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Original article

Embryogenesis in *Quercus petraea*

J Jörgensen

Lower Saxony Forest Research Institute, Department of Forest Tree Breeding, 3513 Staufenberg-Escherode, Germany

Summary — Embryogenesis in *Quercus petraea* was achieved by culture of zygotic embryos and anthers on modified woody plant medium with benzylaminopurine and 2,4 dichlorophenoxyacetic acid. After counting chromosomes and isoenzyme analysis the embryos from anther culture were subdivided into haploid and somatic clones. Protoplasts that had been isolated from haploid and diploid tissues were regenerated to embryos.

somatic embryogenesis / androgenesis / protoplast culture / *Quercus petraea*

Résumé — Embryogenèse chez *Quercus petraea.* *L'embryogenèse de* Quercus petraea *a été réalisée par culture d'embryons zygotiques et d'anthères sur du milieu WP contenant de la BAP et du 2,4 D. L'analyse des isoenzymes et le dénombrement des chromosomes ont montré que les embryons issus de culture d'anthères étaient subdivisés en clones haploïdes et somatiques. Les protoplastes isolés à partir de tissus haploïdes et diploïdes ont été régénérés en embryons.*

***embryogenèse somatique / androgenèse / culture de protoplastes* / Quercus petraea**

INTRODUCTION

In contrast to the breeding of agricultural and horticultural plants, the breeding of oak faces special difficulties: 1) inbred lines, which help to combine attributes exactly, cannot be produced due to the long generation period of oak; 2) the rate of rooting in cuttings of older mother trees is quite low (Spethmann, 1986) so that a sufficient supply of clones of high genetic quality can hardly be achieved; 3) the forests cannot be constantly supplied with oak seeds or plants because oak seeds cannot be stored for more than 3–4 years.

This situation is quite unsatisfactory when confronting simultaneous increases of forest decline and wood demand.

Although at the beginning of this program only shoot cultures had been described for this species (Pevalek-Kozlina and Jelaska, 1986; Meier-Dinkel, 1987),

we tried to find a combination of *in vitro* techniques and conventional methods, for which it would also be easier or even necessary to use physiologically young material, to offer possibilities in tree breeding, comparable to those of traditional breeding of early flowering plants.

Somatic embryos, which are genotypically similar to the mother tree, are ideal for the development of artificial seed, whereas gametophytic embryos are ideal as a basic material for protoplast culture; when the latter are hybridized which shortens the time of breeding and for getting them independent of flowering, they can be used as somatic embryos. Both types can be stored in liquid nitrogen (Jörgensen, 1990) thereby achieving independence of the unsteady seed production.

MATERIALS AND METHODS

Androgenesis and somatic embryogenesis

Anthers with pollen in the single nucleus or tetrade stage of 3 mature and immature zygotic embryos of 5 mother trees of *Quercus petraea* were prepared under sterile conditions, laid on a woody plant medium (WPM) (Lloyd and McCown, 1981) with MS (Murashige and Skoog, 1962) microelements: 1000 mg/l glutamine, 50 mg/l glycine and 50 mg/l serine (modified WPM) with combinations of benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) each at one of the following 6 different concentrations: 0.0, 0.5, 1.0, 2.5, 5.0 and 10.0 µM. Sugar, agar, macro- and microelements without Fe–ethylenediaminetetraacetic acid (FeEDTA) and K_2HPO_4 were autoclaved and FeEDTA, K_2HPO_4, amino acids, vitamins and hormones were sterilized by filtration and added afterwards. The pH was adjusted to 5.8. The tissues were cultivated at 2000–3000 lux and 25 °C ± 1 (zygotic embryos) or 30 °C ± 1 (anthers).

Calli that had developed in anther culture were induced to embryogenesis on modified WPM with 2.5 µM BAP at 2000–3000 lux and 25 °C ± 1.

Culture of androgenic and somatic embryos

Androgenetic and somatic embryos were cloned on modified WPM with 2.5 µM BAP. The embryos developed plants on the same basic medium without hormones.

Protoplast culture

In order to isolate protoplasts, haploid and diploid oak embryos were cut into small pieces with a razor blade and suspended in an enzyme solution consisting of 3% Rohamend PC, 5 mM $Ca^{(2+)}$ and 0.5 M mannitol and cultivated at 800–1000 lux at 25 °C ± 1 for 8–15 h. Afterwards, the isolated protoplasts were concentrated according to a method described by Binding and Kollmann (1985) and Binding *et al* (1988), then washed and embedded at a concentration of about 10^3–10^4 protoplasts/ml in VKM medium (Binding and Nehls, 1977) solidified with agarose. VKM-medium (1 ml) was added and the Petri dish was closed and sealed with parafilm. The cultivation was done at 800–1000 lux and 25 °C ± 1. The liquid VKM medium was renewed every 2–3 days. After the development of 1–2 mm microcalli, the agarose drops with the regenerates were laid on modified WPM with 2.5 µM BAP and further cultivated under the same conditions.

