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Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction

Hely Häggman[1,4], Anne Jokela[2], Jana Krajnakova[3], Anneli Kauppi[2], Karoliina Niemi[1] and Tuija Aronen[1]

[1] *Punkaharju Research Station, The Finnish Forest Research Institute, Finlandiantie 18, FIN-58450 Punkaharju, Finland*

[2] *Department of Biology, University of Oulu, PO Box 3000, FIN-90401 Oulu, Finland*

[3] *Department of Forest Genetics and Tree Breeding, Forest Research Institute, TG Masaryka 22, SK-96022 Zvolen, Slovak Republik*

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Abstract

This is the first report on Scots pine (*Pinus sylvestris* L.) somatic embryo plants regenerated and growing in a greenhouse. The present work focused on improving somatic embryogenesis of the species by studying the factors affecting culture induction. Developmental stage of explants that were immature female gametophytes, including the zygotic embryos with suspensor tissues, was investigated in detail. The genetic background of the material, cold treatments (14 d, 1 or 2 months at +5 °C) of cones including explants, as well as the plant growth regulator composition of the initiation medium, were also examined. When initiation of somatic embryogenesis was successful, the zygotic embryos in the explants were either proembryos or early embryos. Cold treatment of the cones had no significant effect on induction, nor were there any differences among the treatments with different duration, thus improving the practical applicability of the culture technique. The explants in cold-stored cones probably retained their initiation capacity due to the conversion of starch to sugars. This was observed as decreased number and size of starch grains in the megagametophytes compared with the controls. The seed family and the medium significantly affected induction success, the medium with auxin (9.1 or 13.6 μM 2,4-dichlorophenoxyacetic acid) and cytokinin (2.2 μM 6-benzylaminopurine) being better than the medium with cytokinin (5 μM 6-benzylaminopurine) alone. The significance of the genetic background of the explants and the initiation medium indicate that it might be possible to improve the initiation rates by using explants from controlled crossings between competent genotypes, and by developing more specific media for important seed families.

Key words: *Pinus sylvestris*, vegetative propagation, zygotic embryo, megagametophyte, histochemistry.

Introduction

Scots pine (*Pinus sylvestris* L.) is one of the most common conifers in Nordic countries, and it has therefore been one of the main targets of tree breeding efforts. In Scots pine, fascicular shoot production based on cytokinin spraying of young seedlings has proved to be a promising method for vegetative propagation. There are, however, problems caused by poor and genotype-dependent rooting of the shoots (Salonen, 1990). Organogenesis using cotyledons excised from germinated embryos as the explant material has also proved to be suitable for Scots pine, but the success is dependent on the genotype and the average multiplication rate is relatively low (Häggman *et al.*, 1996). The method is therefore applicable for small-scale studies only. However, an efficient vegetative propagation technique is a prerequisite for developing molecular breeding of the species or for realizing the genetic gain obtained through conventional breeding to practical forestry.

Somatic embryogenesis was first reported in conifers with *Picea abies* in 1985 (Hakman *et al.*, 1985). Since then, progress has also been made in the somatic embryo-

[4] To whom correspondence should be addressed. Fax: +358 15 644 333. E-mail: hely.haggman@metla.fi

genesis of pine (*Pinus*) species. So far, embryogenic cultures of pine species have been reported for *P. strobus* (Finér *et al*., 1989; Garin *et al*., 1998), *P. taeda* (Becwar *et al*., 1990), *P. lambertiana* (Gupta and Durzan, 1986), *P. serotina* (Becwar *et al*., 1988), *P. elliottii* (Jain *et al*., 1989), *P. nigra* (Salajova and Salaj, 1992), *P. caribea* (Lainé and David, 1990), *P. radiata* (Smith *et al*., 1994; Chandler and Young, 1995), *P. patula* (Jones and van Staden, 1995), and *P. sylvestris* (Hohtola, 1995; Keinonen-Mettälä *et al*., 1996; Sarjala *et al*., 1997). Somatic embryo plants planted in the field have, however, been reported only in the case of *P. radiata* (Smith *et al*., 1994) and *P. taeda* (Becwar and Pullman, 1995). In Scots pine (*Pinus sylvestris* L.), embryogenic cultures have been established both from precotyledonary embryos isolated from immature seeds (Keinonen-Mettälä *et al*., 1996) and from mature embryos (Hohtola, 1995). To date, however, somatic embryo plants growing in the soil have not been reported.

In earlier studies, attention was focused on the proliferation phase of the somatic embryogenesis of Scots pine and, therefore, the effects of polyamines (Sarjala *et al*., 1997) and ectomycorrhizal fungi on proliferating embryogenic cultures (Niemi *et al*., 1998) were studied. In addition, a cryopreservation method for embryogenic cultures at the proliferation stage was developed, and the genetic fidelity of cryopreserved material was examined (Häggman *et al*., 1998). In Scots pine, as well as in other pine species, immature megagametophytes containing immature zygotic embryos have been used as explant material. The optimum stage for the initiation of embryogenic cultures varies (Roberts *et al*., 1989; Becwar *et al*., 1990, 1991; Keinonen-Mettälä *et al*., 1996) and, generally, the induction rates are rather low, indicating that more information is needed about the factors affecting induction.

