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Factors controlling micropropagation of mature *Fagus sylvatica*

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Abstract

The influence of several endogenous factors on micropropagation of mature elite trees of beech (*Fagus sylvatica* L.) has been investigated. February was the most beneficial month for explantation of dormant buds, since infection with endogenous bacteria was still low and in vitro growth of the plant material was the highest. The genotypes tested gave different results concerning their growth on various tissue culture media. Out of 51 mature grafted genotypes only seven could be established in vitro. Grafting of branches of mature stock plants on juvenile rootstocks resulted in a high increase of multiplication rate compared with corresponding mature material, which could not be subcultured in vitro. Apical buds, larger than 20 mm, from 1-year-old shoots proved to be the most suitable explant source. Plant material could be subcultured for several years and rooted successfully.

Abbreviations: BA – benzyladenine, BTM – Broad-leaved Tree Medium, GD – Gresshoff Doy Medium, IBA – indole-3-butyric acid, MS – Murashige Skoog Medium, PVP – polyvinylpyrrolidone, SH – Schenk Hildebrandt Medium, WPM Woody Plant Medium

Introduction

Beech (*Fagus sylvatica*) is a slow-growing species that belongs to the economically most important deciduous trees in Central Europe. New methods for rapid clonal propagation are required, due to progressive forest decline and the lack of methods for producing superior plant material by vegetative propagation of proven mature trees.

Until now there exist only a few publications on tissue culture of *Fagus sylvatica*. Chalupa (1979) reported the culture and rooting of vegetative axillary buds of various species of broad-leaved deciduous forest trees including beech, and later (1985) described the effect of different growth regulators on in vitro propagation of beech cultures initiated from juvenile plants. Ahuja (1984 a,b) isolated protoplasts from leaves and found limited differentiation in bud explants of mature beech trees and shoot formation in embryonal seed explants. Nadel et al. (1991 a,b) studied medium and plant growth regulator effects on shoot elongation in buds from mature beech. Lang & Kohlenbach (1988) isolated and cultured viable protoplasts from leaves of juvenile and mature *Fagus sylvatica*, which led only to the formation of microcalli. Joergensen (1988, 1991) reported the development of embryos and shoots in anther cultures, but did not produce intact plants. Embryogenic cell suspension cultures and somatic embryos were obtained from immature zygotic embryos by Vieitez et al. (1992), but conversion of embryos into plantlets was low. So far there is no protocol for a large scale micropropagation of mature beech genotypes.

The present study was carried out to investigate the influence of genotype, bud characteristics and reactivation as well as of culture medium and collecting date on in vitro multiplication of mature buds of *Fagus sylvatica*.

Materials and methods

Dormant winter buds (Fig. 1A) from 51 grafted mature beech elite genotypes (35-year old trees) belonging

