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In vitro growth of buds taken from seedlings and adult plant material in *Quercus robur* L.

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Abstract. In vitro growth of *Quercus robur* L. buds taken from 1–7 month old seedlings or adult plant material was studied. The following were investigated for their effects on the establishment of explants and subsequent subcultures: original position and lignification of the primary explant, conditioning and ageing of source plants, incorporation of N^6 benzyladenine (BA) and activated charcoal in the medium.

For bud break the best results were obtained with explants from herbaceous twigs in all tested media. For shoot growth the results depended on the medium used. Medium containing activated charcoal produced episodic growth, leaf organogenesis was reduced, spontaneous rooting occurred, but subculturing from adult plant material failed. On medium containing more than 8.8 μM of BA, all the buds developed abnormally and elongation did not occur. At lower concentrations of BA (4.4 μM) shoots elongated, leaf organogenesis was increased and episodic growth tended to disappear on subcultured seedling explants. No spontaneous rooting was observed, but subculture from adult plant material was managed successfully.

Introduction

Recent data have shown that in *Quercus robur* L, and *Q. petraea* Liebl. some parameters correlated with wood quality are genetically controlled [17, 18]. Therefore, vegetative propagation of selected trees could be used to improve the production of quality wood. Adult oaks (*Q. petraea* Liebl.) can be propagated from cuttings of stump sprouts [9, 14]. However, this method has several limitations, including the need to cut down selected trees and the possible failure of sprouting on the stumps. In addition, the plants grown from the cuttings may exhibit plagiotropic growth.

In vitro propagation might be a means to overcome these limitations. This technique has been reported to induce rejuvenation which can increase rootability and orthotropic growth in plants [12, 19, 26]. However, little has been reported about in vitro propagation of *Quercus* species. *Q. suber* L. [3, 20] and *Q. lebani* L. [23] have been tissue cultured, but viable plantlets have not been obtained. Data concerning micropropagation of *Q. robur* L. are recent [6, 15, 24]. The best results were reported with the mineral solution of Smith and McCown [6, 22], Chalupa [6] and half

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Table 1. Characteristics of source plants grown at 20 ± 2°C (S.20) and at 27 ± 2°C, pruned (S.27.P) or not (S.27). Lignification was assessed through color, appearance, diameter and flexibility of shoots.

	Treatments		
	S.20	S.27	S.27 P
Bud break in growth chamber	April	October November	After pruning on 04/26/84
In vitro establishment	06/13/84	04/26/84	06/19/84
Shoot development when taking the explants	2–3 flushes	5–7 flushes	1–2 flushes
Lignification of explants	moderate	strong	herbaceous

strength Murashige and Skoog (MS) [16] each supplemented with 0.88–4.4 μM N^6 benzyladenine (BA).

This paper deals with the growth pattern of buds of primary or subcultured stem explants derived from seedlings or adult plant material.

Material and methods

Plant material

All experiments were performed with *Q. robur* L. Explants were taken from two types of source plants: (1) One to 7 month old seedlings grown in vermiculite in a chamber at 27 ± 2°C under natural daylight supplemented by continuous fluorescent lamps (Atlas white 3500). Plants were watered every two weeks with the nutritive solution of Coïc and Lesaint [7].

(2) Adult plant material derived from cuttings taken from stump sprouts of a 150 year old forest tree selected for its wood quality. After rooting, the cuttings were grown for 4 months in a greenhouse before being transferred to different growth chambers with the following treatments: (a) S.20: 20 ± 2°C under natural daylight supplemented by continuous lighting from fluorescent lamps (Atlas white 3500); (b) S.27: 27 ± 2°C under lighting conditions as in treatment S.20; (c) S.27.P: resulted from pruning the plants of treatment S.27.

The growth and assessment of shoot lignification under the above culture conditions are given in Table 1.

In vitro culture

Five to 10 days before sampling, source plants were sprayed with a 0.4 g l^{-1} benomyl solution.