In the present study, the aim was to improve somatic embryogenesis of Scots pine by elucidating the factors affecting the induction of embryogenic cultures. Special attention was paid to the developmental stage of the immature seed and the potential role of the megagametophyte therein. The effect of cold treatment of collected cones before inoculation and the response of different seed families on the induction of embryogenic cultures was also examined. This is also the first report on Scots pine somatic embryo plants regenerated and growing in a greenhouse.

Materials and methods

Plant material

The aim of the experiments started in 1995 was to obtain an overall view of somatic embryogenesis (from initiation to regeneration) in Scots pine (*Pinus sylvestris* L.). One-year-old immature seed cones were collected from open pollinated élite Scots pines growing in the Punkaharju clone collection in Finland (61°48′ N; 29°17′ E). The collection period was from 19 June to 13 July, 1995, when the effective temperature sum (i.e. the heat sum unit based on the daily mean temperatures minus the adapted $+5\,^{\circ}C$ base temperature) was between 402 and 626 degree days (d.d.), respectively. The cones were collected from 25 élite clones twice a week, altogether eight times, and the total number of inoculations was 1750 (ten explants per seed family, i.e. mother tree during the first collection dates and five explants per seed family during the two last collection dates). Four of the proliferating cell lines were tested for maturation.

In 1996, the 1-year-old immature seed cones were collected from eight élite Scots pines. Five of these, K779, K800, K884, K908, and K1009 were chosen because they were able to produce embryogenic cell masses in 1995. In addition, cones were collected from pines K395, K942 and E725. During the collection period from 19 June to 31 July 1996, the effective temperature sum was 267 and 646 d.d., respectively. The cones were collected twice a week, altogether 13 times, and the total number of inoculations was 15 168 (Table 1), the number of explants per seed family and treatment combination being 20. Part of the collected cones were cold-treated at $+5\pm2\,^{\circ}C$ for 14 d, 1 or 2 months before inoculation (Table 1).

Initiation of embryogenic cultures

Embryogenic cultures were established using immature zygotic embryos with suspensor tissues surrounded by the immature megagametophyte. The inoculation, induction and proliferation of the embryogenic cell masses were carried out as described previously (Sarjala *et al*., 1997). When the effect of different growth regulators on the initiation of embryogenic cultures was studied, the DCR-based media (Gupta and Durzan, 1985; Becwar *et al*., 1990) were used. DCR1 medium included 13.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.2 μM 6-benzylaminopurine (BA), DCR2 medium included only 5.0 μM BA, and DCR3 medium contained 9.1 μM 2,4-D and 2.2 μM BA. The media were semi-solidified by 2.5 g dm^{-3}

Table 1. *Initiations for somatic embryogenesis of Scots pine in 1996*

All eight seed families (K395, K779, K800, K884, K908, K942, K1009, and E725) were included in the initiation experiments performed at the original cone collection date and also after 28 d cold treatment of the cones. In the initiation experiments after 14 d and 60 d cold treatment of the cones, only the explants from the seed families K800, K908, and K1009 were included. d.d. = degree days.

Time	Collection		Number of initiations at collection date	After cold treatment of		
	Date	d.d.		14 d	28 d	60 d
I	June 19	267	480	180	180	129
II	June 25	314	480	180	402	180
III	June 27	328	480	180	457	180
IV	July 2	375	480	180	358	174
V	July 4	392	480	180	478	180
VI	July 9	442	480	180	439	180
VII	July 11	465	480	180	470	180
VIII	July 16	509	480	180	480	180
IX	July 18	520	480	179	468	180
X	July 23	558	480	180	454	180
XI	July 25	584	480	180	480	180
XII	July 31	646	480	180	480	180
Total			5760	2159	5146	2103

Phytagel™ (Sigma). During proliferation, the embryogenic cell masses were cultivated in the dark at $+25\pm2\,^{\circ}C$.

Maturation, germination and cultivation of somatic embryo plants

For the maturation experiments, the four cell lines K800/6, K884/6, K884/9, and K908/11 from the 1995 inoculations were used. Following proliferation, the embryogenic cell masses were first cultivated on DCR medium with 1% (w/v) activated charcoal for 1 week, after which they were transferred onto DCR medium containing 32 μM abscisic acid (ABA), 50 g dm^{-3} polyethylene glycol 8000 (Aldrich) and 60 g dm^{-3} sucrose for 1 month, and subsequently onto DCR medium with 20 g dm^{-3} sucrose for 1–2 months. The germination medium consisted of half-strength DCR medium with 20 g dm^{-3} sucrose. During maturation, the embryogenic cell masses were cultivated in the dark at $+25\pm2\,^{\circ}C$, and during germination they were grown under a 16/8 h light/dark photoperiod (cool white L36W-642 Airam, Finland; 67–74 μE $m^{-2}\,s^{-1}$) at $+24\,^{\circ}C$. After *in vitro* culture, the plantlets were adapted to greenhouse conditions. Planting was done in non-fertilized horticultural peat : perlite (2 : 1) mixture. For the first 2 weeks in the greenhouse the plantlets were kept under mist. After 1 month the plantlets were transplanted in commercial fertilized peat (VAPO, Finland) containing 1 kg m^{-3} basic fertilizer (basic fertilizer included 9.7% N, 7.5% P, 14.4% K, 5.0% Ca, 6.6% S, 3.8% Mg, 0.27% Fe, 0.13% Mn, 0.04% B, 0.05% Zn, 0.25% Cu, and 0.09% Mo) and 3 kg m^{-3} limestone dust with magnesium. During the growing season, plantlets were fertilized monthly with commercial 0.2% 5-Superex fertilizer (Kekkilä, Finland).

