Fizioloģiskais pamats parastās priedes (*Pinus sylvestris*) pavairošanai audu kultūrā

Promocijas darbs
bioloģijas doktora zinātniskā grāda iegūšanai
augu fizioloģijas apakšnozarē

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KOPSAVILKUMS

Parastās priedes *Pinus sylvestris* L. nobriedušiem audiem raksturīgs ļoti zems morfogēnais potenciāls *in vitro*, īdz šim nav apraktīta praktiski pielietojama mikropavairošanas metode šai komerciāli un ekoloģiski nozīmīgajai sugai. Darba mērķis – veidot fizioloģisko pamatu pieaugušas parastās priedes pavairošanai ar audu kultūru metodi. Ievadīšanai kultūrā izmantoja pieaugušu priežu sānzaru galos esošos pumpurus. Piemērotākais laiks pieaugušu parasto priežu ievadīšanai kultūrā bija no jūlija līdz oktobra beigām, pirms fizioloģiskā miera perioda sākšanās. Fizioloģiskā miera stāvoklī esošu priežu pumpuru morfogēno kompetenci audu kultūrā varēja palielināt, pumpurus pirms ievadīšanas kultūrā uzglabājot pazeminātā temperatūrā. Pazemināta vai stabila oksidatīvo fermentu aktivitāte korelēja ar paaugstinātu audu morfogēno kompetenci *in vitro*. Pazemināta oksidatīvo fermentu aktivitāte skābākā barotnē kultivētiem pumpuriem, mazāka nepieciešamība paskābināt barotni pH līdzsvara sasniegšanai un eksplantu morfoloģiskie rādītāji liecina, ka priežu pumpuru kultivēšanai piemērotāka ir skāba iniciālā barotne. *In vitro* kultivētu pieaugušu priežu audu inkubācija pazeminātā temperatūrā pirms pulsveida apstrādes ar benzilaminopurīnu veicināja audu proliferāciju. Nobriedušu parasto priežu audu juvenilizācijai un to morfogēnās kompetences un proliferācijas nodrošināšanai *in vitro* nepieciešama fizikālu un bioķīmisku faktoru mijiedarbība, kur pazeminātai temperatūrai ir būtiska nozīme. Darba rezultāti norāda virzienu tālākiem pētījumiem sekmīgas pieaugušu parasto priežu mikropavairošanas metodes izstrādei. Tie varētu būt nozīmīgi arī citu veģetatīvi grūti pavairojamu kokaugu sugu in vitro kultūru iegūšanai.

ABSTRACT

Mature tissues of *Pinus sylvestris* L. have a very low morphogenic potential *in vitro*, and so far no practically suitable micropropagation method for this comercially and ecologically important species has been described. The aim of the present study was to create the physiological basis for micropropagation of mature *P. sylvestris* in tissue culture. Buds from tips of lateral branches of mature trees were used for introduction *in vitro*. The most suitable time for introduction *in vitro* of mature P. *sylvestris* bud tissue was from July to late October, when the new buds have matured but dormancy has not started yet. Long cold storage of dormant mature pine buds before introduction *in vitro* increases their morphogenic competence in tissue culture. Lowered or stable activity of oxidative enzymes correlated with increased morphogenic competence *in vitro*. Lowered activity of oxidative enzymes in buds, cultivated on more acidic medium and needing less acidification of medium to reach an equilibrium and morphological parameters, shows, that acidic initial medium is most suitable for cultivation of pine buds. Incubation of *in vitro* cultivated mature pine tissue in lowered temperature before pulse treatment with benzylaminopurine promoted proliferation of the tissue. To achieve the rejuvenation and to provide the morphogenic competence and proliferation of mature pine tissue, it is necessary to secure the interaction of physical and biochemical factors, where lowered temperature is of great significance. The results of the present work shows the direction of further investigations for working out a successful method of *P. sylvestris* micropropogation. They could be significant also for obtaining tissue culture of other tree species, difficult to propagate vegetatively.

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Ievads

Parastā priede (*Pinus sylvestris* L.) ir viena no galvenajām un komerciāli svarīgākajām koku sugām Ziemeļu puslodē. Latvijā *Pinus sylvestris* ir dominējošā suga 37% no kopējās mežu platības, no kurām slavenās Rīgas priedes ir vairs tikai 28%. Skuju koku mežu īpatsvars pēdējā gada laikā sarucis par 9%, attiecīgi paplašinoties lapu koku mežiem (Zirnis 2007). Pēdējo 25 gadu laikā uz Zemes arvien pasliktinās apstākļi sekmīgai priežu mežu attīstībai un to dabiskai atjaunošanai (Toribio, Pardos 1987). Mākslīgai mežu atjaunošanai izcirsto priežu mežu vietā, kā arī lauksaimniecībā neizmantoto zemju apmežošanai Latvijā un arī citur, arvien biežāk izmanto citas koku sugas, kuras ieaudzēt ir vieglāk nekā parasto priedi (Zirnis 2007). Parastās priedes izturība pret vides izraisīto stressu ir kļuvusi par ļoti aktuālu tēmu. Jārada bāze tādai komerciālai meža atjaunošanas materiāla ražošanai, kura pielietošana nodrošinātu augsti produktīvu un kvalitatīvu mežaudžu ierīkošanu, tādējādi paaugstinot meža kapitāla vērtību.

Biotehnoloģija piedāvā vairākas iespējas meža koku īpašību uzlabošanai, no kurām klonālo pavairošanu uzskata par visprogresīvāko. Tomēr skujukoku reģenerācija *in vitro* apstākļos ir grūtāk panākama nekā citiem kokiem (Toribio, Pardos 1987). Parastās priedes kultivēšana un pavairošana *in vitro* vēl joprojām ir ierobežota atbilstošu fizioloģisko pētījumu trūkuma dēļ.

Veikto pētījumu mērķis – izveidot fizioloģisko pamatu pieaugušas parastās priedes *Pinus sylvestris* L. pavairošanai ar audu kultūru metodi.

Mērķa sasniegšanai izvirzīti sekojoši uzdevumi:

• Izpētīt parastās priedes juvenilo audu morfoģenēzi audu kultūrā dažādu fitohormonu klātbūtnē un ar atšķirīgu barotņu sastāvu.

• Noskaidrot sezonālo izmaiņu un aukstumuzglabāšanas ietekmi uz pieaugušas parastās priedes pumpuru audu morfogēno kompetenci *in vitro*. Pētīt morfoģenēzes saistību ar oksidatīvajiem procesiem, analizējot peroksidāzes un polifenoloksidāzes aktivitātes izmaiņas pumpuru audos.

• Izpētīt pieaugušas parastās priedes pumpuru audu morfogēnās kompetences izmaiņas pēc uzglabāšanas saldētavā saistībā ar oksidatīvajiem procesiem un ogļhidrātu metabolisma izmaiņām.

• Noskaidrot barotnes pH izmaiņas pieaugušu parasto priežu audu *in vitro* kultivēšanas laikā un barotnes pH ietekmi uz kultivētu pieaugušu parasto priežu audu morfoģenēzi.

• Pētīt *in vitro* ievadītu pieaugušu parasto priežu pumpuru inkubēšanas pazeminātā temperatūrā ietekmi uz spēju reaģēt uz pulsveida apstrādi ar citokinīniem.

1. Zinātnisko atziņu pārskats pētījumu jomā

Pieaugušu koku materiāla mikropavairošana ir pētnieciski un praktiski nozīmīga, jo tā dod iespēju multiplicēt un strauji izplatīt labākos genotipus, kas izraudzīti lauka apstākļos. Tomēr, audu nobriešana, zaudējot juvelinitāti, rada problēmas, kas kavē plašāku audu kultūru pielietojumu kokaugiem (Pierik 1990). Arī stress, ko izsauc hormonālie un citi faktori *in vitro* kultūras barotnē, var izraisīt audu juvenilitātes pakāpes samazināšanos (Bonga 1987).

Literatūrā atrodami dati tikai par atsevišķu priežu sugu pieaugušo īpatņu veiksmīgu mikropavairošanu – *Pinus radiata* (Horgan, Holland 1989), *Pinus pinaster* (Monteuuis, Dumas 1992), *Pinus brutia* (Abdullah *et al*. 1987), *Pinus lambertiana* (Gupta, Durzan 1985), *Pinus taeda* (Mott, Amerson 1981) un *Pinus nigra* (Jelaska *et al*. 1981). Starp *Pinus* sugām parastā priede (*Pinus sylvestris* L.) ir īpaši nepakļāvīga audu kultūrai (Hohtola 1988).

Pirms šo pētījumu uzsākšanas bija izstrādātas metodes *P. sylvestris* mikropavairošanai, kā eksplantus izmantojot tikai juvenilus audus – dīgstus vai embrijus (Jain *et al*. 1988; Bonga 1991; Supriyanto, Rohr 1994; Häggman *et al*. 1996; Sul, Korban 1998). Līdztekus tam, ir panākts zināms progress parastās priedes somatiskajā embrioģenēzē (Hohtola 1995; Keinonen-Mettala *et al*. 1996; Haggman *et al*. 1999; Lelu *et al*. 1999; Niemi *et al*. 2002, Niskanen *et al*. 2004). Orgānu vai dzinumu reģenerāciju no *P. sylvestris* kallusa nav izdevies iegūt (Laukanen *et al*. 1999; Pirttla *et al*. 2004).

Līdzšim literatūrā nav aprakstīta praktiski pielietojama pieaugušas parastās priedes mikropavairošanas metode. Pieaugušas *Pinus sylvestris* audiem raksturīgs ļoti zems morfogēnais potenciāls *in vitro* (Bonga 1987; Hohtola 1988). Jaunākie pētījumi ir parādījuši, ka parastās priedes audiem piemīt vairākas īpatnības, kas apgrūtina to kultivēšanu *in vitro,* tās ir – augsts endogēnā oksidatīvā stresa līmenis audu kultivēšanas laikā (Laukkanen *et al*. 2000a), stipras ievainojuma atbildes reakcijas, kam seko fenolu daudzuma pieaugums (Hohtola 1988), kā arī endofītisko mikroorganismu - sēņu(*Hormonema dematioides (Ascomycota)),* raugu *(Rhodotorula minuta)* un baktēriju (*Methylobacterium* spp., *Pseudomonas fluorescens* apakšgrupa, un *Mycobacterium* sp.) klātbūtne audos un nekontrolēta vairošanās priežu audu kultūrā , kas aktivizē audu aizsargreakcijas (Laukkanen *et al*. 2000b; Pirttilä *et al*. 2002). Tā rezultātā, *P. sylvestris* dzinumu galotņu pumpuriem, kas ievadīti audu kultūrā, raksturīga strauja audu nobrūnēšana, kam seko šūnu struktūru degradācija un nekroze, kā arī augsts infekciju procents (Lindfors *et al*. 1990).