Stem explants, 10–15 mm long, with at least one bud were surface sterilized as follows:

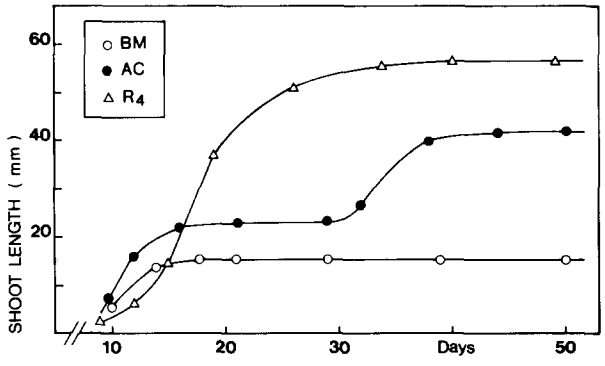

Figure 1. Individual growth curves of shoots from primary explants established on media BM, AC, R_4 (explants derived from first flush seedlings).

— pre-treatment for 15 min in a 10% solution of 'Mercryl Laurylé' a medical disinfectant composed of $0.1\,g\,l^{-1}$ mercurobutol and $40.8\,g\,l^{-1}$ sodium lauryl sulfate.

— fungicide treatment in a solution of $1\,g\,l^{-1}$ benomyl for 10 min.

— disinfestation in a 5% calcium hypochlorite solution (70% active chlorine) for 30 min.

— washing with sterile distilled water.

Surface sterilized explants were placed vertically in test tubes (25 × 200 mm) containing 20 ml of one of the following media:

— BM: basic medium composed of half strength MS macronutrient solution [16], full strength MS micronutrient solution and Fe EDTA, sucrose 88 mM and $7\,g\,l^{-1}$ agar (Merck 1615). The pH was adjusted to 5.7 with NaOH.

— AC: BM + $20\,g\,l^{-1}$ activated charcoal (Merck 2186).

— R: BM + naphthylacetic acid 2.7 μM and BA at different concentrations: 1.8 μM (R_1), 4.4 μM (R_4), 8.8 μM (R_8), 17.6 μM (R_{17}), 44 μM (R_{44}).

All media were autoclaved at 120°C for 20 min.

Established explants were kept in a growth chamber at 26 ± 1°C under 16 h photoperiod (Sylvania Gro-lux fluorescent lamps $40\,\mu E\,m^{-2}\,s^{-1}$).

Presentation of results

Because of insufficient synchronization of growth, average values did not show clearly the episodic (by successive flushes as under natural conditions) or non episodic nature of growth. Consequently, for each experiment average values have been given in tables and the nature of growth is illustrated graphically with individual growth curves of representative shoots.

Table 2. Charactersitics of shoot growth on primary explants established on media BM, AC and R. (Results from samples of 24 explants derived from first flush seedlings).

	Media							Statistical analysis	
	BM	AC	R_1	R_4	R_8	R_{17}	R_{44}	$_{109}^{5}F$	s.d.[1]
% bud break after 30 days	79	88	95	100	95	100	100	—	—
% abnormal growth	0	0	5	5	19	40	100	—	—
Average length after 50 days (mm)									
– main shoot	14	37	53	54	40	17	—	23.0**	15
– lateral sprouts	0	0	25	25	25	2	—	—	—
Average number of leaves (main shoot)									
after = 30 days	6	9	13	15	15	12	—		
50 days	6	11	17	19	18	14	—	30.4**	5
60 days	6	13	18	21	20	14	—	—	—
% of plants with 2 or more flushes									
after = 30 days	0	0	0	0	0	0	—	—	—
50 days	0	33	0	0	0	0	—	—	—
75 days	0	66	4	7	5	0	—	—	—

**effect significant at p = 0.01, [1]significant difference at p = 0.05

Results

Growth pattern of primary explants

Effect of medium. Explants with buds containing 7–12 leaf primordia were taken from first flush seedlings and established on BM, AC and R media. Bud break occurred after 6–15 days in all media, but subsequent growth differed (Figure 1).