Microscopical studies

Female gametophytes were fixed in FAA (ethanol, glacial acetic acid and formalin, 85 : 5 : 10, by vol.) every time when experiments for somatic embryogenesis were started. For microscopy, the fixed female gametophytes either cold-treated for 1 month or without the cold treatment from seed families K884, K908 and K395 were used as explants as presented in Table 2. Generally, the microscopical samples were prepared at the time when both the first somatic embryogenesis initiation occurred (early stage) and somatic embryogenesis initiation occurred for the last time or the collection series ended (late stage). In addition, control material for the cold-treated explants was collected from the same collection days as used for the early and late stage, but without the cold treatment (Table 2).

Female gametophytes were observed under a stereomicroscope (Nikon XN, Japan) and fixed with FAA, dehydrated in an alcohol gradient and embedded in Historesin (Reichert-Jung, Germany, product numbers 70–2218–500–70–2218–502). Microscopical longitudinal sections (4.0 μm) were cut with a glass knife on an LKB-Historange microtome and stained with toluidine blue (Harris *et al*., 1994) for general observation, with Sudan Black B (Harris *et al*., 1994) for lipids, with potassium iodide-iodine (IKI) (Jensen, 1962) for starch, and with Coomassie Brilliant Blue (R-250) (Cawood *et al*., 1978) for proteins. Histochemical sections were observed under a Nikon OPTIPHOT-2 light microscope and the development of embryo and suspensors was classified according to Singh (Singh, 1978). The total number of gametophytes observed was 75 in stereomicroscopy and and 24 in light microscopy, of which 57 and 16 were cold-treated ones.

Both light microscopy and scanning electron microscopy were used to follow the development of the somatic embryos. For scanning electron microscopy, developing somatic embryos were dissolved in 5% (w/v) Ariel washing powder at 38 °C for 16–18 h after which they were fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) for 5 h and dehydrated in a graded ethanol series (Honegger, 1985). The samples in pure ethanol were subjected to a critical point dryer, mounted on aluminium stubs and coated with gold in a sputter coater (Polaron E 5100). Observations were made at 15 kV in a Jeol JSM 35 scanning electron microscope.

Statistical analyses

The effects of seed family, collection date, inoculation medium, and cold treatment of immature cones on the induction of embryogenic cultures in 1996 were analysed by fitting a logistic regression model to the binary data (SE culture induced or not), and then evaluating it with goodness-of-fit χ^2 tests. For modelling, only data originating within the responsive period, i.e. collection dates from 7 July to 31 July, were used in order to reduce the extreme skewness of the data. The odds ratios obtained for the success of SE induction were calculated with 95% confidence intervals (BMDPLR procedure, Engelmann, 1988).

Results

Production of somatic embryo plants

In 1995, the screening of 25 different seed families of Scots pine revealed that 28% of the families had a potential for somatic embryogenesis. The embryogenic cell masses protruded from the explants (Fig. 1A) when the experiments were started between 22 June and 13 July when the temperature sums were 433 and 626 d.d., respectively. Altogether nine cell lines, representing five different seed families (K779, K800, K884, K908, and K1009), were still proliferating 3 months after inoculation. The average success, i.e. percentage of proliferating explants from the total number of explants in all 25 seed families during the responsive period, was 0.6% and the average success rate of the five proliferating families was 3%.

Four of the nine proliferating cell lines of 1995, K800/6, K884/6, K884/9, and K908/11, were tested for maturation of somatic embryos. Cell lines K800/6, K884/6 and K884/9 were able to produce maturating cotyledonary-stage embryos (Fig. 1B). In the case of cell line K884/6, conversion of cotyledonary-stage embryos was successful (Fig. 1C) and a few of them with vigorously elongating root systems were adapted to greenhouse conditions. Figure 1D presents the somatic embryo plant after the first growing season.

Factors affecting the induction of somatic embryogenesis

In 1996, embryogenic cultures were established from 75% of the examined seed families. Approximately 3 months after the initiation, 101 cell lines were proliferating to give an average success of 1.1% during the responsive period (Table 2). One year after inoculations, 67% of the cell lines were still in the culture. The percentage of proliferating explants varied from 0.2% to 4%, depending on the seed family. Induction of the SE cultures took

Table 2. *The number of embryogenic cell lines of Scots pine seed families proliferating three months after inoculation*

The missing values indicate that no embryogenic cultures have been initiated. Initiations were performed from all eight seed families both at the time of cone collection and after 28 d cold treatment. After 14 d and 60 d cold treatment of cones the initiations were done from the seed families K800, K908 and K1009.

