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Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.)

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ABSTRACT

Embryogenic cultures and somatic embryos were obtained from immature zygotic embryos of oak (**Quercus robur** L.) cultured on a modified MS medium and WPM containing BAP (1 mg·l^{-1}) and GA_3 (1 mg·l^{-1}) or BAP and IBA. Germination and conversion of oak somatic embryos into plantlets was achieved on WPM containing a reduced concentration of cytokinin. Linden (**Tilia cordata** Mill.) somatic embryos developed in embryogenic tissues initiated from immature zygotic embryos cultured on a modified MS medium supplemented with 2,4-D (0.3–2.0 mg·l^{-1}). Germination of linden somatic embryos and plantlet formation occurred on MS medium containing a low concentration of IBA. Oak and linden plantlets produced from somatic embryos were successfully established in soil. Somatic embryos and plantlets were also regenerated from embryogenic cultures of **Quercus petraea** and **Tilia platyphyllos**.

ABBREVIATIONS

BAP: 6-benzylaminopurine; GA_3: gibberellic acid; IBA: indole-3-butyric acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; MS: Murashige and Skoog (1962); WPM: woody plant medium

INTRODUCTION

Regeneration of plants via somatic embryogenesis is considered to be an efficient approach for a clonal plant propagation. Somatic embryogenesis also provides the basis for genetic improvement of forest tree species. Transgenic forest trees might be recovered from transformed embryogenic tissues.

In past years we succeeded in micropropagation of oaks and linden by organogenesis (Chalupa 1981, 1983, 1984, 1985). Somatic embryogenesis was observed in oak cultures of **Quercus suber** (El Maataoui and Espagnac 1987), **Quercus ilex** (Féraud-Keller and Espagnac 1989) and **Quercus rubra** (Gingas and Lineberger 1989). However, plant regeneration from oak somatic embryos was not achieved (**Q. suber**, **Q. ilex**), or was achieved in a low frequency (**Q. rubra**). This report describes a system for a high frequency initiation of embryogenic cultures and subsequent development of somatic embryos and plants from **Quercus robur** and **Tilia cordata** cultures.

MATERIALS AND METHODS

Plant material

The fruits of oak (**Quercus robur** L.) were harvested from five open pollinated trees at weekly intervals during July and August. Fruit collection began 9 weeks after pollination which took place first week in May. The fruits of six open pollinated trees of linden (**Tilia cordata** Mill.) were collected at weekly intervals beginning three weeks after pollination which occurred last week in June. Seed sterilization consisted of 70 % ethanol dip followed by a 20 min immersion in calcium hypochlorite (7.5 % w/v). The seeds were then rinced twice in sterile distilled water. Following surface sterilization the immature zygotic embryos were removed using aseptic procedures. The explants were placed in 100 ml flasks (one explant per flask) containing 20 ml of nutrient medium. Each treatment involved 30–60 explants and was repeated twice.

Culture media and conditions

Explants of **Tilia** were cultured on MS medium (Murashige and Skoog 1962) and explants of **Quercus** on MS medium and on WPM (Lloyd and McCown 1980). The basal media were supplemented with glutamine (200 mg·l^{-1}). The media contained varying concentration of cytokinin (BAP 0.2–2.0 mg·l^{-1}) and auxin (2,4-D 0.3–2.0 mg·l^{-1} or IBA 0.1–1.0 mg·l^{-1}) or GA_3 (0.5–1·0 mg.l^{-1}). MS medium contained 3 % and WPM 2 % sucrose. The media were solidified with 0.6 % (w/v) Difco Bacto agar and adjusted to pH 5.8 before sterilization by autoclaving at 121 °C for 20 min. Growth regulators and glutamine were filter-sterilized. Cultures were grown either in the dark or in the light in growth cabinets at 24 °C with a 16 h photoperiod under cool white fluorescent lamps (60 μE $m^{-2}·s^{-1}$).

Somatic embryo development and plantlet regeneration

Explants of **Quercus** were cultured on MS medium and on WPM containing BAP (0.5, 1.0, 2.0 mg·l^{-1}) either alone or in combination with IBA (0.1, 1.0 mg·l^{-1}) or 2,4-D (0.5, 1.0 mg·l^{-1}) or GA_3 (0.5, 1.0 mg·l^{-1}). Embryogenic cultures were transferred every 4–6 weeks to the fresh medium. Embryogenic tissues with induced embryoids were transferred to

WPM containing low concentration of cytokinin (BAP 0.2–0.6 $mg \cdot l^{-1}$). Somatic embryos were also subjected to high osmoticum treatments. Somatic embryos were cultured on MS medium (with 1 $mg \cdot l^{-1}$ BAP) containing 6 % and 9 % sorbitol for 2–3 weeks and then transferred to WPM containing a low concentration of BAP (0.2–0.6 $mg \cdot l^{-1}$).

