Ann Sci For (1993) 50, Suppl 1, 330s-335s

Original article

Rejuvenation of *Quercus robur*

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Summary — Stem sections and some branches, 30 cm in length, of mature *Quercus* were cut to induce the formation of rejuvenated shoots as initial material for *in vitro* propagation. Up to 100-year-old trees were used and topophysical effects were taken into account. *In vitro* rooting of shoot tips taken from this material showed a lower efficiency than embryo-derived cultures, but the fact that rooting occurred indicates some degree of rejuvenation. The influence of stem topophysis as well as genotype remains unclear.

rejuvenation / *Quercus robur* / topophysis / adventitious neoformation

Résumé — Réjuvénilisation de *Quercus robur*. *Des segments de la tige principale ainsi que des branches ont été prélevés sur des chênes âgés de manière à induire la formation de pousses rajeunies destinées à la multiplication* in vitro. *Des arbres de plus de 100 ans ont été retenus et les effets de topophysis ont été pris en compte. L'enracinement* in vitro *de ces pousses est plus difficile que celui produit dans des cultures d'embryons. Mais il indique toutefois un certain degré de rajeunissement. L'influence de la topophysis et du génotype de l'arbre n'a pas été élucidée.*

réjuvénilisation* / Quercus robur / *topophysis* / *néoformation adventive

INTRODUCTION

Micropropagation of woody species has been reported to be limited by rootability in many surveys (*eg*, Bonga and Durzan, 1987) of shoot tips harvested from adult trees. Embryo-derived cultures, used as the juvenile reference, in general proved to be more efficient in growth rate, axillary branching and rooting than adult tree-derived cultures. Rejuvenation strategies have therefore been proposed before (*eg*, Romberger, 1976) but were not so often effectuated. Also in *Quercus robur*, this difference was observed and some rejuvenation attempts implemented (Chalupa, 1984; Meier-Dinkel, 1987; Vermeer and Evers, 1987; Evers *et al*, 1988; Ballester *et al*, 1990). In Dutch forestry, this species is used both for urban and planted stand purposes. For urban areas, selected elite genotypes are used, usually still propagated through grafting. For stands, acorns

are harvested from selected seed stands. This means that the development of micropropagation techniques starting from both adult and juvenile material are required and, at the same time, provide the opportunity to study the consequences of maturation. Maturation is often associated with the loss of rootability and with the success of promotion of flowering (Libby, 1974). The results presented in this paper reflect the attempts to attain rejuvenation through the application of some techniques on the mother trees followed by micropropagation. The rooting results are compared with those of the embryo-derived material. The possibility that the applied techniques have led to the formation of adventitious buds (shoots of epicormic and/or sphaeroblastic origin) in the mother trees is discussed.

MATERIALS AND METHODS

Acorns were harvested from the Ede/de Klomp seed stand. Embryos were pulse treated with 0.5 mM 6-benzyladenine (BA) prior to culture on woody plant medium (WPM). Rooting was done on WPM with 4.9 μM indole-3-butyric acid (IBA) and 8 g/l activated charcoal for 4 weeks. In earlier research, increased sucrose levels proved to inhibit the effect of IBA and, therefore, were not used. Adult trees were 8-, 65- and 100-years old respectively; the 8-year-old trees were defined as 'adult' because *in vivo* rooting was no longer possible but, if the ability to induce flowering is used as a criterion, this stage had not yet been reached.

From the 8- and 100-year-old trees, stem sections of 30 cm in height were sawed in March and incubated upright in peat–potting soil for one-third of their length in the greenhouse after being disinfected with 1% Captan. For this purpose, the trees were cut down, leaving a 30-cm stump remaining. Stem and branch sections were sawed on the forest floor. The stem sections, intact lengths of the stem, were incubated in a plastic tent to maintain high humidity. The cut surfaces did not receive any further treatment. Shoot tips were harvested from one of the remaining stumps in the field 8 weeks later.