Initiation time	Seed family	Cone collection time and date							Number of cell lines
		VI July 9	VII July 11	VIII July 16	IX July 18	X July 23	XI July 25	XII July 31	
	K395			[c]			[c]	1	1
At cone	K779								
collection	K800						1		1
date	K884	[c]	2[a]	4	1[a]		[c]		7
	K908	[c]		5[a]	5	1	4	2[ac]	17
	K942								
	K1009								
	E725								
After 14 d	K800			1			1	1	3
cold	K908		1	3	10	1	1	4	20
treatment	K1009					1			1
After 28 d	L395			3[b]	1	1	1[b]		6
cold	K779								
treatment	K800							1	1
	K884	3[b]	3	2	1	3	1[b]		13
	K908	1[b]	2		4	3	2	2[b]	14
	K942								
	K1009								
	E725				2				2
After 60 d	K800								
cold	K908	2			4	3	2	1	12
treatment	K1009	1		1			1		3
Number of cell lines		7	8	19	28	13	14	12	101

Note: [a] = explants sampled for microscopical observations at the collection date, [b] = explants sampled for microscopical observations after one-month cold treatment, [c] = control samples for the cold-treated material for microscopical observations.

place rather slowly after inoculation. Of the induced cell lines, 37% were proliferating within 4 weeks, 72% within 6 weeks, and 99% within 8 weeks after inoculation.

The optimal stage for cone collection in 1996 was between 7 July and 31 July, when the temperature sum was between 442 and 646 d.d. When the data originating within this responsive period were analysed by fitting a logistic regression model, it was seen that both the seed family and inoculation medium significantly affected the success of SE induction, while the collection dates between 7 July and 31 July and the cold treatment of the cones had no significant effect on induction (Tables 2, 3). Neither were there any differences among the cold treatments of different duration, i.e. the cones kept at $+5\,^{\circ}C$ for 14 d, and 1 or 2 months. The embryogenic capacity of the seed families was different (Tables 2, 3). The greatest number of cell lines ($n=63$) was obtained from K908, and the smallest number of cell lines ($n=2$) from E725. SE induction in K779, K942 and K1009 was totally unsuccessful. The effect of the inoculation media also varied (Table 3), the DCR1 and DCR3 media being comparable with each other and better than the DCR2 medium. There was no statistical interaction between the seed families and media used.

Microscopical observations of the explant material

According to the observations made with a stereomicroscope, 40% of the early stage seeds had a transparent outer part of their megagametophyte, while the centre of the megagametophyte was white. Only the cell nucleus was visible in toluidine blue staining, and the cytoplasm had a large vacuolar area in relation to the cell area, and there were no particles in the cytoplasm (Fig. 2A). Protein-rich vacuoles and vesicles were few in the early stage seeds, but there were large and frequent starch grains in the centre of the megagametophyte (Table 4).

In the late stage, the outer part of the megagametophyte was white and not transparent. The cells were well stained and had a large number of cytoplasmic compartments in the histochemical sections. The protein-rich vacuoles and vesicles frequently contained protein bodies (Fig. 2B). These protein bodies had a few small deposits on their surface, i.e. globoid cavities. In addition, there were large and frequent starch grains in the centre of the megagametophyte.

There were differences between the early and late stage seeds, as well as between the controls and cold-treated seeds in the cold treatment. A transparent outer megagametophyte was observed in the controls at the early stage