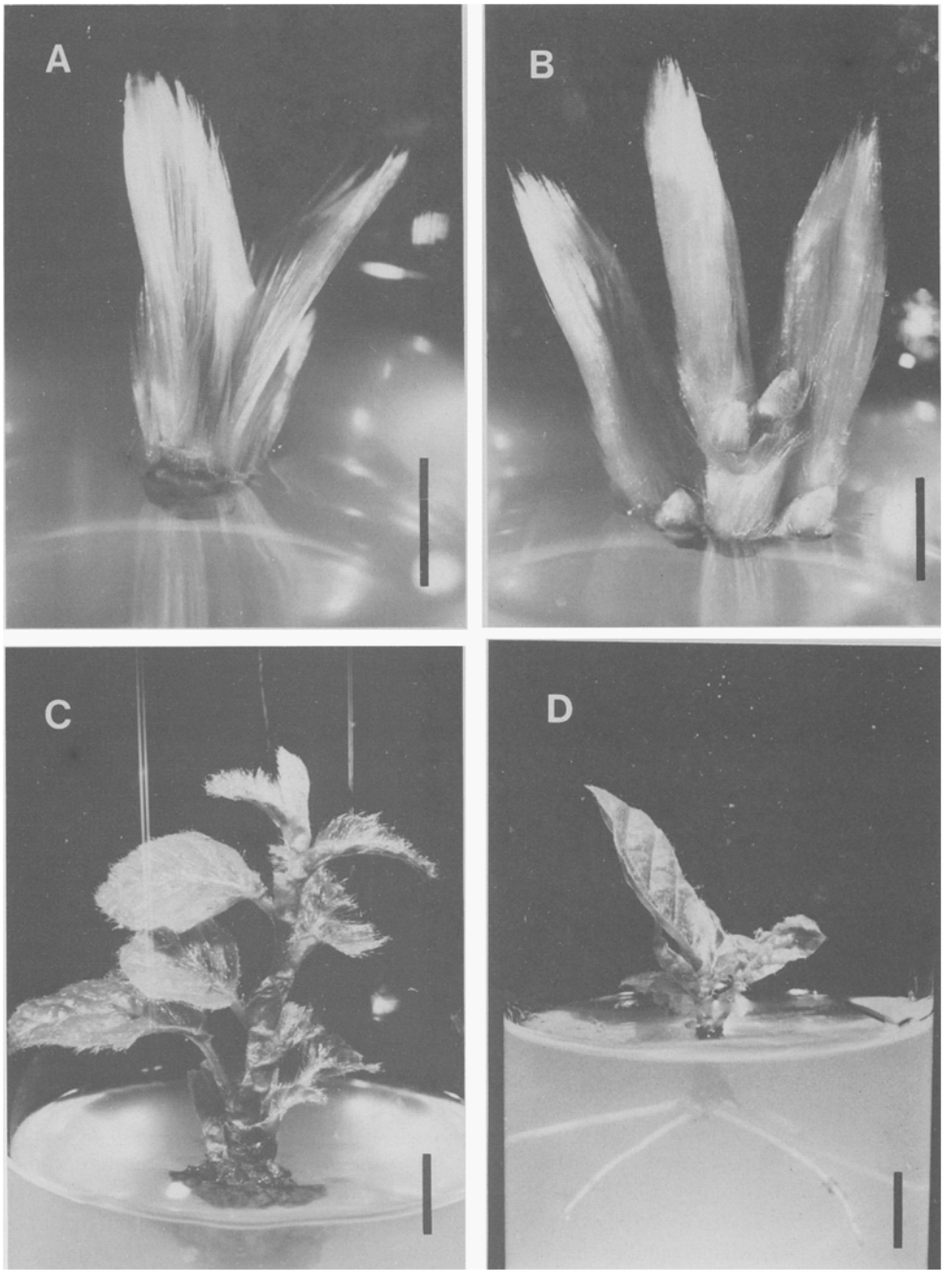

Fig. 1. In vitro development of beech (*A*) primary explant, (*B*) axillary bud and beginning shoot formation, (*C*) shoot regeneration in subcultured basal segments, (*D*) rooted shoot. Bar = 0.5 cm.

to three provenances, Grünberg (Gr), Lich (Li) and Baindt (Ba), were used as primary explants. For comparison three mature genotypes (F-7, F-8, F-9; >100 years old) and two juvenile beech trees (F-1, F-5; about 10 years old) were chosen from the forest in the surroundings of Rüdesheim, Germany. Branches were cut into single bud segments and surface disinfested (see experiment 1). After careful removal of the brown bud scales, buds were excised from the branch and placed on Woody Plant Medium (WPM, Lloyd & McCown

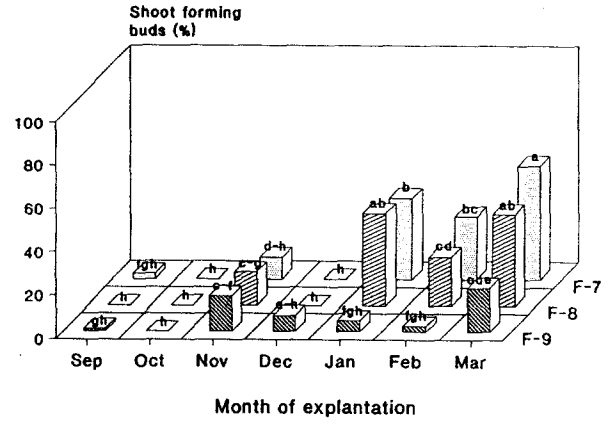

Fig. 2. Effect of collecting date on shoot formation of three mature genotypes of beech (means represent 40 buds per month and genotype; mean separation by Duncan's multiple range test, $p < 0.05$).

