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Seasonal changes in explant viability and contamination of tissue cultures from mature Scots pine

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Abstract. Explants from 10 to 40-year-old Scots pine trees (*Pinus sylvestris* L.) were cultured in vitro. Material was collected from Northern Finland once or twice a week during 1984–1987. Excised shoot meristems and lower parts of the buds formed soft callus on modified MS medium. A seasonal effect was observed in the explant viability and degree of contamination. Callus proliferation was highest from explants collected in December and January and during the growing season from April to July, and lowest in February and during the autumn from September to November. It seemed that the bud metabolism at each particular time was rather persistent and affected the outcome of the experiments. Contamination was significantly higher from December to April. Organogenesis occurred only rarely.

Introduction

Many *Pinus* species have been propagated vegetatively from juvenile tissues using excised embryos and young seedlings as explants [11, 20, 18]. However, there are only a few reports of propagation using tissues from mature conifers [4, 8, 1]. Among *Pinus* species, the Scots pine (*Pinus sylvestris*) appears to be especially difficult to handle in cultures, and attempts to propagate it vegetatively by traditional tissue culture methods have been only partly successful [21, 5, 28, 24]. Some success has been achieved in vegetative propagation by rooting cuttings and microcuttings of Scots pine trees and seedlings [39, 40, 4, 13; M. Salonen, pers. comm.]. At present, none of these methods are in routine use.

A method for vegetative propagation of selected phenotypes from adult trees would be desirable for various forest breeding and reforestation programs. Tissue culture is a very useful method for large-scale cloning of plantlets from selected trees. However, detailed knowledge of the plant material and its requirements for callus proliferation is necessary before mass in vitro propagation can become a reality. Furthermore, tissue cultures

provide homogenous material for studying the molecular biology of coniferous trees.

This study is part of a project which aims at finding a method to propagate Scots pine trees in vitro using tested material, i.e., buds from adult trees. The main purpose of the present work was to find a way for continuous callus production. For this, the seasonal variation in the viability, callus growth and microbial contamination of mature Scots pine tissues in culture conditions was outlined. The possibilities of reducing callus browning were also examined. Furthermore, the suitability of different parts of the buds, different types of buds and buds from different parts of the tree was tested. In addition to callus growth, adventitious shoot formation has been verified in some callus samples.

Materials and methods

Collections were made from 10–40 year old pine (*Pinus sylvestris* L.) trees during 1984–1987 on a natural stand in the district of Oulu (65°N; 23°30′E) from three different heights of the tree. In 1986 the material consisted of 7500 explants. These were obtained from 8–14 monthly samples taken from 3–10 tree individuals, using 9–30 branches per tree. During the other periods the amount of material was about half of the above mentioned.

Buds were surface-sterilized in 3% Na-hypochlorite for 20 min and rinsed four times in sterile distilled water. The buds were peeled and dissected aseptically in a 1% soluble polyvinylpyrrolidone (PVP) solution under a dissecting microscope. In this study, the presentation of the results starts from June when the new buds begin to develop. At the beginning of June, the explants included both a distal part of the elongating one-year-old shoot and the newly activated apex. At the end of June and the beginning of July the explants consisted of the current year's shoot primordium. At other times, three types of explants were used:

- shoot apex consisting of apical meristem with a 1 mm piece of the surrounding tissue,
- a 2–3 mm piece of the tissue under the previous region, and
- a 3–4 mm piece from the basal part of the bud.

Different types of buds were tested: vegetative buds having spur shoot primordia, and male buds having microsporangiate strobilus primordia at the proximal part and spur shoot primordia at the distal part of the bud.

Numerous common nutrient media and modifications of them were used [22, 6, 26, 2, 25]. However, most experiments were performed with modified Murashige & Skoog [28] medium (MS) prepared as described in Table 1. To enhance callus growth, various cytokinins, benzylaminopurine (BAP) and/

Table 1. Composition of the modified Murashige and Skoog medium suitable for year-round callus production started from adult Scots pine trees.