Excised immature zygotic embryos of **Tilia** were cultured on MS medium supplemented with auxin and cytokinin. Auxin 2,4-D was tested at concentrations 0.3, 0.5, 1.0, 2.0 $mg \cdot l^{-1}$ alone or in combination with BAP (0.5, 1.0, 2.0 $mg \cdot l^{-1}$). The embryogenic cultures with developing embryoids were transferred to MS medium lacking 2,4-D and supplemented with IBA (0.1–0.2 $mg \cdot l^{-1}$).

RESULTS AND DISCUSSION

Somatic embryogenesis and plant regeneration in ***Quercus***

Explants from immature acorns collected in July and in the beginning of August gave rise to embryogenic tissues (Table 1). The formation of embryogenic cultures initiated from immature zygotic embryos was stimulated on MS media and WPM supplemented with cytokinin. The immature zygotic embryos cultured in the dark produced white embryogenic tissues within 5–7 weeks. Embryogenic cultures were subcultured to fresh medium of the same composition every 4–5 weeks. Within 6–9 weeks structures resembling embryoids were seen to develop.

Development of embryogenic cultures was best initiated on MS medium and WPM containing BAP (1 $mg \cdot l^{-1}$), BAP (1 $mg \cdot l^{-1}$) plus GA_3 (1 $mg \cdot l^{-1}$), and BAP (1 $mg \cdot l^{-1}$) plus IBA (0.1, 1.0 $mg \cdot l^{-1}$). Substitution of IBA with similar concentration of 2,4-D stimulated callus formation. This callus was friable and non-embryogenic. 2,4-D appears to be ineffective for stimulation of somatic embryogenesis in **Quercus robur** cultures.

Embryogenic tissues produced on MS medium and WPM containing BAP or BAP and GA_3 could easily be maintained and subcultured. Within 8–10 weeks globular structures developed in 60–70 % cultures. The development of globular and torpedo-shaped structures continued in subcultured tissues and the frequency of embryoid formation often increased in subcultured tissues. Globular and torpedo-shaped structures continued in development, later giving rise to embryoids (Fig. 1). Embryoids were often loosely attached to parent tissue at the root pole. Development of somatic embryos showing bipolar structures was observed.

Repetitive somatic embryogenesis was frequent and led to the formation of many small embryoids. Different stages of embryogenic structures, normal and even abnormal, were observed to occur on the same medium. Occurence of various aberration structures, such as leaf-like structures, was observed. Embryogenic tissues kept on medium containing cytokinin, have retained their embryogenic potential for more than two years.

Germination of somatic embryos and formation of plantlets occurred only rarely on the media kept in the dark and containing high cytokinin concentration. The transfer of oak embryogenic tissues with induced embryoids (Fig. 1) to WPM containing a reduced cytokinin concentration (BAP 0.2–0.6 $mg \cdot l^{-1}$) and culturing these explants in the light led to greening and further development of embryoids. Germination of somatic embryos and development of root and shoot was achieved (Fig. 2 and 3). Some embryos formed only long root without shoot, some produced shoot and root (Fig. 3), and some formed short shoot axis with new shoots developing from axillary buds. The frequency of somatic embryo conversion into plantlets was low, germinating somatic embryos were produced from 6–11 % of embryogenic cultures. On the average, one or two plantlets were produced in embryogenic cultures in one flask. The majority of cultures produced embryoids and somatic embryos which did not germinate.

The germination of somatic embryos and production of shoots and plantlets was stimulated after desiccation. Embryogenic tissues with induced embryoids cultured for 3 weeks on MS medium supplemented with cytokinin and sorbitol (6 %) and then transferred to WPM containing a low BAP concentration produced more plantlets (6 plantlets in 30 flasks) than tissues cultured on media lacking sorbitol (2–3 plantlets). Even embryogenic tissues, cultured on the same medium without transfer for 3–4 months, produced after transfer to WPM more plantlets than tissues transferred to a fresh medium every month. Further experiments are needed to achieve a high frequency of somatic embryo conversion into plantlets. Somatic embryos and plantlets were also regenerated from immature zygotic embryos of **Quercus petraea** cultured on the same nutrient media as embryos of **Q. robur**.

