 1—2 cm high with 3—4 leaves. The response of explants on the culture condition was depended on genotype (Tab. 1). The genotype differences in the response

Table 1. Shoot development in birch bud explants in correlation to the genotype and the age of donor plant.

Genotype	Age of stock plant	No. of inoculated buds	% of buds developed in shoots
JV_1	2-year-old	8	75.0
JV_2		8	50.0
JV_3		8	80.0
total		24	67.0
M_1	4-year-old	32	47.0
M_3		26	73.0
M_4		19	80.0
total		77	64.0

of the explants cultured *in vitro* have been reported in related species *Alnus* and in *Salix* (Read et al. 1982, Farnham et al. 1982). The age of the donor plants was not a limiting factor for shoot induction.

The basal region of inoculated buds produced a large amount of green callus on the low concentration of BA (2.2 μM). The highest callus growth was noticed in the bud culture of 2-year-old genotypes (JV_1, JV_2, JV_3). At the same time, in the basal callus of these genotypes the adventitious shoots appeared (Fig. 2). Axillary and adventitious shoots were morphologically different. The axillary shoots were vigorous with short internodes and large leaf surfaces, whereas the adventitious shoots were thinner and had small leaf shapes. The same morphological differences between two kind of shoots in European birch were described by Welander (1988). The formation of adventitious buds in the basal callus in the culture of 4-year-old genotypes (M_1, M_2, M_3) was not noticed. Developed shoots were obtained only as the results of axillary branching of the main shoot. Abbott and Whiteley (1986) described this phenomenon in apple culture. They found that mixed cultures of axillary and adventitious shoots appeared only when buds from juvenile plants were used.

Stem segments with 2—3 axillary buds have been transferring to the same composition medium in 4—5 week intervals. During subcultivation the morpohological characteristics of the cultures noticed in the primary culture were retained. Multiple shoot cultures (Fig. 3) were obtained with 100 per cent success in the tested genotypes, except JV_2. After the fourth subcultivation the number of JV_2 cultures with developed shoots decre-

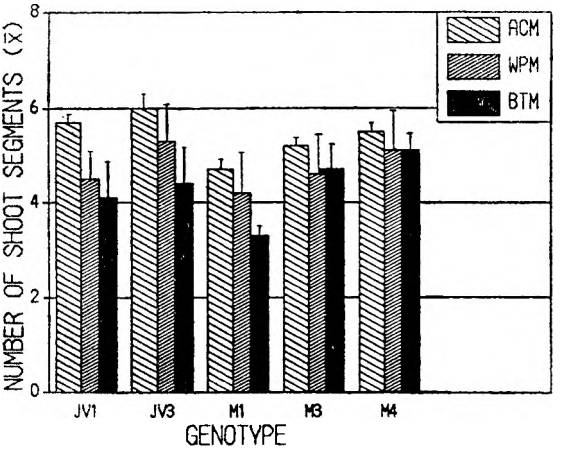


Fig. 1. The effect of genotype and composition of basal medium ACM, WPM and BTM on the multiplication rate in multiple shoot culture of *Betula pendula*. Picture shows average data collected during 6—8 months in culture.

ased while the shoot development in the seventh subculture was not induced. Our results showed that maintenance of multipple shoot culture was affected by the genotype.