On BM growth stopped after one reduced flush. The number of leaves (scale leaves + photosynthetic leaves) obtained was fewer than the number of leaf primordia in the original buds. On AC all explants produced 1 flush within 30 days with the total number of leaves comparable to the original content of the buds (Figure 2.1a). After the 30th day 1 or 2 additional flushes bearing newly formed leaves developed, but at a decreasing frequency. After 75 days in vitro 42% of the explants had 2 flushes, and 24% three (Table 2). On media R a part of the explants developed abnormal shoots exhibiting thickened and reduced internodes, twisted leaves, rosette development and/or vitrification (Figure 2.1b). The percentage of such abnormal growing shoots increased with the concentration of BA (Table 2). On medium R_{44} it reached 100% and 66% of explants died during the experiment. Explants that developed normally produced an extended flush bearing 2 to 3 times more leaves than the buds

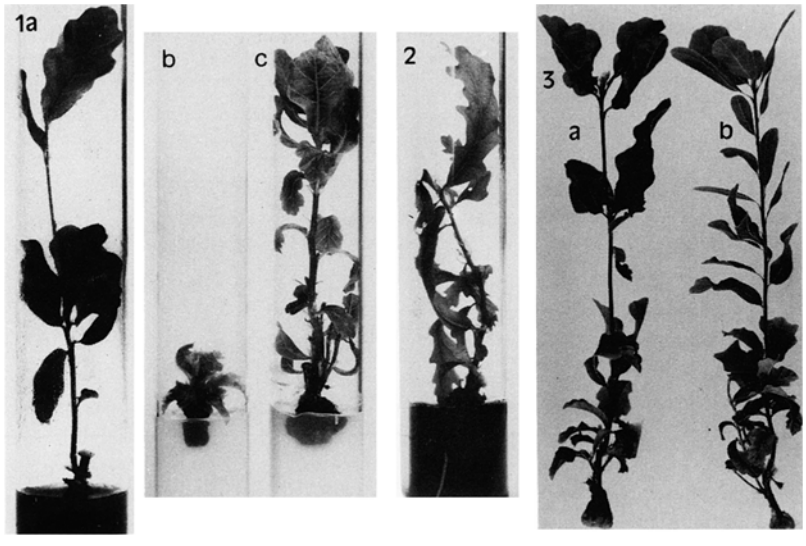

Figure 2. In vitro shoot growth on different media. 1. On primary explants from seedlings: a. Episodic growth on medium AC (60 day old shoot). b. Abnormally growing shoot on medium R_4 (30 day old shoot). c. Extended single flush and basal sprouting on medium R_4 (30 day old shoot).

2. On primary explants from adult plant material: episodic growth and spontaneous rooting on medium AC (60 day old shoot).

3. On subcultured explants from seedlings. a. Continuous elongating shoot with alternating groups of scale and photosynthetic leaves. b. Continuous elongating shoot producing only photosynthetic leaves. (both 60 day old).

initially contained. In a few cases a second flush developed and sprouting of basal buds occurred (Figure 2.1c).

Spontaneous rooting was only observed on AC. However no clear relationship could be detected between rooting and the number of flushes produced.

As R_4 and AC appeared to be the best of the media tested they were selected for use in the further studies.

Effect of position. Growth of explants taken from seedlings after their first flush varied according to their original position. When third or fifth flush seedlings were used, 80–90% of the bud break was obtained for all explants irrespective of origin or test medium. With explants from tenth flush seedlings bud break was 75% on herbaceous explants from the terminal flush, but was less for lignified explants derived from lower flushes (Table 3). This decrease was related to callogenesis which developed throughout the explants, particularly on R_4.

Shoot growth varied according to the origin of the explants. On AC all shoots exhibited episodic growth. Explants taken from the terminal flush

Table 3. Percentage of bud break after 30 days on primary explants taken from different positions on seedlings after their first flush. (Results from samples of 24 explants established on media AC and R_4),

	Position			
	Flush no 1 (basal)	Flush no 3	Flush no 5	Flush no 10
Third flush seedlings				
AC	92	92	—	—
R_4	92	88	—	—
Fifth flush seedlings				
AC	75	96	92	—
R_4	80	96	96	—
Tenth flush seedlings				
AC	41	38	64	75
R_4	27	10	33	75

of 10th flush seedlings produced significantly shorter shoots than explants taken from lower flushes (Table 4). They also produced fewer secondary flushes and 10% gave rise to abnormal growth.

