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Original article

Micropropagation and restricted-growth storage of adult oak genotypes

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Summary — The winter buds of stump sprouts, epicormic shoots and grafts from adult pedunculate and sessile oak trees proved to be valuable sources of shoot tips. Established shoot-tip cultures exhibited long-term viability. Proliferation and vitality of new shoots was best from the base part of shoots if the callus tissue remained at the basal stem segment. Aged callus cells are involved in the process of xylogenesis which inhibits organogenesis. Root initiation depends upon optimum auxin supply but auxin causes side effects on shoot elongation and callus-cell proliferation. Shoot-tip necrosis was prevented if shoots with induced roots were subcultured on cytokinin-containing medium. The labor and expense of repeated subculture can be reduced by lowering growth temperature to 15, 10 or 4 °C. By using abscisic acid (0.1 – 10 μM) and applying polyethylene glycol (mol wt 4000) at concentrations of 4 and 8%, the inhibition of biomass accumulation will continue over 2 regular subculture periods on media without growth retarding substances.

***Quercus* / *in vitro* culture / bud / temperature / conservation**

Résumé — Micropropagation et conservation de chênes âgés sous conditions limitant la croissance. *Les bourgeons de rejets de souche, de pousses épicormiques et de greffes issus de plants âgés de chêne pédonculé et sessile constituent un excellent matériel pour la culture de segments de tiges. Ils manifestent une longue viabilité. Les meilleures viabilité et prolifération ont été obtenues sur du matériel prélevé à la base des segments. Les cellules âgées des cals sont impliquées dans la xylogenèse qui inhibe l'organogenèse. L'initiation des racines dépend d'un niveau optimal d'auxines; mais la production d'auxine a des effets négatifs sur l'élongation de la tige et la prolifération des cals. La nécrose des extrémités racinaires peut être évitée si les pousses issues de racines sont cultivées sur du milieu contenant des cytokinines. Le coût en temps et en main d'œuvre occasionné par les subcultures répétées peut être réduit en diminuant la température jusqu'à 4°C. L'utilisation d'acide abscissique (0,1 à 10 µM) et l'application de polyétylène glycol (poids moléculaire de 4 000) à des concentrations de 4 à 8% permet de prolonger l'inhibition de la production de biomasse pendant 2 périodes de subcultures sur du milieu ne contenant pas de substance de croissances à effet retardant.*

Quercus / *culture* in vitro / *bourgeon* / *température* / *conservation*

INTRODUCTION

By the method of shoot-tip culture, it is possible to preserve oak germplasm but the success of propagation depends upon the degree of juvenility in the starting maretial, on the method of sterilization, specific requirements of nutrients, hormones, cultural conditions and genotype, as described by Chalupa (1985, 1988), Maroti *et al* (1985), Vieitez *et al* (1985), San-José (1986), Pevalek-Kozlina and Jelaska (1986), Civinova and Sladky (1987), Favre and Juncker (1987), Meier-Dinkel (1987), Sasaki *et al* (1988), Meier and Gross (1989) as well as Vermeer and Evers (1990).

Restricted-growth storage of shoot-tip cultures is an effective method for the preservation of forest genetic resources (Gebhardt and Meier-Dinkel, 1990). It is appropriate for oak trees because adult genotypes cannot be propagated by cuttings and long-term seed storage is not possible. The labor and expense of repeated subculture can be reduced by lowering growth temperature (Meier-Dinkel, 1990), the use of chemical growth regulators and the application of hypertonic osmotica.