1980) supplemented with 4.5 µM BA, 1.5 % (w/v) fructose, 0.1 % (w/v) PVP, 1g l^{-1} casein hydrolysate and 100 mg l^{-1} myo-inositol. The pH was adjusted to 6.5 prior to autoclaving (121°C; 100 kPa; 15 min). Culture containers were 50 ml glass jars (100 × 30 mm) sealed with aluminium foil and containing 15 ml of medium solidified with 0.7 % (w/v) Difco Bacto-agar. After 4 weeks, the shoots were cut into shoot-tip and nodal segments and transferred to fresh medium with a reduced BA-level (0.9 µM). Rooting of fully developed shoots (10–15 mm) was achieved by dipping the basal end of the shoot in IBA-solution (4.92 mM) for 30 sec and subsequent culture on WPM without plant growth regulators. Shoot cultures were kept at 23°C in a 16-h photoperiod (Lumilux Warm White Fluorescent lamps, 95, µmol m^{-2} s^{-1}).

At the end of each subculture the following growth indices were determined:

- the in vitro sprouting (percentage of explants with axillary bud development and/or elongation of the shoot axis, new growth $\geq$ 5 mm; Fig. 1B);
- the percentage of shoot forming buds (Fig. 1C);
- and the multiplication rate (number of shoot-tip and nodal segments per primary explant subcultured.

In rooting experiments, the percentage of rooted shoots was recorded. Data from variables 1 and 2 as well as the rooting percentage were transformed by square root (n+0.5). Significant differences among multiplication results were estimated by analysis of variance (ANOVA) and Duncan's multiple range test; $p < 0.05$; values designated by the same letter are not significantly different.

Table 1. Influence of disinfestation method on exogenous contamination, endogenous infection and in vitro growth of beech explanted in February (means represent 35 buds of genotype F-7).

Growth index	Disinfestation	
	ethanol-NaOCl	ethanol-flaming
Contamination (%)	31.4	1.7
Infection (%)	0.0	0.0
Damaged explants (%)	0.0	0.0
Sprouting buds (%)	98.6	100.0
Shoot forming buds (%)	92.9	94.0
Multiplication rate	2.4	2.8

- Experiment 1: For selection of the disinfestation method, two procedures were tested: successive immersion in 70 % (v/v) ethanol (3 sec) and 5 % NaOCl (5 min) followed by three rinses in sterile deionized water (15 min) and dipping in 70 % (v/v) ethanol and pulling through an open flame.
- Experiment 2: For the determination of the most suitable time for explantation, dormant buds of three mature beech genotypes (F-7, F-8, F-9) were collected from September (when fully developed buds of preparable size can be found) to March (just before natural bud break). Per month and genotype, 40 buds were explanted (40 buds x 7 months x 3 genotypes = 840 primary explants).
- Experiment 3: The five culture media investigated, consisted of the salts and vitamins of Broad-leaved Tree Medium (BTM, Chalupa 1981), Gresshoff Doy Medium (GD, Gresshoff & Doy 1972), Murashige Skoog Medium (MS, Murashige & Skoog 1962), Schenk Hildebrandt Medium (SH, Schenk & Hildebrandt 1972) and WPM all supplemented as described above for WPM. A total of 1200 primary explants (5 media x 2 genotypes x 120 buds) were used.
- Experiment 4: To demonstrate the influence of grafting, scions from the upper part of the crown of mature beech elite genotypes (35 years old) were grafted once on seedling rootstocks of beech (1 year old). Corresponding grafted and ungrafted buds of seven mature Baindt-genotypes (Ba-2, Ba-4, Ba-11, Ba-18, Ba-21, Ba-30, Ba-33) were explanted in spring. Multiplication in vitro was recorded 8 weeks after establishment. A total of 280 primary explants (2 bud types x 7 genotypes x 20 buds) were used.

