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Early-flowering Scots pines through tissue culture for accelerating tree breeding

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Abstract Scots pine plantlets were produced via tissue culture using cotyledons excised from germinated embryos as explants. The optimum tissue culture conditions were: ½GD basal medium gelled with agar-Gelrite during shoot formation and with agar during rooting, inclusion of 5.0 μ*M* benzylaminopurine (BAP) and 0.05 μ*M* naphthaleneacetic acid (NAA) for 2 weeks for shoot induction, and repeated 2.7 μ*M* NAA pulses of 1 week for rooting. Micropropagation success was genotype-dependent. Average multiplication rates varied among experiments from 3 to 15 shoots per embryo. The maximum shoot production from a single embryo was 35. Rooting was the most difficult phase in the propagation process. Most of the plantlets had a plagiotrophic and highly branched growth habit when growing in the greenhouse. Some individuals produced megasporangiate strobili at the age of 3 years and microsporangiate strobili with viable pollen at the age of 4 years. Early-flowering clones and the ability to conserve seedlings from which cotyledons have been cultured give new possibilities for accelerated tree breeding.

Key words *Pinus sylvestris* · Scots pine · Tissue culture · Early maturation · Flowering

Introduction

One of the most serious problems confronting Scots pine breeding is the long generation interval (Danell 1993). Scots pine individuals start to produce seed at the age of 8–20 years when growing as single trees (Sarvas 1964). Grafted individuals in the seed orchards form megasporangiate strobili from the age of 4–10 years, with pollen production starting a few years later (Bhumibhamon 1978). While methods such as fertilization, thinning, mechanical injury, and growth regulator treatments have been used to stimulate flowering (Chalupka 1991a), the need still exists for methods that would shorten generation time, and consequently, greatly benefiting forest tree breeding.

Conifers, especially pines, are generally regarded as being difficult to asexually propagate, and *Pinus radiata* is the only species for which vegetative propagation methods have been applied on a practical forestry scale (Aitken-Christie et al. 1988; Gleed 1993). In vitro propagation based on organogenesis (Sommer et al. 1975; Mott and Amerson 1981; Amerson and Mott 1982; Mudge 1986; Gladfelter and Phillips 1987; Baxter et al. 1989; Chang et al. 1991; Bergmann and Stomp 1992; Lambardi et al. 1993; Sen et al. 1994) or somatic embryogenesis (Becwar et al. 1990; Jones et al. 1993; Jones and van Staden 1995) has been successful for several other pine species on a more limited research scale.

Scots pine (*Pinus sylvestris* L.) tissue culture via organogenesis with different types of explants has also been successful for small-scale research purposes (Bornman and Jansson 1980; Mohan Jain et al. 1988; Zel et al. 1988; Chalupa 1989). The further development and characteristics of the plantlets has, however, not been reported in these studies. Supriyanto and Rohr (1994), on the other hand, described the Scots pine plantlets regenerated from a subculturable organogenic line as having the same growth habit as seedlings. Somatic embryogenesis has also been investigated in Scots pine, but attempts to germinate the first somatic embryos that developed failed (Hohtola 1995).

The aim of the investigation presented here was to develop a tissue culture propagation method for Scots pine, i.e. to investigate the effects of different media components, anatomical changes in explant material, and cold storage from the efficiency of adventitious shoot formation, shoot vigor, and rooting as well as growth habit, elongation and maturation of plantlets in greenhouse. The

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approach was taken to identify a combination of variables for the production of Scots pine plantlets suitable for genetic studies and accelerated tree breeding.

Material and methods

Seed source, sterilization and general tissue culture conditions

Scots pine (*Pinus sylvestris* L.) cotyledons excised from germinated embryos were used as explant material. Open-pollinated seed was collected from a seed orchard in Finland. Seeds were surface-sterilized by soaking in 2% calcium hypochlorite for 20 min followed by three rinses with sterile distilled water. Surface-sterilized seeds were germinated in petri dishes containing filter papers moistened with 1% H_2O_2 under continuous light, at 26°C and 70% humidity. Seeds were germinated for 1–7 days in the experiment to test the effect of germination time. Four-day germination was used for all other experiments. After germination, seed coats were removed, and embryos still encased within the megagametophytes were re-sterilized by soaking in 1% Ca-hypochlorite for 8 min, followed by three rinses with sterile distilled water. Embryos were dissected from megagametophytic tissue and the cotyledons (5–7) were excised and dispersed onto tissue culture media.

Explants were grown under 16:8-h light-dark photoperiod (67–74 $\mu E\ m^{-2}\ s^{-1}$) at 24°C. Cotyledons were first maintained on shoot induction media containing 0.05 μM α-naphthaleneacetic acid (NAA) and 5 μM 6-benzylaminopurine (BAP), after which they were transferred to growth regulator-free media. Explants were transferred to fresh media every 3–4 weeks thereafter. Proliferated shoots were excised and transferred to root induction media with 2.7 μM NAA. Roots were allowed to grow out on growth regulator-free media for 4 weeks after the root induction period, and plantlets were then transferred to greenhouse conditions. For shoots that had not developed roots after the first NAA treatment, the root induction pulse followed by a root growth period was repeated twice. Root induction and rooting media were always solidified with 1% agar. Following cotyledon growth and differentiation, cotyledon length was measured, emerging shoots were counted, and frequency of rooted shoots was noted.