Macronutrients ($mg\,l^{-1}$)		
NH_4NO_3	825	1/2 MS
KNO_3	950	1/2 MS
$CaCl_2 \cdot 2H_2O$	220	1/2 MS
$MgSO_4 \cdot 7H_2O$	370	1 MS
KH_2PO_4	340	2 MS
Micronutrients ($mg\,l^{-1}$)		
KI	1.66	2 MS
H_3BO_3	12.40	2 MS
$MnSO_4 \cdot 4H_2O$	22.30	1 MS
$ZnSO_4 \cdot 7H_2O$	8.60	1 MS
$Na_2MoO_4 \cdot 2H_2O$	0.50	2 MS
$CuSO_4 \cdot 5H_2O$	0.025	1 MS
$CoCl_2 \cdot 6H_2O$	0.05	2 MS
Na_2-Fe-EDTA	39.00	
Other components ($mg\,l^{-1}$)		
Nicotinic acid	0.75	1 1/2 MS
Pyridoxine HCl	0.75	1 1/2 MS
Thiamine HCl	0.15	1 1/2 MS
Myo-inositol	100.00	1 MS
Glycine	3.00	1 1/2 MS
L-arginine	100.00	
L-glutamine	200.00	
Arabinose	100.00	
Sucrose	8000.00	
Glucose	8000.00	
Agar granulated	6000.00	
Hormones ($mg\,l^{-1}$)		
2iP	0.2–0.5	
BAP	0.2–0.5	
NAA	0.2–0.5	
IBA	0.2–0.5	
pH	*5.6–5.7*	

or N^6-(2-isopentenyl)adenine (2iP) and auxins, naphtalene-1-acetic acid (NAA) and/or indolebutyric acid (IBA) were used alone or in combination with each other.

Explants were grown under a 16-h light period (white fluorescent Osram 18 W tubes, at 1.8 $W\,m^{-2}$, 400–700 nm) at +26 °C and a 8-h dark period at +23 °C.

The viability and growth rate of explants were estimated visually and by measuring the diameter of the tissues once a week over a two-month period. Samples with a diameter under 3 mm after 6 weeks of culture were regarded as non-viable. Samples were subcultured once during that time, using the same basal medium containing less or no hormones. The dry-weight gain was estimated from a sample of explants (+ 105 °C, overnight). Anatomical studies were performed on tissues fixed in FAA (formalin: acetic acid: ethanol = 10:5:85) and embedded in paraffin. Series of sections (thickness 10–15 μm) were cut by LKB Historange microtome and stained with toluidine blue (0.05% in H_2O) or safranin-fast green [13].

Because the measurements were made in relative units (percentage), non-parametric methods (Kruskal–Wallis-test) [27] were used for statistical inference.

Results

Callus initiation

Good callus growth was supported by a modified MS medium (Table 1) containing the following growth regulators: BAP (0.3 mg l^{-1}), 2iP (0.5 mg l^{-1}), NAA (0.2 mg l^{-1}) and IBA (0.5 mg l^{-1}). Callus from the three types of explants was induced on this medium within 2–3 weeks in culture. Callus also appeared on media lacking cytokinins but not without auxins. After 5–6 weeks in culture, the calli could be transferred to a growth-regulator-free medium. Full-strength macronutrients of MS medium and other tested media resulted in the browning and death of explants in 2–3 weeks in

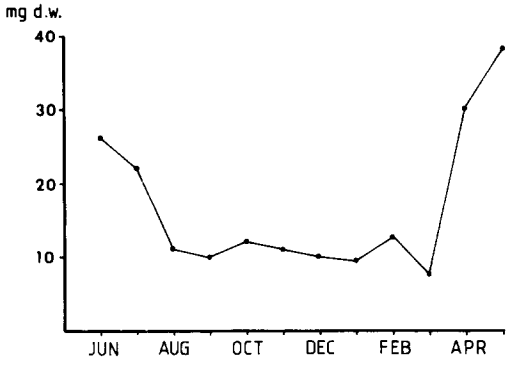

Fig. 1. Effect of collection date of Scots pine buds on explant regenerative repsonse. Dry weight is measured from samples after 8–12 weeks in culture. Each point represents 5–12 samples from 2–4 monthly collections.

