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Somatic embryogenesis in cell cultures of birch (*Betula pendula* Roth.)

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Abstract

Somatic embryogenesis was induced in cell cultures of birch (*Betula pendula* Roth.) derived from juvenile tissue of seed embryos and from mature leaf tissue. Embryos were formed in liquid and on solidified medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (kinetin). Sometimes somatic embryos formed only after transfer to medium devoid of growth regulators. The embryos germinated on hormone-free medium and were potted in soil and grown in the greenhouse.

Introduction

Birch (*Betula pendula* Roth.) is an important raw material for the Finnish wood refining industry. The cultivation of birch is, however, disturbed by mammalian herbivores that can cause severe losses in plantations. Attempts are, therefore, being made to develop herbivore-resistant birches. The resistance varies considerably between different genotypes and so far it has been impossible to estimate its heritability [7]. Tissue culture methods offer the possibility of vegetative propagation of desired genotypes. Micropropagation of several birch species has been reported [2, 3, 9] but has not yet been applied commercially for plantlet production.

We are interested in the possibility of using somatic embryogenesis for large scale propagation of herbivore resistant genotypes of birch. Embryo formation has been observed in many cell and tissue cultures of dicotyledons [1] including several woody perennials [11].

Fully expressed somatic embryogenesis in cell cultures of birch has, to our knowledge, not been reported before, although there is one publication of early stage embryogenesis of birch [10]. The present study reports the successful induction and development of somatic embryos from callus cultures of birch.

Materials and methods

Plant material

The seed and plant material of *Betula pendula* Roth. was provided by the Finnish Forest Research Institute. In the experiments eight different birch families, obtained by crossing parent plants with different herbivore resistances, were used. Four of the families were provided as seeds and four as one-year-old plants grown in the greenhouse.

The following material was used: seeds, one-week-old seedlings and leaves of one-year-old plants.

This provided explants of different developmental stages, which were used in order to find the most appropriate type of tissue for inducing embryogenic calli. The plant material was surface sterilized with 70% ethanol (5 min) and sodium hypochlorite (for leaves 10 min in 0.6% available chlorine and for seeds 30 min in 8.0% available chlorine) and thoroughly rinsed with sterile water.

From each family 60–500 seeds were tested

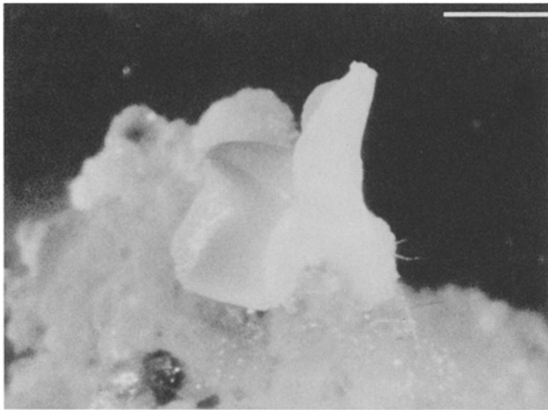

Fig. 1. A somatic birch embryo and embryogenic callus growing on N70 medium. Bar = 1 mm.

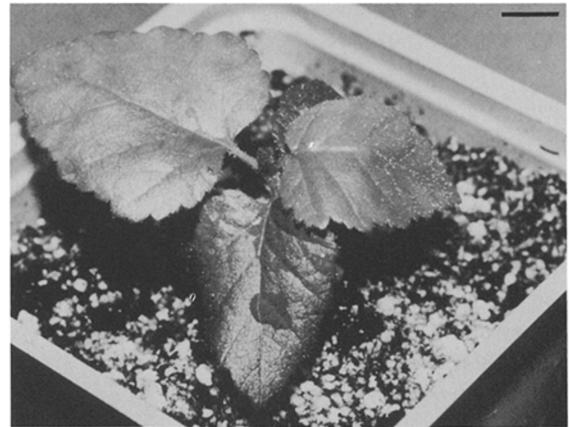

Fig. 3. A potted birch plant derived from a somatic embryo. Bar = 1 cm.

Fig. 2. Cross section of an embryo at cotyledonary stage, showing shoot and root apex. Bar = 100 μm.

(Table 1). Prior to plating for callus induction the mature seeds were cut in half. Each callus culture was derived from one seed.

Seedlings were obtained by germinating surface sterilized seeds on agar plates (0.8% agar in water). The seedlings were used at an age of about one week, when the first foliage leaf had emerged. From each family 200–400 seedlings were used. The seedlings in each family were not tested individually but in groups of ten. One seedling pool consisted of ten seedlings, randomly cut into pieces. Each callus culture was derived from one seedling pool (Table 1).

Leaves of one-year-old plants were used for callus induction. Each leaf was transversely cut across the midrib resulting in pieces approximately 2 × 0.5 cm in size. Each callus culture was derived from the segments of one leaf. Thirty plants per family were tested, except for family 6, of which only 3 plants were available. Approximately six leaves per plant were taken (Table 1).

The explants were plated on agar media in plastic petri dishes (ϕ 9 cm) for callus induction.

Culture media and growth conditions

Callus induction and growth

The medium used for callus induction and for supporting callus growth was N7 basal medium [8]

Table 1. The effect of family and explant type on the induction of callus on solid N7 growth medium and on the embryogenic competence of these callus cultures.

