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Spontaneous somatic embryogenesis and plant regeneration from root cultures of *Peucedanum palustre*

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ABSTRACT

The regeneration of *Peucedanum palustre* (L.) Moench (milk parsley) was established for the first time via somatic embryogenesis from primary root cultures. Callus formation occurred on the root cultures and showed spontaneous embryogenic capability on B5 basal medium supplemented with a low concentration of indoleacetic acid (5.5×10^{-7} M). 2,4-Dichlorophenoxyacetic acid was not needed for the initiation of embryogenesis. The somatic embryos germinated and formed plantlets on hormone-free B5 medium. These plantlets were easily transferable to pots, and are presently passing their second growing season in the greenhouse.

Development of the somatic embryos progressed through the globular, heart-shaped, torpedo-shaped, and cotyledonary stages, typical of zygotic embryos. Synchronization performed by sieving the embryos did not affect the development time. The culture has retained its embryogenic capacity for 25 months.

Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid; IAA = indoleacetic acid; IBA = 3-indolebutyric acid; BAP = 6-benzylaminopurine.

INTRODUCTION

The main criterion for somatic embryogenesis is that the somatic cells behave like zygotes with certain abilities to go through the similar development steps which are characteristic for embryogenesis *in vivo*. Use of 2,4-D, high light intensity or darkness are known to induce somatic embryogenesis (Ammirato 1983, Vasil 1984). Embryogenesis has been achieved using various plant tissues as explants *e.g.* epidermal cells of leaves, hypocotyls, microspores or stems but seldom roots (Vasil 1984, Jia *et al.* 1989).

Synchronization of the cell and somatic embryo cultures can greatly increase the efficiency of somatic embryogenesis for *in vitro* propagation (Nadel *et al.* 1990). Thus, if all the embryos of a similar size could be synchronized, this would greatly shorten the culture time and increase the propagation efficiency and uniformity.

There have been several reports of embryogenesis on *Daucus carota*, and the carrot has often been used as a model plant for studies on this phenomenon. Studies have also been reported on some other plants belonging to the Apiaceae family, *e.g. Petroselinum crispum, Apium graveolens* (Ammirato 1983, Furmanowa *et al.* 1991), *Levisticum officinale* (Jia *et al.* 1989) and *Carum carvi* (Collin and Isaac 1991).

Peucedanum palustre (L.) Moench (Apiaceae) is common throughout Finland, apart from northern Lapland where it is found only sporadically. The major active compounds in *P. palustre* are coumarins, and they have been found to exhibit calcium antagonistic effects on rabbit smooth muscle preparations (Vuorela 1988) and on prolactin release in GH_3 rat pituitary cells (Vuorela *et al.* 1988). This report describes for the first time spontaneous, indirect somatic embryogenesis from primary root cultures of *P. palustre* and rapid plant regeneration of the embryos. Although the auxin 2,4-D is often needed to stimulate the somatic embryogenesis (Dudits *et al.* 1991), supplementation of 2,4-D was not necessary to promote embryogenesis in this case. We also show that embryogenic capacity can be retained for an unexceptionally long time.

MATERIALS AND METHODS

Peucedanum palustre (L.) Moench plants originated from Kirkkonummi, Finland (60°09'N, 24°32'E), and had been growing for one year in the greenhouse. The seeds were sterilized with 0.01% $HgCl_2$ + Tween 80 for 15 min, rinsed with sterile water four times, and germinated on 0.6% water agar containing 1% sucrose in the dark or according to the photoperiod cycle described below. Conventional root cultures of *P. palustre* were obtained from seedlings grown from surface-sterilized seeds. Three-week-old root tips were cultured in 20 ml of liquid Gamborg B5 basal medium [Gamborg *et al.* 1968, modified as described elsewhere (Oksman-Caldentey *et al.* 1991)] containing 3% sucrose (w/v) supplemented with IAA (5.5×10^{-7} M) referred as B5-A, or IBA (4.7×10^{-7} M, referred from now on as B5-B_1) in a 100 ml conical flask. Tips were cut from the third transfer passage after seven weeks, and transferred to 21 conical flasks (5 tips in each) containing B5-A (12 flasks) or B50+IBA (9 flasks; B50 = B5 basal medium, hormone-free). The IBA concentration of the medium into which the root tips were transferred was decreased to 4.7×10^{-9} M (referred as B5-B_2). Three flasks of both cultures were kept in the dark.

