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Alleviation of browning in oak explants by chemical pretreatments

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Abstract

Meristems from 25 - 90-year-old oak (*Quercus robur* L. and *Q. petraea* Matt.) trees and seed embryos were pretreated with polyvinyl pyrrolidone, ascorbic acid, cysteine and citric acid solutions. Tissues were cultured mostly on a WPM medium supplemented with different combinations and concentrations of growth regulators. All the different pretreatments showed a positive effect against the otherwise very rapid and harmful browning of the explants, but ascorbic acid (100 mg dm^{-3}) proved to be the most effective. Shooting was induced from seed embryos and meristems originating from adult trees. Rooted plantlets were obtained from explants of seed embryos.

Introduction

Oaks (*Quercus robur, Q. petraea*) appear to be among the most difficult broad-leaved tree species to propagate *in vitro*. Some progress has been reported with explants taken from embryos or seedlings (Chalupa 1984, Vieitez *et al.* 1985, 1989, Favre and Juncker 1987, Tóth 1989) or stem sprouts (Favre and Juncker 1987, San-José *et al.* 1990, Vieitez *et al.* 1985). The only reports so far on *in vitro* propagation of *Quercus robur* and *Q. petraea* from explants taken from adult trees are those of Chalupa (1988) and San-José *et al.* (1988), but large scale micropropagation of the trees is still problematic. For the silvicultural purposes, propagation of selected trees of high value would be in greatest demand.

One of the reasons for the failure in *in vitro* culturing of the oak tissues is the rapid blackening of the explants. This is at least partly caused by oxidation of polyphenols which are abundant in various oak species and occur in most parts of the trees (Basden and Dalvi 1987, Scalbert *et al.* 1988). Also, strong clonal effects are typical at different steps of the *in vitro* propagation (Juncker and Favre 1989).

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Abbreviations: BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - naphthalene-1-acetic acid; 2iP - N^6-2-isopentenyl adenine
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The goal of the present study was to alleviate, by using various pretreatments, the harmful effects of phenolic compounds and thus increase the capability of the explants to grow in *in vitro* conditions. Explants were taken from adult trees belonging to several clones of two oak species in vastly separated areas, Finland and Hungary. Oak in Finland is in its northern border of distribution and there are only few very old trees, while in Hungary it is widely distributed and economically important. For comparison, embryonic material was used.

Materials and methods

Plant material: For the meristem cultures, twigs from 25-, 40-, 75-, 80- and 90-year-old trees (*Quercus robur* L. and *Q. petraea* Matt.) were collected in the area of Szombathely (Hungary), and from Loppi (southern Finland), Turku (western Finland) and Oulu (northern Finland). The twigs were collected in autumn and in February, and prepared for culture immediately or after couple of week's storage in +5 °C. The buds were surface sterilized in 3.5 % Na-hypochlorite for 20 min and rinsed four times with sterile distilled water. Shoot tips consisting of apical meristem with about 1 mm piece of surrounding tissue were dissected under microscope, soaked in ascorbic acid (100 mg dm^{-3}, pH 2.9), citric acid (100 mg dm^{-3}, pH 2.5), L-cysteine hydrochloride (10 mg dm^{-3}, pH 3.1) or PVP, (polyvinyl pyrrolidone, soluble, 5 g dm^{-3}, pH 4.7) solution for 30 - 60 min and transferred to media. Untreated meristems and meristems rinsed in H_2O were used as control material. Pretreatment with ascorbic acid and citric acid was made to all the explants but only half of the material was treated with cysteine and PVP. Altogether 760 meristems were treated.

For the embryo cultures, acorns of *Q. robur* and *Q. petraea* were collected from the area of Szombathely, Hungary, and stored in cold room (+2 °C) until use. Seeds were surface sterilized with or without seed coats in 3.5 % Na-hypochlorite for 20 min and rinsed four times with sterile distilled water. Embryos were dissected under microscope, pretreated like meristems and transferred to media. Control material was treated as mentioned above. Altogether 120 embryos were tested.