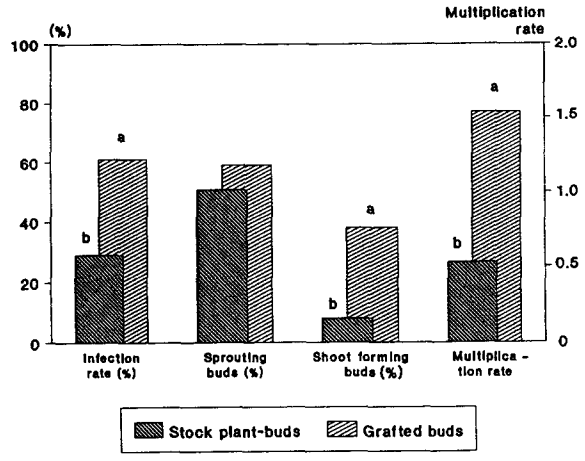

Fig. 3. Grafting effects on micropropagation of mature beech explants 8 weeks after establishment (means represent 140 buds of seven Ba-genotypes with 20 buds each; mean separation within growth index by Duncan's multiple range test, $p < 0.05$).

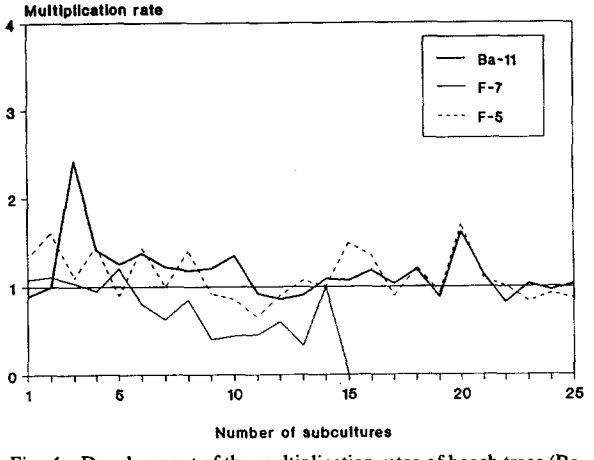

Fig. 4. Development of the multiplication rates of beech trees (Ba 35 years old, grafted; F-7: >100 years old; F-5: 10 years old) over 2 years in vitro.

– Experiment 5: For determination of the most beneficial bud characteristics, buds from various positions along the 1-year-old shoot were tested as well as apical buds of three different lengths. Each experiment was carried out with 20 buds of mature genotype F-7 per variant.

Results

Actively growing herbaceous cuttings of beech collected from bud break to the beginning of dormancy were not suitable explant sources since infection with endogenous bacteria was high and disinfestation with sodium hypochlorite led to severe damage of the bud tissue. The apical and axillary buds from these cuttings were smaller than 10 mm and neither isolated meristems nor complete buds with a small original stem segment showed any growth response in vitro. However, dormant buds gave better results in starting tissue culture. Surface disinfestation by flaming reduced exogenous contamination compared with chemical disinfestation usually employed in tissue culture (Table 1). Because of the firmly closed bud scales, no damage to the primary explants was observed by this technique. It also had no negative effect on the further in vitro development of the transplanted segments. Rate of infection with endogenous bacteria, not influenced by the disinfestation method, increased from September to March. No contamination of primary explants collected in September November, 2–3 % contamination in December - January and about 4 % in February March was observed. There was little variation among the genotypes tested. Buds explanted in February and March also showed higher shoot formation in vitro compared with winter buds (Fig. 2).

The rate of sprouting and shoot-forming buds was relatively low in both genotypes and on all culture media tested (Table 2); however, the mature F-8 gave nearly the same or even better results than the juvenile F-5. The highest survival rate and growth response of buds from the juvenile genotype were found on GD, whereas bud development of the mature F-8 was best on MS. This applies also to the percentage of buds surviving after 4 weeks in vitro. In the following subculture steps WPM proved to be more suitable for micropropagation of mature beech material, followed by SH and GD.

For activation of the regeneration potential, branches from the crown of seven mature beech trees were grafted onto seedlings used as rootstocks. Buds from 2-year-old graftings were compared in vitro with bud explants from mature donor plants of the same genotype (Fig. 3). Grafted buds were highly infected with endogenous bacteria. Although the vigour of explants was almost the same in both bud types, grafting resulted in a significant increase of shoot formation and of the multiplication rate of subcultured nodal segments and shoot tips. However, from the corresponding mature material a continuous decrease of the survival rate was observed, leading to total loss of the cultures.