The culture conditions were as follows unless otherwise indicated. The cultivation temperature was 26°±2°C, and a photoperiod cycle of 16 hours light (25 µmol m^{-2} s^{-1};

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Lumilux® L 36 W/31, warm white, Osram) followed by 8 hours darkness. The suspension cultures were agitated on rotary shakers at 110 rpm.

The embryos were removed using forceps under a stereomicroscope and placed in liquid or on solidified [0.2% (w/v) Gelrite® gellan gum] B5-A or B5-B_2 medium. After two weeks the embryos were further cultivated on solid B50 or supplemented with BAP (4.4×10^{-6} M, referred as B5-C). The regenerated plantlets were then transferred from sterile conditions into a mixture of Vermiculite® and soil (1:1) and incubated for the first 2 weeks in a growth chamber to maintain a high humidity. The plants were subsequently transferred into soil and grown under greenhouse conditions, *i.e.* in a room without heating (temperature in winter not less than 5°C) under natural daylight.

For maintaining the embryogenic callus, small pieces were transferred onto solidified B50 supplemented with 2,4-D. The concentrations used were 9.0×10^{-6} M (B5-D_1), 1.35×10^{-5} M (B5-D_2) and 2.3×10^{-5} M (B5-D_3).

The embryos were fractionated using 1000 -125 µm sieves (F0; ø >1000 µm, F1; 1000-500 µm, F2; 500-250 µm, F3; 250-125 µm, F4; <125 µm) and kept in B5-A medium for the growth rate studies on the embryos. Embryo development was recorded every day on fraction F2 until the plantlet stage was reached. This was done in duplicate.

Growth was determined using the filter paper growth assay (Horsch and King 1990). Embryogenic cell mass was determined (100.0 mg/plate; n = 6) on growth media plates (ø = 9 cm; B50 solidified as above) overlaid with filter paper. Growth was followed by measuring the fresh weight of the embryogenic cell mass growing on the filter paper once or twice a week (see Fig. 2A). The filter paper was aseptically removed for weighing and then replaced on the medium. At every measurement a small amount (about 20-50 mg) of embryogenic mass was removed from the same plate for dry weight determination. The embryogenic mass was lyophilized and the dry weight was determined.

Growth was also determined on the suspension cultures. 100 ml conical flasks containing 20.0 ml B50 medium were inoculated with 100.0 mg/flask of embryogenic cell mass. Three flasks were removed once or twice a week (see Fig. 2B), filtered (fresh weight) and lyophilized (dry weight).

RESULTS AND DISCUSSION

Proliferation of the embryogenic callus

The *Peucedanum palustre* seeds started to germinate on average six days after sterilization. Darkness and seed coat removal were found to promote germination, as well as using Petri dishes instead of *e.g.* beakers. Conventional root cultures were established from the roots of these seedlings. Different concentrations of IAA and IBA, as well as no hormone supplementation in the basic B5 medium, were used in optimizing the medium for the root cultures. The best root growth was obtained in B5-A (IAA 5.5×10^{-7} M), in B50 no growth occurred. Higher hormone concentrations induced so much callus formation that this inhibited the growth of the roots.

Green roots were typical of all the conventional *P. palustre* root cultures grown in the light. Callus formation occurred on all the root cultures (Fig. 1A). Embryos developed spontaneously from the callus that was produced in the third passage in one of the flasks grown in the light in B5-A. White, embryo-like globular structures started to appear (Fig. 1B). These grew rapidly at the same time as new proembryogenic structures continued to appear on the callus surface, as well as on the previously formed ones. Thus clusters of embryo structures at various stages of development were found on the same callus within 2 weeks. To our knowledge root material has not been used to produce embryogenesis in the Apiaceae family, except for *Daucus carota*, where almost any part, including the roots (Smith and Street 1974), has been reported to produce somatic embryos in culture.