***In vitro* culture:** Explants were placed in 20 × 150 mm test tubes containing 15 cm^3 of culture medium. Most explants were cultured on a WPM (Lloyd and McCown 1980) media. Also SH (Schenk and Hilderbrand 1972) or half-strength MS (Murashige and Skoog 1962) media were tested but with a smaller amount of explants. Different combinations of growth regulators were tried (BAP 1 - 2 mg dm^{-3}, kinetin 1 - 2 mg dm^{-3}, 2iP 1 - 2 mg dm^{-3}, NAA 0.01 - 0.5 mg dm^{-3}, IAA 0.01 - 0.5 mg dm^{-3}, GA_3 0.5 mg dm^{-3}, adenine sulphate 20 mg dm^{-3}). Sucrose, 30 g dm^{-3} was used as a carbon source. Difco Bacto agar (7 g dm^{-3}) was used to solidify nutrient media. Rooting medium was hormone-free 1/2 MS to which 20 - 30 mm long shoots were transferred after dipping them for 5 s in IBA (1 mg dm^{-3}). Cultures were maintained in a growth room with a 16/8 h photoperiod (irradiance of 1.8 W m^{-2}, 400 - 700 nm, fluorescent tubes, *FLUORA L36W/77*) with day/night temperatures of 25/20 °C, respectively. The explants were transferred to a fresh

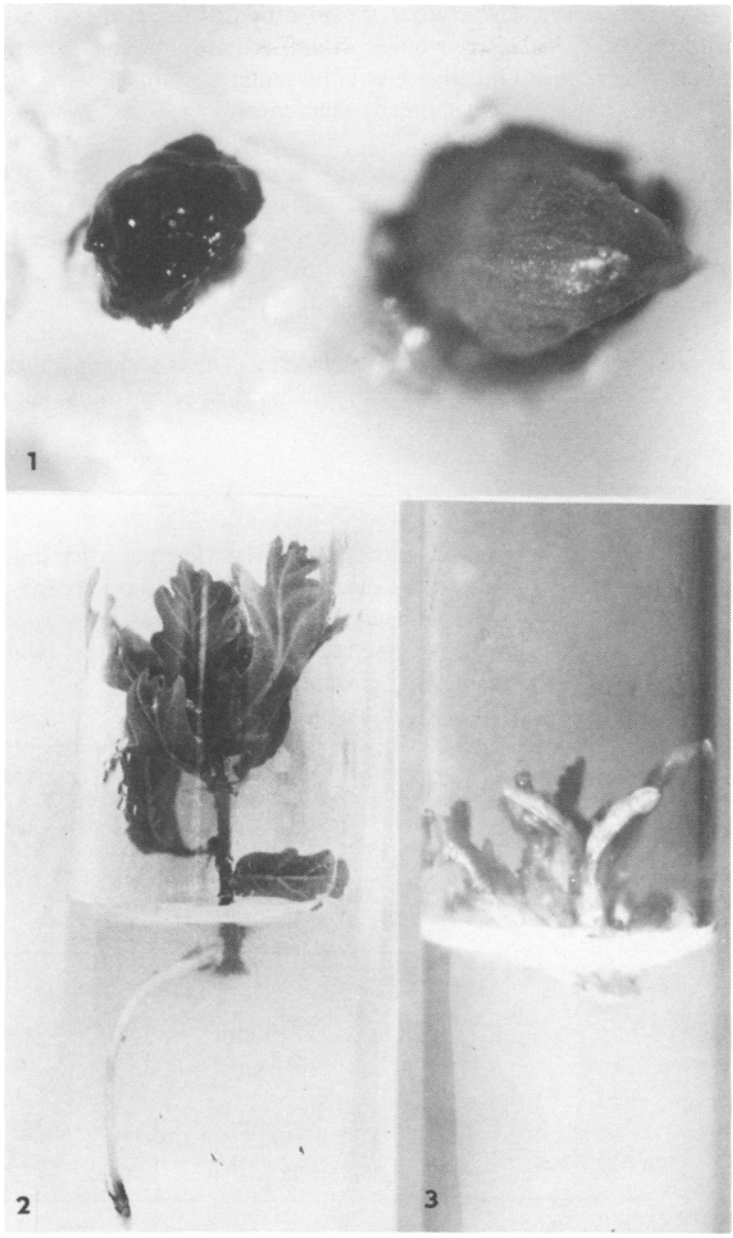

Fig. 1. Untreated (left) and ascorbic acid treated meristem of *Quercus robur* after one week in culture. Explants taken from 40-year-old tree in northern Finland.

Fig. 2. Rooted shoot of micropropagated *Quercus robur*. Culture started from seed embryo.

Fig. 3. Shoots proliferating from ascorbic acid pretreated meristem of *Quercus petraea* after 3 months in culture. Explant taken from 25-year-old tree in western Finland.

medium every three weeks. The number of explants was 10 - 25 in each experiment.