In vitro kultūru nobrūnēšanā piedalās oksidatīvie fermenti – peroksidāzes un polifenoloksidāze (Dowd, Norton, 1995; Tang, Newton, 2004). No pieaugušu parasto priežu dzinumu galiem iegūtām kallusu kultūrām brūnēšana ir kultivēšanas apstākļu izraisītā oksidatīvā stressa rezultāts (Laukkanen *et al*. 2000a). Augu audiem ar pazemināt oksidatīvo reakciju aktivitāti pēc ievadīšanas *in vitro* kultūrā

varētu būt mazāk izteikta nobrūnēšana un, tā rezultātā, labāka morfogēnā kompetence.

Kā rāda sezonālās izmaiņas parastās priedes pumpuru šūnu metabolismā (Hohtola 1988), pastāv iespēja, ka oksidatīvo reakciju kapacitāte mainās augšanas sezonas laikā. Tāpēc *in vitro* kultūrā ievadīšanai paredzētā augu materiāla ievākšanas laiks varētu būt izšķirošs faktors sekmīgai kultūras uzsākšanai (Bonga 1987).

Ir aprakstītas vairākas metodes pieaugušu skujukoku audu priekšapstrādei pirms ievadīšanas kultūrā, lai panāktu to rejuvenilizāciju vai palielinātu morfogēno potenciālu. Ilgstoša aukstumuzglabāšana var tikt izmantota kā līdzeklis oksidatīvā metabolisma ietekmēšanai. Tā piemēram, četrus mēnešus ilga parastās priedes sakņu aukstumuzglabāšana būtiski samazina audu peroksidāzes aktivitāti (Ahonen *et al*. 1989).

Iespēja ietekmēt embrioģenēzi un morfoģenēzi, augu materiālu uzglabājot saldētavā, aprakstīta citām koku sugām (Bonga 1996). Tomēr, neskatoties uz augu audu kriokonservācijas plašo pielietošanu ģenētiskā materiāla uzglabāšanai (Sakai 2000; Mathur *et al*. 2003), praktiski nav pieejama informācija par bioķīmiskajām izmaiņām intaktā augu materiālā, glabājot to saldētavā.

Priežu veģetatīvajos pumpuros temperatūrā zem nulles notiek ārpussūnas sasalšana, pretstatā ārpusorgānu sasalšanai, kas raksturīga citu skujukoku lapu pumpuriem (Ide et al. 1998). Tieši tas izraisa priežu pumpuru ārkārtīgi augsto sala izturību.

Laikā, kad augu audi pielāgojas aukstumam, tajos notiek šķīstošo ogļhidrātu, pārsvarā saharozes, satura pieaugums, paralēli ar nešķīstošo ogļhidrātu satura samazināšanos (Oleksyn et al. 2000). Uzskata, ka cukuru daudzuma pieaugums ir tieši saistīts ar cietes sadalīšanos (Fischer and Höll 1991). Šķīstošo ogļhidrātu uzkrāšanās var pasargāt membrānas to sasalšanas laikā (Fujikawa and Jitsuyama 2000). Sakņu dārzeņu uzglabāšanas laikā pēcnovākšanas periodā to audos palielinās reducējošo cukuru (heksožu) daudzums, bet samazinās saharozes daudzums, pie kam, kopējais cukuru daudzums saglabājas relatīvi nemainīgs (Nilsson 1987). Saharozes/heksozes satura attiecības izmaiņas bija novērotas lucernas augos, kuros notika sala izturības attīstība, kas bija saistīta ar saharozes uzkrāšanos uz glikozes, fruktozes un cietes līmeņu pazemināšanās fona (Castonguay et al. 1995). Reducējošo cukuru daudzuma pieaugums var izraisīt arī relatīvi augstāku iespēju veidoties nelabvēlīgām mijiedarbībām starp ogļhidrātiem un proteīniem.

Bez tam, uzglabājot augu audus zemās temperatūrās, tiem rodas brīvo radikāļu izsauktais oksidatīvais stress (Hendry 1993). Tieši šī iemesla dēļ pretoksidatīvo fermentu aktivitātes pieaugums ir nepieciešams komponents, lai pielāgotos salam (Tao et al. 1998).

Barotnes pH ir viens no nozīmīgiem fizikāli-ķīmiskās vides faktoriem augu audu attīstībai *in vitro* kultūras apstākļos (Williams et al. 1990). Ir izpētīts, ka optimālais barotnes pH līmenis dažādiem morfoģenēzes posmiem kokaugiem variē (von Arnold, Eriksson 1982; Williams *et al*. 1985; Saborio *et al*. 1997; Ostrolucka *et al*. 2004). Suboptimāls pH var izraisīt anomālijas eksplantu attīstībā (Gurel, Gulsen 1998; Laukkanen *et al*. 2000b).

Audu kultūru praksē barotnes pH noregulē pirms tās autoklavēšanas un pH izmaiņas autoklavēšanas un audu kultivēšanas laikā parasti neņem vērā. Izmaiņas autoklavēšanas laikā ir atkarīgas no sākotnējā pH un barotnes sarecēšanu nodrošinošās vielas (Williams *et a*l. 1990; Van Winkle, Pullman 2003). Pretēji parastajam uzskatam, barotnes pH izmaiņas kultivēšanas laikā nav atbilde uz ievainojumu. Lai

arī ievainojums izraisa pH samazināšanos pirmo dažu dienu laikā, šis efekts nav būtisks (Williams *et al*. 1990).

Augu audi spēj uzturēt relatīvi konstantu citoplazmas pH, ja ārējais pH ir intervālā no 4 līdz 9 (Caponetti *et al*. 1971; Gudrupa *et al*. 2002). Augu šūnas spēj arī mainīt vides pH – eksplanti palielina vai samazina pH, atkarībā no tā, kādā pH diapazonā notiek kultivēšana, līdz iestājas līdzsvars (Mac AntSaoir, Damvoglou 1994). Lokālas pH izmaiņas notiek auga audu un augšanas vides kontakta vietā gan audu kultūrā, gan augsnē (Constable 1963; Haussling et al. 1985), izdaloties noteiktiem, pH modificējošiem savienojumiem no auga audiem, vai augam uzņemot specifiskus jonus (Butenko 1964).

Literatūrā aprakstīto *Pinus* ģints sugu audu kultūrām barotnes pH ir minēts robežās no 5.5 līdz 6.0 (Durzan, Chalupa 1976; Gupta, Durzan 1986; Halos, Go 1993; Sen *et al*. 1994; Saborio *et al*. 1997; Tang 2001; Sul, Korban 2004; Tang *et al*. 2004). *P. sylvestris* parasti kultivē barotnē ar pH 5.5 līdz 5.8 (Bornman, Jansson 1980; Hohtola 1988; Mohan *et al*. 1988; Žel *et al*. 1988; Hohtola, Kvist 1991; Supriyanto, Rohr 1994; Hohtola 1995; Keinonen-Mettala *et al*. 1996; Laukkanen *et al*. 1997; Laukkanen *et al*. 1999; Lelu *et al*. 1999; Laukkanen *et al*. 2000a; Laukkanen *et al*. 2000b). Skujukoki *in vitro* kultivēšanas laikā spēj izmainīt barotnes pH (Van Winkle, Pullman 2003; Durzan, Chalupa 1976).

Pieaugušu skujukoku audu rejuvenilizāciju, palielinot to morfogēno potenciālu, iespējams panākt arī ar sērijveida potēšanu (Huang *et al*. 1992; Ewald, Kretzschmar 1996; Centeno *et al*. 1998) vai apsmidzinot donoraugus ar citokinīniem (Salonen 1991). Ir labi zināms, ka pumpuru veidošana vai adventīvā zarošanās parasti tiek panākta, palielinot citokinīnu līmeni audos. Tomēr, ļoti strauja vairošanās audu kultūrā augsta citokinīnu līmeņa ietekmē var izraisīt ģenētiskas izmaiņas (Peschke, Phillips 1992). Tāpat ir zināms, ka augsta citokinīnu koncentrācija, stimulējot orgānu veidošanos, vienlaikus inhibē to stiepšanos garumā un attīstību (Salonen 1991). Tāpēc pulsveida apstrādi ar citokinīnu grupas savienojumiem starp subkultivēšanām var izmantot, lai sasniegtu strauju vairošanos bez nevēlamiem ģenētiskiem efektiem. Eksperimentos ar pieaugušiem intaktiem skuju kokiem juvenilizācijai izmanto atkārtotu citokinīnu apstrādi, kas izraisa jaunu dzinumu veidošanos (Krikorian 1982).

2. Pārskats par darbā izmantotajām metodēm

Darbs izstrādāts LU Bioloģijas institūtā, Nacionālajā botāniskajā dārzā un LU Bioloģijas fakultātes Augu fizioloģijas katedrā laikā no 1998. līdz 2008. gadam.

Parastās priedes juvenilo audu morfoģenēzes panākšanai audu kultūrā *P. sylvestris* sēklas dezinficēja un diedzēja sterilos apstākļos. Primārie eksplanti sastāvēja no hipokotila augšējās daļas, dīgļlapām un epikotila. Sekundārie eksplanti bija no mātesauga atdalīti mikrodzinumi. Eksplantus kultivēja uz agarizētas barotnes, kas saturēja 50 % Murašiges - Skūga minerālsāļus (Murashige, Skoog 1962) un 50 % Borgina - Nitča organiskos komponentus (Bourgin, Nitsch 1967) (1/2 MSBN) un saharozi 10 g L^{-1} (pamatbarotne), 23±3 °C temperatūrā ar 16 h fotoperiodu. Apgaismojumu nodrošināja dienasgaismas spuldzes kombinācijā ar saules gaismu.

Lai inducētu mikrodzinumu veidošanos, primāros un sekundāros eksplantus kultivēja barotnēs, kur 1/2 MSBN papildināts ar citokinīnu 6-benzilaminopurīnu (BAP) (5 vai 10 mg L^{-1}) un auksīnu indol-3-sviestskābi (IBA) (1 mg L^{-1}) vai kokosriekstu pienu (0.5 %).

Lai panāktu mikrodzinumu attīstību un augšanu garumā, eksplantus pārvietoja uz pamatbarotni (1/2 MSBN) vai atšķaidītu pamatbarotni (1/6 MS 1/2BN), kas bagātināta ar aktīvo ogli (2 g L^{-1}) vai giberelskābi (1 mg L^{-1}) kombinācijā ar IBA (1 mg L^{-1}) vai BAP (0.1 mg L^{-1}) vai ar priežu skuju ūdens ekstraktu.

Eksperimentus morfogēnās kompetences sezonālo izmaiņu noteikšanai un aukstumuzglabāšanas ietekmes uz morfoģenēzi pētījumiem veica no janvāra beigām līdz jūlija beigām. Miera periodā esošu veģetatīvo pumpuru augšējo daļu, jauno dzinumu galiņus vai jaunizveidotos pumpurus (atbilstoši augšanas fāzei) ievāca divreiz mēnesī no pieaugušām priedēm sēklu plantācijā, kas atrodas netālu no Rīgas. Augu materiālu ņēma pēc nejaušības principa no dažādiem kokiem, no vainagu apakšējās daļas. To izmantoja ievadīšanai audu kultūrā un fermentu analīzēm.