Basal media and gelling agents

Four different tissue culture media were tested: (1) GD-medium was the same as described by Gresshof and Doy (1972). (2) ½GD-medium included half of the micronutrients, macronutrients, and organics of those in GD-medium, with sucrose concentration the same (%) in both media, (3) L-medium was prepared according to Litvay and co-workers (1981), and (4) modified MS-medium (Murashige and Skoog 1962) included micronutrients, macronutrients, and sucrose as described by Brown and Lawrence (1968) and the following organic compounds, 9.7 μM nicotinic acid, 1.4 μM pyridoxin HCl, 10 μM thiamine HCl, 0.1 μM biotin, 0.5 μM D-Ca-pantothenate, 0.5 μM riboflavin, 0.5 μM ascorbic acid, 0.5 μM choline chloride, 2.5 μM glysine, 17 μM L-cystine, and 150 μM inositol. Each medium was solidified with either 1% agar or 0.06% agar and 0.19% Gelrite (Merck). Both shoot and root induction pulses lasted for 2 weeks. A randomized design was used to test basal media and gelling agent. Cotyledons from 10 embryos (genotypes) were plated on each of the eight medium/gelling agent combinations in each of three replications. Thus, a total of 240 embryos were used. Cotyledon viability and growth and number of shoots were observed for 8 weeks.

Effect of growth regulator concentration and exposure time on shoot formation

Different growth regulator combinations were tested using ½GD-medium solidified with agar and Gelrite during shoot induction. The growth regulator concentrations tested were: 0, 1, 5, 10, and 20 μM BAP and 0, 0.05, and 0.5 μM NAA. Explants remained on shoot induction media for 2 weeks before transfer to growth regulator-free medium. The root induction pulse lasted for 1 week. The experiment was a full-factorial design. Cotyledons from 12 embryos were plated on each of the 15 BAP/NAA combinations. There were two replications, and a total of 360 embryos were used. Cotyledon viability and number of adventitious shoots ready for rooting was followed for 20 weeks.

The effect of the cytokinin-pulse duration on shoot induction was tested by using GD-medium containing 0.05 μM NAA and 5 μM BAP that was solidified with agar and Gelrite. Cotyledons were kept on induction medium for 1, 2, 4, 6, 8, or 10 weeks before being transferred to growth regulator-free medium. The 1-week root induction pulse and rooting period were performed on ½GD-medium. Cotyledons from 20 seeds, divided over 5 plates, were used for each of the six induction times. There were two replications, and a total of 240 embryos were used. Cotyledon viability and number of adventitious shoots ready for rooting was followed for 20 weeks.

Effect of activated charcoal on shoot formation and rooting

The effect of activated charcoal on shoot formation and rooting was tested using three basal media, MS, GD and ½GD. MS and GD were solidified with agar and Gelrite, and ½GD with agar only. The shoot induction period was 4 weeks, and the root induction pulse was 1 week. ½GD was used for root induction and rooting. Cotyledons from 8 seeds were cultured on each of 16 plates for each of the three media. Thus, a total of 384 embryos were used. Cotyledons from 48 seeds (6 plates) were treated for 1 week on media containing 0.1% activated charcoal in the third passage. Cotyledon differentiation, number of shoots, and root formation were followed for 24 weeks.

Anatomical observations of cotyledon differentiation

The anatomical structure of differentiating cotyledons was examined using cotyledons fixed in FAA (formalin: acetic acid: 95% ethanol, 10:5:85, v/v/v), embedded in paraffin and stained with safranin-fast green (Gerlach 1984). Surface structures of differentiating cotyledons were studied using a scanning electron microscope (SEM) JEOL JSM-820. The glutaraldehyde (6.25%)-fixed material was dehydrated in an alcohol gradient, dried by the Critical Point method, mounted on SEM adapters with double-sided adhesive tape, and coated with gold according to the manufacturers' instructions.

In vitro cold storage of rooted plantlets

The effect of in vitro cold storage was studied by maintaining rooted plantlets (n=242) on ½GD medium for 5–7 months in +2°C, in the dark. After cold treatment the plantlets were taken back to normal tissue culture conditions through an 8-day adaptation programme. Over an 8-day period photoperiod and temperature were increased gradually from 2 h to 16 h and from +3°C to 24°C, respectively. New nutrients were provided to the plantlets by adding liquid ½GD-medium to the tissue culture medium (20% of medium volume). Plantlet growth was followed under tissue culture conditions for 1 month, after which the material was transferred to the greenhouse.