Out of 51 grafted mature beech trees belonging to three provenances (Grünberg, Lich, Baindt) only seven genotypes could be propagated in vitro for more than

Table 2. Influence of culture medium on in vitro propagation of beech (F-5: juvenile, 10 years old; F-8: adult, ≥ 100 years old; 120 buds per variant; mean separation within each growth index by Duncan's multiple range test, $p < 0.05$).

Growth index	Genotype	Culture medium				
		BTM	GD	MS	SH	WPM
Sprouting buds (%)	F-5	35.4 cd	37.7 c	34.8 cd	37.1 cd	28.6 d
	F-8	37.7 c	47.6 ab	50.7 a	41.8 bc	41.9 bc
	x̄	36.6 b	42.7 a	42.7 a	39.5 ab	35.3 b
Shoot forming buds (%)	F-5	3.3 c	11.3 ab	5.5 bc	5.5 bc	7.4 abc
	F-8	4.4 c	10.8 ab	12.8 a	2.6 c	7.7 abc
	x̄	3.8 b	11.1 a	9.2 a	4.1 b	7.6 ab
Rate of survival (%) (4 weeks)	F-5	67.4 cd	74.6 abc	67.7 cd	61.5 de	51.9 e
	F-8	67.1 cd	73.8 bc	85.1 a	81.1 ab	77.4 abc
	x̄	67.2 bc	74.2 ab	76.4 a	71.3 abc	64.6 c
Rate of survival (%) (8 weeks)	F-5	40.0 ab	55.4 a	15.4 d	17.9 cd	36.6 abc
	F-8	28.6 bcd	39.7 ab	27.8 bcd	49.3 a	50.0 a
	x̄	34.3 bc	47.5 a	21.6 c	33.6 bc	43.3 ab

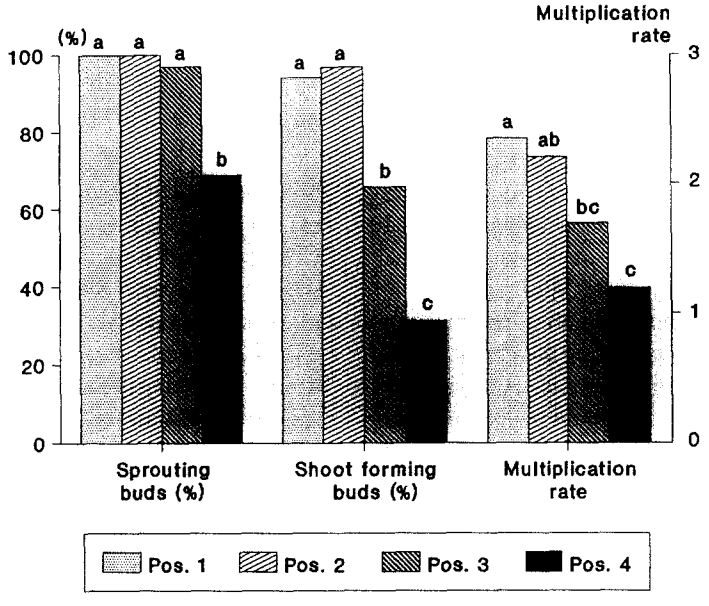

Fig. 5. Influence of bud position along the 1-year-old shoot-forming sprouting, shoot-forming capacity and multiplication rate of beech in vitro. Position 1 means the apical bud, position 4 the most basal bud. (20 explants of mature genotype F-7 per variant; mean separation within growth index by Duncan's multiple range test, $p < 0.05$).

1 year; percentage of sprouting buds varied from 50 to 68 % and multiplication rates from 0.9 to 1.2 within 4 weeks. Within 4 weeks Ba-11 produced up to four shoots per transplanted shoot-tip or seven new segments per transplanted modal segment within 4 weeks. Figure 4 represents the long-term multiplication rates of F-5 (10 years old), Ba-11 (35 years old, grafted) and F-7 (>100 years old) over a period of 2 years. For the

