

Large Scale Micropropagation of *Quercus robur* L. Using Adenine-Type Cytokinins and Thidiazuron to Stimulate Shoot Proliferation

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Abstract. High rates of oak shoot multiplication were achieved on low salt nutrient media (BTM, WPM) supplemented with adenine type cytokinin PBA or BAP. Low concentration of PBA ($0.2-0.6 \text{ mg l}^{-1}$) stimulated formation of a large number of axillary shoots and their elongation. Thidiazuron in very low concentration ($0.001-0.002 \text{ mg l}^{-1}$) promoted shoot proliferation, in high concentration stimulated formation of large callus. More than twelve thousand micropropagated shoots were rooted in low salt agar media supplemented with low concentration of auxin (IBA or NAA $0.2-0.5 \text{ mg l}^{-1}$). High rooting percentages (81 %) were obtained. Survival of the micropropagated plantlets transplanted into soil was high (78 %). Micropropagated trees planted in the field withstood severe winter frosts without significant losses. At the end of the fifth growing season trees attained considerable size.

A rapid vegetative propagation of selected trees of *Quercus robur* is very desirable for the acceleration of the tree improvement programs. In past years we have paid considerable attention to a rapid *in vitro* propagation of oak. We have succeeded in multiplication of oak shoots using nodal segments and shoot tips as initial explants (CHALUPA 1979). In order to improve shoot multiplication rate new low salt nutrient media were developed (CHALUPA 1981) which stimulated rapid growth and proliferation of axillary shoots. Our further experiments showed that various low salt media are suitable for promoting rapid shoot proliferation and rooting of *Quercus robur* (CHALUPA 1983, 1984). Our first *in vitro* propagated trees of *Quercus robur* were planted in the field in 1980 and from that time micropropagated trees of *Quercus robur* have been produced every year and planted in the field.

For further improvement of oak shoot proliferation we have tested some new compounds which show a high cytokinin activity. The present communication describes the effects of different cytokinins on *in vitro* shoot multiplication of *Quercus robur* and the field growth of *in vitro* propagated trees.

MATERIALS AND METHODS

Initial explants were taken from seedlings and from adult trees. More than 90 seedlings (3—6 months old) grown in the greenhouse were used as the source of juvenile material. As the source of material of adult trees,

stump sprouts, epicormic shoots and shoots from lower branches of the crown were used. 24 adult trees (30–50 years old) were used for experiments.

In order to obtain initial explants, all leaves were removed from seedling or shoot and axis was cut into shoot tip and nodal segments 10–15 mm long. Shoot tips and nodal segments were surface sterilized in 0.1 % of mercuric chloride solution for 20–40 min. After three successive rinses in sterile distilled water the segments were placed on agar nutrient media. Segments were cultured in 100 ml flasks containing 20 ml of nutrient media.

Cultures were grown at 25 °C, under 16 h photoperiod of cool white fluorescent light with an illuminance of 4–8 klx. Cultures were grown on the following nutrient media (Table 1): a modified GRESSHOFF and DOY (1972) medium (GD), woody plant medium (WPM — SMITH and McCOWN 1983) and broad-leaved tree medium (BTM — CHALUPA 1981) and its modification (BTM-1).

In order to study the effect of different cytokinins on oak shoot multiplication, four cytokinins were tested: kinetin (6-furfurylaminopurine), BAP (6-benzylaminopurine), PBA (N-(phenylmethyl)-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine) and thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea). Kinetin, BAP and PBA were tested in concentrations 0.1, 0.2, 0.4, 0.6, 1.0, 2.0 and 5.0 mg l⁻¹, thidiazuron in concentrations 0.001, 0.002, 0.004, 0.006, 0.01, 0.02, 0.05 and 0.1 mg l⁻¹. For rooting NAA (1-naphthaleneacetic acid) and IBA (indol-3-ylbutyric acid) were used in concentrations 0.1–1.0 mg l⁻¹.

Difco Bacto agar (6 g l⁻¹) was used to solidify nutrient media. Sucrose (20 g l⁻¹) was used as a carbon source. Media were autoclaved for 20 min at 121 °C. Each treatment involved 24–40 flasks and was repeated at least twice.

Rooted plantlets were transplanted into Jiffy-pots or into small containers containing a mixture of peat, soil and perlite (1 : 1 : 1 v/v/v) and were grown under a plastic sheet at high air humidity (90–95 %) for 2–3 weeks. The plantlets were grown under long photoperiod (16 h illumination) or under continuous light at temperature 20–25 °C. After 2 weeks, humidity was gradually reduced to normal values. After formation of new adapted leaves the plants were placed outdoors and grown for 2–3 months in partial shade.