Ievāktos miera periodā esošos pumpurus no ārpuses dezinficēja un ievietoja slēgtos konteineros pie 5 °C. Divreiz mēnesī aukstumā uzglabātos pumpurus ievadīja audu kultūrā un izmantoja fermentu analīzēm. Pumpuru dezinfekcija: pumpurus stundu mazgāja saimniecības ziepju šķīdumā un stundu skaloja tekošā krāna ūdenī, tad 20 min dezinficēja uz pusi atšķaidītā balinātājā ACE (*Procture & Gamble*), kas satur 5-15 % nātrija hipohlorīta, 10 min skaloja sterilā destilētā ūdenī, vēlreiz dezinficēja 15 % ūdeņraža peroksīda šķīdumā un trīs reizes pa 10 min skaloja sterilā destilētā ūdenī. Nodezinficētos

pumpurus sterilos apstākļos atpreparēja. Aukstumā uzglabātos pumpurus dezinficēja divas reizes – pirms un pēc aukstumuzglabāšanas.

Eksplantus kultivēja mēģenēs ar vates-marles aizbāžņiem, kurās bija iepildīti pa 10 mL agarizētas barotnes. Kultivēšana notika 23±3 °C temperatūrā ar 16 h fotoperiodu. Apgaismojumu nodrošināja ar dienasgaismas spuldzēm kombinācijā ar saules gaismu (fotonu plūsmas blīvums 40-45 μmol m^{-2} s^{-1}).

Barotne saturēja kokaugu barotnes minerālsāļus (Lloyd, Mc Cown 1981) (WPM), vitamīnus, saharozi 45 g L^{-1} un sauso olu pulveri, kokosriekstu pienu, BAP, IBA un naftiletiķskābi (NAA) (mikrodzinumu veidošanai aukstumā uzglabātiem pumpuriem, kā arī kallusa inducēšanai uz eksplantiem) vai adenīnu, kinetīnu un NAA (skuju veidošanai).

Pētījumiem par saldēšanas ietekmi uz pumpuru morfogēno kompetenci sēklu plantācijā februāra beigās ievāca ap 15 cm garus zaru galus ar galotnes pumpuriem un skujām. Zarus kopā ar dažām saujām sniega ievietoja noslēgtos plastmasas maisiņos un ātri nogādāja saldētavā –18 °C. Desmit uzglabāšanas mēnešu laikā daļu zaru ik pa laikam izņēma no saldētavas un izmantoja ievadīšanai audu kultūrā un bioķīmiskajām analīzēm.

Lai sagatavotu pumpurus ievadīšanai audu kultūrā, tos nogrieza no zariem, dezinficēja un atpreparēja kā aprakstīts iepriekš. Katru reizi pusi no eksplantiem likta uz kallusa veidošanos inducējošās barotnes, bet otru pusi uz brahioblastu un skuju veidošanos inducējošās barotnes.

Pumpurus kultivēja tādos pašos apstākļos, kā iepriekšējā eksperimentā. Mēģenes noslēdza ar vates-marles korķiem, ko vēl pārklāja ar pārtikas plēvi, nostiprinot to ar gumiju. Kallusa iniciācijai eksplantus kultivēja uz modificētas MS barotnes (Hohtola 1988), kas bagātināta ar 2,4-dihlorfenoksietiķskābi (2.4-D), BAP un kinetīnu. Barotnes pH bija 5.6-5.7. Brahioblastu un skuju veidošanās iniciācijai pumpurus kultivēja uz WPM barotnes, kas bagātināta ar NAA, adenīnu un kinetīnu, kā arī mio-inozitolu, tiamīna hidrohlorīdu, piridoksīna hidrohlorīdu un nikotīnskābi.

Barotnes pH ietekmes pētījumos eksplantus kultivēja agarizētā barotnē 40×100 mm mēģenēs ar 20 mL barotnes, ja pH mērīja pēc autoklavēšanas, vai 20×200 mm mēģenēs ar 10 mL barotnes, ja pH mērīja pirms agara pievienošanas un autoklavēšanas, 20±5 °C dabiskā apgaismojumā (ja nav norādīts citādi). Pumpurus kultivēja uz modificētas WPM barotnes, kas bagātināta ar vitamīniem, adenīnu, kinetīnu un NAA. Tā saturēja 0.57 % augu agara.

Barotnes pH nostabilizēja pirms agara pievienošanas un autoklavēšanas. Pētot pumpuru audu ietekmi uz barotnes pH, to nostabilizēja uz 5.8 (1. eksperiments). Pētot barotnes pH ietekmi uz pumpuru attīstību audu kultūrā, ar 1N HCl vai 1N KOH palīdzību pH nostabilizēja uz 3.0 līdz 7.0 ar 0.5 pH intervāliem (2. eksperiments).

Lai noteiktu barotnes pH izmaiņas tās pagatavošanas gaitā, pēc minerālsāļu un organisko savienojumu pievienošanas nostabilizēja pH (no 2.5 līdz 7.0 ar intervalu 0.5), tad pievienoja agaru, salēja barotni kultivēšanas traukos un autoklavēja 121 °C temperatūrā un 0.1013 MPa 20 min. Kultivēšanas traukos pH mērīja divas dienas pēc barotnes pagatavošanas.

Lai izpētītu *in vitro* kultivētu pieaugušas parastās priedes pumpuru ietekmi uz barotnes pH (1. eksperiments), kultivējamos pumpurus pārvietoja uz svaigu barotni ik pēc 1, 2, 3, 4 vai 5 nedēļām. Trešdaļu pumpuru kultivēja audzēšanas kamerā ar 16 h fotoperiodu, kur apgaismojumu nodrošināja ar fluorescentajām spuldzēm OSRAM L 36/W77, bet pārējos audzēja dabiskā apgaismojumā laboratorijā pie loga, kur tos saule apspīdēja īsāku vai garāku laiku. Barotņu pH mērīja pēc autoklavēšanas un pēc pumpuru pārstādīšanas ik pēc 1, 2, 3, 4 vai 5 nedēļām. Barotnes pH izmaiņas pumpuru kultivēšanas rezultātā noteica pirmās 6-8 kultivēšanas nedēļas. Pumpurus kultivēja 10 mēnešus. Pumpuru morfoloģisko stāvokli (brahioblastu un skuju attīstību, nekrozes) reģistrēja kultivēšanas laikā un eksperimenta beigās.

Citokinīnu atbildes kompetences eksperimentiem augu materiālu (pieaugušu priežu jaunizveidotie pumpuri) ievāca jūlija otrajā pusē un augusta pirmajā pusē. *In vitro* kultūras uzsākšanai izmantoja M1 barotni, kas saturēja WPM minerālsāļus, vitamīnus, NAA, adenīnu un kinetīnu. Četru stundu ilgu pulsveida apstrādi ar augstām citokinīnu koncentrācijām veica uz M2 barotnes (ar BAP) vai uz M3 barotnes (ar kinetīnu un adenīnu). Pēc apstrādes ar citokinīnu pumpurus kultivēja uz pamatbarotnes (BM) ar WPM minerālsāļiem un vitamīniem, bez augšanas regulatoriem. Visā kultivēšanas laikā pumpuri saņēma dabisko apgaismojumu. Aukstumuzglabāšanas laikā temperatūra bija 8±3 °C; kontroli kultivēja 22±5 °C temperatūrā. Izmēģināja sešus dažādus variantus citokinīnu apstrādei ar vai bez aukstumuzglabāšanas:

• citokinīnu apstrāde ar M2 vai M3 pēc 5 mēnešus ilgas kultivēšanas uz M1, pēc tam kultvēšana uz BM 22±5 °C temperatūrā (kontrole);

• citokinīnu apstrāde ar M2 vai M3 pēc 5 mēnešus ilgas kultivēšanas uz M1, pēc tam 4 mēnešus ilga kultvēšana uz BM 8±3 °C temperatūrā, kam seko M1 22±5 °C temperatūrā (aukstumuzglabāšana pēc apstrādes ar citokinīnu);

• 5 mēnešus ilga kultivēšana uz M1 pie 22±5 °C temperatūrā, tad 4 mēnešus ilga kultivēšana uz M1 pie 8±3 °C, kam seko mēnesis 22±5 °C temperatūrā, tad pulsveida apstrāde ar M2 vai M3 un BM 22±5 °C temperatūrā (aukstumuzglabāšana pirms apstrādes ar citokinīnu).

Fermentu analīzēm pumpurus bez zvīņām (0.5 g) sasaldēja šķidrajā slāpeklī un smalki saberza porcelāna tīģelī ar piestu. Fermentus ekstrağēja 15 min 4 °C temperatūrā ar 25 mmol L^{-1} HEPES/KOH buferi (pH 7.2), kas saturēja 1 mmol L^{-1} EDTA, 3 % polivinilpirolidonu (PVPP) un 0.8 % *Triton X-100*, homogenātu centrifugēja 20 min ar paātrinājumu 15 000 g, ekstraktu lietoja analīzēm. Olbaltumvielas noteica pēc Bradforda (1976) metodes.

Peroksidāzes aktivitāti mērīja spektrofotometriski 470 nm gaismas viļņu garumā. Reakcijas maisījums sastāvēja no 2 ml 50 mmol L^1 nātrija fosfāta bufera (pH 7.0) ar 10 mmol L^{-1} gvajakola, 0.5 mL 0.03 mol L^{-1} H_2O_2 un 0.01 mL fermentu ekstrakta. Reakcijas maisījumu bez H_2O_2. izmantoja kā atskaites punktu.

Polifenoloksidāzes aktivitāti mērīja spektrofotometriski pie gaismas viļņu garuma 410 nm. Reakcijas

maisījums sastāvēja no 20 mmol L^{L1} nātrija fosfāta (pH 6.5) ar 25 mmol L^{-1} pirokatehola (3 mL) un fermentu ekstrakta (0.01 mL).

Cietes un cukuru daudzuma noteikšanai atpreparētus pumpurus nofiksēja, 20 min apstrādājot ar karstu tvaiku un izžāvēja. Cietes daudzumu noteica ar Bertrama metodi (Strong, Koch 1974). Formula cietes satura aprēķināšanai:

$$X = \frac{0{,}675 \times B \times k \times (a - b)}{v \times n} = \frac{0{,}675 \times 50 \times 0{,}1 \times (a - b)}{8 \times 0.5} = 0{,}844 \times (a - b)$$

X – cietes saturs (%),

B – kopējais pētāmā šķīduma tilpums (ml),

v- šķīduma tilpums, kas ņemts cietes izgulsnēšanai ar J_2 (ml),

a – 0.1 n tiosulfāta tilpums, kas ņemts kontroles titrēšanai (ml),

b – 0.1 n tiosulfāta tilpums, kas ņemts analīzes titrēšanai (ml),

k – tiosulfāta normalitāte (0.1 n)

n – iesvars (g).

0.675 – cietes normālais titrs, pareizināts ar 100, pārrēķināšanai uz procentiem.

Noteicamais cietes daudzums 1 – 14 mg cietes.