A total of 126 plantlets regenerated from **Q. robur** somatic embryos were produced and 74 plantlets with well developed root were transplanted into potting mixture. Plantlets were grown for two months at high air humidity under continuous light to achieve a fast stem elongation and formation of new leaves. High humidity was gradually reduced to normal values. 58 plantlets (78 %) survived (Fig. 4) and after hardening off they were transferred outdoors and planted in the field.

Table 1. Effect of collection date and nutrient medium on the formation of embryogenic tissues from cultured immature embryos of **Quercus robur**.[a]

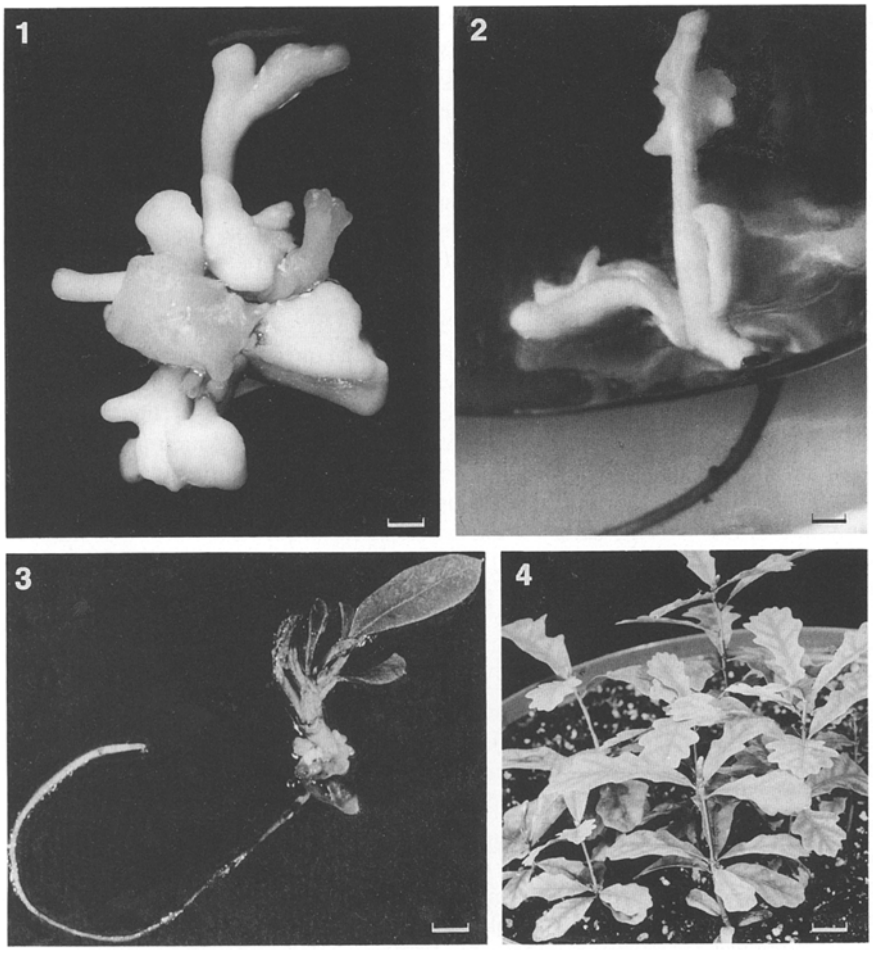
Collection date	Embryogenic cultures (%)	
	MS	WPM
July 5	62	54
July 12	80	76
July 19	82	74
July 26	76	70
August 2	75	72
August 9	61	65

[a]Nutrient medium consisted of modified MS medium or WPM supplemented with glutamine (200 $mg \cdot l^{-1}$), BAP (1 $mg \cdot l^{-1}$) and GA_3 (1 $mg \cdot l^{-1}$). For each treatment 50–60 immature embryos were used.

Somatic embryogenesis and plant regeneration in ***Tilia***

Immature zygotic embryos when cultured on MS medium supplemented with 2,4-D (0.3–2.0 $mg \cdot l^{-1}$) gave rise to embryogenic cultures within 5–8 weeks. The optimum seed collection date for the induction of embryogenic cultures was the last week of July and the first half of August.