From the 65-year-olds, stem and branch sections were taken. All of the 8-year-old and two-thirds of the 100-year-old sections were taken to the greenhouse due to a lack of space; for the 65-year olds, 3 sections out of each of the total number of sections present in each fourth portion of the stem height were randomly selected. This reduction was again due to the lack of greenhouse space.

For the 65-year-olds, a comparison was made between 6 genotypes that form epicormic shoots (defined as any stem shoot occurring in intact trees) readily and those that do not. In the regenerated or activated shoots from the sections, usually occurring within 9 days, a distinction was made between shoots presumably of sphaeroblastic (adventitious) origin and shoots of accessory origin (axillary shoots occurring near branch scars). The average quarter height part of the stem contained an average of 17 sections. One of every 3 sections was incubated in the greenhouse. The 5 stem height sections of the 100-year-old trees were defined simply by dividing the height into 5 parts. Some of the 100-year-old trees in the same stand were severely branch-pruned *in situ* to induce shoot formation in the field. All mechanical pretreatments were done in March. Shoot tips were cultured on WPM with 20 g/l activated charcoal which was lowered to 5 g/l in the next subculture. The regulators were: 6-BA, 4.4 μM in the initial phase and 2.2 μM in the multiplication phases thereafter; for rooting (attempted from the 5th *in vitro* cycle onwards on different macromedia), IBA at 4.6 μM without activated charcoal was used. Plantlets were planted out in potting soil and gradually adapted to the lower humidity in the greenhouse by opening the plastic tent.

RESULTS

The reference material, embryo-derived shoot clusters showed a multiplication rate of 3.4–3.7 (Standard error of difference of means 2.4) after 3 subcultures. The average rooting efficiency *in vitro* depends upon the method (especially the sucrose concentration when IBA is not applied) and genotype, but can be as high as 100% on a medium with 30 g/l sucrose.

The results of shoot regeneration *in vivo* showed a large variation between the segments and the genotypes. The segment shoot production of the different mother tree according to age was: 195 for 8-year-old trees (whole stem in 13 segments, all used), 240 for 65-year-old trees (1/3 of the segments used, estimated 720 for the whole tree) and 893 for 100-year-old trees (2/3 of the segments used, estimated 1340 for the whole tree). In the 100-year-old trees, 21% of the shoots may be adventitious, since they did not occur close to branch scars; for the 65-year-olds and 8-year-olds the percentages were 1.6 and 35%, respectively. In the 65-year-olds, the difference between the epicormic-rich and epicormic poor was 393 and 87, respectively. The number of presumably adventitious origin on trees of the 3 ages from oldest to youngest were: 68, 4 and 183, respectively, again on the above mentioned portion of segments. In preliminary experiments, branch shoot regeneration showed comparable results, especially when the bark surface area was taken into account. Shoots from the *in situ* treatments (telegraph post, branch) were not counted and were impossible to isolate *in vitro* due to contamination problems.

The data obtained from the 100-year-old (figs 1, 2), 65-year-old (fig 3) and 8-year-old trees showed that no clear stem topophysical effects in regenerating adventitious or axillary shoots were apparent. Of the average total number of shoots regenerated on segments from 65-year-old trees, 55% were found in the lower half of the stem, a part presumed to be more juvenile as a result of ontogenesis. However, in the epicormic-rich group, this percentage was 63%, while in the epicormic-poor group it was only 16%.

The juvenility parameter 'maximum *in vitro* rooting' did not differ significantly be-