Adaptation and growth characteristics of plantlets in the greenhouse

After in vitro culture the rooted plantlets (n=154) were adapted to greenhouse conditions. Planting was done in either garden peat: pine mull:perlite (1:1:1) or garden peat:perlite (1:1). For the first 2 weeks in the greenhouse, plantlets were kept under mist and watered daily with liquid ½GD-medium. During the growing season, plantlets were fertilized twice monthly with commercial 0.2% 9- and 5-Superex fertilizers (Kekkilä). Between growing seasons the plantlets were over-wintered for approximately 6 months at 1°–6°C. Plantlet height, growth habit, and flower formation were observed during

several growing seasons. Pollen was collected from microsporangiate strobili.

In vitro pollen germination

Pollen collected from microsporangiate strobili was suspended in liquid medium for in vitro germination tests. The in vitro germination medium (pH 5.6) consisted of 1.62 m*M* H_3BO_3, 1.27 m*M* $Ca(NO_3)_2$*4 H_2O, 0.81 m*M* $MgSO_4$*7 H_2O, 2.20 m*M* KH_2PO_4, and 0.3 *M* sucrose following the method of Brewbaker and Kwack (1963) as refined by Muren et al. (1979). Pollen suspensions were aliquoted in 24-well plates and placed on an orbital shaker (Infors AG, 180 rpm) at +28°C in the dark for 24 h. Pollen viability was evaluated microscopically with pollen grains being regarded as germinated when the tube length exceeded the pollen diameter.

Embryo rescue for conserving original genotypes

The ability to conserve the original genotypes (seedlings) from which cotyledons were used as explants for tissue culture was tested. Approximately half of the cotyledons from each germinated embryo were excised, and the embryos were dispersed on ½GD agar medium without growth regulators in vitro, followed by greenhouse culture. Seeds were surface-sterilized and germinated, and the embryos were excised from megagametophytes. Germination ability of the seed lot under in vitro conditions was tested by dispersing surface-sterilized seeds on ½GD agar-medium without growth regulators. As control treatments, intact germinated seeds were replaced on the medium after re-sterilization in 1% Ca-hypochlorite for 8 min, or the seed coats were removed from germinated seeds, which were then surface-sterilized and dispersed on the medium. One hundred embryos were used for each of the four treatments (10 embryos per each of 10 tissue culture jars). The viability, size, and growth habit of the rescued embryos were followed for 2 weeks in vitro and for 4 months in the greenhouse.

Statistical analysis

Statistical comparisons among treatments were made by analysis of variance, and means were compared by the Tukeys test or by Student-Newman-Keuls multiple range test. A nonparametric Kruskal-Wallis-test followed by multiple mean comparisons were used when the data were not normally distributed. Pearson's correlation coefficients were calculated (BMDP 1988) in some cases.

Results

Across all of the experiments, explant genotype had a significant effect on shoot production and rooting. Average percentage of responding genotypes varied from 13% to 60% in the different experiments when ½GD with 5 μ*M* BAP and 0.05 μ*M* NAA gelled with agar and Gelrite were used. Shoot production varied from 3 to 15 from the cotyledons of a single embryo, with 35 being the maximum number observed. Rooting ranged from 0 to 100% across genotypes but averaged only 6%, indicating that root induction was the most problematic phase in the procedure.

The first adventitious shoots could usually be excised from the cotyledons and transferred to root induction medium approximately 6 weeks after their appearance (Fig. 1a). At this stage shoots look vigorous but rarely elongate properly. If the first pulse in the root induction is successful, the plantlets are ready to be transferred to ex

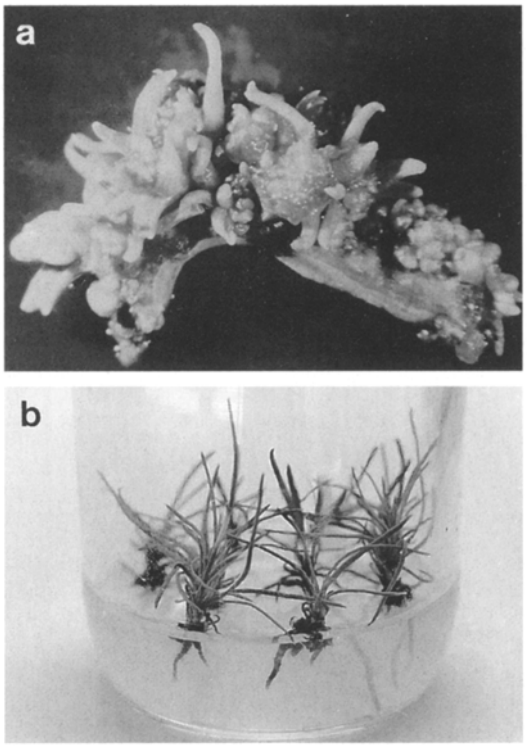

Fig. 1a, b Tissue culture production of Scots pine plantlets. **a** Cotyledon with adventitious buds after 2 months in culture, **b** rooted shoots in tissue culture jar

vitro conditions 4 months after the beginning of propagation (Fig. 1b). Apical bud formation was observed in some of the plantlets under tissue culture conditions.