The explants on 2,4-D containing MS media produced white globular embryogenic tissues loosely attached without a vascular connection to the parent tissue. In the next 2–4 weeks the



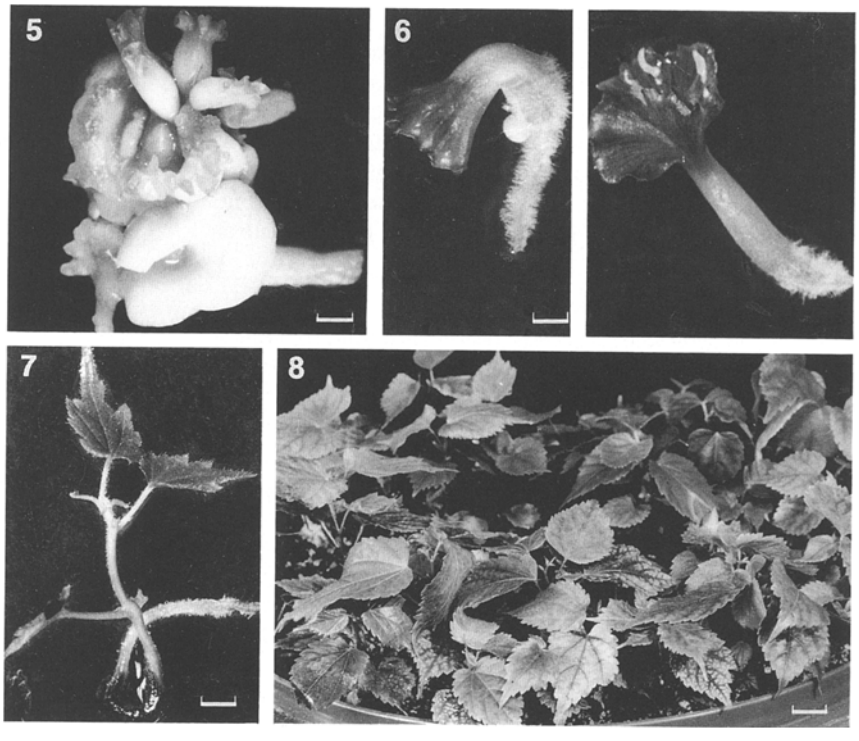
Figs. 1–4. Somatic embryogenesis and plant regeneration in **Quercus robur**.

Fig. 1. Somatic embryoids arising from the embryogenic culture (bar = 3 mm).

Fig. 2. Germinating mature somatic embryo (bar = 2 mm).

Fig. 3. Plantlet with expanding leaves and growing root, regenerated from somatic embryo (bar = 4 mm).

Fig. 4. Plants originated from somatic embryos, growing in a greenhouse (bar = 12 mm).



Figs. 5–8. Somatic embryogenesis and plant renegeration in **Tilia cordata**.

Fig. 5. Development of somatic embryos in embryogenic culture (bar = 1 mm).

Fig. 6. Conversion of somatic embryos into plantlets: enlarged cotyledons and growing root (bar = 2 mm).

Fig. 7. Plantlet with growing shoot and root, regenerated from somatic embryo (bar = 5 mm).

Fig. 8. Plants regenerated from somatic embryos, growing in soil (bar = 10 mm).

Table 2. Effect of 2,4-D on the formation of embryogenic tissues from cultured immature embryos of **Tilia cordata**. [a]

2,4-D ($mg \cdot l^{-1}$)	Embryogenic cultures (%)	
	August 2	August 14
0.3	76	68
0.5	84	72
1.0	82	73
2.0	72	67

[a]Nutrient medium consisted of modified MS medium supplemented with glutamine (200 $mg \cdot l^{-1}$) and 2,4-D. For each treatment 50–60 immature embryos were used.

embryoids acquired shapes ranging from globular to torpedo stage, and well-formed bipolar embryos bearing cotyledon and root initials were observed (Fig. 5).