After lowering the IBA concentration from B5-B_1 to B5-B_2, embryos also started to form in one of the flasks kept in the dark. The embryos formed tight, spherical aggregates which did not develop further. Their developmental stage remained the same even after being transferred into the light. The embryos appeared to be abnormal when viewed under phase contrast and stereomicroscopes. One way of inducing embryos is to use high concentrations of auxins. The hormone concentration then has to be lowered drastically or removed in order to induce the development of the embryos and plant regeneration (*e.g.* Mizuta *et al.* 1987, Pierik 1987). Transfer to hormone-free medium did not result in any changes in the embryos formed in IBA in our study.

Characterization of the embryogenic culture

The cells from which embryos are derived show a number of common features (Fig. 1C). These are small size, dense cytoplasmic contents, large nuclei with prominent enlarged nucleoli, small vacuoles and a profusion of starch grains (Pierik 1987).

Embryo formation was further investigated under a stereomicroscope and the different embryogenic stages were observed (Fig. 1D-H). Globular, heart-shaped, torpedo, cotyledonary and germinating embryos were present in the culture. These different stages were fractionated from a mature culture using a series of metal sieves. The rate of embryo development was followed (Table 1; for development rate of F2, see materials and methods). Within sixteen days some of the globular stage embryos had already developed into plantlets, which could then be transferred into beakers for further plant regeneration. Increased aggregation of the embryos and the formation of secondary embryos made it difficult to alter the growth stages after sixteen days.

Table 1 Development rate of globular stage embryos (number of different embryogenic stages / day).

Day	Globular stage	Heart stage	Torpedo stage	Cotyledonary stage	Germinating embryos	Secondary embryos	Abnormal
0	196	-	-	-	-	-	-
2	131	6	-	-	-	-	5
4	23	8	-	-	-	-	6
7	113	13	4	-	-	-	5
10	88	15	1	13	-	-	10
12	51	9	9	20	-	14	9
14	35	10	4	32	1	25	8
16	20	12	10	40	4	27	8

Since size fractionation of the embryogenic mass would also assist in achieving synchronized growth of the embryos, the growth of the embryos in fraction F1-F4 was followed in the same way as the development of the embryos. The cell fraction <125 µm (F4) showed delayed growth (contained embryogenic and normal cells as well as cell debris), but the other fractions developed normally. All the cell fractions produced new embryos; evident as the increasing number of secondary embryos formed in Table 1. After 2-3 weeks all the fractions, except F4, contained embryos and plantlets of different stages.

An interesting feature in the growth curve shown in Fig. 2A is the continuous growth of the embryogenic mass. Growth seemed to cease due to the lack of space in the Petri