RESULTS

In vitro Shoot Multiplication

Nutrient media of different composition, supplemented with low concentration of cytokinin, were tested (Table 1). Shoots were growing fast and elongated significantly when cultured on low salt media. Of tested media, shoot proliferation was best stimulated on WPM, BTM and BTM-1. On these media shoots elongated considerably and leaves were well developed. Some clones proliferated better on WPM than on BTM, the others on BTM. Multiplication rates obtained on WPM and BTM were not significantly different. Explants grown on high salt media (MS, DKW) were short and thick and exhibited abnormalities in leaf morphology.

Shoot proliferation greatly depended on the type and concentration of cytokinin in nutrient medium. The number of shoots produced on media containing low concentration of cytokinin was significantly larger than on

TABLE 1

Composition of used nutrient media

Compound	GD	WPM	BTM	BTM-1
	[mg l ⁻¹]			
KNO ₃	1000	—	190	475
NH ₄ NO ₃	—	400	165	412
CaCl ₂ . 2H ₂ O	150	96	44	44
Ca(NO ₃) ₂ . 4H ₂ O	—	556	640	640
(NH ₄) ₂ SO ₄	200	—	240	—
K ₂ SO ₄	—	990	860	—
KCl	300	—	—	—
MgSO ₄ . 7H ₂ O	250	370	370	370
KH ₂ PO ₄	—	170	170	170
NaH ₂ PO ₄ . H ₂ O	90	—	—	—
Na ₂ HPO ₄	30	—	—	—
Na ₂ EDTA	37.3	37.3	37.3	37.3
FeSO ₄ . 7H ₂ O	27.8	27.8	27.8	27.8
H ₃ BO ₃	3.0	6.2	6.2	6.2
MnSO ₄ . H ₂ O	10.0	22.3	22.3	2.3
ZnSO ₄ . 7H ₂ O	3.0	8.6	8.6	8.6
KI	0.75	—	0.15	0.15
Na ₂ MnO ₄ . 2H ₂ O	0.25	0.25	0.25	0.25
CuSO ₄ . 5H ₂ O	0.25	0.25	0.25	0.25
CoCl ₂ . 6H ₂ O	0.25	—	0.02	0.02
Myo-Inositol	10.0	100.0	100.0	100.0
Thiamine . HCl	1.0	1.0	1.0	1.0
Nicotinic acid	0.1	0.5	0.5	5
Pyridoxine HCl	0.1	0.5	0.5	5
Glycine	2.0	2.0	2.0	0
Glutamine	2.0	2.0	2.0	2.0

media lacking cytokinin (Tables 2, 3). Of tested cytokinins, PBA, BAP and thidiazuron exhibited a high cytokinin activity.

BAP stimulated formation of new axillary shoots more effectively than kinetin (Table 2). Low concentration of BAP (0.1–0.2 mg l⁻¹) promoted shoot proliferation. With the increasing concentration of BAP (0.1–0.6 mg l⁻¹) the number of shoots increased. The large number of new shoots was produced on media containing 0.4–0.6 mg l⁻¹ BAP. Shoots produced on media containing a higher concentration of BAP (2 mg l⁻¹) were short and a large callus was formed at the basal part of shoots. Kinetin was less effective in promoting shoot multiplication than BAP.

The multiplication rate achieved on WPM supplemented with BAP was high. Each shoot 20 mm long may be cut into three or four segments (including shoot tip). As is evident from Table 2, the multiplication rate ranged from 6.3 to 8.4 for the media supplemented with low concentration of BAP (0.1–0.6 mg l⁻¹).

A high cytokinin activity exhibited adenine-type cytokinin PBA and a phenylurea derivative thidiazuron (Table 3). PBA promoted rapid shoot growth and stimulated formation of new axillary buds and their elongation. Shoot number of cultures grown on media supplemented with PBA was large. Most tested clones produced more shoots on media supplemented

TABLE 2

The effect of BAP and kinetin on shoot number and length of shoots of *Quercus robur* (cultured five weeks on WPM)