Family code	Starting material	Number of seeds/leaves/ seedling pools[a]	Number of callus cultures	
			Total	Embryogenic
18	Seed	558	11	0
	Seedling	25	3	
20	Seed	222	2	0
	Seedling	45	9	0
33	Seed	168	0	0
	Seedling	33	0	0
24	Seed	66	28	2
	Seedling	40	26	2
6	Leaf	18	7	2
3	Leaf	185	71	7
102	Leaf	200	5	0
30	Leaf	149	6	0

[a] One seedling pool contained 10 seedlings (see Materials and methods).

supplemented with 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg l^{-1} 6-furfurylaminopurine (kinetin) and 1.0 g l^{-1} casein hydrolysate (Sigma, acid hydrolysate). This medium is hereafter referred to in the text as N7 growth medium. For callus cultures the medium was solidified with 0.8% agar (Difco, Bacto-agar). The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The callus cultures were transferred to fresh medium once a month.

Suspension cultures
The N7 growth medium (without agar) was also used for suspension cultures. Approximately 2 g of cells were suspended in 50 ml of medium in 250 ml erlenmeyer flasks capped with aluminium foil. Suspension cultures were transferred to fresh medium once a week using 10 ml of cells (measured as packed cell volume). Suspensions were grown on a horizontal shaker (133 rpm, stroke radius 2.5 cm).

Growth conditions
All cultures were grown at 23 °C in a 16 h–8 h light-dark regime (light intensity 125 μE $m^{-2}.sec^{-1}$, white fluorescent lamp 30 W Airam/Fluora 36 W Osram, 1:1).

The test for embryogenic potential of cell cultures
The embryogenic competence was estimated by transferring 1 g pieces of callus on N7 basal medium [8] solidified with 0.3% Gelrite[R] (Kelco) instead of agar in petri dishes (ϕ 5 cm). This medium is called N70 medium in the text. The presence or absence of somatic embryos was recorded one month after the transfer. This test was repeated twice for each cell line during subsequent generations.

Germination of somatic embryos
Somatic embryos, individually picked from the cell cultures, were germinated on N70 medium. In 2–3 weeks time the embryos developed into plantlets.

Multiple shoots
After germination of the somatic embryos, adventitious shoots, if subsequently formed, were excised and rooted on half strength N7 basal medium solidified with 0.3% Gelrite[R].

Potting and transfer to greenhouse
The plantlets of somatic embryo and adventitious shoot origin were planted in a peat:perlite mixture (1:1) and grown under greenhouse conditions, at 22 °C in a 16 h–8 h light-dark regime. The light intensity at the level of the pot was 125 μE.$m^{-2}.sec^{-1}$. The plants were covered with plastic hoods and watered by spraying every second day. After 2–3 weeks the hoods could be removed.

Histological preparations

Samples were fixed in formalin-acetic acid-50% ethanol (1:1:18), dehydrated in an ethanol-butanol series and embedded in paraffin. Sections of 10 μm were stained in hematoxylin-safranine (1:9) and fast green for microscopic studies.

Results and discussion

Callus cultures

Preliminary media screening for callus induction from seed- and seedling explants were made with three basal media, MS [5], B5 [4] and N7 [8], with various concentrations of the two growth regulators 2,4-D and kinetin, in concentrations ranging from 0.1–5.0 $mg\,l^{-1}$ and 0–2.0 $mg\,l^{-1}$ respectively (data not shown). N7 basal medium supplemented with 2.0 $mg\,l^{-1}$ 2,4-D, 0.5 $mg\,l^{-1}$ kinetin and 1 $g\,l^{-1}$ casein hydrolysate (N7 growth medium) was chosen for all further callus inductions. This was the only medium that could support continuous callus growth beyond the second callus generation.

In a series of further experiments callus cultures were successfully initiated on N7 growth medium from eight different birch families using all explant types described in Materials and methods. The frequency of callus induction is shown in Table 1. The family used caused more variation in the callus formation than the type of the explant.

Embryogenesis

The embryogenic potential of the calli were tested on N70 medium after they had reached the third callus generation. Embryogenic calli formed somatic embryos on the callus surface 3–4 weeks after transfer on N70 medium (Fig. 1). The embryos were first seen as creamy white globular structures. When these proembryos developed further, distinct shoot and root poles were seen in longitudinal section (Fig. 2).

Embryogenic calli were induced in three birch families out of eight (Table 1). As in callus induction, the family had a more pronounced effect on somatic embryogenesis than the type of explant. Embryogenically competent cell cultures could be derived from seed-, seedling- as well as leaf tissue. The embryogenic calli were friable and grew well on N7 growth medium. Abundant root formation and anthocyanin production were observed. The calli consisted of small, densely cytoplasmic cells. There were no visible morphological differences between embryogenic and non-embryogenic callus cultures. The non-embryogenic cultures also formed anthocyanins.

In two cell lines, from family 6, with a strong embryogenic potential, white globular shaped embryos appeared on the callus surface even without transferring calli on N70 medium. The somatic embryos did not, however, develop beyond the heart shaped stage on N7 medium. There are several reports on the ability of cell cultures to form embryos on media containing auxin. Our results correspond with those obtained in cell cultures of *Corylus avellana*, which belongs to the family *Betulaceae* [6]. When our cell lines were transferred to suspension culture of the same medium composition as used for callus culture (N7 growth medium) and subcultured every week, fast growing suspensions were established. When grown without transferring for 2–3 weeks, white embryos appeared in the suspensions.

Embryo germination

Embryos, which were initially formed on solid N70 medium or later transferred to it, developed normally and germinated. So far 49 plantlets derived from callus cultures of birch family 6 have been potted in soil and transferred to greenhouse conditions (Fig. 3). The germination ability of the embryos formed in suspension cultures has not yet been tested.

Some abnormal embryos and embryos germinating with multiple shoots were also observed. Cup-shaped embryos with fused cotyledons and germinating embryos with swollen hypocotyl and non-functioning shoot apices were found. The multiple shoots were cut and rooted in vitro before potting in soil. Whether the shoots were formed from adventitious buds is not known. A total of 127 plantlets originating from such shoots have been potted and grown in the greenhouse.

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