MATERIALS AND METHODS

Shoot-tip cultures were established from closed winter buds of adult trees as described earlier (Gebhardt *et al*, 1991). In order to prevent the browning of the shoot tips, ascorbic acid was added to the disinfectant. Shoot tips were placed on GD-medium (Gresshoff and Doy, 1972) supplemented with 0.2 mg/l benzyladenine (BA), 2% sucrose, 100 mg/l myo-inositol. Prior to autoclaving the pH was adjusted to 5.7. The media were solidified with 0.28% Gelrite (Kelco). Shoot-tip cultures were kept in a growth chamber at 26 °C in a 16 h photoperiod supplied by cool white fluorescent lamps (1500 lux). Elongated shoots were dissected from developing shoot clusters and subcultured monthly. The low temperature storage was examined with 5 genotypes (n = 300) cultivated on GD- and woody plant (WP)-medium (Lloyd and McCown, 1980) supplemented with 0.5 mg/l BA with 5 replicates for each temperature (4, 10, 15 °C) at reduced light (100–300 lux). For each storage period (8 and 20 wk) the accumulation of biomass (fresh weight of shoots) was determined before and after the test period and after subsequent periods of subculture. Abscisic acid (ABA) was added to WP-medium supplemented with 0.5 mg/l BA at final concentrations of 0.1, 1.0 and 10 μM. After a test period of 4 weeks, the biomass accumulation was compared (n = 48). Two subculture periods of 4 weeks followed and, with regard to the amount of callus cells, the recovery of shoot tips was determined on a control medium without ABA. Polyethylene glycol (PEG) was used as hypertonic osmoticum at concentrations of 4 or 8% in a WP-medium supplemented with 0.5 mg/l BA.

Microscopy

Callus tissue was fixed and stained with 0.25% safranin as described by Gebhardt and Goldbach (1988). Specimens were embedded in Rotiplast (Roth, 6642), sectioned at a thickness of 20 μm and mounted in Roti-Histokitt (Roth no 6638) after removal of the embedding material with Rotihistol (Roth, 6640). In UV light (excitation 436 nm) lignified cell walls exhibit green fluorescence, while cellulose stains yellow.

RESULTS

Adults trees provide buds from different positions with a varying degree of juvenility. To compare the regeneration capacity of different bud sources, the current years' shoots of the tree crown, epicormic shoots and stump sprouts were used as sources of shoot tips. Overall, 54 sterile cultures of different genotypes and bud sources were established but most of them remained non-viable for more than 3 subcultures. As demonstrated en figure 1, the viability of shoot-tip cultures was related to the origi-

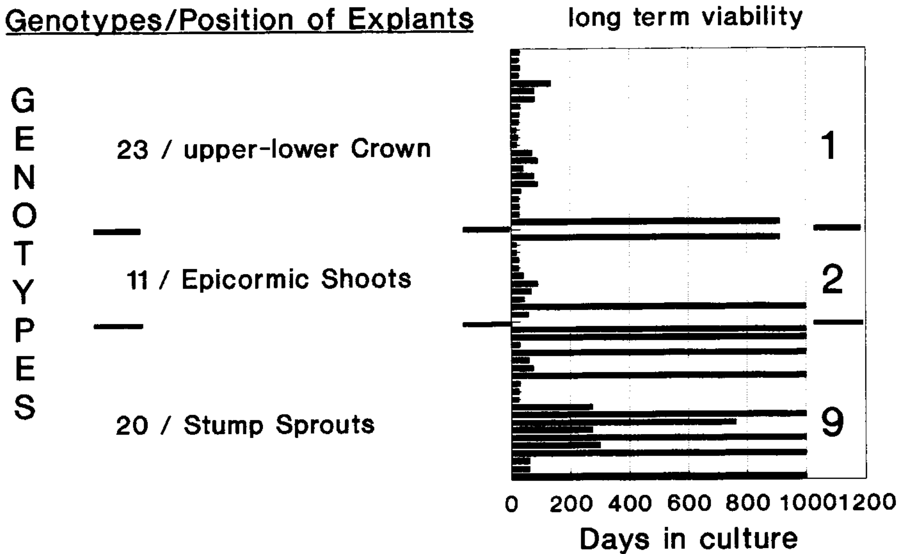

Fig 1. Long-term viability of shoot-tip cultures of adult oaks in relation to topophysis. Shoot-tip cultures derived from 54 genotypes and 3 sources of explants differing in position of topophysis. Shoot-tip cultures from 12 genotypes remained viable for a period of more than 800 days by repeated subculture (4-wk periods).