BAP [mg l ⁻¹]	Number of shoots*	Mean shoot length* [cm]	Kinetin [mg l ⁻¹]	Number of shoots*	Mean shoot length* [cm]
0.0	0.6 ± 0.5	0.8 ± 0.4	0.0	0.6 ± 0.5	0.8 ± 0.4
0.1	2.1 ± 0.9	1.8 ± 0.8	0.1	1.2 ± 0.6	1.6 ± 0.6
0.2	2.5 ± 1.1	1.8 ± 0.8	0.2	1.3 ± 0.6	1.7 ± 0.6
0.4	2.7 ± 1.3	1.9 ± 0.9	0.4	1.4 ± 0.7	1.8 ± 0.8
0.6	2.8 ± 1.3	1.9 ± 0.9	0.6	1.7 ± 0.8	1.8 ± 0.8
1.0	2.7 ± 1.2	1.8 ± 0.8	1.0	1.7 ± 0.8	1.7 ± 0.7
2.0	2.5 ± 1.2	1.6 ± 0.7	2.0	1.6 ± 0.8	1.7 ± 0.7

* Based on 12 juvenile clones, 24 segments per clone, mean of four subcultures, Mean ± SE

with PBA than on media containing BAP or kinetin. The shoots elongated significantly (Table 3) and exhibited normal morphology (Fig. 1). The number of shoots produced increased with increasing concentration of PBA. Shoots cultured on media containing higher concentration of PBA (2 mg l⁻¹) were shorter and a large callus formed at the basal part of shoots.

Thidiazuron affected elongation and morphology of oak shoots very significantly. Very low concentrations of thidiazuron (0.001–0.004 mg l⁻¹) promoted shoot formation. Higher concentrations (0.01–0.02 mg l⁻¹) stimulated formation of callus tissue at the basal part of shoots and the shoots produced were short and less numerous. The callus tissue produced on media containing thidiazuron exhibited organogenic potential and the formation of new adventitious shoots from the callus tissue was observed. Shoots cultured on media supplemented with a high level of thidiazuron (0.05–0.1 mg l⁻¹) formed large callus and the shoot elongation was inhibited.

We have also tested effects of various combinations of different cytokinins on shoot multiplication. Shoot proliferation of some clones was more stimu-

TABLE 3

The effect of PBA and thidiazuron on shoot number and length of shoots of *Quercus robur* (cultured five weeks on WPM)

PBA [mg l ⁻¹]	umber of shoots*	Mean shoot length* [cm]	Thidiazuron [mg l ⁻¹]	Number of shoots*	Mean shoot length* [cm]
0.0	0.6 ± 0.5	0.8 ± 0.4	0.0	0.6 ± 0.5	0.8 ± 0.4
0.1	2.3 ± 1.1	1.9 ± 0.8	0.001	1.5 ± 0.6	1.7 ± 0.7
0.2	2.6 ± 1.2	1.9 ± 0.9	0.002	1.8 ± 0.8	1.7 ± 0.7
0.4	2.8 ± 1.3	2.0 ± 0.9	0.004	1.7 ± 0.8	1.6 ± 0.6
0.6	3.1 ± 1.4	1.9 ± 0.9	0.006	1.5 ± 0.7	1.4 ± 0.6
1.0	2.8 ± 1.3	1.8 ± 0.8	0.01	1.4 ± 0.7	1.2 ± 0.5
2.0	2.5 ± 1.2	1.6 ± 0.7	0.02	1.3 ± 0.6	1.0 ± 0.5

* Based on 12 juvenile clones, 24 segments per clone; mean of four subcultures Mean ± SE

TABLE 4

The effect of nutrient media and auxins on rooting of juvenile microshoots of *Quercus robur*

Nutrient medium	Auxin [mg l ⁻¹]	Rooting* [%]		Mean number of roots*
		mean	range	
GD (half strength)	IBA 0.3	78	69–88	1.8 ± 0.4
	IBA 0.5	82	62–94	2.0 ± 0.5
	IBA 1.0	82	75–94	2.2 ± 0.7
	IBA 0.3 + NAA 0.1	80	69–88	2.3 ± 0.6
WPM (half strength)	IBA 0.3	80	69–94	1.9 ± 0.4
	IBA 0.5	81	75–100	2.1 ± 0.3
	IBA 1.0	84	75–94	2.3 ± 0.7
	IBA 0.3 + NAA 0.1	82	69–88	2.3 ± 0.6
WPM (full strength)	IBA 0.3	77	69–88	1.7 ± 0.3
	IBA 0.5	81	62–94	2.1 ± 0.6
	IBA 1.0	80	75–88	2.3 ± 0.7
	IBA 0.3 + NAA 0.1	82	75–94	2.1 ± 0.6

* Data based on 12 clones, 16 shoots per clone.
Mean ± SE

lated on media containing PBA, other clones on media supplemented with BAP. Media containing both cytokinins (PBA and BAP) in low concentration (0.1–0.3 mg l⁻¹) stimulated rapid shoot multiplication. Combinations of PBA or BAP with thidiazuron promoted formation of callus and adventitious shoots.