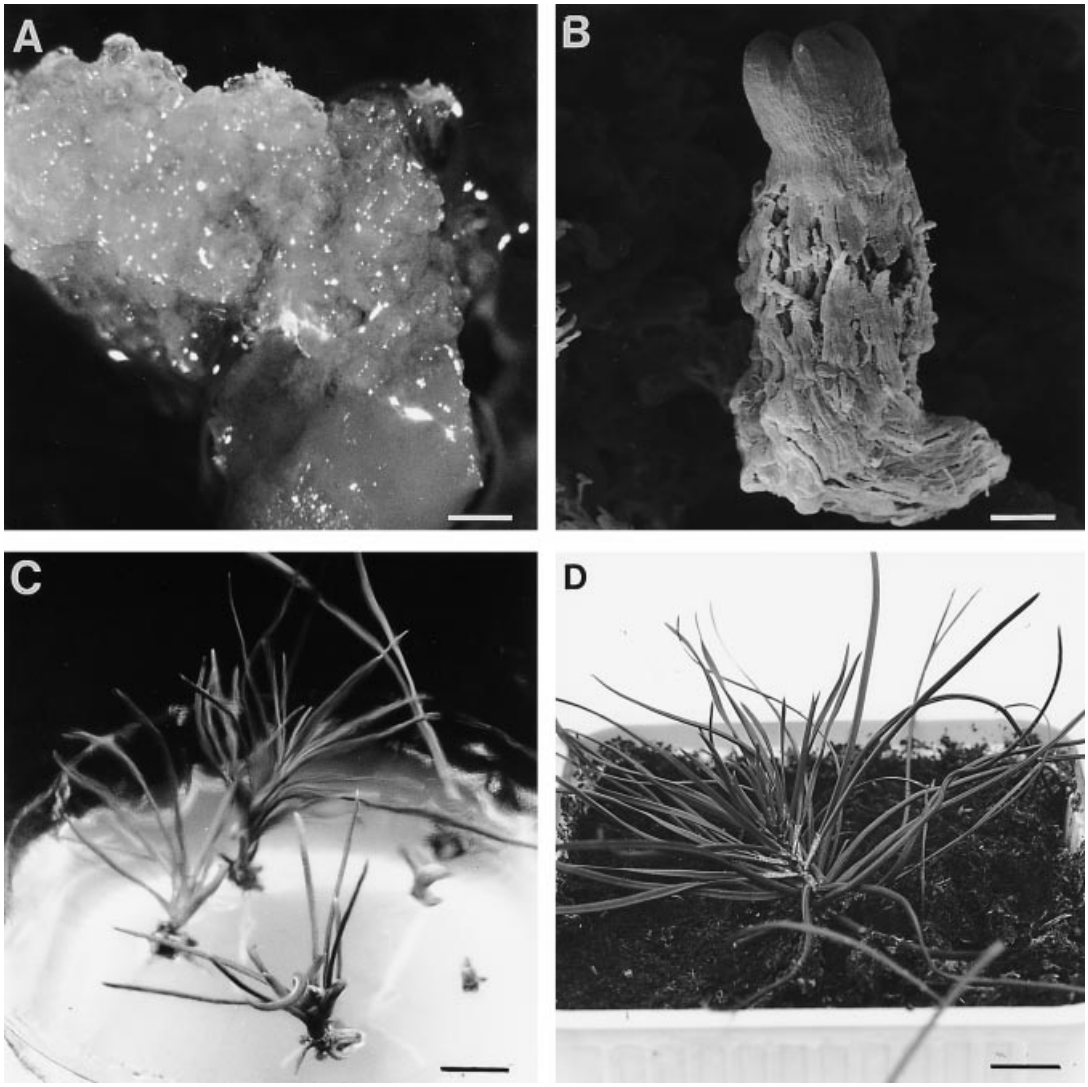

Fig. 1. Production of somatic embryo plants in Scots pine, cell line K884/6. (A) Embryogenic cell mass protruding from an explant 6 weeks after culture initiation. Scale bar represents 0.8 mm. (B) A scanning electron micrograph representing a maturing cotyledonary-stage somatic embryo. Scale bar represents 0.25 mm. (C) Germinating somatic embryos with elongating root system in the tissue culture jar. Scale bar represents 1.8 cm. (D) A somatic embryo plant in the greenhouse after the first growing season. Scale bar represents 0.9 cm.

(72% of seeds), but seldom in the other treatments where it was white and the cells contained abundant cytoplasmic compartments. The cold treatment did not affect the number of protein-rich vacuoles and vesicles, which were most frequent in the late stage, but there were differences in the occurrence of starch grains (Table 4). The starch grains were large and abundant in the controls of early as well as late stage seeds (Fig. 2C), but small and few in the cold-treated ones.

The developmental stages of the embryo and embryonal suspensors are presented in Table 4. Female gametophytes with a proembryo have no developed suspensors (Fig. 2D), while in gametophytes with an early embryo the suspensor system already exists and elongates and the corrosion cavity develops at the same time (Fig. 2E). Polar meristems (root and shoot) already exist in the late embryo stage. The primary embryonal suspensors have one crop of suspensors (representing Es_1 in Fig. 130C in Singh, 1978), as seen also in Fig. 2E, and the secondary ones also have secondary crops of suspensors (Es_2 in Singh, 1978).

There was a proembryo or early embryo in the gametophytes in the early stage (Table 4). The early embryos contained only two cells, as seen in the longitudinal sections (Fig. 2E). The embryonal suspensors were primary ones and the maximum length of the corrosion cavity was one-third that of the megagametophyte (Fig. 2E). The early embryos in late stage were larger, with 2–10 cells. The embryonal suspensors were primary or secondary, and the maximum length of the corrosion cavity was half that of the megagametophyte. The cold-treated samples had a proembryo or early embryo in the early

Table 3. *Multivariate association between the success of induction of somatic embryogenesis, seed family, collection date within the period from July 7 to July 31, initiation medium, and cold treatment of cones prior to initiation*

The logistic regression model generated was log $(p/1–p) = -4.47 - 1.23s_1 - 17.2s_2 - 0.814s_3 + 0.925s_4 + 1.59s_5 - 17.2s_6 - 17.2s_7 - 1.28i_1 + 0.009i_2$. In the model s_1, s_2, s_3, s_4, s_5, s_6, and s_7 are the design variables for the seed families E725, K779, K800, K884, K908, K942, and K1009, respectively, and i_1 and i_2 are the design variables for the initiation media DCR2 and DCR3, respectively.