Genetic investigations of the androgenic embryos

After fixing the material in a mixture of 4 parts ethanol and 3 parts acetic acid, the tissues were smashed and stained in carminoacetic acid, and the chromosomes were counted. Isoenzyme investigations were carried out by Dr Müller-Starck (Müller-Starck and Jörgensen, 1991) at the Forest Genetic Institute of the University of Göttingen.

RESULTS

Androgenic and somatic embryogenesis

Two to 3 months after cultivation of the anthers on a modified WPM with 0.5 to 10 μM BAP and 0.5 to 10 μM 2,4 D, the walls of 1–3 of 50 anthers, from 2 trees in which the pollen was in the tetrade or for the 1st 2 days in the single-nucleus stage, were ruptured by the growing calli. It is not yet possible to give a statistical analysis, because the reactions of the anthers differed from clone to clone ranging from 0 to 6%. On the other hand no organgenesis occurred in 2 of 6 years, whereas in other years up to 6% of the cultivated anthers of 2 of the 3 tested mother trees showed organogenesis. This difference in reaction seems to depend upon the environmental conditions of the mother trees. These calli were totally transferred to embryos on modified WPM with 2.5 μM BAP. Secondary embryos developed on the surface of the primary embryos on the same medium (fig 1). Embryos resulting from tetrades and from pollen being in the single-nucleus stage for the 1st day (3 clones) proved to be haploid, whereas embryos from pollen being in the single-nucleus stage for the 2nd day were somatic (3 clones) (see below). We could not assign the clones from this year to haploid or somatic, because to date there has not been enough material to evaluate. On modified WPM without hormones, embryos of the last years developed plants (fig 2) that were transferred to greenhouse conditions.

Zygotic embryos of 4 mother trees cultivated on modified WPM with 0.5–5 μM BAP and 2.5–10 μM 2,4 D developed somatic embryos in the lower 3rd of the cotyledon and on the hypocotyl. Here too, the regeneration rate on the different media varied from clone to clone between 1 and 4 of 5 acorns in a year during which the regeneration rate was very high, whereas, in other years, the regeneration rates of the same mother trees were between 0 and 3 of 5 acorns, placed in one Petri dish. The first plants were regenerated on modified WPM without hormones.

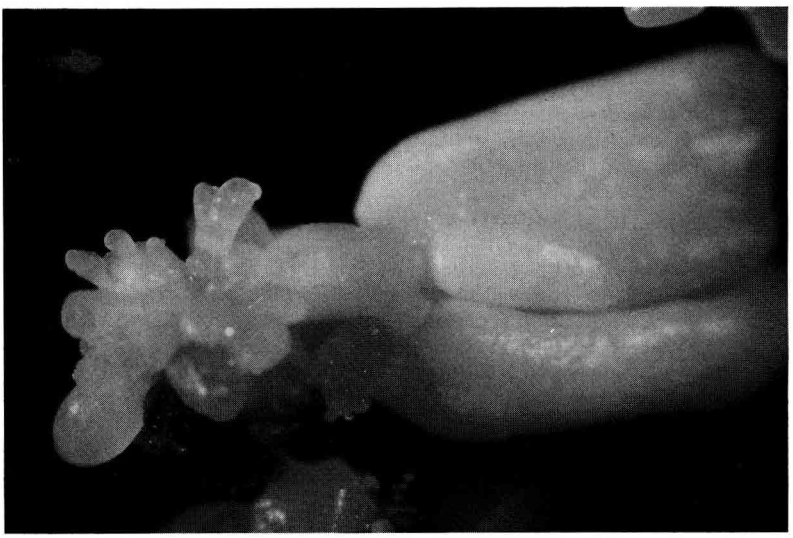

Fig 1. Androgenic embryos of *Quercus petraea*.

Fig 2. Androgenic plants of *Quercus petraea.*

Protoplast culture

Three to 5 days after the beginning of the protoplast culture, the first divisions were observed. Altogether, 0.5–3% of the protoplasts divided. About 70% of these cells developed through sustained divisions to 1-to-2-mm microcalli within 3–4 weeks. After the transfer of the agarose drops with the microcalli to modified WPM with 5% coconut milk and 2.5 μM BAP, between 5 and 24% of the microcalli grew to calli of about 4–5 mm diameter within 2–3 weeks (fig 3); development of embryos occurred after 11 weeks. The evolution of the 4 repetitions was very similar. The same experiments in beech showed that a period of at least 2 months was necessary for the development of the first embryos (Jörgensen, in press).