Basal media and gelling agents

Of the four tested basal media (modified MS, GD, ½GD and L), ½GD proved superior on the basis of cotyledon viability and the number of shoots produced per seed (Table 1). The increase in cotyledon length was greatest on modified MS (Table 1). However, no correlation was observed between an increase in cotyledon length and shoot production (Pearson's r=0.145; P=0.113).

Agar and agar-Gelrite combinations were tested as gelling agents for tissue culture media during the shoot induction and proliferation periods. When cotyledon length was examined in the different basal media (Table 1) the greatest increase was observed on media gelled with agar-Gelrite. Also, agar-solidified media supported less shoot formation relative to media solidified with agar-Gelrite (Table 1).

Effect of growth regulator concentration and exposure time on shoot formation

The effect of different BAP and NAA combinations on shoot induction is presented in Fig. 2. Shoot induction was great-

Table 1 Effect of basal tissue culture media on survival, growth (after 4 weeks in vitro) and shoot production (after 20 weeks in vitro) of Scots pine cotyledons. The best means (±SE) are boxed (*MS* modified Murashige and Skoog, *GD* Gresshoff and Doy, *A* agar, *AG* agar and gelrite, *ND* not determined)

Medium		MS		GD		½GD		L	
Gelling agent	*n*	A	AG	A	AG	A	AG	A	AG
Viability (%)	240	69±6	74±6	75±6	67±7	83±4	86±4	70±6	22±5[d]
Growth (mm)	976	2.20±0.09[a]	3.20±0.13[b]	1.96±0.08	2.26±0.09[a]	1.67±0.06	2.38±0.09[a]	1.12±0.04	1.55±0.07
Shoots per seed	120	0	1.35±0.74	0	1.35±0.69	0.10±0.10	4.55±2.09[c]	ND	ND

[a] Growth of cotyledons was significantly worse than on the MS/AG medium, but better than on the other media, $P<0.05$
[b] Growth of cotyledons was significantly better than on the other media, $P<0.05$
[c] Significantly more shoots than on the other media, $P<0.05$
[d] Viability of cotyledons was significantly worse than on the other media, $P<0.01$

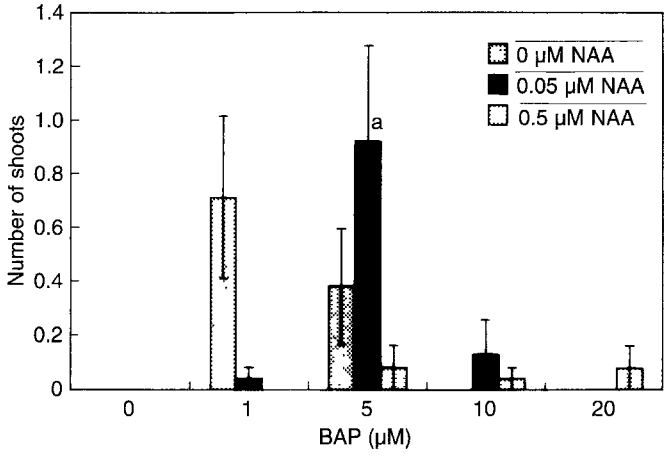

Fig. 2 Effect of different growth regulator combinations on shoot formation in Scots pine cotyledons after 20 weeks in vitro. **a** Significantly ($P<0.05$) more shoots than in other treatments except in 0 µ*M* NAA/1 µ*M* BAP and 0 µ*M* NAA/5 µ*M* BAP

est on medium containing 0.05 µ*M* NAA and 5 µ*M* BAP (Fig. 2). The duration of the shoot induction period had no effect on cotyledon viability but did affect shoot formation. Shoot induction using 1- or 2-week cytokinin exposures was significantly better than a long exposure to cytokinin ($P<0.05$), producing 1.9±0.59 and 1.2±0.28 shoots per seed, respectively. Shoot production was greatly reduced (0.35±0.18) using a 4-week cytokinin induction period, and no shoots were formed using 6-, 8-, or 10-week exposures.

Effect of activated charcoal on shoot formation and rooting

Differentiating cotyledons were treated in the third-medium passage by 1 week on shoot proliferation medium including 0.1% activated charcoal. The inclusion of charcoal caused a small but significant ($P=0.0227$) increase in shoot induction: 1.1±0.37 per seed when cotyledons were grown on the charcoal medium compared with 0.95±0.38 on the control medium. Rooting did not differ among treatments.