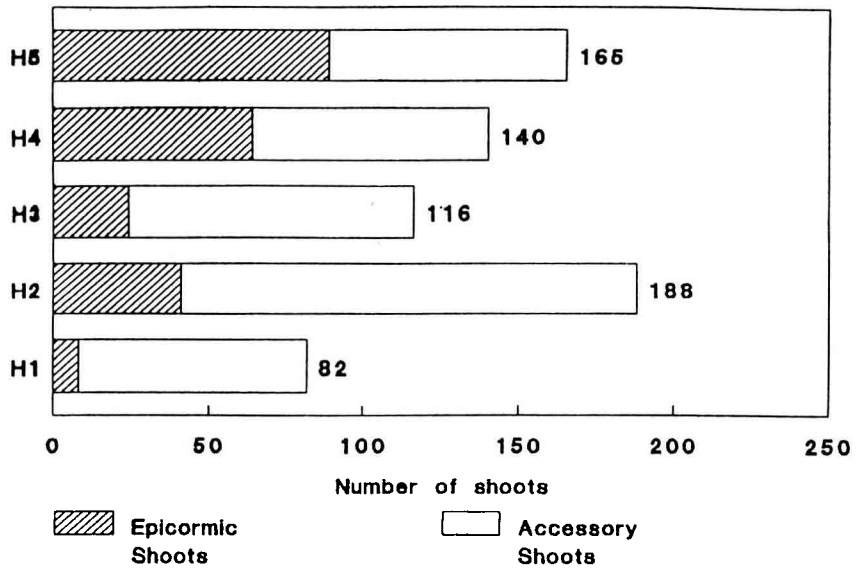

Fig 1. Regeneration of stem shoots on segments of the stem of a single 100-year-old genotype in 5 height sections (2/3 of the sections). Epicormic shoots are defined as not being associated with a branch scar and are therefore considered to be aventitious. H1 is the lowest section.

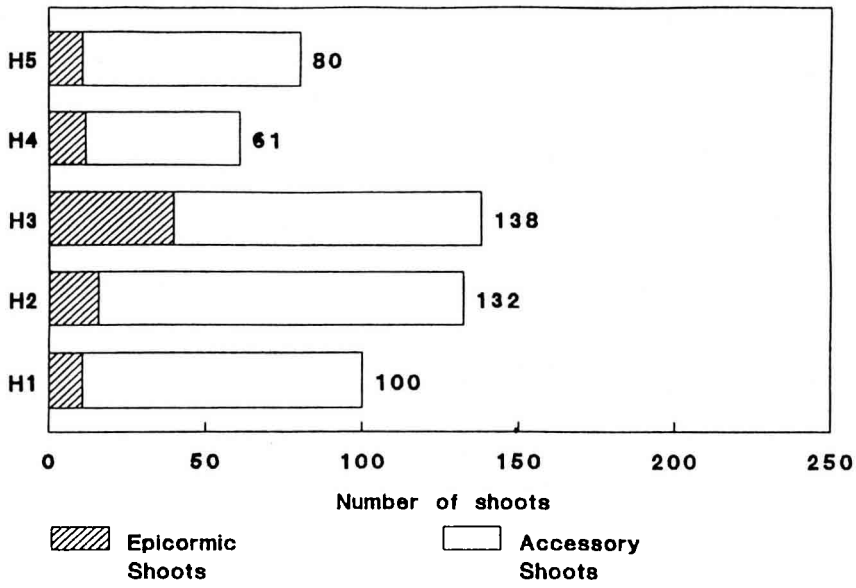

Fig 2. As for figure 1, another model tree.