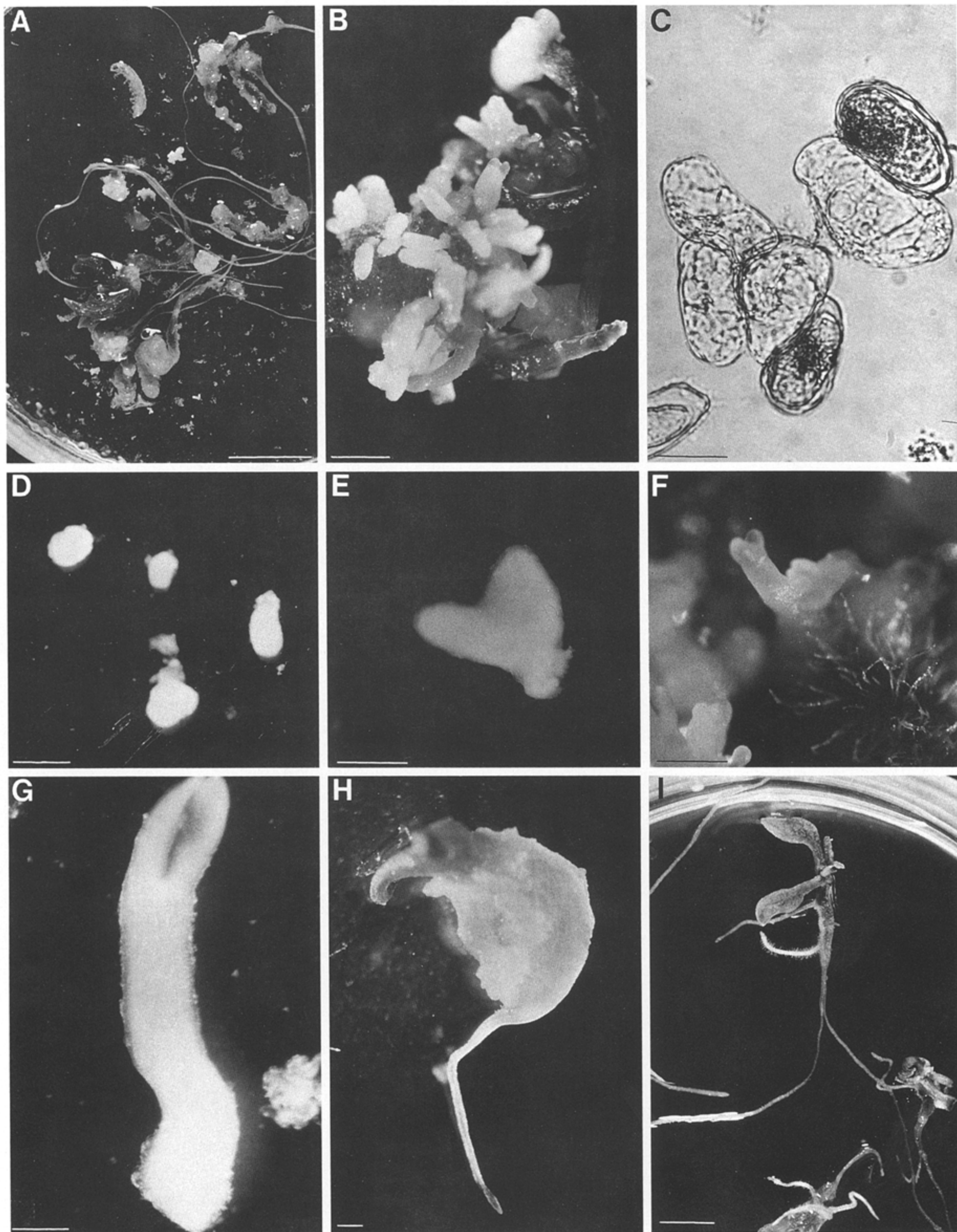

Fig. 1. A. Callus formation on the green roots. (Bar ≈ 1 cm); **B.** Polyformation of the embryos on callus tissue. (Bar = 1 mm); **C.** Large, vacuolated cells and typical densely cytoplasmic embryogenic cells. (Bar = 50 µm); **D.** The globular stage embryos appear so smooth because of the surrounding epidermal layer. (Bar = 1 mm); **E.** Heart-shaped embryo in which the cotyledonary primordia first begin to appear. (Bar = 0.5 mm); **F.** A torpedo stage embryo surrounded by callus tissue. (Bar = 1 mm); **G.** A developing cotyledonary stage embryo. (Bar = 0.5 mm); **H.** Germinating embryo in which vascular tissue has begun to form. The connection between root and shoot is direct (bipolaric). Secondary embryos can be seen on the germinating embryo. (Bar = 1 mm); **I.** Regenerated plantlet on solid B50 medium. (Bar = 10 mm).

dish rather than to a lack of nutritients after four months. On the other hand, the growth in the suspension cultures showed normal behaviour, reaching a maximum at 28 days (Fig. 2B).

The embryogenetic capacity has been retained on B50 and B5-A media, although it slowed down surprisingly during the winter. During the summer the embryogenic potential of these cultures has increased and remained stable. The normal growing season is probably still strong in these cell cultures since they have been in *in vitro* cultivation for less than two years.