nal position of the explants. From 20 genotypes established by the use of stump sprouts, 9 (45%) remained viable for a period of more than 800 days. If epicormic shoots were used as a bud source, the success rate dropped to 18% of the genotypes. Only 1 of 23 genotypes from buds out of the tree crown exhibited long-term viability. In order to micropropagate selected trees of specific oak stands, chip-budded grafts were established and provided a bud source with a long-term viability of 37 (*Quercus robur*) to 56% (*Q petraea* Matt Liebl).

The proliferation of new shoots from leaf axils, at stem base or along the shoot axis, was promoted by the addition of 0.2 or 0.5 mg/l BA. It is assumed that new shoots develop from axillary as well as from trace buds which are released by the apical meristem but remain dormant for a certain period of time. In older trees, the activity of trace buds leads to the formation of epicormic shoots. In shoots derived from shoot-tip culture, the formation of new shoots is related to the activity of the shoot apex which can be very different, even if shoots are placed on the same media. Elongated and rooted shoots frequently exhibited shoot-tip necrosis and stopped elongation growth. Shoot-tip necrosis was stimulated by subculture on cytokinin-free media. It was prevented by a dip treatment of the shoot apex using 50 mg/l BA (15 s) or by subculture on BA-containing media. In order to prevent shoot-tip necrosis caused by an auxin treatment, we removed shoots from an auxin-containing medium (GD-medium with 50% macro- and microelements, 0.5 mg/l BA, 1.0 mg/l indole-3-butyric acid (IBA) after 9, 11, 13, 16 and 18 days of root induction and subcultured

them on WP-meduim supplemented with 0.5 mg/l BA. The mean number of roots/shoot increased from 1.6 after 9 days incubation to 4 roots/shoot after 16 days incubation on auxin-containing medium. Shoot elongation was also best after 16 days on auxin-containing medium. Callus cell proliferation at stem base was lowest after 13 days of auxin treatment.

Callus tissue remained partly green if subcultured on BA-containing medium. If shoots with a large callus at stem base were cut back and subcultured on BA-containing media, new shoots arose from the stem base. These shoots exhibited vigorous growth, long internodes and small leaves with juvenile character. This suggests that the callus tissue at stem base can partly compensate for the lack of a root system because of its large surface. If the callus tissue was subcultured twice, the release of polyphenols, as indicated by the browning of cells and surrounding medium, was enhanced. As demonstrated by