Anatomical observations on differentiation of cotyledons

When comparing newly excised cotyledons (Fig. 3a), we observed that differentiation of adventitious buds initiates at the areas near the cotyledon tip and proceeds towards the basipetal end of the cotyledon. After 1 week on tissue culture medium, the triangular shape of the cotyledon begins to change. Epidermal walls have thickened, mesophyll cells have begun to autolyse, leading to an increase in intercellular spaces, and a central differentiated vascular system is clearly visible (Fig. 3b, d). At this time, the first cell divisions leading to the development of adventitious buds can be found in the superficial cell layers. In some cases cell divisions leading to adventitious buds initiates next to the developing stomata (Fig. 3c) or in the mesophyll (Fig. 3d). After 2 weeks on tissue culture medium, the initials of adventitious buds and single needles are recognizable in SEM (Fig. 3e) and paraffin sections (Fig. 3f). At this stage the nucleus:cell volume ratio is high in the newly developed tissues. After 3 weeks, elongation and further differentiation of the adventitious buds proceeds (Fig. 3g,h), and the nucleus:cell volume ratio decreases.

In vitro cold storage of rooted plantlets

Micropropagated, rooted plantlets survived cold storage in vitro: 98% of the plantlets (236/242) were viable after 5–7 months. Further growth of these plantlets started from the root systems. Actively growing root tips were observed 3 weeks after transfer to a warm environment. At the time of transfer to greenhouse conditions, plantlet elongation had just started.

Adaptation and growth characteristics of plantlets in greenhouse

Most of the Scots pine plantlets produced through tissue culture (139 of 154) were plagiotrophic and ramified (Fig. 4a), with only a portion of the plantlets exhibiting a juvenile growth habit (Fig. 4b). The growth habit, either