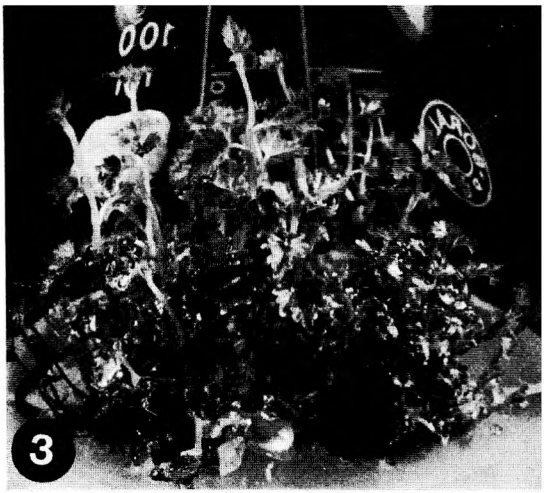
Shoot multiplication

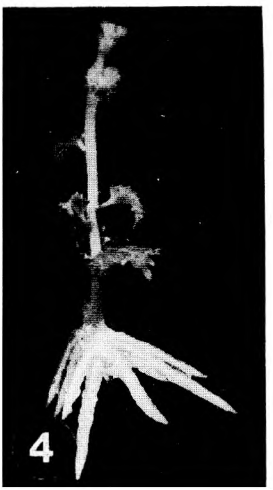
During 6—8 months, the multiplication rate, presented as a number of shoot segments per inoculum, was investigated. The rate of multiplication was analysed on ACM, WPM and BTM media supplemented with 2.2 μM BA. Shoot multiplication was possible on all the three media tested, but the ACM medium seemed to be superior to WPM and particularly in relation to BTM (Fig. 1). Chalupa (1983) reporteded the WPM and BTM media supplemented with a similar concentration of BA, were suitable for rapid clonal propagation of birches. In our investigation the multiplication rate achieved on the mentioned media was lower. The shoots obtained on WPM medium were yellowish green. When the concentration of Na_2EDTA and $FeSO_4 \cdot 7H_2O$ was doubled (200 μM) normal green-leaved shoots developed.

The genotypic effect on the multiplication rate was noticed in all tested media, but the deffirences between genotypes were not signifficant (Fig. 1). We also observed that the age of the stock plants used in our investigations was not a limiting factor for rapd multiplication. Results obtained could be explained by the strong effect of ecotypes as well as genotypes as suggested by Farnham at al. (1982) in their work with *Alnus rubra*.

Rooting of shoots

Microshoots excised from the shoot multiplication culture were rooted on the ACM medium supplemented with 1.0 μM IBA. Rooting of 100% of the microcuttings and complete plant regeneration were





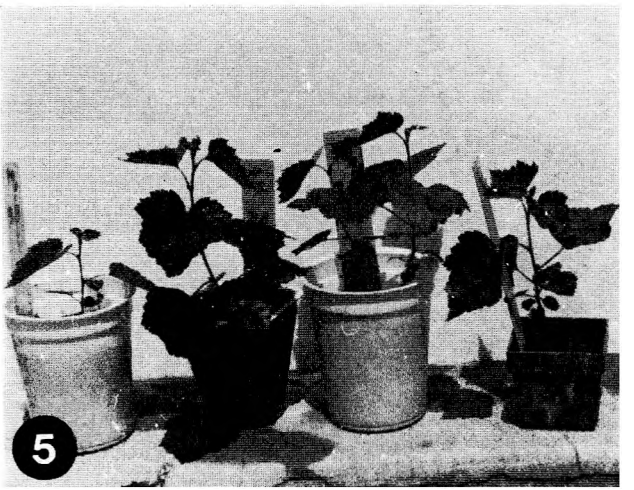


Fig. 2. The elongation of axillary shoot (white arrow) and adventitious shoot development (black arrow) in basal callus of the explant in primary bud culture, genotype JV_3, on ACM medium supplemented with 2.2 μM BA.

Fig. 3. Multiple shoot culture of *Betula pendula*, genotype JV_3, on modified ACM medium supplemented with 2.2 μM BA.

Fig. 4. Complete plants, genotype M_4, regenerated *in vitro*. The rooting of microcuttings obtained on ACM medium supplemented with 1.0 μM IBA.

Fig. 5. Plantlets after 2 months of acclimatization and greenhouse cultivation.

accomplished on this medium after 10 days (Fig. 4). The successful root induction using low concentration of IBA was described by Chalupa (1981, 1983) and Perez and Postigo (1989) in cultures of the same or related species.

The regenerated plants were transferred to an artificial horticultural peat/perlite substrate and subsequently acclimatized to greenhouse conditions (Fig. 5). After 2—3 months of cultivation in a greenhouse the plantlets were successfully transferred to a field.

Our results indicate that there is a considerable potential for rapid *in vitro* propagation of *Betula pendula* when using the multiple shoot culture method. With a proper selection of the genotype and nutrition media it has been possible to produce complete plants. The high efficiency of this method suggests the possibility of its commercial utilization.

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References

Abbott, A. J., E. Whiteley, 1976: Culture of *Malus* tissues *in vitro.* Multiplication of apple plants from isolated shoot apices. Sci. Hort. *4,* 183—189.

Ahuja, M. R., 1983: Somatic cell differentiation and rapid clonal propagation of aspen. Silvae Genet. *32,* (3—4), 131—135.

Besendorfer ,V., B. Kolevska-Pletikapić, D. Papeš, M. Krsnik-Rasol, 1989: Plant regeneration in the root callus culture of *Betula pendula* Roth. Biol. Vestn. *37,* (3), 13—22.