The viability and colour (green or browning), number of buds or shoots, size, growth and development and the degree of vitrification or callusing were inspected 1, 3, 4, 8 and 10 weeks after the beginning of experiments.

Analysis of phenolics: In order to obtain approximate values of range of total phenolic content, phenolics were analyzed by Folin-Denis test (Burns 1963). Twigs (length of 5 cm) from one-year-old shoots originating from seven Hungarian clones of 40-year-old *Quercus robur* trees were dried for 24 h at 80 °C and homogenized with mechanic homogeniser through a 20 mesh metal net. Samples 40 mg of powder were extracted 3 times with 50 % methanol at 80 °C. Total phenolic concentration in the extract was calculated using tannic acid as a standard. The absorbance of the reaction products was read at 750 nm using a *DU-64 (Beckman)* spectrophotometer.

Results

In the explants the first signs of blackening were visible right after dissecting. In many cases meristems were dark at the moment when they were transferred to the media. The viability of pretreated meristems and embryos was in every case better than that of untreated material. The degree of browning of explants was much lower (Fig. 1) and subsequent growth was more pronounced.

Table 1. Percentage of green explants after pretreatments with ascorbic acid, citric acid, cysteine and PVP. Shoot tip explants of *Quercus robur* from two adult oak clones (Finnish and Hungarian) and seed embryos were evaluated after one and three weeks on the media WPM6 (BAP 1, NAA 0.01, adenine sulphate 20 mg dm^{-3}) and WPM7 (BAP 0.5, IAA 0.5, adenine sulphate 20 mg dm^{-3}); n = 25.

	Ascorbic acid		Citric acid		Cysteine		PVP		H_2O	
	1 w	3 w	1 w	3 w	1 w	3 w	1 w	3 w	1 w	3 w
WPM6										
Fin	55	50	45	25	45	20	15	5.0	1.5	0.1
Hun	95	75	80	20	-	-	-	-	2.0	0.6
WPM7										
Fin	80	70	70	55	-	-	-	-	20.0	0.0
Hun	75	60	80	20	35	20	25	15	5.0	0.0
Embryos	80	80	75	75	70	70	70	60	60	50

Ascorbic acid was the most effective and long lasting treatment (Table 1). In about half of the material growth was prominent already after three weeks in culture and after six weeks 20 % of the meristems treated with ascorbic acid showed moderate multiplication. Only 10 % of the cultures died. The clone or tree age did not significantly affect the degree of browning.

The effect of citric acid pretreatment against the browning was almost as good as that of ascorbic acid, in some cases even better, but later, the viability of the

meristems treated with citric acid was lower.

The effect of cysteine and PVP was lower and rapidly decreasing (Table 1). Meristems pretreated with cysteine and PVP showed no growth during the first three weeks and only about 6 % of the whole material showed real growth. On the other hand, mortality was much lower than in untreated material (treated 20 %, untreated 46 %).

Rinsing in the pretreatment media did not always prevent or stop the browning process in the later state of *in vitro* culture. Rinsing the newly developed shoots with antioxidants was not beneficial for further growth.

Pretreatment with antioxidants was more beneficial for the meristems than for the seed embryos. After ascorbic acid rinsing the embryos started to grow a little slower and after citric acid rinsing a little later than the meristems. The pretreatments resulted in a 15 to 20 % higher viability compared to controls. New shoots originating from embryo explants formed roots on a hormone-free medium (Fig. 2). The percentage of rooting was 65 ± 25 %. Rooting of shoots originating from meristems did not occur.

The pretreatment media had also a beneficial effect against bacterial and fungal contamination; the degree of contamination of untreated meristems was about 40 % and that of treated meristems about 15 % for the whole material. Different pretreatments had different effect on degree of contamination; after ascorbic acid, citric acid and cysteine contamination was 10 % and after PVP treatment it was 23 %. Contamination was the most frequent problem with embryo explants. Of acorns sterilized with intact seed coats about 90 % contaminated, while the degree of contamination was about 50 % for pealed acorns.