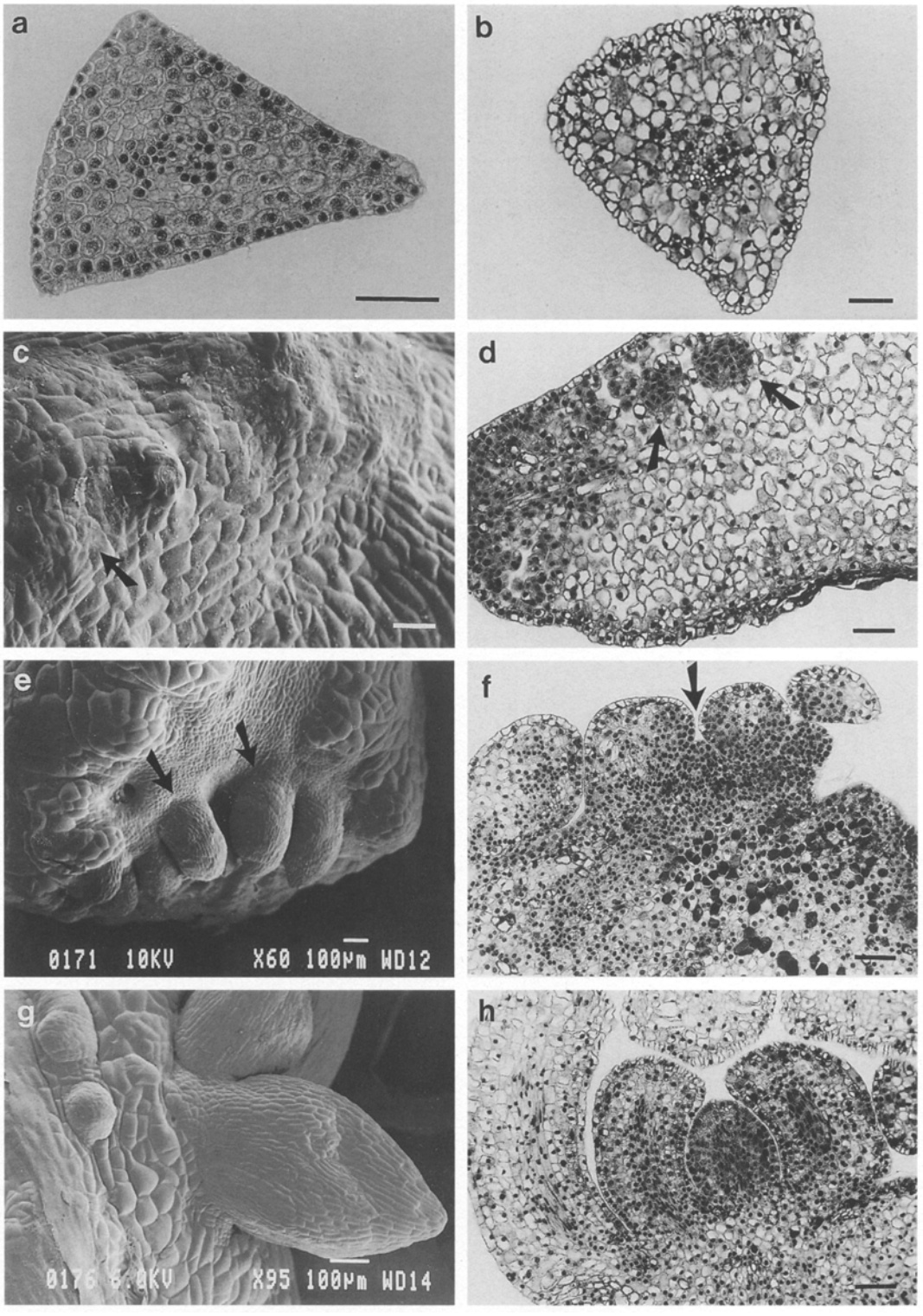

Fig. 3a–h Anatomical observations of differentiation in Scots pine cotyledons in tissue culture. **a** Transection of a cotyledon excised from an embryo allowed to germinate for 4 days, **b** transection of a cotyledon after 1 week on tissue culture medium. **c** scanning electron micrograph showing the initiation of differentiation adjacent to a stomate located near the tip of the cotyledon (*arrow*), **d** differentiation in mesophyll (*arrows*) after 9 days in vitro, **e** and **f** initials of single needles (*arrows*) and a bud (*arrow*), respectively, after 2 weeks as seen in SEM (**e**) and in paraffin sections (**f**), **g** and **h** growth and differentiation of adventitious buds after 4 weeks as seen in SEM and paraffin sections respectively. *Bars*: 100 µm

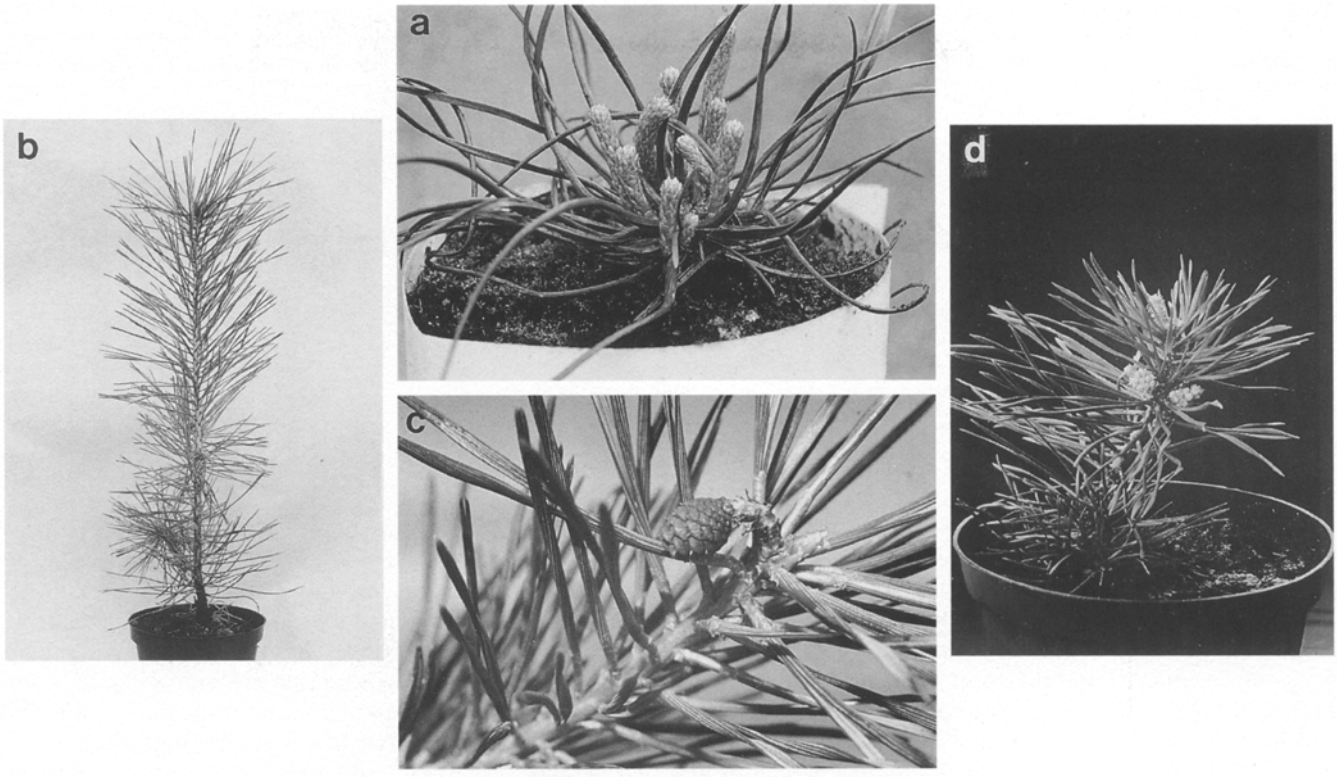

Fig. 4a–d Characteristics of Scots pine plantlets in greenhouse **a** Plagiotrophic, ramified growth habit of a plantlet under greenhouse conditions. **b** a plantlet having a juvenile growth habit at the age of 3 years. **c** a 3-year-old plantlet with a megasporangiate strobilus. **d** a 4-year-old plantlet with microsporangiate strobili

maturate or juvenile, was usually maintained for several years. The proportion of orthotropically growing plantlets was 10%, and half of the plantlets grew at an angle of 45 degrees or less to the horizontal plane. The proportion of plantlets with a single stem was 47%. The average height of the plantlets was 7 cm (±SE 0.5) after 1½ years in the greenhouse, and 22 cm (±1.0) after 3½ years.