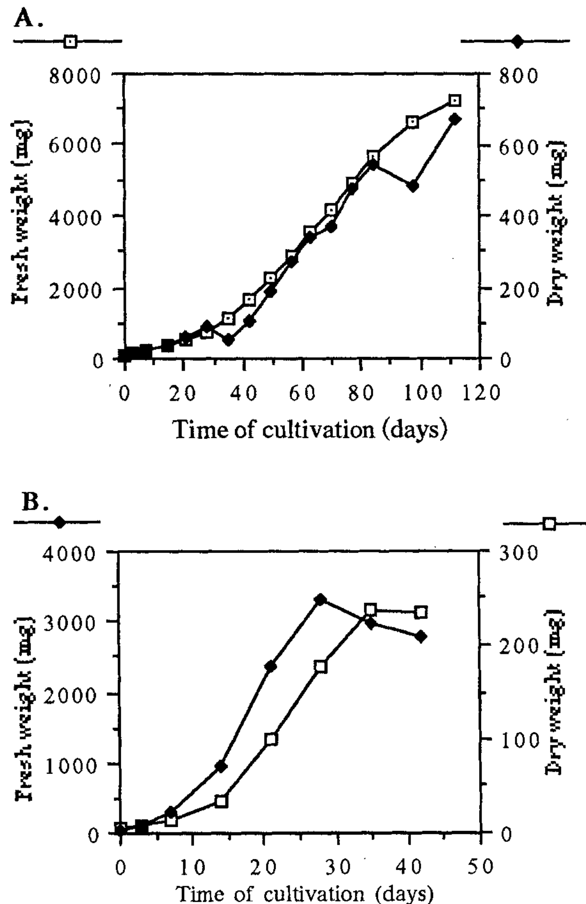

Fig. 2. A. Growth of embryogenic culture determined by filter paper growth assay on solidified B50-medium, shown as fresh and dry weight. **B.** Growth in embryogenic suspension cultures (B50), shown as fresh and dry weight.

Effect of 2,4-D on the embryogenic cell line

The embryos developed and grew so rapidly that the callus almost disappeared. For maintaining the embryogenic line as callus, the B50 medium was supplemented with 2,4-D. This auxin often initiates embryogenic capacity, but prevents further development of the embryos (*e.g.* Ammirato 1983, Borkird *et al.* 1986). 2,4-D is also widely used as a callus-promoting auxin for many plant species. In our case 2,4-D was not needed to promote embryogenesis.

Overall the growth slowed down, and the globular stage embryos remained in this stage probably as a result of inhibition by 2,4-D. The callus and embryos were healthy looking. However, adaption to the medium apparently occurred after 6 months and the embryos started to develop and spontaneous organogenesis was observed.

To see if the embryogenic capacity had retained, callus grown on 2,4-D for 4-5 months was transferred to B50 or B5-A media. After a delay of one week the pre-existing embryos started to develop and subculturing on the fresh medium speeded up the development. However, examination under the microscope revealed abnormal embryos and a disturbed further development. The globular stage embryos were swollen, and some of them had a greenish appearance. Elongated torpedo stage embryos with multiple tips occurred. There was no root formation and the leaves were elongated and hard, with abnormal tips. One month later the leaves had softened on both media, and root formation had started on B5-A medium. The regeneration was successful of plantlets developed on B5-A medium, but very slow on B50, whereas before 2,4-D treatment the development was best on B50.

Plant regeneration

The cotyledonary stage embryos (grown in B5-A) which had elongated to form bipolar structures, as well as small plantlet stage embryos, were transferred to solid, auxin-free medium (B50) or medium supplemented with BAP (B5-C). The latter was used because cytokinins usually promote shoot formation. However, in this study the shoot and root formation "exploded" on the auxin-free medium, *i.e.* the development was rapid and no special media was necessary. Polyembryogenesis frequently appeared on the germinating embryos (Fig. 1H). In such cases the plantlets were kept overnight in suspension in a rotary shaker after isolation in order to separate the embryos before planting. After the embryos had attained sufficient size (plantlet stage), they were transferred from the Petri dishes (Fig. 1I) to beakers (containing solidified B50 medium). The regeneration capability of the germinating embryos was almost 100%. Plants survived the transfer to soil and grew well through their second growing season.

The regenerated plants were further grown in the greenhouse, and their morphology was followed. The 141 regenerated plants did not show any differences from normal plants as regards leaf, stem or root form. *P. palustre* is a perennial plant, and it usually flowers the first time during the second or third year. During the second growing season the plants remained quite small (up to 50 cm), and no flowering occurred.

It is known that the regenerative capacity of the cells and callus tissue may be reduced or be completely lost if growth is continued for too long. Our cell line has kept its embryogenic and regenerative capacity for 25 months already. New embryos are still being formed and the plants derived are growing well in soil. Further studies will mainly focus on the coumarin contents during embryogenesis and in fullgrown plants.

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