On R_4, no clear effect of position could be noted. Frequency of abnormally growing shoots increased up to 50–66% on explants taken from terminal flushes. A single extended flush was obtained within 50 days.

Effect of source plant ageing. On explants derived from adult plant material (treatments S.20, S.27, S.27.P) bud break occurred 8 to 20 days after the establishment in vitro. As with seedlings, the same differences existed

Table 4. Characteristics of shoot growth on primary explants taken from different positions on tenth flush seedlings. (Results from samples of 24 explants established on medium AC).

	Position				Statistical analysis	
	Flush 1 (Basal)	Flush 3	Flush 5	Flush 10 (terminal)	$_{43}^{3}F$	s.d.[1]
% abnormal growth	0	0	0	10	—	—
Average length after 50 days (mm)						
– main shoot	79	59	67	26	34.9**	20
– lateral sprouts	—	—	—	—	—	—
Average number of leaves (main shoot)						
after = 30 days	12	11	10	8	—	—
= 50 days	19	17	15	9	21.2**	5
% of plants with 2 or more flushes						
after = 30 days	0	0	0	0	—	—
50 days	56	62	66	17	—	—
75 days	100	100	100	41	—	—

**effect significant at p = 0.01, [1]significant difference at p = 0.05.

Table 5. Characteristics of shoot growth on primary explants from adult plant material grown under different conditions: 20 ± 2°C (S.20), 27 ± 2°C pruned (S.27.P.) or not (S.27). Results from samples of 24 explants established on media AC and R_4.

	Medium AC			Medium R_4			Statistical analysis	
	S.20	S.27	S.27.P	S.20	S.27	S.27.P	$^{2}_{40}F$	s.d.[1]
% bud break after 30 days	50	52	90	50	48	92	—	—
% abnormal growth	20	0	0	100	36	100	—	—
Average length after 30 days (mm)								
– main shoot	13	41	8	—	37	—	28.2**	14
– lateral sprouts	—	—	—	—	13	—	—	—
Average number of leaves (main shoot)								
after = 40 days	6	10	5	—	14	—	—	—
60 days	7	15	5	—	17	—	40.6**	4
% of plants with 2 or more flushes								
after = 40 days	0	0	0	—	0	—	—	—
60 days	12	64	6	—	0	—	—	—
75 days	12	75	12	—	0	—	—	—

**effect significant at p = 0.01, [1]significant difference at p = 0.05.

between lignified and herbaceous explants. In S.27.P where all shoots were herbaceous (Table 1), bud break reached 90% whereas on explants taken from S.20 and S.27, which were all lignified, it reached only 50% and intense callogenesis was observed. No significant differences were noted between explants on AC and R_4 (Table 5). However, growth differed according to the medium as with explants from seedlings.

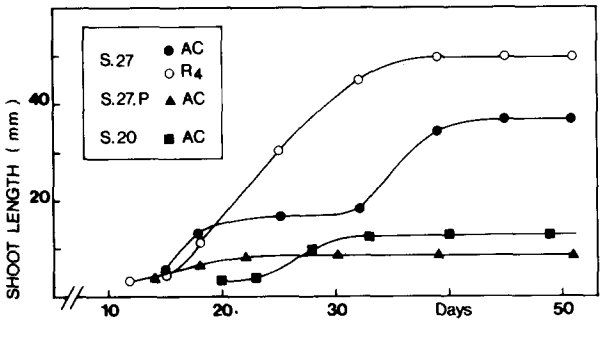

Figure 3. Individual growth curves of shoots from primary explants derived from adult plant material grown at 20 ± 2°C (S.20), and at 27 ± 2°C, pruned (S.27.P) or not (S.27). Explants established on media AC or R_4.

Table 6. Characteristics of shoot growth on explants subcultured on media AC and R_4 derived from primary explants themselves previously cultured on media AC and R_4. (Results from samples of 24 explants. Source plants = seedlings).