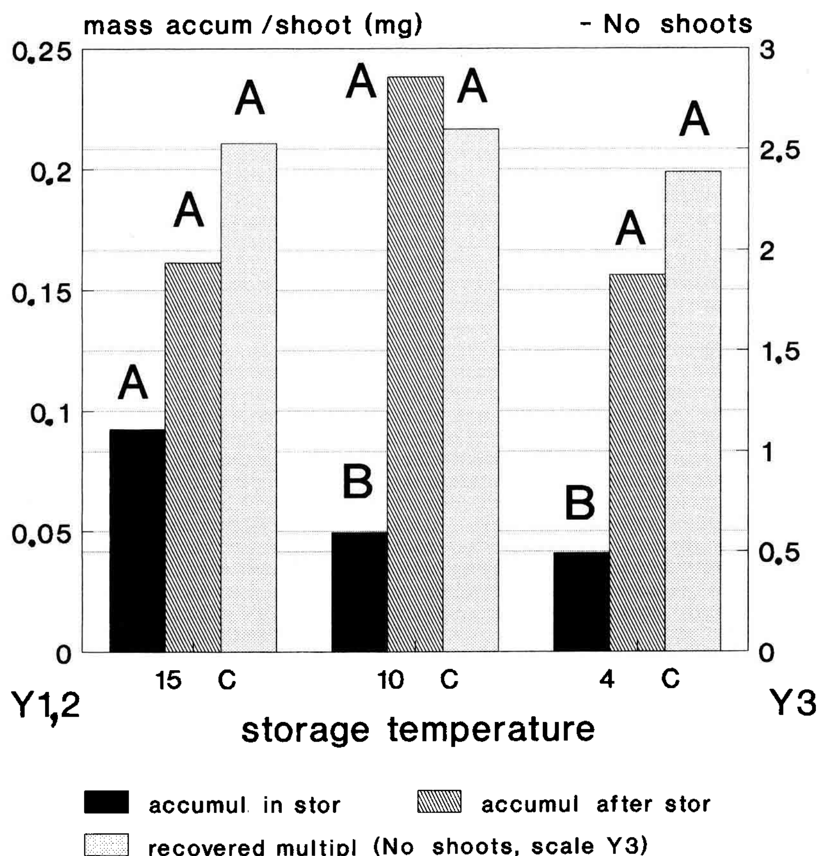

Fig 2. Effect of temperature on stored and recovered oak shoot tips. The accumulation of biomass (fresh weight of shoots) was determined before and after storage, and after subsequent periods of recover, the number of recovered shoots was also counted. Data from 190 stored samples were subjected to a multivariate analysis using the General Linear Models procedure of SAS. A Ryan-Einot-Gabriel-Welsch-test on variables y 1–3 revealed significant differences between temperatures as indicated by letters. Means with the same letter are not significantly different.

microscopy, callus cells are converted into tracheary elements. Tracheary elements exhibit aberrant secondary wall deposition and are subjects to autolysis (Roberts, 1976). Irregular patterns of lignified vascular tissue were formed but cambial activity was not observed.

In order to reduce labor and expense of repeated subculture, we lowered the growth temperature of shoot-tip cultures from the normal of 26 °C to 15, 10 and 4 °C. The subculture period of 5 genotypes on 2 media was prolonged from the normal of 4 weeks to 8 and 20 weeks. To separate the effects of temperature, media and subculture period, a multivariate analysis of variance was calculated. As shown in figure 2, the accumulation of biomass (fresh weight) decreased significantly at 10 and 4 °C. Cold temperatures in storage stimulated considerably the accumulation of biomass after storage. Accumulation of biomass after storage was less if shoots were stored on WP medium instead of GD medium but the number of shoots developed after storage was the same (fig 3, A). The extension of the subculture period resulted in a smaller number of regenerated shoots. The recovered mass accumulation was not significantly less (fig 3, B). When we compared the mass accumulation of shoots differing in the amount of callus at stem base during the course of storage, it became obvious that shoots with a large amount of callus cells may decrease in fresh weight even during a short storage period. This might be due to the process of xylogenesis and the resulting conversion of tissue.

Abscisic acid was added to WP-medium at final concentrations of 0.1, 1.0 and 10 μM. At 1 and 10 μM, significant inhibition of growth became obvious when the biomass accumulation after storage was compared. After a subculture period of 4 weeks, the