Shoot multiplication rates of cultures initiated from adult trees were lower than those initiated from seedlings. Great differences existed between clones in shoot proliferation potential. Only 29 % of tested trees produced proliferating cultures. The average number of shoots of adult trees produced on WPM supplemented with low concentration of BAP (0.2–0.4 mg l⁻¹) ranged from 0.8 to 1.9 depending on the clone.

Rooting

Microshoots excised from proliferating cultures have been rooted either *in vitro* or in nonsterile substrate. In most cases we have rooted microshoots in agar nutrient media (Fig. 2) supplemented with low concentration of IBA or NAA (0.2–0.5 mg l⁻¹). Low salt nutrient media (GD, BTM or WPM, half strength or full strength) were used for rooting. High rooting percentages (Table 4) were achieved when microshoots initiated from juvenile material were used. Rooting percentages of different clones varied considerably. The effect of nutrient media used for rooting and the effect of auxin concentration on rooting percentages was less significant than the effect of clones (Table 4). The mean rooting percentages were high on all media used. The mean number of roots formed increased with the increasing auxin concentration, however the high auxin concentration promoted formation of callus tissue at the basal part of microshoots.

Rooting percentages of microshoots initiated from adult trees were significantly lower than those produced from juvenile explants. The average rooting percentage of microshoots of 12 adult clones was 43 %. Large differences in rooting ability between individual clones were found. Rooting percentages of 12 adult clones varied from 14 % to 86 %.

Microshoots excised from juvenile cultures were also rooted in nonsterile substrate. The basal part of excised shoots was first immersed in a 0.5 g l⁻¹ IBA solution for a period of 10 min. After this treatment the shoots were transplanted into substrate and were kept under a plastic sheet in a high air humidity. Roots started to develop within 2–3 weeks. Rooting percentages of 8 clones ranged from 23 % to 76 %.

Transplanting and Acclimatization

Plantlets rooted in agar medium were carefully removed from the medium and agar was washed from roots. Plantlets were transplanted to a potting mixture (peat, soil, perlite 1 : 1 : 1 v/v/v) into Jiffy-pots or small containers and were covered with clear plastic to maintain a high relative humidity. Containers with plantlets were kept in a warm greenhouse (20–25 °C) shaded against direct sun radiation and illuminated for 16 h (or continuously) to stimulate stem elongation. Plantlets were grown under high relative humidity (90–95 %) for 2–3 weeks, then humidity was gradually reduced to normal values. A rapid stem elongation and formation of new leaves that were adapted to normal relative humidity was important to achieve high percentages of plantlet survival. After new adapted leaves had formed and plants attained the height of 8–12 cm, they were placed outdoors and grown for 2–3 months in partial shade.

High survival rates were achieved for *Quercus robur* plantlets. The survival varied depending on the clones and ranged from 68 % to 93 %. Most plantlets were transplanted into substrate during the winter period and in early spring. In May or June the plants were transferred from the greenhouse to outdoor conditions and grown in partial shade. In June or September they were planted in the field.

Field Growth

Survival of micropropagated trees planted in the field was high (86 %–98 %). Planted trees withstood severe winter frosts without significant losses and in spring started to flush and grow. The majority of micropropagated trees planted in the previous year in spring continued in growth and losses during the first winter were very low (2–8 %) or none. The height increment during the second growing season was considerable and trees attained the height of 20–30 cm at the end of the second growing season. Even in the following years the height and diameter increment of planted trees was high. At the end of the fifth growing season micropropagated trees attained the height of 110–175 cm.

Trees regenerated from the same mother tree showed uniformity in the form and size of leaves and in the date of spring flushing and autumn coloration of leaves. No abnormalities in the growth and morphology of micropropagated trees were observed.

DISCUSSION

Micropropagation of oak is greatly dependent on the use of suitable cytokinin. *Quercus robur* is one of the forest tree species which reacts sensitively to added cytokinin and even a low concentration of cytokinin significantly affects elongation and morphology of shoots. Nodal segments cultured on media lacking cytokinin usually formed short shoots with a small number of leaves. The addition of low concentrations of cytokinin into nutrient media promoted elongation of shoots and stimulated formation of shoots from axillary buds. Nodal segments on media supplemented with a low concentration of cytokinin produced elongated shoots bearing more leaves than the original bud contained initially. Cytokinin stimulated development of shoots from axillary buds. The number of new shoots produced from axillary buds increased with increasing concentration of cytokinin. Nodal segments cultured on media containing a high concentration of cytokinin produced abnormal shoots with twisted leaves, and large callus at the basal part of shoot. In our experiments proliferation of oak shoots was best stimulated on media containing a low concentration of cytokinin.