	Growth medium of primary explants				Growth medium of subcultured explants				Stastistical analysis	
	AC	AC	R_4	AC	AC	R_4	R_4	R_4	$_{83}^{3}F$	s.d.[1]
% bud break	80		100		96		100		—	—
% abnormal growth	5		0		10		0		—	—
Average length after 60 days (mm)										
– main shoot	34		32		30		66		16.4**	18
– lateral sprouts	—		—		—		—		—	—
Average number of leaves (main shoot)										
after = 30 days	4		4		6		7			
60 days	7		7		7		17		45.9**	3
% of plants with 2 or more flushes										
after = 30 days	0		0		0		8		—	—
60 days	70		75		20		84		—	—

**effect significant at p = 0.01, [1]significant difference at p = 0.05.

On R_4, abnormal growth occurred especially on explants taken from twigs of treatments S.27.P and S.20. In both cases 100% of shoots were abnormal. Normal shoots growing on explants derived from S.27 produced a single increased flush and lateral sprouts within 60 days (Table 5, Figure 3).

On AC abnormal growth did not occur (S.27, S.27.P) or was limited to 20% of explants (S.20). Growth was episodic (Figure 2.2), but depended on the lignification of explants. When lignified (S.27), 75% of them developed at least 2 flushes within 75 days, the first flush corresponding to the elongation of the preformed tissues in the original buds which contained 5–10 leaf primordia. In contrast, when herbaceous or slightly lignified twigs (S.20, S.27.P) were used, only 12% of explants developed 2 flushes in the same period. Consequently the shoots were 4 times shorter than on lignified explants (Table 5).

However, on AC as well as on R_4 shoot length remained lower than that obtained on explants derived from seedlings.

On AC, 50% of explants rooted spontaneously after 75 days in vitro.

Growth pattern of subcultured explants

Subculture of shoots derived from seedling explants. Explants subcultured to AC exhibited episodic growth irrespective of whether they were derived from shoots grown on R_4 or AC. The first flush developed within 30 days bearing an average number of leaves [2–5] equivalent to the primordia content of the buds formed on in vitro grown shoots. After 2 months 60%

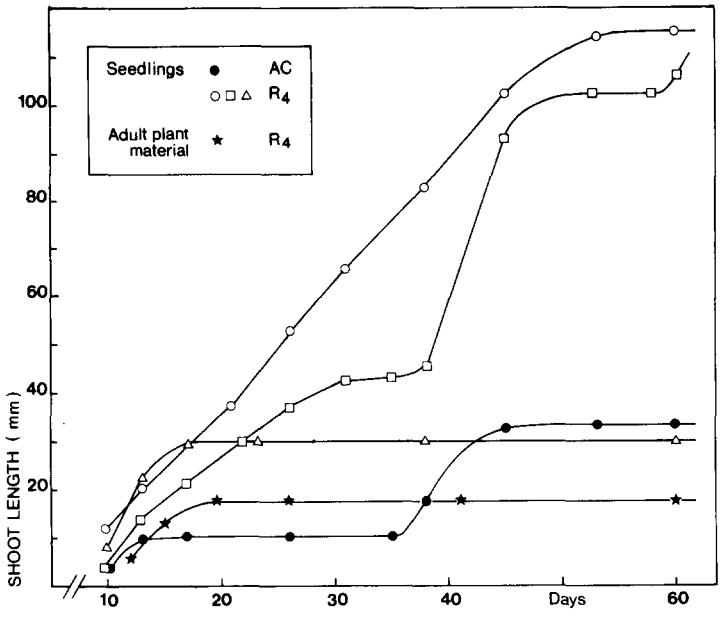

Figure 4. Individual growth curves of shoots from subcultured explants derived from seedlings or adult plant material established on media AC or R_4.

of explants had 2 flushes and 10% three. Total shoot length was comparable to that obtained on primary explants established on the same medium (Table 6). Three successive subcultures were performed every 2 months, each giving similar results.

When the subcultured explants were established on R_4, the results differed according to their origin. Subcultured explants taken from AC had a limited growth: 80% of them produced a single extended flush. Only 20% developed a second flush, none developed a third flush. After 2 months the average shoot length represented only half of that obtained with primary explants cultured on the same medium. Lateral sprouts were not observed (Table 6).