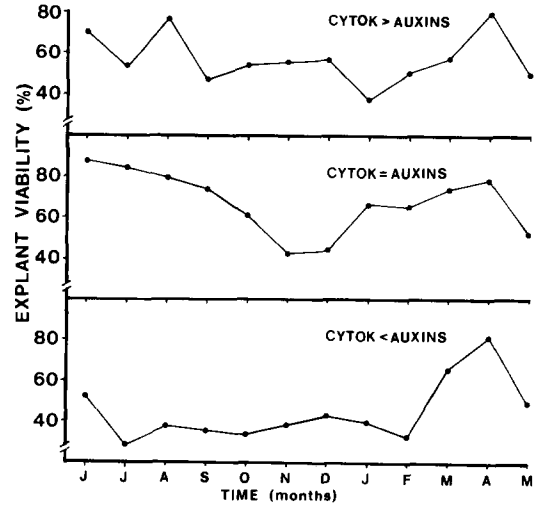

Fig. 3. Effect of the cytokinin/auxin ratio on explant viability and callus growth of Scots pine during different seasons in 1986. Each point represents 2–8 dates and 20–80 explants per experiment.

different months: it was high in April, May and June, decreased during the autumn and rose again in December and January (Fig. 2A). There was a transient fall in February. Differences were statistically highly significant ($P < 0.001$). About 70% of the explants collected in the autumn and in February became brown and eventually died after 2–3 weeks in culture without growing at all or only 1–2 mm. The high variation in viability results can partially be explained by increasing/decreasing trends within the monthly data (Fig. 2b).

Callus growth showed a seasonal response to the cytokinin/auxin ratio of the media; during the winter, spring and autumn, callus growth did not significantly respond to changes in the hormone ratio, but during early summer, a cytokinin/auxin ratio of under or over 1 was harmful for growth as compared to a cytokinin/auxin ratio equalling 1 (Fig. 3). These differences are significant ($P < 0.01$).

Browning

A fairly rapid browning of the calli was very common. Scots pine tissues contain phenolic compounds (A. Hohtola, unpubl.) which are easily oxidized [1]. This is often assumed to result in the death of explants. To prevent polyphenol oxidation, citric acid (20–50 mg l^{-1}, 150 mg l^{-1} for rinsing), ascorbic acid (10–20 mg l^{-1}, 150 mg l^{-1} for rinsing), activated charcoal (1–

2 g l^{-1}) and PVP (0.5–7 g l^{-1}, 10 g l^{-1} for rinsing) were added separately to the medium or were used in a rinsing medium. Of these, 1% PVP gave the best result against browning, but it did not provide a final solution to the problem. Activated charcoal was very harmful for the explants; without exception, all the explants died within 2 weeks.

Effect of tree age and type and part of the bud

There were no statistically significant differences in viability of explants from 10 to 19 and 20 to 40-year-old trees. The age of the branch had an influence on the growth rate; the best proliferating calli were obtained from buds taken from the youngest part of a tree, while the buds from the lowest (oldest) branches mostly gave the poorest results ($P < 0.1$). Differences between individual trees were great. Fresh weight was 30% higher in calli started from buds from upper branches after 4 weeks in culture. Differences were more obvious at the beginning of the explant growth than after 2 months.

Shoot apex explants from both vegetative and male buds gave equal parts in summer ($P < 0.05$) and autumn ($P < 0.001$) and the growth rate of the middle part of the bud was equally good with shoot apex in the spring. The basal part of the bud had the best callus proliferation in April compared to other months and the poorest in October ($P < 0.001$). Fig. 2 shows the mean viability of the combined pieces.

Shoot apex explants from (both vegetative and male buds gave equal results in culture. However, most of the experiments were performed using vegetative buds.