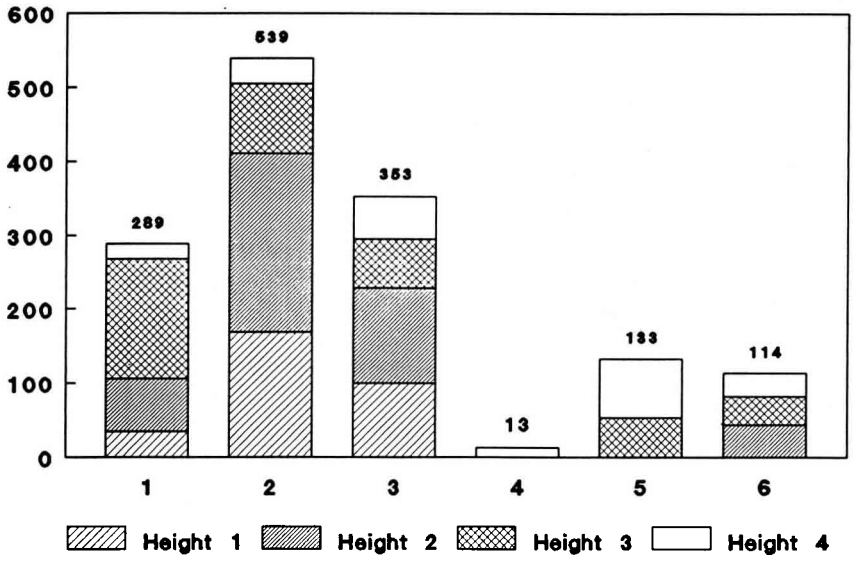

Fig 3. Shoot regeneration on stem sections of 3 epicormic shoot-rich genotypes (1, 2, 3) and 3 epicormic shoot poor genotypes (4, 5, 6) in 4 stem-height sections. Height 1 is the lowest section. The bars represent the numbers of shoots.

tween the embryo-derived material (100% maximum, 72% average) and 8-year-old-derived material (93% maximum, 87% average). The percentage in the old trees is at present < 20% on the average, but is expected to go up once the *in vitro* age of this material increases. In some experiments, it was as high as 37%, especially when regenerants from the most basal root part of stumps from the forest were used.

The multiplication rate of the 8-year-olds was not lower than that of embryo-derived material (4.0 *vs* 3.4 mo). The 65-year-old trees were not in culture long enough to give conclusive results. The highest multiplication rates (5.4, 100-year-old trees) were found in shoot-tip material in vitro harvested from regenerates on the basal part of the main roots of felled trees; the lowest rate occurred in tips from accessory buds from the highest part of the stem (no multiplication at all). From 2 model 100-year-old trees, a total of 858 and 466 shoot tips for tissue culture could be harvested (2/3 of the segments).

Acclimatization in the greenhouse of 8-year-old-derived material did not differ from that of the embryo-derived plantlets. However, growth in the greenhouse and the nursery of plantlets derived from the 8-year-old material was much slower.

DISCUSSION

The principle of rejuvenation in *Q robur* has been proven, if rooting *in vitro* is used as a parameter. The *in vivo* reference, the ability to root crown branch cuttings was not successful. The *in vitro* reference, embryo-derived shoot tips, proved to be no more efficient than the tips from 8-year-old segment-derived material. The question is whether 8-year-old trees can be called adult or whether they are in a transition phase from juvenility. Some scientists consider the ability to flower as transition to the adult stage; in that sense, 8-year-olds are not yet adult. It is however clear that, with increasing age of the mother tree, shoot material is regenerated that is in terms of *in vitro* behavior not like the shoots derived from embryo cultures. This apparently incomplete rejuvenation may be full rejuvenation (comparable to germinating seedlings), because it can be obscured by non-optimal *in vitro* conditions causing a more 'adult' type of morphogenesis. Also, expression of rejuvenation may depend upon morphogenesis. Also, expression of rejuvenation may depend upon *in vitro* age: it is a well-known phenomenon that material coming from older trees has a slower 'acceleration' in initial morphogenesis. On the other hand, it may simply be a changing metabolism, even in flushing resting buds that were not formed in the previous season (Libby, 1974).

Based on theories of Nozeran *et al* (1971), the observed rejuvenation could also be a topophysically based difference in the level of differentiation. Our earlier results indicated a relationship between *in vitro* morphogenesis and mother plant topophysis (Evers, 1984; Evers *et al*, 1988). This hypothesis, considering the lack in stem segment discrepancies in the results presented in this paper, seems not to be the case. According to Halle *et al* (1978), the observed regeneration might also be called reiteration, indicating a more adventitious nature. The definitions given in the literature for regenerating stem and branch shoots are very unclear in terms of being adventitious or not. This distinction is of utmost importance to be able to estimate risks of the occurrence of genetic aberrations. Histological studies may yield more conclusive results in the near future.

The results for the 65-year-olds may indicate a more discouraging aspect: a relationship between epicormic-rich genotypes, less desirable for many applications,

and the ability to regenerate stem and branch shoots from segments. The genotypic differences are not well understood. Some of the differences thought to be genotypic may, in the end, turn out to be growth-site-induced in comparable genotypes. This last factor will be a major item in our research.

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