Reducējošo cukuru daudzumu ekstraktos noteica ar vara jodometrisko titrēšanu pēc Šaffera un Somodži (1933) metodes (Strong, Koch 1974). Formula reducējošo cukuru (glikozes un fruktozes) satura aprēķināšanai:

$$B = \frac{A \times [248 - (a - b)] \times (a - b)}{v \times n \times 10000} = \frac{0{,}1 \times A \times f}{v \times n} (\%)$$

B – reducējošo cukuru daudzums (%),

A – ekstrakta tilpums (ml),

a– $Na_2S_2O_3$ tilpums kontroles titrēšanai (ml),

b - $Na_2S_2O_3$ tilpums analīzes titrēšanai (ml),

n – iesvars (g),

v – analīzei ņemtais ekstrakta tilpums (ml),

248 – (a – b) – invertcukura daudz., kas atbilst 1 ml 0.01 n $Na_2S_2O_3$ (mkg),

10000 – pārrēķināšani no mkg uz g un %,

f – faktors (248 – (a – b)) × (a – b) / 1000 (tabulā),

Lai noteiktu saharozes daudzumu, vispirms noteica kopējo cukuru daudzumu. Kopējo cukuru daudzumu noteica tāpat, kā reducējošos cukurus. Saharozes daudzumu aprēķināja, atņemot reducējošo cukuru daudzumu no kopējā cukuru daudzuma un rezultātu reizinot ar 0.95. Cukuru daudzums pēc inversijas:

$$C = \frac{4A \times [248 - (a - b)] \times (a - b)}{v \times n \times 10000} = \frac{0{,}4 \times A \times f}{v \times n} (\%)$$

Saharozes daudzuma aprēķināšana:

$$D = 0{,}95 \times (C - B)$$

C – cukuru daudzums pēc inversijas,

D – saharozes daudzums,

0.95 – koeficients, lai pārrēķinātu glikozi saharozē.

Cietes un cukuru koncentrāciju izteica kā sausās masas procentu.

Veiktajos pētījumos katrā audu apstrādes variantā bija 10-20 eksplanti divos vai trijos atkārtojumos. Barotnes pH mērījumi veikti trijos atkārtojumos. Fermentu aktivitātes analīzēm un cietes un cukuru satura analīzēm veikti trīs bioloģiskie un trīs ķīmiskie atkārtojumi. Datu statistiskajā apstrādē aprēķināti aritmētiskie vidējie lielumi un noteikta standartkļūda (±SE), sezonalitātes, aukstumuzglabāšanas un saldēšanas eksperimentu rezultātu analīzei izmantots Stjudenta tests.

3. Audu kultūra, izmantojot juvenilus parastās priedes audus

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EFFECT OF HORMONAL AND NUTRITIONAL FACTORS ON MORPHOGENESIS OF *Pinus sylvestris* L. *in vitro*

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The morphogenesis of Pinus sylvestris *L.* in vitro *was investigated. Pine seedlings grown on medium containing 50 % Murashige-Skoog mineral salts plus 50 % Bourgin-Nitsch organic compounds plus sucrose without hormones formed shoots with needles. Transfer of hypocotyl cuttings to cytokinine-containing medium induced microshoot development. An inverse relationship between the number of newly formed microshoots and the length of the hypocotyl shoots before transplantation was established. Growth of buds was stimulated by transfer to a hormone-free medium. On shoot explants microshoot density was related to microshoot length. For initiation of elongation of newly formed microshoots, explants were tranferred to a hormone-free medium with diminished amounts of minerals and supplemented with activated charcoal.*

Key words: Pinus sylvestris *L., in vitro culture, morphogenesis, hormonal factors.*

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is one of the major forest species in Northern Hemisphere. In Latvia, *P. sylvestris* occupies up to 40 % of the total forest area. However, within the last 25 years, the conditions are becoming more and more inadequate for successful development and natural renewal of pine forests (Toribio and Pardos, 1987). Therefore, environmental stress resistance of pines has become a topic of vital interest.

Biotechnology offers several possibilities for forest tree improvement, clonal propagation being the most advanced at present. However, regeneration of coniferous species under *in vitro* conditions has been more difficult than for other trees (Toribio and Pardos, 1987). Therefore, the work carried out with *P. sylvestris* cultured *in vitro* has been limited up to date. Micropropagation of *P. sylvestris* has been established via organogenesis from cultivated seedlings (Supriyanto and Rohr, 1994) and some progress in initiation of somatic embryogenesis in *P. sylvestris* (Lelu *et al.*, 1999) has been made. However, successful regeneration through callus culture has not been achieved yet (Laukkanen *et al.*, 1999).

We report here on the establishment of *in vitro* culture of *P. sylvestris*, and the hormonal and nutritional factors, and cultivation conditions, required for successful morphogenesis. The long-range objective of the current project is to develop a convenient method for *in vitro* multiplication of selected clones of mature pine trees.

MATERIALS AND METHODS

Plant material. Pine (*Pinus sylvestris* L.) seeds were obtained from Mežaparks, Rīga (harvested in 1998). Experiments were carried out in 1999. Seeds were surface sterilised in commercial bleach ACE for 15 min, then in 70 % ethanol for 3 min, and finally were rinsed three times with sterile water. The seeds were germinated in aseptic conditions on Petri dishes on sterile wet filter paper for 45 to 110 days.

Establishment of cultures. Primary explants consisted of the upper part of the hypocotyl (1 cm in length), the cotyledons, and the epicotyl (such explants were named hypocotyl cuttings). Secondary explants were excised newly formed microshoots of differing length (3 to 17 mm). Explants were cultivated in 20 × 200 mm glass Pyrex test tubes containing 10 mL of agarised nutrient medium. The basal medium consisted of 50 % Murashige-Skoog minerals (MS) (Murashige and Skoog, 1962) and 50 % Bourgin-Nitsch organic compounds (BN) (Bourgin and Nitsch, 1967) with sucrose 10 $g \cdot L^{-1}$ (further called 1/2 MSBN). The pH was adjusted to 5.8 with 1 M NaOH or 1 M HCl, and the medium was autoclaved for 20 min at 0.1013 MPa. Hormones were added before autoclaving. Tubes were closed with cotton-wool plugs.

The cultures consisted of one explant per tube. They were cultivated in 23±3 °C with a 16 h photoperiod. In all experiments, illumination was provided by fluorescent tubes (LB80-1 and LB80-7) combined with sunlight.

Microshoot induction. For induction of microshoots, primary and secondary explants were placed on induction medium consisting of basal medium (1/2 MSBN) which was supplemented with cytokinine 6-benzylaminopurine (BAP, Lachema, Czech Republic), 5 or 10 $mg \cdot L^{-1}$, and auxin indole-3-butyric acid (IBA, 1 $mg \cdot L^{-1}$, Janssen Chimica, Belgium). As an alternative, coconut milk taken from a ripe seed (0.5 %, v/v) was used as a source of phytohormones. Explants were cultivated on the induction medium for 1 month.

Microshoot development and elongation. To achieve development and elongation of microshoots, explants were transferred from the induction medium to the basal medium (1/2 MSBN) or to a diluted basal medium (1/6 MS 1/2 BN plus sucrose 10 $g \cdot L^{-1}$) for 2 to 3 months. The medium was supplemented with activated charcoal (2 $g \cdot L^{-1}$).

As alternatives for promotion of shoot bud elongation, the basal medium was supplemented with gibberellic acid (1 $mg \cdot L^{-1}$) in combination with IBA (1 $mg \cdot L^{-1}$) or BAP (0.1 $mg \cdot L^{-1}$). Pine needle extract was also tested as a supplement to basal medium. Freshly cut current year pine shoots were ground in a blender and boiled in water for 2 h. After cooling and filtration, the water extract was added to the medium.

Statistical analysis. 15 primary explants were chosen for the establishment of cultures. 36 secondary explants were excised and used for further experiments. Replication of tests to determine induction, development and elongation of microshoots on appopriate media was 10 to 15 explants. Experiments were repeated twice with similar results.

RESULTS

Induction of microshoot formation. Pine seedlings cultivated on 1/2 MSBN medium without hormones formed shoots with needles (Figure 1A). When primary or secondary explants were transferred to a medium with cytokinine and auxin, development of axillary buds was induced, in parallel with inhibition of elongation of the apical shoot (Figure 1B). After another transfer of explants to the hormone-free basal medium supplemented with activated charcoal, the growth of microshoots continued. This led to the appearance of a cluster of shoots, usually half in height as cotyledons for hypocotyl cuttings or needles for excised shoots. However, presumably because of genetic diversity, not all the explants produced axillary shoots (Table 1). Two months after being placed on the hormone-free basal medium with activated charcoal, each shoot-forming hypocotyl cutting produced 4.6 shoots on average and each shoot-forming shoot explant produced 3.2 microshoots on average.

Effect of shoot length on morphogenesis. It was important to determine the optimum morphological characteristics for selection of explants for further multiplication. Firstly, an inverse relationship between the number of newly-formed

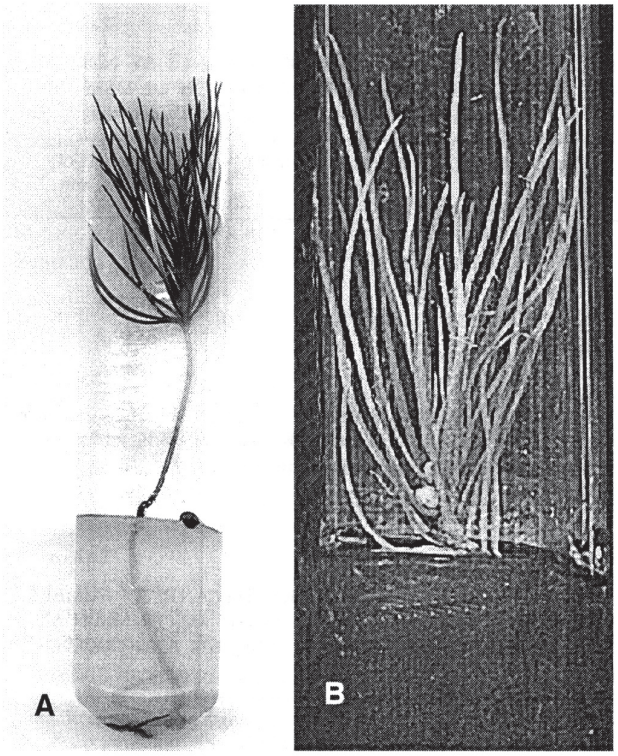

Fig. 1. **A**, Morphology of a *Pinus sylvestris* seedling cultivated on medium containing 50 % Murashige-Skoog minerals plus 50 % Bourgin-Nitsch organic compounds plus sucrose 10 $g \cdot L^{-1}$ without hormones for 5 months. **B**, Initiation of microshoot development from the axillary buds on a 5-month-old hypocotyl cutting on medium with 10 $mg \cdot L^{-1}$ 6-benzylaminopurine and 1 $mg \cdot L^{-1}$ indole-3-butyric acid.