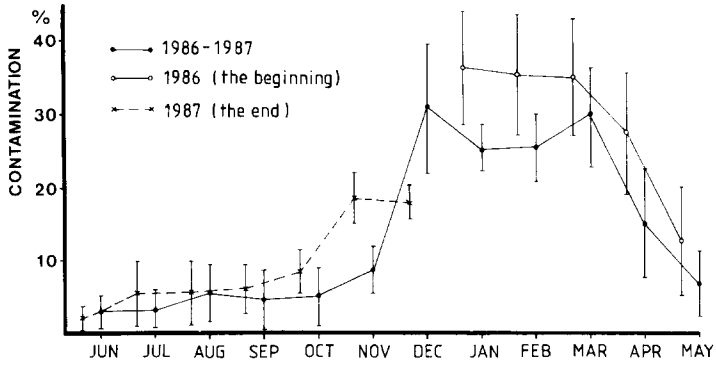

Fig. 4. Effect of collection date on explant contamination. Each point in 1986 represents 8–14 dates and 40–90 samples per collection and 4–8 dates and 40–150 samples per collection in 1987. Vertical bars denote standard error.

Contamination

Contamination was low from May to October and significantly higher ($P < 0.001$) from December to April (Fig. 4). Explants were usually infected by fungi and bacteria and contamination was normally visible after a week in culture. The basal part of the bud was readily and rapidly contaminated, while the shoot tip explants with meristem were infected very rarely ($P < 0.001$). Fig. 4 represents the extent of microbial contamination for the entire bud.

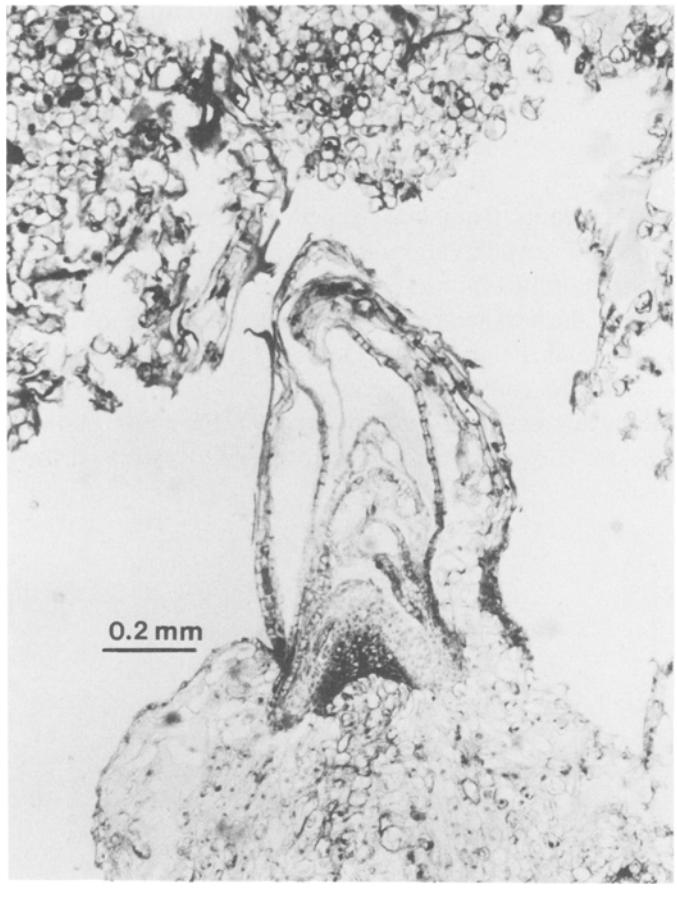

Fig. 5. Cross section of an 11-week-old callus culture grown on a modified MS medium supplemented with hormones (cytokinins $2\,mg\,l^{-1}$ and auxins $1\,mg\,l^{-1}$). Shoot primordium has differentiated from the callus tissue. Shoot tip explant was taken at the end of May from a 15-year-old tree.