Of the micropropagated plantlets 3% produced megasporangiate strobili (Fig. 4c) for the first time at the age of 3 years, and 1 year later the number of plantlets with megasporangiate strobili had doubled. The first microsporangiate strobili (Fig. 4d) were observed at the age of 4 years, when 3% of the plantlets were flowering. In vitro viability of the pollen collected from these microsporangiate strobili was 67%.

Embryo rescue for conserving original genotypes

Results of the embryo rescue experiment are shown in Table 2. Fifty percent of the embryos survived surface-sterilization, removal of megagametophyte, and excision of cotyledons, and grew as seedlings in the greenhouse for 4 months after embryo rescue. Surface-sterilization decreased the number of viable seedlings in the control treatments when compared with the germination ability of the seed lot. The decrease in viability was more severe when the seed coats were removed before surface-sterilization. During in vitro culture, some of the seedlings in every treatment lost normal polarity. Under greenhouse conditions, seedlings from which a portion of the cotyledons had been excised were smaller than seedlings grown from control intact germinated seeds.

Discussion

The success of Scots pine organogenesis is strongly dependent on the genotype of the explants. The present study used seed orchard-produced, open-pollinated seeds to examine applicability of this method to a wide range of genotypes. Sen and co-workers (1994) were able to produce more shoots in Afghan pine using half-sib seed as explants than when using mixed orchard seed. In radiata pine, in vitro shoot production from cotyledon explants could be improved approximately 100% through family selection for tissue culture ability (Bergmann and Stomp 1994). Given the variability in in vitro performance observed in our work with Scots pine, the use of seed from certain controlled superior crosses or half-sib seed from selected elite trees may result in higher in vitro shoot production. Improved tissue culture ability may be due to either genotypic effects or better seed quality, or both. Abo El-Nil and Wochok (1986) found that Douglas fir cotyledons from full-sib families gave substantially more bud induction than cotyledons from wild stock, and a strong positive correlation between seed weight and in vitro bud induction was ob-

Table 2 In vitro development and greenhouse survival of rescued Scots pine embryos

Explant material on tissue culture media	Development of seedlings in vitro (%)				Viability of seedlings in greenhouse (%)	
	Normal	Abnormal[1]	Slow germination	Dead	2 weeks	4 months
Rescued embryos	39	61	0	0	50	50
Control treatments:						
Seeds	88	0	6	6	Not determined	
Germinated seeds	33	28	14	25	56	52
Germinated seeds without seed coats	27	45	8	20	46	36

[1] Seedlings lacking normal polarity that grew upside down or had a horizontal growth habit

served in the same data. However, Bergmann and co-workers (1995) found no relation between tissue culture ability and field performance traits.

Explant material for Scots pine organogenesis has in all earlier reports been relatively young, derived from embryos or seedlings (Bornman and Jansson 1980; Mohan Jain et al. 1988; Zel et al. 1988; Chalupa 1989; Supriyanto and Rohr 1994). Mohan Jain and co-workers (1988) reported adventitious bud formation from the cotyledons of embryos germinated for 3–4 days, thus supporting the present results with excised cotyledons.

GD medium has been used successfully for tissue culture of *Pinus palustris* (Sommer et al. 1975), *Pinus taeda* (Mott and Amerson 1981), and *Pinus virginiana* (Chang et al. 1991), as well as for rooting of *Pinus radiata* (Reilly and Washer 1977). ½GD was proven to be best for Scots pine. Shoot and root formation were successfully induced in Scots pine by the pulsed growth regulator treatments that have been previously reported to be superior to constant treatments in *Pinus monticola* (Amerson and Mott 1982). The combination of agar and Gelrite provides the benefits of both gelling agents while minimizing the disadvantages of each (Bonga and von Aderkas 1992), as seen in the cases of radiata pine (Nairn 1986) and larch (McLaughlin and Karnosky 1989) micropropagation. In Scots pine it was necessary to combine agar and Gelrite to obtain shoot formation. Inclusion of activated charcoal has been found to stimulate the development of adventitious buds (Mehra-Palta et al. 1978; Martinez Pulido et al. 1990) and shoot elongation (Mudge 1986; Baxter et al. 1989; Chang et al. 1991) in several pine species. Also in Scots pine, activated charcoal caused an increase in shoot induction.