Table 1

EFFECT OF EXPLANT TYPE AND HORMONE COMPOSITION IN BASAL MEDIUM ON AXILLARY SHOOT PRODUCTION OF *Pinus sylvestris*

Type of explant and hormone composition in basal medium	Percentage of explants undergoing multiplication, %	Average number of microshoots per explant
Hypocotyl cuttings, BAP 10 $mg \cdot L^{-1}$, IBA 1 $mg \cdot L^{-1}$ (n=10)	61.6	4.6±0.7
Excised shoots, BAP 5 $mg \cdot L^{-1}$, 0.5 % coconut milk (n=10)	71.4	3.2±0.5

BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid

microshoots and the length of the microshoots from primary explants was established (Table 2). To determine further which length of newly formed microshoots is most suitable for further multiplication, the relationship between the number of microshoots, their length, and the length of microshoot-forming secondary explants was investigated (Table 3). The number of newly-formed microshoots was directly proportional to the length of microshoot-forming shoots. In addition, secondary explants that formed the highest number of microshoots also had the highest length

Table 2

CHARACTERISATION OF PRIMARY EXPLANTS BY NUMBER AND LENGTH OF FORMED MICROSHOOTS ON 1/2 MSBN MEDIUM SUPPLEMENTED WITH BAP (10 mg·L^{-1}) AND IBA (1 mg·L^{-1})

Number of newly-formed microshoots	Percentage of primary explants, %	Average length of microshoots, mm
1	38	11.2±0.4
2	23	10.3±0.3
3–4	15	8.6±0.2
5–6	8	7.8±0.2
7	16	5.8±0.2

½ MSBN, basal medium containing 50 % Murashige-Skoog minerals and 50 % Bourgin-Nitsch organic compounds with sucrose 10 g·L^{-1}; BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid

Table 3

CHARACTERISATION OF SECONDARY EXPLANTS BY NUMBER AND LENGTH OF FORMED MICROSHOOTS ON 1/2 MSBN MEDIUM SUPPLEMENTED WITH BAP (5 mg·L^{-1}) AND COCONUT MILK

Number of formed microshoots	Average length of secondary explants at the time of transplantation, mm	Average length of newly-formed microshoots, mm	Percentage of secondary explants, %
0	7.0±0.5	–	29
1	10.2±0.5	1.4±0.1	36
2	12.3±0.8	2.2±0.1	14
3	15.8±0.7	2.4±0.1	14
4 and more	35.0±2.0	2.7±0.1	7

Abbreviations as in Table 2

of microshoots. Thus, the relationship between the number and the length of the microshoots was inverse for primary explants but positive for secondary explants. Microshoot-non-forming shoots had an average length of 7 mm, in comparison with more than 10 mm for bud-forming shoots. Microshoot-forming shoots formed on primary explants which were maintained on the 1/2 MSBN medium with activated charcoal for 2 months formed 2 to 4 new microshoots.

The survival of secondary explants for different microshoot-forming genotypes was extremely variable. For 29 % of genotypes, none of the transplanted microshoots survived, although for the remaining 71 % of genotypes forming new buds, the survival was about 50 % (Table 4). Further it was observed that a direct relationship exists between the survival of microshoots after transplantation and their length before transplantation (Table 5).

Microshoot elongation. Microshoots that formed straight needles were most suitable for further multiplication as it was possible to achieve their successful elongation. Circular

Table 4

RELATIONSHIP BETWEEN MICROSHOOT LENGTH BEFORE TRANSPLANTATION AND SURVIVAL AND FURTHER BUD FORMATION ON SHOOT EXPLANTS ON 1/2 MSBN MEDIUM SUPPLEMENTED WITH BAP (10 mg·L^{-1}) AND IBA (1 mg·L^{-1})

Shoot explants	Average length of microshoot before transplantation, mm	Percentage of secondary explants, %
Microshoot-non-forming, non-surviving	–	29
Microshoot-forming, non-surviving	5.3	35.5
Microshoot-forming, surviving	13.4	35.5

Abbreviations as in Table 2

Table 5

RELATIVE SURVIVAL OF MICROSHOOTS AFTER TRANSPLANTATION TO HORMONE-FREE BASAL MEDIUM WITH ACTIVATED CHARCOAL, IN RELATION TO THEIR LENGTH BEFORE TRANSPLANTATION

Length of microshoots, mm	Relative survival, %
2–3	64.9
4–5	75.7
6–7	81.0
8–9	83.8
10–12	94.6
> 12	100.0

or elliptic microshoots did not show any further elongation of their needles in the same cultivation conditions.

For succesful elongation of newly formed microshoots, different media were tested. The best results were achieved on basal medium with a diminished amount of minerals (1/6 MS 1/2 BN) and supplemented with activated charcoal (Table 6). Addition of gibberellic acid in the medium led to complete suppression of microshoot elongation.

Table 6

EFFECT OF MEDIA COMPOSITION ON THE SURVIVAL AND ELONGATION OF MICROSHOOTS

Medium composition	Elongation, mm per month	Relative survival, %
1/2 MSBN + charcoal 2 g·L^{-1}	0.4±0.1	86.0
1/6 MS 1/2 BN + charcoal 2 g·L^{-1}	2.8±0.5	92.1
1/2 MSBN + gibberellic acid 1 mg·L^{-1} + BAP 0.1 mg·L^{-1}	0	83.0
1/2 MSBN + gibberellic acid 1 mg·L^{-1} + IBA 0.05 mg·L^{-1}	0	33.0

Abbreviations as in Table 2

In addition, shoot elongation was promoted on shoots kept on hormone free medium (1/2 MSBN or 1/6 MS 1/2 BN plus activated charcoal) for 5 months, for shoots that already had formed real needles (about 35 mm) after transfer

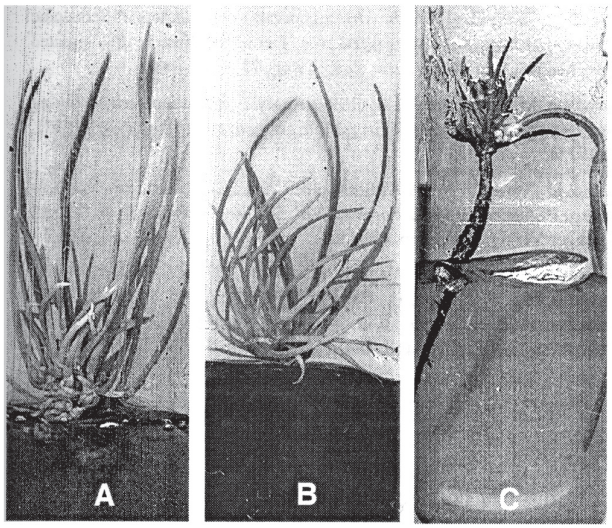

Fig. 2. **A**, Microshoot induction on secondary explants cultivated on a hormone-free medium for 5 months by transfer to medium with 6-benzylaminominopurine and indole-3-butyric acid. **B**, Promotion of microshoot elongation on the 1/2 MSBN medium supplemented with activated charcoal. **C**, Formation of real needle pairs on non-elongating root-forming primary explants with circular-shaped microshoots after transfer to the basal medium supplemented with pine needle extract.

to the basal medium (1/2 MSBN) supplemented with BAP 10 mg·L^{-1} and IBA 1 mg·L^{-1} (Figure 2A). New microshoots were formed in the centre of every needle pair. After excision of the new microshoots together with the appropriate needle pair and their transfer to 1/2 MSBN supplemented with activated charcoal, extremely active shoot elongation was promoted (Figure 2B). By using this protocol, it was possible to obtain within 4 months 13 new microshoots with an average length of 16.5 mm per initial secondary explant.

When non-elongating root-forming primary explants (with circular-shaped microshoots) were transferred to basal medium supplemented with pine needle extract (10 mL·L^{-1}), formation of shoots with real needle pairs was observed (Figure 2C). The elongation of the real needles was extremely intense, reaching up to 100 mm in length.

DISCUSSION

The present experiments represent the first step towards establishing a complete protocol of *in vitro* multiplication of *P. sylvestris* L. The microshoot induction on epicotyl cuttings of young seedlings seems to be the most reliable method for its micropropagation.

Our experiments support previous results that the presence of auxin in the induction medium is not necessary for microshoot induction in conifers (Sen *et al.*, 1994; Supriyanto and Rohr, 1994). The results confirmed the need for a specific length of microshoots (at least 10 mm) (Table 3). In contrast, it was reported earlier that a length of 8 mm might be appropriate for further multiplication of Scots pine *in vitro* (Supriyanto and Rohr, 1994).

Other authors have shown that the age of hypocotyl explants before further multiplication is of particular importance (Toribio and Pardos, 1987). In the present study, the most successful multiplication was observed from 5-month-old secondary explants after transfer to basal medium supplemented with BAP and IBA (Figures 1 and 2).

The differences in survival of secondary explants among various seedlings most probably reflect genotype-related differences among various mother trees. Genetical heterogeneity has been reported to cause differences in the initiation frequency of somatic embryogenesis (Keinonen- Mettala *et al.*, 1996). Thus, the parental effect on successful micropropagation of *P. sylvestris* should be taken into account in further studies.

Physiological age of seedlings is a very important factor for successful micropropagation. Our results supported the previous observation that organogenic potential is better if the cultured tissues are younger (Toribio and Pardos, 1987). In order to achieve micropropagation of material from mature trees, morphological rejuvenation of explants should be forced. However, another problem with *in vitro* culture of mature pine tissues is related to sterilisation (Toribio and Pardos, 1987).

Thus, the present data describing successful micropropagation of *P. sylvestris* through microshoot induction on epicotyl cuttings, form the basis for further experiments leading to multiplication of tissues from mature pine trees.

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HORMONĀLO UN BARĪBAS FAKTORU IETEKME UZ *Pinus sylvestris* L. MORFOĢENĒZI *in vitro*

Aprakstīti *Pinus sylvestris* morfoģenēzes pētījumi *in vitro*. Priežu dīgsti veidoja dzinumus ar skujām uz 50 % Murašige-Skūga / 50 % Borgina-Nitča organiskās barotnes saharozes klātbūtnē, bet bez hormonu pievienošanas. Hipokotilu spraudeņu pārvietošana uz citokinīnus saturošu barotni inducēja dzinumu pumpuru attīstību. Novērojām apgrieztu sakarību starp hipokotila dzinumu garumu pirms pārvietošanas un jaunveidoto dzinumu pumpuru daudzumu. Pumpuru augšanu stimulēja sekojošā pārvietošana uz barotni bez hormoniem. Eksplantiem, kas veidoja lielāko pumpuru daudzumu, bija raksturīgs arī vislielākais pumpuru garums. Lai stimulētu jaunveidoto dzinumu stiepšanos, eksplantus pārvietoja uz bezhormonu barotni ar samazinātu minerālu daudzumu, kurai bija pievienota aktīvā ogle.