Shoot formation capacity

Organogenesis was verified from paraffin sections of calli. Shoot formation, such as shown in Fig. 5, was observed in ca 5% of the samples examined. The age of the organ-forming calli varied from two to eight months and in most cases the material tested was collected during the early summer. On the media used in the present experiments, the newly formed adventitious shoots failed to grow beyond the microscopic level. In some cases, from August to October, the original shoot tip meristem started to grow and green needles surrounded by callus tissue could be seen.

Discussion

The present results indicate that callus production from the buds of adult Scots pine trees is possible throughout the year. The time of year at which Scots pine explants are dissected and placed in culture influences their regenerative response. Similar observations have been reported from other gymnosperms [23, 1]. Strong seasonal differences in the rooting capacity have also been noticed [30].

The seasonal variation in this study can be explained by earlier studies with Scots pine trees made at the same laboratory. The higher level in explant viability and growth rate in June and July represents the normal active growth period in the local natural stand. The second peak in January and the following decline coincide with the previously noticed change in the cytosolic ribosome content [12]. This is the point where the tree breaks down the winter metabolism at the cellular level [17]. The next peak in April correlates with the first cell divisions in buds in Finland [16]. The low explant viability and growth rate in the fall are simultaneous with the commencement of the dormant period. The last cell divisions can be seen in October.

The unexpectedly strong variation in the explant viability during the cold season apparently reflects the natural course of cellular metabolism. Changes in the wintertime activity of the buds have been noticed previously [12], and studies at the fine-structural level have revealed changes in bud cells at natural stands during the cold season [10]. In the present study, it seems that the season-dependent metabolism is retained in tissue culture conditions and, consequently, it is hard to break down the dormant state for instance. The same difficulty may explain the lack of organogenesis; it is difficult to switch off the callusing type of the growth in the cultures.

In agreement with this study, Rogozinska [21] also noticed that in the medium BAP alone resulted in very poor growth in the tissues of Scots pine.

It was suggested that callus tissue synthesizes cytokinins in sufficient amounts. Tranvan [29] observed that the BAP concentration necessary for bud induction depends upon the physiological age of the explant. Similarly, the physiological stage of the explant could explain the different responses to the cytokinin/auxin ratio in the medium (Fig. 3). Kopcewicz et al. [15] studied 10 to 140-year-old Scots pine trees. They found that the oldest trees showed the smallest amount of auxins and the highest level of inhibitors.

The inclusion of PVP reduced browning caused by the accumulation of phenolic compounds. On the other hand, PVP is also known to reduce the rate of regeneration [1]. Contrary to previous studies [7], a harmful (inhibitory) effect of activated charcoal was observed in this study. Probably activated charcoal adsorbs an element essential for Scots pine growth from the medium.

Seasonal variation on the extent of microbial contamination is also known from other works [14]. The sterilization of explants from mature conifer trees is difficult and injuries are common [8]. Seasonal variation in the contamination may be a result of better resistance of the tissue against the microbes during the active period and/or resistance of microbes to the decontaminants during the winter.

At present, the *Pinus* trees which can be propagated successfully are juvenile and untested, while mature trees with favourable characteristics have very seldom regenerated from tissue cultures. In addition to ageing, the stress induced by culture and large individual and local differences in physiological traits should be considered. For instance, *Pinus radiata* has been successfully propagated in New Zealand [11]. Compared to other *Pinus* species, the northern Scots pine trees probably have particularly strong stress reactions when dissected for tissue cultures.

The present investigation shows that there is a difference in explant viability and contamination degree of cultures of mature Scots pine trees during different seasons. The best times to start callus cultures seem to be the normal growing period and mid-winter, and the best material for culture is the shoot apex from the upper parts of the tree. However, callus production is possible throughout the year. Hormonal requirements differ only slightly during different seasons. The browning and death of the calli is still a problem which hampers long-time mass propagation. Although organogenesis in subcultured callus occurred only occasionally, it does however indicate that some cells have retained their regenerative potential.

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