WPM medium gave the best results. In SH and MS media, meristems failed to grow or produced callus. Cytokinins (BAP 1 mg dm^{-3}, kinetin 1-2 mg dm^{-3}, adenine sulphate 20 mg dm^{-3}) together with low amount of auxins (IAA, NAA) are beneficial for the growth of oak meristems. On the other hand, high levels of BAP (2 - 5 mg dm^{-3}) caused vitrification. GA_3 (0.5 mg dm^{-3}) induced elongation of the existing shoots. The media WPM4 (kinetin 2 mg dm^{-3}, NAA 0.02 mg dm^{-3}), WPM6 (BAP 1 mg dm^{-3}, NAA 0.01 mg dm^{-3}, adenine sulphate 20 mg dm^{-3}) and WPM7 (BAP 0.5 mg dm^{-3}, IAA 0.5 mg dm^{-3}, adenine sulphate 20 mg dm^{-3}) were mainly used for meristem explants in subsequent experiments. The decrease of explant blackening was dependent on pretreatments, not on the composition of media.

The total phenolic contents of various clones were: 5.06 % (dry mass) in Ny572, 4.40 % in Ny901, 2.04 % in Ny902, 5.10 % in Zs122, 8.30 % in Zs125, 7.10 % in Td51 and 7.00 % in G96. It was found that the *Q. robur* clone G96 was well propagable *in vitro* and by traditional rooting of cuttings. The vegetative multiplication *in vitro* of a clone Zs122 was satisfactory but the rooting capacity of cuttings was very low. Thus, no connection between the propagation and rooting capacity and the phenolic content of the tissues was found.

The growth was strongly affected by the position to which the explants were placed on the medium. For the meristems and the embryos the most favourable position was the natural right side up. Sidewise position induced callus growth or other undesirable phenomena. The meristems of adult trees produced about 5 shoots

(Fig. 3) and seed embryos 10 - 20 shoots per explant in about 15 weeks. The maximum multiplication rate was obtained with WPM2 medium (BAP 1 mg dm^{-3}, NAA 0.02 mg dm^{-3}), while the media WPM6 and WPM7 proved to be good at the beginning of the culture. When the *in vitro* grown shoot was dissected to segments of 1 cm and put onto a new media, it was found that only uppermost 3 segments were able to produce new shoots.

Discussion

The present results indicate that harmful oxidation of phenols can be avoided to great extent by treating the explants with antioxidants right after dissection. The effect was more beneficial for shoot tip explants than for seed embryos.

Phenolic compounds are known to accumulate in high quantities to various part of the oaks including bark, wood, leaves and calli grown *in vitro* (Scalbert *et al.* 1988) as well as to acorns (Basden and Dalvi 1987), and the main polyphenols of pedunculate oak have been characterized. In this study marked differences were noticed in total phenolic content between different clones, but there was no correlation between high content of phenolics and poor shooting. The onset of tissue browning has been found to be associated with changes in protein pattern, amino acid content, ethylene production and the occurrence of saccharose and accumulation of starch (Lindfors *et al.* 1990).

An increase in phenol content is often a reaction to injury or infection. Tissue blackening occurs through the action of copper-containing oxidase enzymes such as polyphenol oxidase, which are released or synthesized in oxidative conditions when tissues are wounded (Scalbert *et al.* 1988). Of the compounds used for pretreatment in the present experiments polyvinylpyrrolidone absorbs phenols through hydrogen bonding by thus preventing oxidation of phenols (Loomis and Battaile 1966). PVP prevented browning of the explants but growth was poor. Similarly, Hilderbrandt and Harney (1989) found that PVP had an inhibitory effect on normal shoot development of *Geranium*. Ascorbic acid, citric acid and cysteine are reducing agents. In the present experiment ascorbic acid gave the best result as pretreatment agent against browning of oak explants. Soaking explants in solution of ascorbic acid or citric acid not only exposes them to reducing agents but also to a lower pH. Polyphenol oxidase activity is maximal at pH 6.5 and reduced at lower pH (Kurkdjian and Guern 1989).

In the present study, a lower degree of contamination was noticed after rinsing meristems in ascorbic acid, citric acid or cysteine. The efficacy of pH adjustment in control of bacterial contamination has been investigated by Gibbons and Westby (1988), who maintained pH between 2.0 - 2.2.

The beneficial effect of the pretreatment agent was recognized right after the dissection but not in the later stages of the growth, indicating that the mechanism of browning in wounded tissues is different from intact plants. The losses of the explants are greatest at the beginning of the growth. Alleviating these losses was the major stimulus for the present work. As the propagation of tissues *in vitro* is most difficult to achieve in adult material, the greatest attention was paid to samples from

mature trees. Further research will be necessary to achieve higher multiplication rate and rooting of the explants.

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