On subcultured explants derived from shoots developed on R_4, the following growth types were observed (Figure 4):

— development of a single extended flush (15% of explants)

— episodic growth (2–3 flushes within 2 months). (50% of explants)

— continuous elongation with production either of alternating groups of scale and photosynthetic leaves, or of photosynthetic leaves only (20% of explants), (Figure 2.3).

Intermediate forms (15%) existed between these 3 types of growth. Figures presented in Table 6 are the averages for the three types of shoots observed. Similar growth types were noted in subsequent subcultures.

Subculture of shoots derived from adult plant material. All attempts to subculture to AC failed. Bud break did not occur and the explants died after 3 weeks.

In contrast, on R_4 buds grew irrespective of whether they were derived from shoots grown on R_4 or AC. A first flush developed within 1 month, but in comparison with subcultured explants derived from seedlings, it elongated poorly (average shoot length after 2 months: 16 mm). However, the number of leaves obtained [6, 7] surpassed the primordia content of the buds. In 80% of explants growth stopped after developing the first flush. In 20% a second flush started. The same results were obtained after a second subculture.

Discussion

Our results indicate two areas that need consideration when micropropagating selected oak trees.

Initial growth from explants

Shoot elongation from the primary explant can be prevented by failure of the buds to expand, callogenesis on the axillary zones, or abnormal shoot growth.

The occurrence of these phenomena on explants taken from seedlings or adult plant material dependend on several factors:

— Concentration of BA: a significant number of shoots gave rise to abnormal growth and callogenesis occurred on medium containing over 4.4 μM BA. This negative effect of BA also was noted by Vieitez et al. [24] who were unable to obtain shoot growth from nodal segments of *Q. robur* L. established on a medium containing 4.4 μM BA. Thus, media with concentrations of BA lower than 4.4 μM should be used for oaks as for other woody species such as wild cherry [8].

— Activated charcoal: on medium containing activated charcoal, callogenesis and abnormal growth occurred at low frequency and shoots elongated normally. This positive effect of activated charcoal on growth is similar to that observed in several other woody species [4, 5, 10]. It is generally attributed to the adsorption of inhibitor compounds released in the medium by the explants and/or by a stimulating effect of impurities contained in the charcoal itself [1, 2, 11, 13, 25, 27].

— Lignification of the primary explant: herbaceous explants more frequently gave rise to abnormal growth than did lignified explants. But, lignification reduced the % of bud break. By controlling the culture conditions (temperature regime, photoperiod) and pruning, the number of suitable explants from source plants can be optimized.

Growth pattern

Under natural conditions growth of oaks is episodic. Each flush is composed of leaves that were formed before bud break (60%) and leaves formed during bud break (40%) [21].

In vitro growth patterns varied according to the medium. On medium containing activated charcoal growth remained highly episodic. On suitable lignified primary explants derived from seedlings, 3 flushes could be obtained before the shoots needed to be subcultured. The average number of flushes and shoot length were reduced when herbaceous explants and/or adult plant material were used. Spontaneous rooting occurred. However, both on primary and subcultured explants, the number of leaves per flush corresponded to the original preformed leaves contained in the buds (i.e. no leaf organogenesis occurred during bud break). Thus, on this medium, leaf organogenesis preceded the elongation of a determinate flush. Shoots from explants derived from adult plant material could not be subcultured onto AC.

On medium containing 4.4 μM of BA, 60–80% of the shoots grown on primary explants derived from seedlings or adult plant material produced a single extended flush before they needed to be subcultured. Also, shoot elongation and leaf organogenesis were stimulated. The first flush bore 2 to 3 times more leaves than the buds initially contained. Spontaneous rooting than the buds initially contained. Spontaneous rooting was never recorded. On subcultured explants derived from seedlings, episodic growth tended to disappear. Twenty % of the shoots elongated continuously, some always producing alternating groups of scale and photosynthetic leaves, while others only produced photosynthetic leaves. Subculture of shoots derived from adult plant material could be managed successfully and episodic growth was not lost.

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