Boulay M., 1987: *In vitro* propagation of tree species. In: C. E. Green, D. A. Somers, W. P. Hackett, D. D. Biesboer (Eds.): Plant Tissue and Cell Culture, Alan R. Liss, Inc., New York.

Chalupa, V.: 1981: Clonal propagation of broad-leaved forest trees *in vitro.* Commun. Inst. Forest. Čechosl. *12,* 255—257.

Chalupa, V., 1983: Micropropagation of conifers and broadleaved forest trees. Commun. Inst. Forest. Čechosl. *13,* 7—39.

Chalupa, V., 1987: European hardwoods. In: J. M. Bonga and D. J. Durzan (Eds.): Cell and Tissue Culture in Forestry, Vol. *3,* pp. 224—246. Martinus Nijhoff Publishers, The Netherlands.

Farnham, R. S., Garton, K. A. Louis, P. E. Read, 1982: Propagating and establishing bioenergy plantations. Energy Conservation and Use of Renewable Energies in the Bio-industries (2nd), Proceedings, pp. 274—283. Trinity College, Oxford.

Grellier, B., R. Letouze, D. G. Strullu, 1984: Micropropagation of birch and mycorrhizal formation *in vitro.* New Phytol. *97,* 591—599.

McCown, B., R. Amos, 1979: Initial trials with commercial micropropagation of birch selections. Comb. Proc. Int. Plant Prop. Soc. *29,* 387—393.

McCown, B., D. G. Lloyd, 1981: A mineral nutrient formulation for microculture of woody plant species. Hort. Sci. *(16* (3), 453.

McCown, D. D., B. H. McCown, 1987: North american hardwoods. In: J. M. Bonga and D. J. Durzan (Eds.): Cell and Tissue Culture in Forestry, Vol. *3,* pp. 247—260. Martinus Nijhoff Publishers, The Netherlands.

Perez, C., P. Postigo, 1989: Micropropagation of *Betula celtiberica.* Ann. of Bot. *64,* 67—69.

Périnet, P., M. Lalonde, 1983: *In vitro* propagation and nodulation of the actinorhizal host plant *Alnus glutinosa* (L.) Gaertn. Plant Sci. Lett. *29,* 9—17.

Périnet, P., M. Lalonde, 1983: Axenic nodulation of *in vitro* propagated *Alnus glutinosa* plantlets by *Frankia* strains. Can. J. Bot. *61* (11), 2883—2888.

Read, P., S. Garton, K. A. Louis, E. S. Zimmermann, 1982: *In vitro* propagation of species for bioenergy plantations. Proc. 5th Intl. Cong. Plant Tissue and Cell Culture. Plant Tissue Culture, 757—758.

Suzska, B., 1979: Razmnožanie wegetatywne. Nasze Drzewa Lesne, Tom 7. Brzozy, 119—218. Polska Akademia Nauk. Warszawa — Poznan.

Tremblay, F. M., M. Lalonde, 1984: Requirements for *in vitro* propagation of seven nitrogen-fixing *Alnus* species. Plant Cell Tissue and Organ Culture *3,* 189—199.

Welander, M., 1988: Biochemical and anatomical studies of birch (*Betula pendula* Roth.) buds exposed to different climatic conditions in relation to growth *in vitro.* In: W. Hanover and D. E. Keathley (Eds.): Genetic Manipulation of Woody Plants. Basic Life Science, Vol. *44,* pp. 79—100. Plennum Press. New York.

SAŽETAK

INDUKCIJA MULTIPLIH IZDANAKA I REGENERACIJA BILJAKA VRSTE *BETULA PENDULA IN VITRO*

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Razvitak multiplih izdanaka i regeneracija biljaka dobivena je kulturom aksilarnih pupova obične breze (*Betula pendula* Roth.). Za indukciju izdanaka korištena je modificirana hranidbena podloga ACM uz dodatak 2,2 μM BA. Kod dvogodišnjih genotipova za razliku od četverogodišnjih, inducirani su osim aksilarnih i adventivni izdanci. Mogućnost uspostavljanja kulture multiplih izdanaka ovisila je o genotipu. Utvrđeno je da je podloga ACM s 2,2 μM BA u odnosu na WPM i BTM s istom koncentracijom citokinina bila pogodnija za umnažanje izdanaka. Izdanci su uspješno zakorjenjivani na ACM hranidbenoj podlozi uz dodatak 1,0 μM IBA. Kompletne biljke uspješno su se prilagodile na vanjske uvjete uzgoja.

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