The major obstacle in the present method was the poor average rooting percentage. The addition of activated charcoal into the rooting medium (Grönroos and von Arnold 1985), the careful control of environmental conditions together with pretreatments (Horgan and Holland 1989), and the addition of mycorrhizae (Supriyanto and Rohr 1994) may be used to stimulate the root formation and to improve the transfer to ex vitro conditions. In Scots pine cuttings, rooting ability decreases with increasing age, as reviewed by Salonen (1990). Our difficulties in inducing roots in micropropagated Scots pine shoots may reflect the early maturation of the shoots.

Scots pine plantlets produced through tissue culture were relatively ontogenetically mature as evidenced by their plagiotrophic growth habit and early flowering. Our results agree with previous reports on conifer plantlets derived from adventitious buds induced on tissue-cultured zygotic embryos or cotyledons (Pierik 1990; Monteuuis and Dumas 1992; Frampton and Foster 1993). Anderson and co-workers (1992) showed that ontogenetic aging of the loblolly pine plantlets is induced by shoots and not by the indirect effect of the poorly developed plantlet root system.

Supriyanto and Rohr (1994) were able to regenerate juvenile Scots pine plantlets, but they started meristematic tissue cultures from a second generation of adventitious buds induced on the needles of in vitro axillary microcuttings. The in vitro rejuvenation of coniferous explants by increasing the length of in vitro culture or the number of sub-cultures has been described previously (reviewed by Hackett 1985). By choosing the tissue culture procedure it is possible to obtain juvenile or early-maturating Scots pine plantlets, depending on the goal.

In addition to the effect of the explant and tissue culture method, maturation might also be caused by stress-related factors. The root systems of the plantlets may not be fully developed, thereby causing reduced water and nutrient uptake or a deficiency in the growth regulators synthesized in roots, as seen in loblolly pine (McKeand and Allen 1984) and Douglas fir (Timmis et al. 1992). An independent study showed that the in vitro and greenhouse growth conditions for Scots pine roots used in the present study may be oxygen poor (Aronen and Häggman 1994). As reviewed by Hackett (1985), investigations with conifers suggest that stress caused by drought, high temperature, low soil oxygen levels, or any treatment that inhibits root growth tends to promote flowering.

Conservation of the original genotype after in vitro cotyledon excision was successful. Seedlings derived from bare embryos were smaller than intact seedlings, probably due to nutrient deficiency. This might be due to premature excision of the embryo from the haploid megagameto-

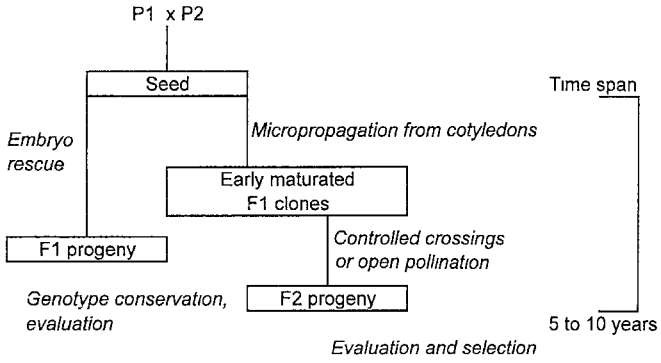

Fig. 5 Scheme for integration of the present vegetative propagation method into Scots pine breeding programmes

phytic storage tissue that provides carbon and energy for the germinating seedling. The loss of strong polarity in the bare embryos and control seedlings was presumably due to root damage caused by sterilization. It is well-known that the removal of the root cap abolishes graviresponsiveness in most plant species (Taiz and Zeiger 1991). Growth habit problems could be avoided by germinating seeds aseptically.

Shortening of the juvenile phase would be advantageous for obtaining progeny and establishing tests earlier in Scots pine breeding. There have been many attempts to shorten the generation interval by stimulating flowering. All these methods, e.g., regulation of light and heat conditions, mechanical methods, hormonal stimulation, and combined treatments, have drawbacks. The effect of these approaches is temporary, mechanical injuries diminish the vigour of grafts, and strobili abortion limits the effect of growth regulator applications (reviewed by Chalupka 1991a, b). In some cases, Scots pine grafts may flower 1 year after seed orchard establishment, after which they revert to vegetative growth for several years (T. Nikkanen, personal communication). The characteristics of the micropropagated plantlets in this study, like poor elongation growth and bud formation in vitro, rooting difficulties, plagiotrophic growth habit, and early formation of both female and male strobili without any artificial stimulation, suggest irreversible maturation.

From the forest tree breeding point of view, the present Scots pine organogenesis method offers new possibilities. Breeding strategies can be based on progeny testing or on phenotypic selection. The potential for increasing the genetic gain per year is greater when progeny testing is included, but the delay in the creation of a new generation can be considerable (Wilhelmsson and Andersson 1993). The option of early flowering clones could accelerate breeding programmes (Fig. 5), even those including progeny evaluation. The long-term seed production capability of plantlets produced through micropropagation remains to be demonstrated, but the production of viable pollen by the plantlets opens the door to controlled pollinations. In the optimum case, our method provides material to study the characteristics of the parent and progeny genotypes differing only a few years in age.

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