In our previous experiments with *Quercus robur* (CHALUPA 1979, 1981, 1983, 1984) we have observed that BAP is an effective cytokinin which stimulates oak shoot multiplication more than some other cytokinins. Even experiments of VIEITEZ *et al.* (1985) and FAVRE and JUNCKER (1987) showed that BAP significantly affects growth and proliferation of oak shoots. VIEITEZ *et al.* (1985) used for shoot multiplication a low concentration of BAP (0.1 mg l^{-1}). FAVRE and JUNCKER (1987) found that concentration of BAP 1 mg l^{-1} promoted shoot elongation. In our previous (CHALUPA 1981, 1984) and present experiments good results with oak shoot multiplication were obtained on media containing $0.2\text{--}0.6 \text{ mg l}^{-1}$ BAP.

In our recent experiments we have tested the effect of some new compounds which show cytokinin activity with the aim of achieving rapid oak shoot multiplication. One of the new compounds which show high cytokinin activity is thidiazuron (MOK *et al.* 1982). This highly cytokinin-active phenylurea derivative promotes callus formation of *Phaseolus lunatus* (MOK *et al.* 1982, MOK and MOK 1985) and as was shown in our experiments (CHALUPA 1987) also stimulates shoot multiplication of some forest tree species. Our recent experiments have shown that even a very low concentration of thidiazuron significantly affects shoot growth and callus formation of *Quercus robur* culture. However, multiplication rate of oak shoots grown on media containing thidiazuron was lower than on media containing BAP. Shoots grown on media containing thidiazuron formed large callus at the basal part of shoot. Formation of new adventitious shoots from the callus was observed.

High multiplication rate of oak shoots was found on media supplemented with adenine-type cytokinin PBA. This cytokinin stimulates shoot growth and promotes formation of new axillary shoots. Of tested cytokinins PBA stimulated proliferation of oak shoots best. Shoot number produced on media containing PBA was large and shoots were well developed. PBA is a very active cytokinin suitable for rapid clonal propagation of oak.

Our last and present experiments with *in vitro* propagation of oak show that shoot multiplication rate is sufficiently high for large scale propagation of oak. Our long-term experiments with the field growth of *in vitro* propagated oak tree showed that survival rate of micropropagated tree is high.

Planted trees withstood severe winter frosts without significant losses. The growth of micropropagated trees was rapid and at the end of the fifth growing season trees attained a considerable size.

REFERENCES

- CHALUPA, V.: *In vitro* propagation of some broad-leaved forest trees. — Commun. Inst. Forest. Czechosl. **11** : 159—170, 1979.
- CHALUPA, V.: Clonal propagation of broad-leaved forest trees *in vitro*. — Commun. Inst. Forest. Czechosl. **12** : 255—271, 1981.
- CHALUPA, V.: Micropropagation of conifer and broadleaved forest trees. — Commun. Inst. Forest. Czechosl. **13** : 7—39, 1983.
- CHALUPA, V.: *In vitro* propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* MILL.). — Biol. Plant. **26** : 374—377, 1984.
- CHALUPA, V.: Effect of benzylaminopurine and thidiazuron on *in vitro* shoot proliferation of *Tilia cordata* MILL., *Sorbus aucuparia* L. and *Robinia pseudoacacia* L. — Biol. Plant. **29** : 425—429, 1987.
- FAVRE, J. M., JUNCKER, B.: *In vitro* growth of buds taken from seedlings and adult plant material in *Quercus robur* L. — Plant Cell Tissue Organ Cult. **8** : 49—60, 1987.
- GRESSHOFF, P. M., DOY, C. H.: Development and differentiation of haploid *Lycopersicon esculentum* (tomato). — Planta **107** : 161—170, 1972.
- MOK, M. C., MOK, D. W. S.: The metabolism of (¹⁴C)-thidiazuron in callus tissues of *Phaseolus lunatus*. — Physiol. Plant. **65** : 427—432, 1985.
- MOK, M. C., MOK, D. W. S., ARMSTRONG, D. J., SHUDO, K., ISOGAI, Y., OKAMOTO, T.: Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron). — Phytochemistry **21** : 1509—1511, 1982.
- SMITH, M. A. L., McCOWN, B. H.: A comparison of source tissue for protoplast isolation from three woody plant species. — Plant Sci. Lett. **28** : 149—156, 1983.
- VIEITEZ, A. M., SAN-JOSE, M. C., VIEITEZ, E.: *In vitro* plantlet regeneration from juvenile and mature *Quercus robur* L. — J. hort. Sci **60** : 99—106, 1985.

Figures at the end of the issue.