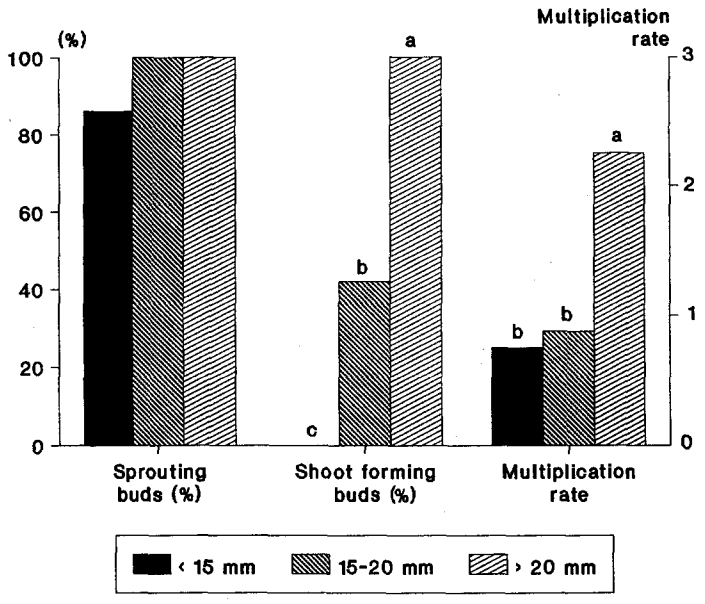

Fig. 6. Effect of bud size on sprouting, shoot formation and multiplication rate of beech. (20 buds of mature genotype F-7 per variant; mean separation within growth index by Duncan's multiple range test, $p < 0.05$).

juvenile F-5 and the grafted mature Ba-11, this growth index did not show any change except for monthly deviations, values for the mature genotype F-7 could be maintained in vitro for only 15 subcultures.

There was a strong influence of the bud position along an 1-year-old shoot on the in vitro establishment and micropropagation of excised buds (Fig. 5). The apical buds (position 1) were more reactive and formed more shoots and new segments than buds from the basal part of the branch (position 4). Micropropagation of beech also depended on the length of the primary bud explant (Fig. 6). Survival during the first 4 weeks in vitro was the same in all variants. Although 69 % of buds smaller than 15 mm showed axillary bud development, they did not produce shoots and new segments. Increasing bud size led to a strong increase in the number of shoot-forming explants and in the multiplication rate.

Meanwhile some of the mature genotypes were subcultured for more than 6 years without showing any decline in regeneration capacity. Four week old shoots, 10–15 mm in length, could be rooted by dipping the basal end in IBA-solution (4.92 mM, 30 sec) according to a rooting procedure for *Quercus* followed by transfer to growth regulator-free WPM (Fig. 1D). Prior to root development a soft white callus was formed at the shoot base. Root formation depended on genotype and age of the stock plant, with values of 65 % for two 10 year old clones (F-1, F-5) and 75 % for a 38-year-old grafted clone (Ba-21).

Discussion

Tissue culture of beech could be started only with dormant buds. When buds were disinfested with NaOCl, about 70 % were obtained without contamination, which agrees with the results of Nadel et al. (1991 a). By soaking in NaOCl solution, the microorganisms adhering to the surface could penetrate through the bud scales. Replacing this method by dipping in ethanol and subsequent flaming led to a decrease in contamination without damage to the tissues if the bud scales were entirely closed. Buds explanted in February and March just before natural bud break could be forced to grow and form new shoots in vitro without any severe infection. Nadel et al. (1991 b) also found a seasonal growth response of beech to application of plant growth regulators and corresponding changes in proteins. Buds sampled in November elongated only slightly, whereas spring buds showed considerable shoot elongation and leaf expansion.

Genotypes of different age showed non-uniform growth response on various culture media. WPM proved to be more suitable for the mature genotype, whereas the juvenile clone grew better on GD,

which was also successfully used for tissue cultures of *Quercus* species (Meier-Dinkel 1987). WPM has also been used by Ahuja (1984 b) for the cultivation of bud explants of mature beech trees and of embryonal explants derived from seeds. Out of three mature genotypes, only one showed slight growth and only 10 % of the buds exhibited a limited differentiation within 10 to 12 weeks. Vieitez et al. (1992) used WPM as solid primary medium for embryo axis and cotyledon fragments. Cell suspension cultures were kept in modified MS, whereas embryos at the cotyledonary stage could be cultured either on WPM or on MS. MS was also used for embryogenesis in anther cultures (Jörgensen 1988). Nadel et al. (1991 a) compared growth of beech buds on MS or on AC (Aspen Culture Medium, Ahuja 1983) and found enhanced but development on AC as compared with MS. Therefore, suitability of different basal media seems to depend on genotype, age and type of explant and the periodic adjustment of the mineral and growth regulator composition of the culture medium to the actual degree of rejuvenation (Franclet 1991).