Variable	*P*-value	Odds ratio	95% confidence interval
Seed family	<0.0001		
K395		1	
K725		0.294	0.061–1.42
K779		0[a]	
K800		0.443	0.114–1.72
K884		2.52	1.05–6.08
K908		4.90	2.15–11.2
K942		0[a]	
K1009		0[a]	
Collection date	0.8633		
Initiation medium	0.0004		
DCR1		1	
DCR2		0.277	0.126–0.613
DCR3		1.01	0.589–1.73
Cold treatment of cones	0.6087		

[a]With these seed families the induction of somatic embryogenesis was unsuccessful, i.e. only negative response was observed causing the odds ratios to approach nought.

stage, but always an early embryo in the late stage. Early embryos had up to 12 cells in the cold-treated samples in the late stage (Fig. 2F). The embryonal suspensors in the cold-treated early stage samples were primary, while in the cold-treated samples in the late stage secondary ones were also observed. The length of the corrosion cavity was up to half that of the megagametophyte in cold-treated samples in the late stage, but in other cases only up to one-third.

Discussion

The present study is the first report on the regeneration of somatic embryo plants in Scots pine. In pines, explants including immature zygotic embryos have so far been the most successful material for initiating embryogenic cultures. In *Pinus elliottii* (Newton *et al*., 1995) the immature zygotic embryos have been used as explants, in *P. taeda* (Becwar *et al*., 1990), *P. pinaster* (Bercetche and Pâques, 1995), *P. radiata* (Smith *et al*., 1994), and *P. strobus* (Kaul, 1995) both immature zygotic embryos and female gametophytes have been used, and in *P. lambertiana* (Gupta, 1995) and in *P. nigra* (Salajova *et al*., 1995) the female gametophytes have turned out to be the explant material of choice. In the case of Scots pine, the use of female gametophytes as explant material is, in practice, the only choice, owing in part to the small seed size (thousand seed weight between 3 and 4.5 g).

The optimum stage of immature zygotic embryo development for the initiation of embryogenic cultures in *Pinus* species has been the precotyledonary stage, when fertilization has occurred but the cotyledons have not yet been formed (Gupta and Durzan, 1987; Newton *et al*., 1995; Keinonen-Mettälä *et al*., 1996). In the present study, the window for initiation in 1996 was a period lasting for about 3 weeks in July. During this period, the developmental stage of the zygotic embryos was either proembryo or early embryo (up to 12 cells). Early embryos with up to 100 cells were found at the time of initiation (Keinonen-Mettälä *et al*., 1996). In the present study, the last collection date in 1996 when initiation of embryogenic cultures was found was 31 July. Thus, if the collections in the present study had been continued, later initiations might also have been possible.

In the present study, the megagametophyte was often transparent and had only a few cellular compartments in the early stage seeds indicating an early developmental stage. A few starch grains in the megagametophyte of *Pseudotsuga menziesii* collected in the beginning of June soon after fertilization has been observed (Owens *et al*., 1993). It was reported that, before the beginning of embryogenesis in *Pinus resinosa*, the megagametophyte appears to be filled with transparent, viscous gel and is made up of very thin-walled cells (Gains and Greenwood, 1991). In contrast, the megagametophytes in the present study were transparent even when early embryos were present in early-stage samples. The protein-rich vacuoles and vesicles were still developing, and no fully developed protein bodies in the cells of the megagametophyte (as described by Simola,1974; Krasowski and Owens,1993) were observed in the present samples. It has been reported that there is a marked decline in the ability of the embryo to form somatic embryos as the storage proteins accumulate (Roberts *et al*., 1989). The protein bodies in the embryo that have been observed (Flinn *et al*., 1991) contained two differently stained protein-zones as in the