4. Priežu pumpuru attīstības fāzes ietekme uz morfogēno kompetenci *in vitro* un nobriedušu pumpuru uzglabāšanas aukstumā ietekme uz to morfoģenēzi un fermentu aktivitāti

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SHORT COMMUNICATION

Changes of Morphogenic Competence in Mature *Pinus sylvestris* L. Buds *in vitro*

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The effects of season and cold storage on morphogenic competence in mature *Pinus sylvestris* buds were investigated. Peroxidase and polyphenol oxidase activity were measured as markers of oxidative metabolism. No growth *in vitro* was observed on explants detached from the end of January until the beginning of March. Brachioblasts, each with a couple of needles, formed on 11 % of the buds without macrostrobili that were detached in early April and introduced immediately into culture. Of the explants detached in late July, 15 % formed shoots with brachioblasts and needles. The lowest activity of peroxidase and polyphenol oxidase in pine buds was observed from the end of April until the beginning of June when morphogenic competence of tissues started to increase. Development of bud explants detached in January was achieved by cold storage for 5 months. Low polyphenol oxidase and peroxidase activity coincided with increased morphogenic potential. Results suggest that reduced or stable activity of peroxidase and polyphenol oxidase is associated with an increased ability of tissues to start growth *in vitro*.

Key words: *Pinus sylvestris* L., Scots pine, buds, morphogenic competence, peroxidase, polyphenol oxidase, seasonal changes, cold storage.

INTRODUCTION

Micropropagation of mature trees is important because it allows multiplication of superior genotypes identified in the field. Successful *in vitro* regeneration of conifers has been achieved using embryos or young seedlings (Bonga, 1991). However, maturation appears to be a problem that prevents wider application of tissue culture technology among woody species (Pierik, 1990). Also, stress due to hormonal and other stimuli in the nutrient media can cause accelerated maturation (Bonga, 1987).

Successful micropropagation of mature *Pinus* is reported for few species, including *Pinus radiata* (Horgan and Holland, 1989), *P. pinaster* (Monteuuis and Dumas, 1992), *P. brutia* (Abdullah et al., 1987), *P. lambertiana* (Gupta and Durzan, 1985), *P. taeda* (Mott and Amerson, 1981) and *P. nigra* (Jelaska et al., 1981). Of the *Pinus* species, Scots pine (*P. sylvestris* L.) is especially difficult to deal with in culture (Hohtola, 1988). Successful micropropagation of Scots pine has been established only via organogenesis from young seedlings (Jain *et al.*, 1988; Supriyanto and Rohr, 1994; Häggman *et al.*, 1996; Sul and Korban, 1998; Andersone and Ievinsh, 2000). In the case of shoot tip cultures from mature Scots pines, tissue browning and slow deterioration of the cellular ultrastructure, which finally leads to necrosis, makes tissue culture work difficult (Lindfors *et al.*, 1990).

Oxidative enzymes, e.g. peroxidases and polyphenol oxidases, participate in browning of *in vitro* cultures (Dowd and Norton, 1995). In callus cultures derived from shoot tips of mature Scots pine, browning was a consequence of high oxidative stress (Laukkanen *et al.*, 2000). It is possible that after introduction into *in vitro* culture, plant tissues with a reduced capacity for oxidative reactions will show less browning and, as a consequence, better morphogenic competence. As pronounced seasonal changes in cellular metabolism have been described for Scots pine buds (Hohtola, 1988), it is possible that the capacity for oxidative reactions changes during the growth season. On the other hand, prolonged cold storage could be used as a tool for affecting oxidative metabolism. It has been shown that cold storage of Scots pine roots for 4 months significantly decreases peroxidase activity (Ahonen *et al.*, 1989).

The aim of the present work was to investigate changes of morphogenic competence in mature *P. sylvestris* buds due to seasonal effects or cold storage. Peroxidase and polyphenol oxidase activities were measured as markers of oxidative metabolism.

MATERIALS AND METHODS

Plant material

Experiments were performed from the end of January (average temperature –5·5 °C) until the end of July (average temperature +17 °C). Buds were collected twice a month from mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Riga, Latvia. Plant material was taken randomly from different trees from the lower part of the crown. The upper part of resting vegetative buds, tips of the new shoots or newly formed buds (according to the growth phase) were used. Each time, six replicates of 20 buds per replicate were used as explants and three replicates were used for enzymatic analysis.

To investigate the effect of cold storage on morphogenesis, resting buds were collected in winter (January) and spring (April). Buds were surface sterilized and placed in

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closed containers at 5 °C. Twice a month, three replicates of 20 cold-stored buds per replicate were used as explants and three replicates were used for enzymatic analysis.

For surface disinfection, buds were washed in a solution of household soap for 1 h and then rinsed in tapwater for 1 h. They were surface sterilized with a half-diluted commercial bleach ACE (Procter & Gamble, Riga, Latvia; containing 5–15 % sodium hypochlorite) for 20 min, rinsed for 10 min in sterile distilled water, sterilized again in 15 % hydrogen peroxide and rinsed three times for 10 min in sterile distilled water. The buds were peeled and dissected aseptically.

Cold-stored buds were sterilized twice, once before cold storage when they were washed, sterilized with bleach and rinsed with distilled water, and again after cold storage when they were sterilized with bleach, rinsed, sterilized with hydrogen peroxide and rinsed again.

TABLE 1. *Media used for cultivation of* Pinus sylvestris *explants*

	Medium no. 1	Medium no. 2
Woody Plant Medium mineral salts	+	+
$AgNO_3$ 20 mg l^{-1}	+/–	+/–
Myo-inositol	100 mg l^{-1}	100 mg l^{-1}
Thiamine hydrochloride	30 mg l^{-1}	30 mg l^{-1}
Pyridoxine hydrochloride	10 mg l^{-1}	10 mg l^{-1}
Nicotinic acid	10 mg l^{-1}	10 mg l^{-1}
Dry egg powder	100 mg l^{-1}	–
Coconut milk	0·5 % v/v	–
Benzyladenine	5 mg l^{-1}	–
Adenine	–	10 mg l^{-1}
Kinetine	–	1 mg l^{-1}
Indole-3-acetic acid	0·2 mg l^{-1}	–
Naftylacetic acid	0·2 mg l^{-1}	0·1 mg l^{-1}
Sucrose	45 g l^{-1}	45 g l^{-1}
Agar	7 g l^{-1}	7 g l^{-1}
pH	5·8	5·8

Culture conditions and media

Explants were cultivated in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium. Tubes were closed with cotton wool plugs. Cultures consisted of one explant per tube. They were cultivated at 23 ± 3 °C with a 16 h photoperiod. Illumination was provided by fluorescent tubes (LB80–1 and LB80–7) combined with sunlight.

Explants were placed on medium no. 1 or medium no. 2 with Woody Plant Medium mineral salts (Lloyd and McCown, 1981), vitamins, and other components and hormones (Table 1). Medium no. 1 was used for microshoot formation on cold-stored explants as well as for callus induction. Medium no. 2 was used for induction of needle formation.

Measurement of peroxidase and polyphenol oxidase activity

For polyphenol oxidase and peroxidase measurement, buds without scales (0·5 g) were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. Enzymes were extracted with 25 mmol l^{-1} HEPES/KOH buffer (pH 7·2) containing 1 mmol l^{-1} EDTA, 3 % (w/v) PVPP and 0·8 % (v/v) Triton X-100 for 15 min at 4 °C. The homogenate was centrifuged at 15 000 *g* for 20 min. The supernatant was used for assays. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Peroxidase activity was measured spectrophotometrically at 470 nm in reaction mixture containing 2 ml 50 mmol l^{-1} sodium phosphate buffer (pH 7·0) with 10 mmol l^{-1} guaicol, 0·5 ml 0·03 mol l^{-1} H_2O_2 and 0·01 ml enzymatic extract. The reaction mixture without H_2O_2 was used as a reference.

The activity of polyphenol oxidase was determined spectrophotometrically in a reaction mixture (3 ml) containing 20 mmol l^{-1} sodium phosphate (pH 6·5) with 25 mmol l^{-1} pyrocatechol and the enzymatic extract (0·01 ml). The change in absorbance was monitored at 410 nm.

TABLE 2. *The effect of developmental phase on morphogenic competence of* Pinus sylvestris *bud explants* in vitro

Date of introduction into culture	Sterile explants (%)*	Sterile explants with macrostrobili on medium no. 1 (%)†	Sterile explants forming callus on medium no. 1 (%)†	Sterile explants forming brachioblasts with needles on medium no. 2 (%)‡
29 January	100 ± 0^{a}	87 ± 3^{a}	0	0
18 February	39 ± 2^{b}	69 ± 3^{b}	0	0
4 March	35 ± 1^{b}	67 ± 2^{b}	0	0
23 March	27 ± 1^{c}	96 ± 2^{c}	88 ± 3^{a}	0
3 April	24 ± 1^{c}	94 ± 2^{c}	98 ± 2^{a}	11 ± 2^{a}
15 April	32 ± 1^{b}	83 ± 2^{a}	80 ± 3^{a}	0
29 April	37 ± 3^{b}	89 ± 3^{a}	92 ± 4^{a}	0
18 May	40 ± 2^{b}	0	0	0
2 June	21 ± 2^{c}	0	0	0
20 June	29 ± 2^{bc}	0	0	0
6 July	23 ± 1^{c}	0	0	0
21 July	42 ± 2^{b}	0	0	15 ± 2^{a}

Values within a column with the same superscript are not significantly different at $P = 0{\cdot}05$ among dates using Student's *t*-test.

* Sterilization of this material was very difficult. Values (± s.e.) are means of six independent replicates, 20 explants each (three replicates on medium no. 1 and three on medium no. 2).

† Values (± s.e.) are percentage means from sterile explants of three independent replicates.

‡ Values (± s.e.) are percentage means from sterile explants of three independent replicates.