Genetic investigations of the androgenic embryos

As somatic cells, *ie*, anther walls were generative cells, and pollen were cultivated together in the framework of the anther culture, the origin of the regenerates had to be examined. The haploid clones were identified as pollen regenerates by counting the chromosomes, whereas the diploid clones proved to be regenerates of the anther wall. Isoenzyme analysis carried out by Dr Müller-Starck (Müller-Starck and Jörgensen, 1991) showed that a duplica-

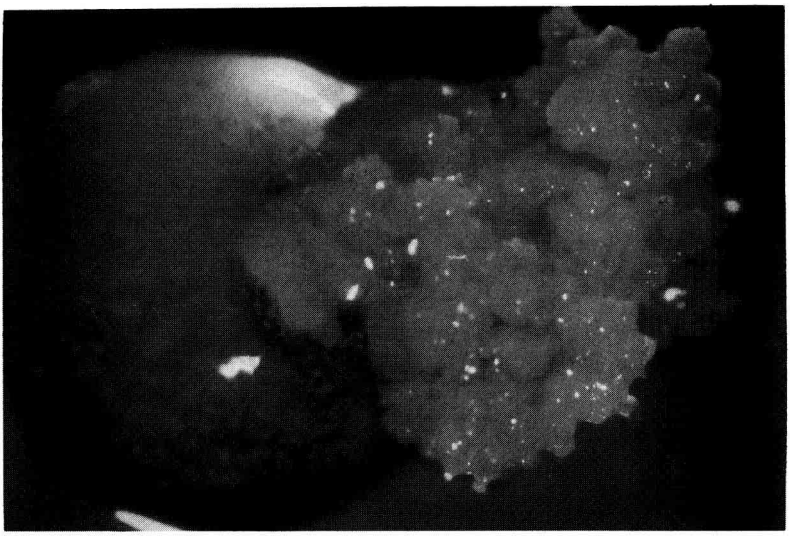

Fig 3. Callus formation of protoplasts embedded in agarose.

tion of the haploid chromosome set could be excluded (fig 4).

DISCUSSION

The results described above represent an important step forward in the breeding of *Quercus petraea* in a shorter time, but the number of different clones obtained within the short period of this project were not sufficient to realize crossing experiments useful for the practice of oak breeding. The fact that the experiments to produce androgenic embryos can be done only a few days per year and only in years in which the trees are flowering seems to be the biggest obstacle to generating enough haploid clones for plant breeding *in vitro*.

In addition to androgenesis, it is important to multiply valuable adult trees. To do so, somatic embryogenesis is a useful method, because it reactivates attributes which are typical of young material, like

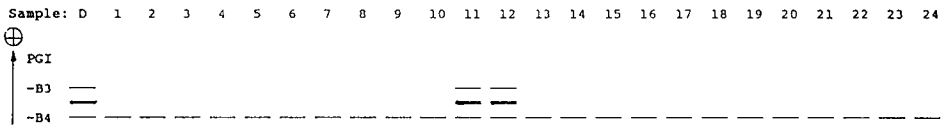

Fig 4. Allelic polymorphism at the gene locus PGI-B observed in young leaves of the *Quercus petraea* donor tree (D) and in 24 embryoid samples from anther cultures. The origin of samples 11 and 12 was the anther wall, because they have the same PGI-pattern as the donor tree (Müller-Starck and Jörgensen, 1991).

rooting ability, growth etc. In *Quercus acutissima* (Sasaki *et al,* 1988), *Q petraea* (Jörgensen, 1990), *Q rubra* (Gingas and Lineberger, 1989) and *Q robur* (Chalupa, 1990), somatic embryos were observed, but they are only of scientific interest, because they were induced on zygotic embryos, which means on genetically indefinite material. Only Feraud-Keller and Espagnac (1989) with *Q ilex* and Jörgensen (1988, 1990) with *Q petraea* could induce somatic embryogenesis with tissues of adult trees.

Protoplasts from embryogenic material can be regenerated to embryos and, consequently, to plants in *Quercus petraea* and *Fagus sylvatica* (Jörgensen, in press), so that the hybridization of these species should be possible, whereas, as shown only in *Fagus sylvatica*, the use of mesophyll protoplasts leads only to callus formation (Lang and Kohlenbach, 1988).

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