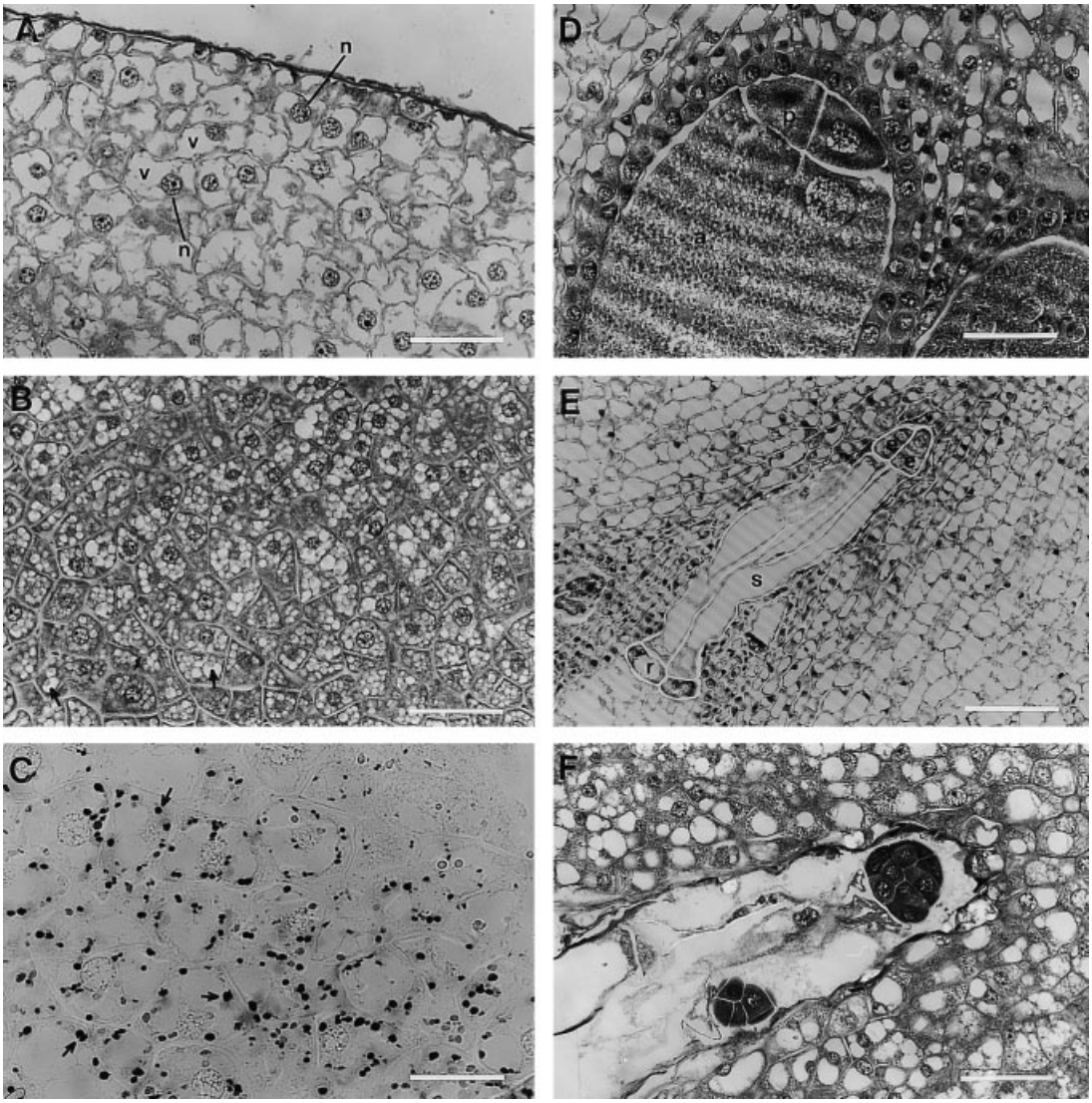

Fig. 2. Microscopical observations on immature female gametophytes, used as explants for embryogenic cultures, showing the development of the zygotic embryo and suspensor tissue as well as the megagametophyte during the collection period. (A) Megagametophyte cells with nucleus (n) and large vacuole (v) of the 11 July sample from seed family K884. No other cellular compartments are visible in the collection dates when the first somatic embryogenesis initiation occurred. (B) Megagametophyte cells of the 25 July sample from seed family K884 are well stained and have a large number of cytoplasmic compartments such as protein bodies (arrow). These compartments are typical for those collection dates when the initiation of somatic embryogenesis occurred for the last time or the collection series ended. (C) The large abundant starch grains (arrow) in the central cells of the megagametophyte of the 25 July sample from seed family K395, IKI-staining. (D) Proembryo in archegonia (a) of the 11 July sample from seed family K884. There are no suspensors, p, proembryonal cell. (E) Two early embryos (e) with primary embryonal suspensors (s) in a gametophyte of the 16 July sample from seed family K395, cold-treated for 1 month. Corrosion cavity length is about one-third that of the megagametophyte. Rosette cells (r) are visible in the archegonia. (F) The largest early embryo observed in this study with 12 cells in longitudinal section in a gametophyte of the 31 July sample from seed family K908, cold-treated for 1 month. Scale bars in (A), (B), and (F) represent 100 μm, in (C) and (D) 50 μm and in (E) 200 μm.

present study. This was also observed in electrophoresis as two dominant sizes of protein in the megagametophyte (Flinn *et al*., 1991). The megagametophyte can probably provide extra nutrients and/or growth regulators, which may be suboptimal in the culture medium (Becwar and Pullman, 1995), and therefore the developmental stage of this tissue is also important for the success of embryogenic culturing.

The initiation of somatic embryogenesis in *Pinus lambertiana* (Gupta and Durzan, 1986), *P. taeda* (Becwar *et al*., 1990) and *P. pinaster* (Bercetche and Pâques, 1995) is suggested to originate from cell divisions at the interface of the suspensor and embryogenic cells, or are a result of continued cleavage of the embryogenic area. In the present samples, the embryonal suspensors were most developed at the end of the collection period, indicating that the

Table 4. *Anatomical observations of the development of immature female gametophytes during the responsive period of induction of somatic embryogenesis*

Early stage means the collection dates when initiation of somatic embryogenesis was successful for the first time, and late stage means the dates when initiation was successful for the last time or the collection series ended. In the one-month cold treatment the control samples originate on the same collection dates as treated ones but without cold storage.