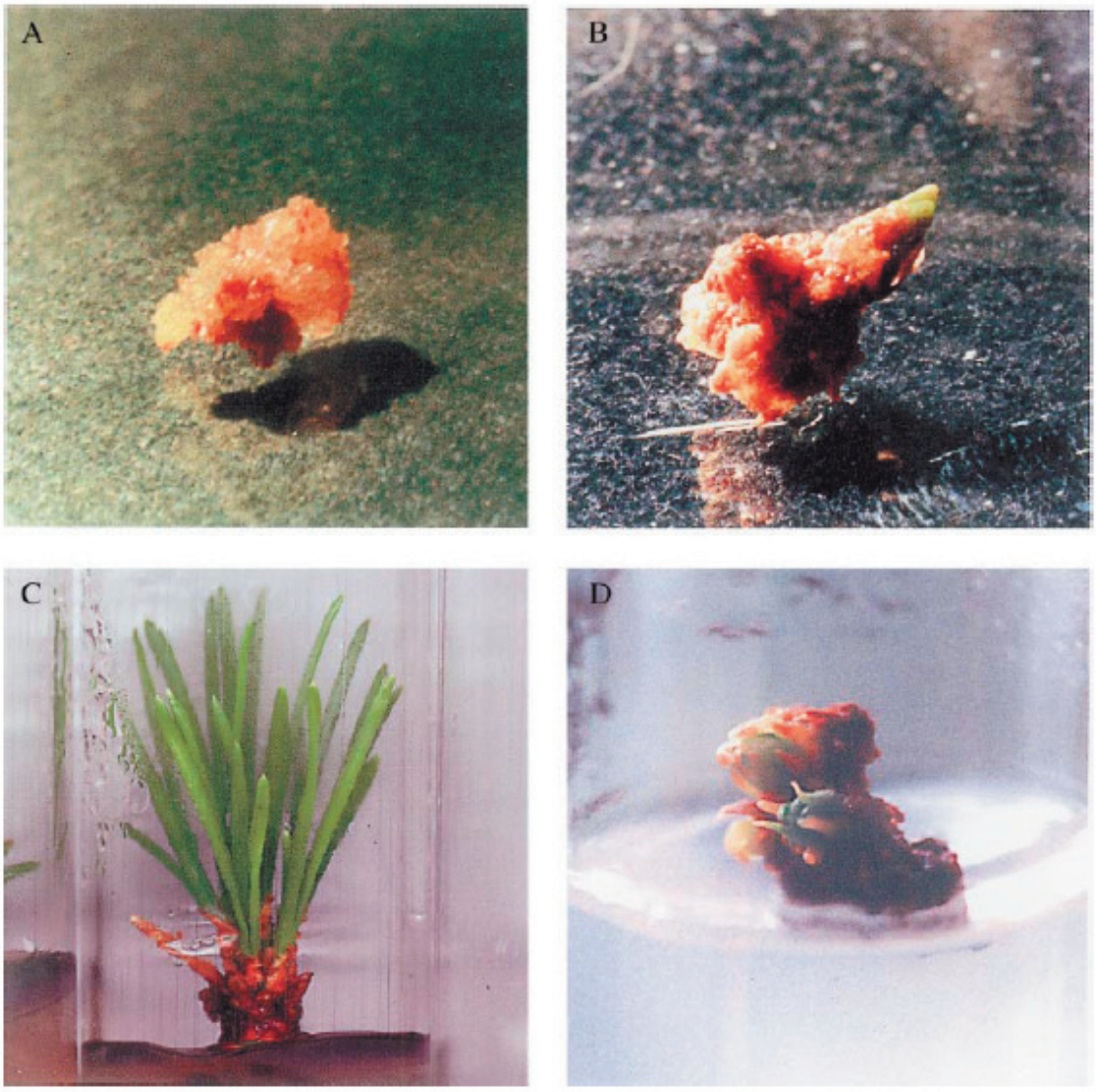

FIG. 1. A, Isolated microstrobilus initial of mature *Pinus sylvestris* with callus 2 weeks after introduction *in vitro*. The bud was excised at the end of March. Medium no. 2. B, Mature *P. sylvestris* explant with brachioblast and a pair of needles 2 months after introduction *in vitro*. The bud was excised on 3 April. Medium no. 1 for first month; medium no. 2 for second month. C, Mature *P. sylvestris* explant with shoots consisting of brachioblasts and needles 2 months after introduction *in vitro*. The bud was excised in late July. Medium no. 2. D, Mature *P. sylvestris* explant with microshoots 6 weeks after introduction *in vitro*. The bud was excised in late January and stored at 5 °C for 5 months. Medium no. 1.

RESULTS

The effect of developmental phase on morphogenic competence in vitro *and enzyme activities*

The parts of the buds, used as explants, had macrostrobili (Table 2). No growth response of macrostrobili could be observed *in vitro* on explants detached from the end of January until the beginning of March. They exhibited browning, without any changes in size and structure. Rapid formation of callus on macrostrobili was observed when buds were introduced into culture between the end of March and the end of April, i.e. just before rapid growth of macrostrobili (Table 2; Fig. 1A). Callus was formed on about 90 % of macrostrobili near the tip of the bud as well as on those isolated from the bud and placed directly on the surface of the medium. The callus was larger in the latter case. Browning of the callus began within 2 weeks and led to necrosis after 1 month.

On 11 % of the buds detached in early April and introduced immediately into culture, formation of brachioblasts with a couple of needles was observed (Table 2; Fig. 1B). Needles were up to 4 mm long. The rest of the explants did not show any signs of development and turned brown.

In late July, when new buds had already formed on the tips of branches, 15 % of explants detached at that time formed shoots with brachioblasts and needles up to 3 cm in length on medium no. 2 (Table 2; Fig. 1C).

Activity of peroxidase and polyphenol oxidase was monitored in intact pine buds during the growth season. The activity of peroxidase gradually decreased from the end of January until the end of April, and was lowest from the

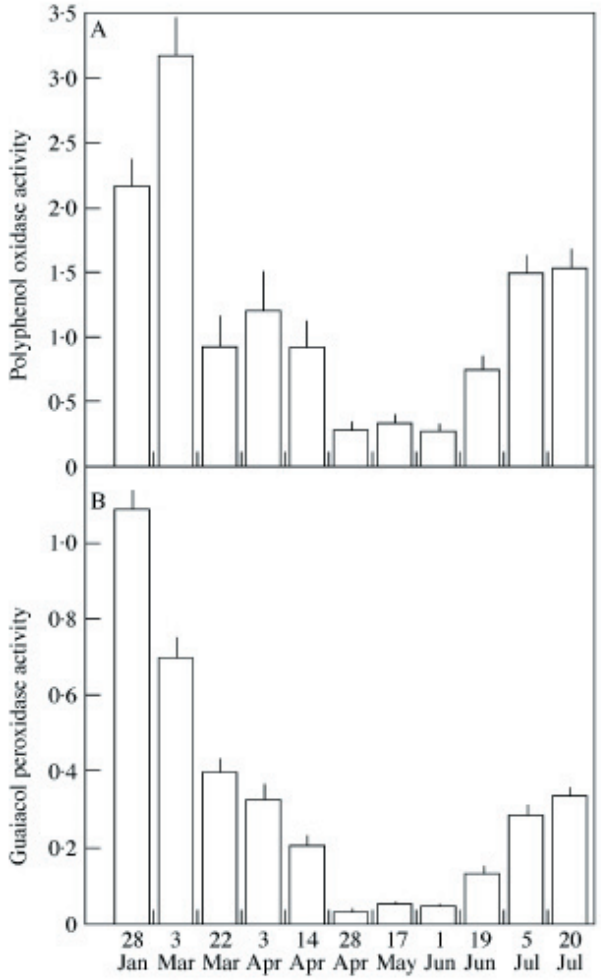

FIG. 2. Time course of polyphenol oxidase activity (A) and guaiacol peroxidase activity (B) in intact *P. sylvestris* buds during the growth season. Enzyme activities are expressed as $\Delta A\ min^{-1}\ mg^{-1}$ protein. Values (± s.e.) are means of three independent measurements for each date.

end of April until the beginning of June (Fig. 2A), increasing again from mid-June. The level of polyphenol oxidase fluctuated more than that of peroxidase during the growth season (Fig. 2B). At the beginning of April, when the buds started to open, the activity of polyphenol oxidase decreased. The period of lowest activity was the same for both enzymes—from the end of April until the beginning of June. During this period the most active new shoot growth occurred. In the middle of June, when maturation of new shoots began, the activity of the enzymes increased, but in July the increase stopped.

The effect of cold storage on morphogenesis and enzyme activities

Buds detached in winter and spring did not develop *in vitro* without any special treatment and gradually browned over the course of a few weeks. Development of such explants was achieved by using cold storage. A considerable increase (about two-fold) of the apical part of the bud was seen on 15 % of buds detached in January and stored at 5 °C for 2 weeks before being introduced into culture, but they did not form shoots (Table 3). When buds detached in January were stored in the cold for 5 months and then introduced into culture in late June, 30 % of them produced microshoots on medium no. 1 (Table 3; Fig. 1D).

During cold storage of mature resting buds the activity of polyphenol oxidase decreased from April to June and increased slightly from June to July (Fig. 3A). The activity of peroxidase in cold-stored buds fluctuated during the period of investigation (Fig. 3B). The activity was lowest at the beginning of May and at the beginning of July, but increased rapidly towards the end of July.

DISCUSSION

By using cold storage as a means of affecting oxidative metabolism, it was possible to achieve direct morphogenesis

TABLE 3. *The effect of cold storage on morphogenic competence of* Pinus sylvestris *bud explants* in vitro

Date of introduction into culture	Sterile explants (%)*	Sterile explants exhibiting bud growth on medium no. 1 (%)†	Sterile explants forming microshoots on medium no. 1 (%)‡
30 January	100 ± 0[a]	0	0
14 February	64 ± 2[b]	15 ± 1	0
5 March	54 ± 2[c]	0	0
24 March	53 ± 4[bc]	0	0
5 April	21 ± 2[d]	0	0
21 April	16 ± 2[d]	0	0
6 May	21 ± 5[d]	0	0
20 May	3 ± 2[e]	0	0
4 June	4 ± 1[e]	0	0
21 June	16 ± 2[d]	0	32 ± 2
7 July	0	0	0
26 July	0	0	0

Buds were detached on 30 January.

* Sterilization of this material was very difficult. Values (± s.e.) are means of three independent replicates, 20 explants each.

† Values (± s.e.) are percentage means from sterile explants of three independent replicates.

‡ Values (± s.e.) are percentage means from sterile explants of three independent replicates. Values within a column with the same superscript are not significantly different at $P = 0{\cdot}05$ among dates using Student's *t*-test.

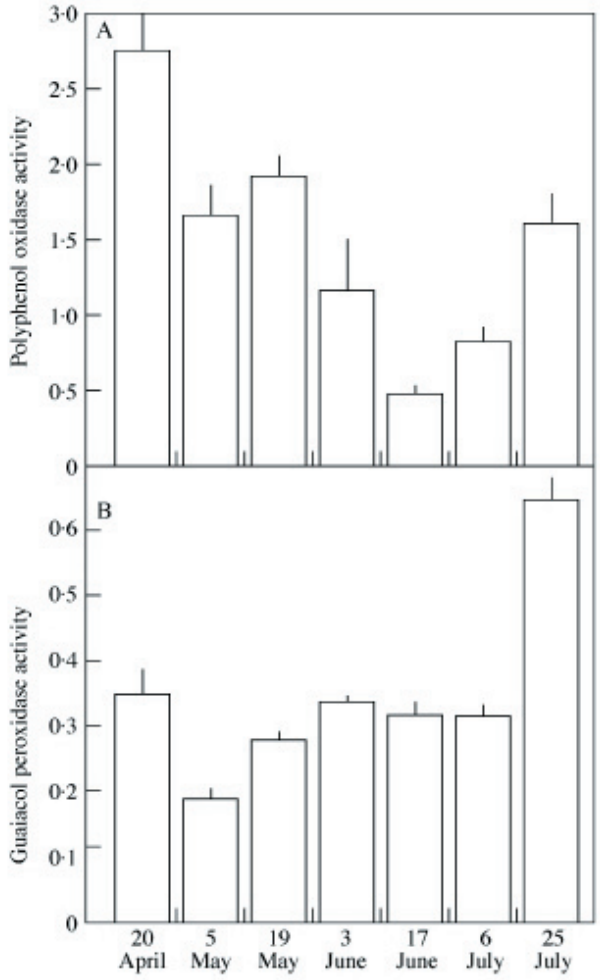

FIG. 3. Time course of polyphenol oxidase activity (A) and guaiacol peroxidase activity (B) during cold storage (5 °C) of mature resting *P. sylvestris* buds detached in January. Enzyme activities are expressed as ΔA min^{-1} mg^{-1} protein. Values (± s.e.) are means of three independent measurements for each date.

on mature pine buds. To our knowledge, this is the first successful case of morphogenesis of mature Scots pine tissues apart from callus formation. In previous experiments, the newly formed adventitious shoot primordia have failed to grow beyond the microscopic level (Hohtola, 1988).