The selection of genotypes is very important for the micropropagation of recalcitrant species; 18 % of the tested mature clones could be established in vitro. This agrees with our own results for other slow-growing deciduous species such as *Quercus robur* L. (20%) and *Quercus petraea* Matt. (16%) (Meier unpublished). Meier-Dinkel (1987) was able to establish five of 20 mature oak genotypes in vitro, while Juncker & Favre (1989) improved micropropagation by genotype-specific variation of the culture conditions. Vieitez et al. (1992) found differences between the embryogenic capacities and maturation frequencies of cultures of different beech genotypes and stated that the genotype was a significant factor also for embryogenic cultures. In our experiments both juvenile and grafted mature genotypes could be propagated successfully in vitro. The continuous decline of the multiplication rate of the mature F-7 resulted in total loss of the plant material. As has been described for mature explants of several tree species (Franclet et al. 1987), this character could not be changed in the course of subculturing of mature genotypes of beech. Similar results were found by Ahuja (1984 b), who only achieved growth and differentiation in embryonal explants of beech.

The techniques employed for rejuvenation of mature trees include application of a cytokinin either during or immediately before explants are placed in culture, propagation of stump sprouts, severe pruning or serial grafting (Bonga 1987; Greenwood 1987). Grafting of mature scions on juvenile rootstocks generally results in a short-term rejuvenation. However, rejuvenation manipulations are useful as pretreatments for further rejuvenation in vitro (Franclet et al. 1987).

Physiological activation of mature beech genotypes by grafting once on juvenile seedling rootstocks resulted in a significant increase in shoot formation and multiplication, whereas the multiplication rate of bud explants from corresponding mature trees declined continuously up to the total loss of the in vitro material. Positive results were also recorded when mature scions were grafted in succession on juvenile rootstocks of *Hedera, Eucalyptus, Platanus, Cupressus, Sequoia, Thuja, Pinus* and *Sequoiadendron*. One possible explanation is that the close proximity of the mature shoot apex to the cytokinin-producing juvenile roots may account for the rejuvenation obtained (Franclet et al. 1987; Pierik 1990).

The position along the branch as well as the length of the bud had a significant influence on the establishment of beech primary explants in vitro. Although bud break occurred in all variants to a high degree, even in small basal explants, shoot-forming capacity and multiplication rate differed remarkably. Completely elongated shoots that could be segmented and subcultured in vitro were formed only in apical buds longer than 20 mm. This result agrees with the results of Nadel et al. (1991a), who used only the upper four to five buds, since the small basal buds did not respond well in culture. In *Quercus robur* L. and *Q. suber* L. also, only terminal buds of seedlings could be established in vitro and the mid-stem nodal explants failed to develop (Vieitez et al. 1985; Pardos 1981). However, it has been reported that both the frequency of axillary shoot outgrowth and the rate of axillary shoot elongation were higher for basal primary explants of mature material and subcultured basal shoot segments of oak seedlings (San José1986; San José et al. 1988; Volkaert et al. 1990).

It seems necessary, therefore, to select primary explant material carefully according to genotype, season, bud position and length because of the observed differences in subsequent development in vitro. For the establishment of tissue cultures from mature beech trees, dormant bud explants longer than 20 mm from the apical region of 1-year-old shoots of grafted genotypes collected in spring should be used.