Tissue	Sampling at cone Collection date		Cold treatment			
			Early stage		Late stage	
	Early stage	Late stage	Control	Cold-treated	Control	Cold-treated
Megagametophyte						
Protein rich vacuoles and vesicles	Few	Frequent	Few	Few	Frequent	Frequent
Starch grains	Large and frequent	Large and frequent	Large and frequent	Small and few	Large and frequent	Small and few
Zygotic embryo						
Developmental stage	Proembryo/ early embryo	Early embryo	Proembryo/ early embryo	Proembryo/ early embryo	Early embryo	Early embryo
Number of cells, longitudinal section	2	2–10	2	2–5	2–5	2–12
Suspensors						
Developmental stage	Primary	Primary/ secondary	Primary	Primary	Primary	Primary/ secondary
Corrosion cavity						
Length in relation to megagametophyte	Max 1/3	Max 1/2	Max 1/3	Max 1/3	Max 1/3	Max 1/2

suspensors were moving the early embryos towards the chalazal end of the gametophyte. The corrosion cavity was developing at the same time. Proliferation of SE cultures from the explant was visible 4–8 weeks after culture initiation, indicating that induction of these tissues has probably taken place only after a time lag on the initiation medium. The zygotic proembryos or early embryos with suspensors found in the microscopical samples during this induction period may have continued their development within the megagametophyte, thus providing a suitable origin for the embryogenic cultures.

Cold treatment of the cones or the duration of cold treatment before inoculation had no effect on the SE induction frequency in the present study. This observation is encouraging for practical reasons. The cones can be collected and stored for at least 2 months without losing the ability to initiate somatic embryogenesis. The observation under the microscope revealed, however, that cold treatment of the cones sampled at the end of the collection period slightly enhanced embryo development. During the cold treatment the gametophytes were still in the cones, and it appears that a temperature of $+5\,^{\circ}C$ was high enough for the continuing development of female gametophytes. Owens *et al*. reported that starch is abundant in the area of the megagametophyte, which breaks down to form the corrosion cavity, and that the starch is rapidly hydrolysed as the early embryos are forced into the megagametophyte by the elongating suspensors (Owens *et al*., 1993). In the present study, abundant large starch grains were correspondingly observed in this part of the megagametophyte especially in the controls of the cold treatment. Conversion of starch to sugars, inducing the increase in the osmotic potential of the cells, is typical at low temperatures, while the metabolism of sugars also provides energy for the plant (Sakai and Larcher, 1987).

The percentages of initiation of embryogenic cultures in pine species are typically low. As reviewed earlier (Becwar and Pullman, 1995), they vary between 1% and 5%. In some species, the initiation percentages for somatic embryogenesis have been slightly higher, 15% in *Pinus pinaster* (Bercetche and Pâques, 1995) and varying in *P. strobus* from 2.6% to 23% (Garin *et al*., 1998). The earlier report of initiation percentages in Scots pine being between 0.2% and 9% (Keinonen-Mettälä *et al*., 1996) is in agreement with the average initiation percentages varying from 0.2% to 4% obtained in the present study.

The fact that the tissue culture medium has an effect on the initiation success has been pointed out in several reports. In *P. caribaea* (David *et al*., 1995) different cell lines were found to express different nutritional requirements. Several initiation media have been tested for the induction of somatic embryogenesis in pines. One of the most common media used is the DCR basal medium (first described by Gupta and Durzan, 1985). In *P. taeda* (Becwar *et al*., 1990) and *P. patula* (Jones *et al*., 1993) the media with both 2,4-D and BA as growth regulators were better than media without growth regulators or only 2,4-D. In the present study, cytokinin as the sole growth regulator in the DCR-initiation medium for Scots pine was not as effective as the media with both 2,4-D (13.6 μM or 9.1 μM) and BA (2.2 μM) for the induction of somatic embryogenesis.

The results of the present study emphasize the effect of seed family on the success of somatic embryogenesis induction in Scots pine. In 1995, the initiation succeeded in 28% of the unknown seed families, and in 1996 the initiation succeeded in 75% of the seed families selected partly according to the success during the previous year. The effect of mother tree genotype has also been pointed out in *P. taeda* (Becwar *et al.*, 1990), *P. pinaster* (Bercetche and Pâques, 1995) and *P. strobus* (Garin *et al.*, 1998).

In the present study, a somatic embryogenesis protocol for Scots pine with somatic embryo plants in the greenhouse has been developed. The relatively low initiation percentages and the significant effect of the genetic background of the explants and initiation medium reveal that it might be possible to improve the initiation rates by using explants from controlled crossings between competent genotypes and by developing more specific media for important seed families. As is also the case for other pine species, improvement of the maturation and conversion phases of somatic embryogenesis is needed in order to develop a method applicable for practical forestry.

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