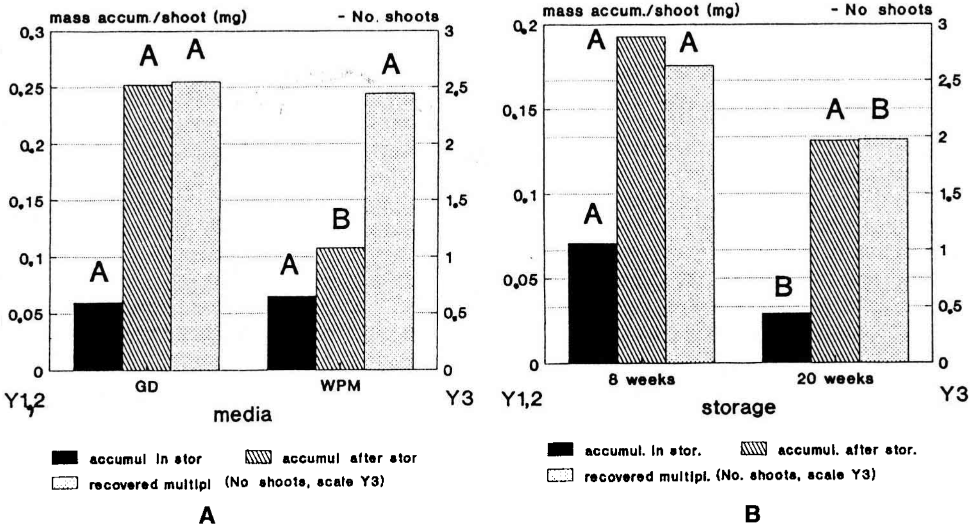

Fig 3. Effect of media (A) and time (B) on stored and recovered oak shoot tips. The accumulation of biomass (fresh weight of shoots) was determined before and after storage, and after subsequent periods of recovery the number of recovered shoots was also counted. Data from 190 stored samples were subjected to a multivariate analysis using the General Linear Models procedure of SAS. A Ryan-Einot-Gabriel-Welsch-test on variables y 1–3 revealed significant differences between media and storage period as indicated by letters. Means with the same letter are not significantly different.

recovery of shoot-tips was tested on a control medium without abscisc acid. Inhibition of shoot elongation and shoot multiplication remained significant after a second subculture period of recovery. Shoots with a large amount of callus cells at the stem base accumulated more biomass and multiplied significantly better than shoots with small calli.

PEG was used as hypertonic osmoticum at a concentration of 4 or 8% and was added to WP -medium. At both concentrations, the biomass accumulation was significantly decreased. Although biomass accumulation was not restored completely after 2 subculture periods without PEG, the mean weight shoot increased with time. In contrast to the ABA -treatments, a large amount of callus cells at the stem base evoked a negative effect on biomass accumulation and shoot multiplication during the course of recovery.

DISCUSSION

As mentioned by Cheliak and Rogers (1990), tree improvement is a process of managing genetic resources. Conservation is directed toward both wild and managed germplasm resources. Time to sexual maturity directly affects the efficiency of artifical selection and recombination. In most heterozygote tree populations, the performance of single trees depends upon age. From progeny tests, we can estimate that age-stable field performance of oak trees cannot be expected before age 30. Therefore it is important to be able to propagate a large number of genotypes from stands over 30 years old. From genetic studies, it remains to be clarified whether regeneration of individuals *in vitro* is linked to the genetic organization or to the methylation of DNA. The distribution of phenolic compounds could possibly mark the degree of juvenility in specific tissue (Scalbert *et al*, 1988). To maintain the genetic stability of cultures *in vitro*, it is recommended to use highly differentiated shoot tips but labor and expense of repeated subculturing should be reduced by the methods of restricted growth storage described above. Cold storage would allow the storage period to be extended over 19 months (Meier-Dinkel, 1990) but clonal differences must be identified. The recovery of shoot tips after storage must be assisted by specific physiological and environmental conditions, especially if hypertonic osmotica or growth regulators are used for growth reduction. Application of abscisic acid could increase cold hardiness and would induce stunted growth which allows storage of cultures in small vessels. Storage of meristems in liquid nitrogen could possibly increase genetic stability for conserved material. However, cryopreservation is still an empirical process and depends upon specific cellular activities and stages of development (Grout, 1990), which might be related to the juvenile character of somatic embryos (see Joergensen,1988, 1990).

True-to-type propagated and well-rooted plantlets could be used to compensate for the depletion of genetic resources in the original forest stands. In this case, the reproduced clone number would be small. Mass-propagated selected oak trees would allow the creation of multiclonal varieties that guarantee a high genetic variability as well as considerable genetic gain related to specific characters like wood density or flushing time. The functioning of a root system developed *in vitro* will be critical for further development.

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