Acknowledgements

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References

Ahuja MR (1983) Somatic cell differentiation and rapid clonal propagation of aspen. Silvae Genet. 32: 131–135

Ahuja MR (1984 a) Isolation and culture of mesophyll protoplasts from mature beech trees. Silvae Genet. 33: 37–39

Ahuja MR (1984 b) In vitro induction of organogenesis in juvenile and mature beech. Silvae Genet. 33: 241–242

Bonga JM (1987) Clonal propagation of mature trees: problems and possible solutions. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol 1 General Principles and Biotechnology (pp 249–271). Martinus Nijhoff Publ, Dordrecht

Chalupa V (1979) In vitro propagation of some broad-leaved forest trees. Commun. Inst. For. Cechosl. 11:159–170

Chalupa V (1981) Clonal propagation of broad-leaved forest trees in vitro. Commun. Inst. Forest. Cechosl. 12: 255–271

Chalupa V (1985) In vitro propagation of *Larix, Picea, Pinus, Quercus, Fagus*, and other species using adenine-type cytokinins and thidiazuron. Commun. Inst. For. Cechosl. 14: 65–90

Franclet A (1991) Biotechnology in 'rejuvenation': hope for the micropropagation of difficult woody plants. Acta Hort. 289: 273–282

Franclet A, Boulay M, Bekkaoui F, Fouret Y, Verschoore-Martouzet B & Walker N (1987) Rejuvenation. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol 1 General Principles and Biotechnology (pp 232–248). Martinus Nijhoff Publ, Dordrecht

Greenwood MS (1987) Rejuvenation of forest trees. In: Kossuth SV & Ross SD (Eds) Hormonal Control of Tree Growth (pp 1–12).Martinus Nijhoff Publ, Dordrecht

Gresshoff PM & Doy CH (1972) Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta 107: 161–170

Joergensen J (1988) Embryogenesis in *Quercus petraea* and *Fagus sylvatica*. J. Plant Physiol. 132: 638–640

Joergensen J (1991) Androgenesis in *Quercus petraea, Fagus sylvatica* and *Aesculus hippocastanum*. In: Ahuja MR (Ed) Woody Plant Biotechnology (pp 353–354). Plenum Press, New York

Juncker B & Favre JM (1989) Clonal effects in propagating oak trees via in vitro culture. Plant Cell Tiss. Org. Cult. 19: 267–276

Lang H & Kohlenbach HW (1988) Callus formation from mesophyll protoplasts of *Fagus sylvatica* L. Plant Cell Rep. 7: 485–488

Lloyd G & McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb. Proc. Intl. Plant Prop. Soc. 30: 421–427

Meier-Dinkel A (1987) In vitro Vermehrung und Weiterkultur von Stieleiche (*Quercus robur* L.) und Traubeneiche (*Quercus petraea* (Matt.) Liebl.)). Allg. Forst- u.J.-Ztg 158:199–204

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nadel BL, Altman A, Pleban S & Hüttermann A (1991 a) In vitro development of mature *Fagus sylvatica* L. buds. I. The effect of medium and plant growth regulators on bud growth and protein profiles. J. Plant Physiol. 138: 596–601

Nadel BL, Altman A, Pleban S, Kocks R & Hüttermann A (1991 b) In vitro development of mature *Fagus sylvatica* L. buds. II. Seasonal changes in the response to plant growth regulators. J. Plant Physiol. 138: 136–141

Pardos JA (1981) In vitro plants formation from stem pieces of *Quercus suber* L. In: AFOCEL (Ed) Colloque International sur la Culture 'In Vitro' des Essences Forestieres (pp 186–190). Fontainebleau, France

Pierik RLM (1990) Rejuvenation and micropropagation. Proceedings of the VIIth International Congress on Plant Tissue and Cell Culture (pp 91–101).Amsterdam

San José MC (1986) Influencia de la situacion del explanto en la planta y del tamaño del tubo de cultivo en la multiplicaciõn in vitro de *Quercus robur* L. Phyton 46: 33–38

San José MC, Ballester A & Vieitez AM (1988) Factors affecting in vitro propagation of *Quercus robur* L. Tree Physiol. 4: 281–290

Schenk RU & Hildebrandt AC(1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199–204

Vieitez AM, San-José MC & Vieitez E (1985) In vitro plantlet regeneration from juvenile and mature *Quercus robur* L. J. Hort. Sci. 60: 99–106

Vieitez FJ, Ballester A & Vieitez AM(1992) Somatic embryogenesis and plantlet regeneration from suspension cultures of *Fagus sylvatica* L. Plant Cell Rep. 11: 609–613

Volkaert H, Schoofs J, Pieters A & De Langhe E (1990) Influence of explant source on in vitro axillary shoot formation in oak seedlings. Tree Physiol. 6: 87–93