Of the various media containing different auxin-cytokinin combinations, the MS medium containing 2,4-D induced the formation of embryogenic cultures. The presence of cytokinin (BAP) in the medium was not essential, immature embryos produced embryogenic tissues and embryoids on MS media containing only 2,4-D. Explants cultured on MS media containing both auxin (2,4-D) and cytokinin (BAP) mostly produced non-embryogenic callus. Experiments demonstrated that only 2,4-D was essential for the induction of embryogenesis in **Tilia** cultures. The majority of immature zygotic embryos inoculated on MS medium produced somatic embryos in the presence of 2,4-D. The number of embryogenic cultures produced from immature zygotic embryos varied with the concentration of 2,4-D (Table 2). 2,4-D at 0.5 $mg \cdot l^{-1}$ was found to be optimum for the induction of somatic embryos. No significant differences in the formation of embryogenic cultures were observed with 0.5 and 1.0 $mg \cdot l^{-1}$ 2,4-D.

In **Tilia** cultures, the process of development from the initial globular stage to the bipolar somatic embryos occurred on MS medium containing 2,4-D, however 2,4-D generally arrested fast development of embryoids and only embryoids that were not in direct contact with the medium exhibited a rapid development. The fast development of somatic embryos was stimulated when embryogenic cultures with induced embryoids obtained on MS media containing 2,4-D were subcultured on MS media lacking 2,4-D and supplemented with a low concentration of IBA (0.1–0.2 $mg\ l^{-1}$).

Repetitive somatic embryogenesis was frequent. Numerous adventive embryos were formed on hypocotyl and root of somatic embryos. These adventive embryos developed gradually into mature somatic embryos. When the produced somatic embryos were subcultured on MS medium containing 2,4-D (0.3–1.0 $mg \cdot l^{-1}$), the formation of a second generation of embryogenic tissue and somatic embryos was induced.

After transfer of mature somatic embryos on a fresh MS medium containing a low concentration of IBA (0.1–0.2 $mg \cdot l^{-1}$), the somatic embryos germinated and within 2–4 weeks produced roots and shoots. The embryos with developing root and shoot were subcultured individually on MS medium of the same composition to produce complete plantlets with well developed roots and shoots with leaves (Fig. 7). The majority (63–78 %) of mature somatic embryos developed shoots and roots and formed plantlets. More than 450 growing plantlets were regenerated from somatic embryos of **Tilia cordata**. Somatic embryos and plantlets were also regenerated from immature zygotic embryos of **Tilia platyphyllos** cultured on the same MS media as embryos of **T. cordata**, however, the percentage of initiated embryogenic cultures and somatic embryos produced in **T. platyphyllos** cultures was significantly lower than in **T. cordata** cultures.

The plantlets with growing roots and shoots were transferred to potting mixture and grown under a high air humidity. 76–84 % of transplanted plantlets survived the transfer into soil and continued to grow. Following acclimatization, the plantlets were moved to the greenhouse, where they maintained fast growth (Fig. 8). After 3–4 months of growth, they formed vigorous plants with long axis, leaves, and root system. In total, 125 acclimated plants, regenerated from somatic embryos of **Tilia cordata**, were planted in the field.

Experiments with somatic embryogenesis in **Quercus robur** and **Tilia cordata** cultures showed that each species required different phytohormones and conditions for the induction of embryogenic tissues and development of somatic embryos. In **Quercus robur** cultures, somatic embryogenesis was induced in the dark on nutrient media lacking 2,4-D. Also somatic embryos of **Quercus suber** (El Maataoui and Espagnac 1987) and **Quercus ilex** (Féraud-Keller and Espagnac 1989) were induced on calluses grown on media lacking 2,4-D. Gingas and Lineberger (1989) observed that somatic embryoids of **Quercus rubra** arose from explants cultured on various combinations of 2,4-D and BAP, however, the highest percentages of normal embryoids were produced by explants cultured on growth-regulator-free media. In **Tilia cordata** cultures, the presence of 2,4-D in nutrient media was necessary for the induction of somatic embryogenesis. The linden embryogenic cultures were initiated in the light.

REFERENCES

Chalupa V (1981) Commun Inst For Cechosl 12: 255–271

Chalupa V (1983) Commun Inst For Cechosl 13: 7–39

Chalupa V (1984) Biol Plant 26: 374–377

Chalupa V (1985) Commun Inst For Cechosl 14: 65–90

El Maataoui M, Espagnac H (1987) C R Acad Sci Ser 3, 304: 83–88

Féraud-Keller C, Espagnac H (1989) Can J Bot 67: 1066–1070

Gingas VM, Lineberger RD (1989) Plant Cell Tissue Organ Cult 17: 191–203

Lloyd G, McCown B (1980) Comb Proc. Int Plant Prop Soc 30: 421–427

Murashige T, Skoog F (1962) Physiol Plant 15: 473–497