Our experiments involving cold storage of buds before introducing them into culture suggest that prolonged cold storage of detached material could be used to affect metabolic processes in Scots pine tissues. The decreased activity of polyphenol oxidase and peroxidase during cold storage indicates the possibility of increased morphogenic activity due to a lowered capacity for oxidative metabolism.

Particularly high peroxidase activities in callus tissues initiated from mature trees cause rapid and early browning and possibly lead to cell death (Laukkanen *et al*., 1999). It is well known that conifer tissues are especially rich in phenolic substances (Nyman, 1985). Obviously, the damage during *in vitro* manipulation of cultivated plant material causes mixing of the contents of cellular compartments, leading to oxidation of various phenolic compounds by peroxidases and polyphenol oxidases. Comparing the growth activity of mature pine buds *in vitro* with changes in the activity of peroxidase and polyphenol oxidase during the growth season showed that the period of decreased enzyme activity coincided with rapid formation of callus tissues on macrostrobili explants. At the time when explants were able to form shoots with brachioblasts and needles, the increase in enzyme activity stopped. These results suggest that reduced or stable activity of peroxidase and polyphenol oxidase leads to an increased ability of tissues to start growth *in vitro*. It should be mentioned that peroxidases are a diverse group of enzymes that participate in many physiological processes. Therefore, not all the changes of peroxidase activity during cold storage or the growth season could be attributed to tissue browning and deterioration.

However, it is possible that factors other than peroxidases and polyphenol oxidase may contribute to loss of morphogenic competence in mature pine tissues. It has been shown that *P. sylvestris* has especially strong wounding reactions, including increased activity of enzymes involved in oxidative metabolism that in turn inhibit differentiation and growth (Hohtola, 1988). On the other hand, a relatively high percentage of infections during storage at 4 °C (Table 3) supported the idea that endophytic microbes in Scots pine buds are a potential cause of the defence reactions (Pirttilä *et al.*, 2002). In addition, *P. sylvestris* may have some specific requirements in the culture medium or growing conditions that have not yet been identified. Further research is therefore needed to optimize conditions for successful multiplication of established cultures.

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5. Nobriedušu audu uzglabāšanas sasaldētā stāvoklī ietekme uz to morfogēno potenciālu, fermentu aktivitāti, cietes un cukuru saturu

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BRIEF COMMUNICATION

In vitro regeneration of mature *Pinus sylvestris* buds stored at freezing temperatures

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Abstract

Changes of morphogenic competence in mature *P. sylvestris* L. buds due to frozen storage were investigated. The highest callus formation was registered on explants stored at -18 °C for three months, but on explants stored for five months, it was also higher than in the control. Budding and development of needles *in vitro* was observed only for buds frozen three to five months. Peroxidase activity was lowest in these buds. In contrast, polyphenol oxidase activity in bud tissues continually increased during frozen storage. Within 10 months of frozen storage the content of starch and sugars in resting buds changed. It may be concluded that changes in composition of non-structural sugars in pine buds after five months of frozen storage are part of metabolic changes leading to loss of morphogenic capacity.

Additional key words: peroxidase, polyphenol oxidase, Scots pine, sugars.

Scots pine (*Pinus sylvestris* L.) is one of commercially most important conifer species in Northern Hemisphere. Tissue culture provides an important tool for practical propagation of selected genotypes of mature trees. However, no practically suitable method has been described so far for *P. sylvestris*.

Recent investigations have revealed that several peculiarities make mature Scots pine tissue culture difficult, *e.g.*, high oxidative stress during cultivation (Laukkanen *et al.* 2000), strong wound reactions accompanied by increase in phenolic substances (Hohtola 1988), and endophytic microbes (Pirttilä *et al.* 2002). As a result, rapid tissue browning, followed by deterioration of cellular ultrastructure and necrosis, as well as high percentage of infections usually occurs during tissue culture of *P. sylvestris*.

Our previous experiments with mature pine tissues revealed that morphogenic potential could be increased by cold storage of pine buds at 5 °C (Andersone and Ievinsh 2002). However, a relatively high percentage of infections during storage and a low rate of microshoots-forming explants were among major drawbacks of the method making effective propagation difficult.

A possibility to affect embryogenesis and morphogenesis by frozen storage of plant material was shown for other tree species (Bonga 1996). Yet, in spite of intensive practical use of cryopreservation of plant tissues for germplasm conservation (Sakai 2000, Mathur *et al.* 2003) there is practically no information available on biochemical changes during freezing storage of detached plant material.

The aim of the present work was to investigate the changes of morphogenic competence in mature *P. sylvestris* buds due to frozen storage. Peroxidase and polyphenol oxidase activities were measured as markers of oxidative metabolism. Starch and sugars content in buds was measured to investigate biochemical processes during storage as well as to elucidate the further nutritional demands of buds *in vitro*.

Terminal sections with buds and needles, about 15 cm long, were cut from branches in the lower half of the crown randomly from different mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Riga, Latvia. Plant material was collected in winter of 2001 and 2002 (end of February with average daily temperature of -5 °C). The branches, together with a few handfuls of snow, were enclosed in plastic bags, which were quickly taken to a freezer running at -18 °C. Within 10 months of

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frozen storage branches were periodically in 1 to 4 months interval removed from the freezer. Buds were cut off from the branches and used as explants and for biochemical analyses. Each time three replicates of 20 buds per replicate were used as explants and three replicates were used for biochemical analysis.

To prepare the buds for tissue culture the following procedure was used. Buds were immersed in a solution of household soap for 1 h then rinsed in tap water for 1 h and surface sterilized with a partially diluted commercial bleach ACE (*Proctore and Gamble*, Riga, Latvia; containing 5 - 15 % sodium hypochlorite) for 20 min, rinsed again for 10 min in sterile distilled water, sterilized once more in 15 % H_2O_2 and rinsed three times for 10 min in sterile distilled water. Finally, the buds were peeled and dissected aseptically. It should be mentioned that only the upper part of buds was used as explants. For biochemical analyses peeled buds were used. For peroxidase and polyphenol oxidase measurements buds without scales (0.5 g) were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. For analyses of starch and sugars buds without scales (0.5 g for each replicate) were fixed in a hot steam for 20 min and dried.

Bud explants were cultivated in 20 × 200 mm glass test tubes containing 10 cm^3 nutrient medium solidified with agar. Tubes were closed with cotton-wool plugs and covered with thin polythene film fixed with a rubber band. Each tube contained one explant. They were cultivated at temperature of 23 ± 3 °C, 16-h photoperiod (irradiance of 40 - 45 μmol m^{-2} s^{-1}, fluorescent tubes *LB80-1* and *LB80-7* combined with sunlight).

Each time half of the explants were placed on medium for callus induction, and the other half were placed on medium for brachioblast and needle formation. For callus initiation explants were cultivated on a Murashige and Skoog medium modified by Hohtola (1988) with the exception that the only inorganic nitrogen source was 2 mM NH_4NO_3. As growth regulators, 4.5 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 1.7 μM benzyladenine (BA), and 1.8 μM kinetin were used. A pH of the medium was adjusted to 5.6 - 5.7. For brachioblast and needle formation explants were cultivated on a Woody Plant Medium (WPM, Lloyd and McCown 1981) as modified by Andersone and Ievinsh (2002). 0.5 μM naphtyl acetic acid (NAA), 54 μM adenine, and 4.7 μM kinetin were used as growth regulators. The medium was supplemented with 0.6 mM myo-inositol, 88.9 μM thiamine hydrochloride, 48.6 μM pyridoxine hydrochloride, and 81.2 μM nicotinic acid. Explants were transferred to a fresh medium monthly.

To determine starch content, dried buds were ground with 10 cm^3 of solution containing 80 % (m/v) $Ca(NO_3)_2$ and boiled for 3 min to pass starch into the colloidal solution. The amount of starch was measured by Berthram method of bichromate-sodium thiosulfate titration (Strong and Koch 1974).

For measurement of non-polymeric sugars dried buds were ground to fine powder to which 10 cm^3 98 % (v/v) ethanol and 50 cm^3 of hot distilled water was added. Extraction was made in a water bath at 70 - 80 °C for 45 min. After extraction the material was supplemented with $Pb(CH_3COO)_2$ for sedimentation of proteins, fats and tannins. For the sedimentation of the remaining Pb ions 2 cm^3 of saturated Na_2SO_4 was added. The solution was filled up to 100 cm^3 with distilled water and filtered. The amount of reducing sugars in extracts was measured by Shaffer and Somogyi (1933) method of copper-iodometric titration (Strong and Koch 1974).

For determination of sucrose content, the total amount of sugars was measured first. Three cm^3 of 8 % (m/v) oxalic acid was added to 25 cm^3 of filtrate. To hydrolyze the sucrose, the solution was heated in boiling water for 10 min. After cooling a drop of methylene red was added and the solution was neutralized with 1 M NaOH until color became yellow. Distilled water was added up to 50 cm^3. The total amount of sugars was measured as described previously for reducing sugars. The content of sucrose was calculated by subtracting the content of reducing sugars from the total content of sugars and multiplying the result by 0.95. Sugar concentration was expressed as percentage of dry mass attributable to sucrose, starch, reducing sugars (hexoses glucose, and fructose), and total nonstructural sugars.

Enzymes were extracted from ground bud tissues with 25 mM HEPES/KOH buffer (pH 7.2) with addition of 1 mM EDTA, 3 % (m/v) insoluble polyvinylpyrrolidone, and 0.8 % (v/v) Triton X-100 for 15 min at 4 °C. The homogenate was centrifuged at 15 000 *g* for 20 min. Peroxidase and polyphenol oxidase activity was measured in the supernatant as described previously (Andersone and Ievinsh 2002).

Formation of callus on Hohtola's medium in control explants was observed within a month of cultivation (57 %, Table 1). The highest callus formation was registered on explants, stored for 3 months at -18 °C (100 %), but on explants, stored for 5 months, it was still higher (67 %) than in the control. Explants stored for six or more months did not form callus.

Budding and development of needles *in vitro* was observed only for buds frozen 3 to 5 months (20 - 30 % of explants on Woody Plant Medium, Table 1). Buds without freezing or those frozen for 6 or more months did not form any needles. Peroxidase activity was lower in buds, stored for 3 to 5 months, than in control (Table 1). Polyphenol oxidase activity in bud tissues continually increased during frozen storage (Table 1).

Within 10 months of frozen storage the content of starch and sugars in resting buds of mature *P. sylvestris* had changed. There were no statistically significant

Table 1. Changes of morphological characteristics *in vitro* and biochemical characteristics during frozen storage of mature resting pine buds. The ability to form callus was estimated for explants cultivated on Hohtola's medium. Needle formation was estimated on explants cultivated on Woody Plant Medium. Pine bud explants were taken from frozen material at the times indicated and placed on appropriate media. Morphological characteristics and sterility were evaluated a month later. Enzyme activities and sugar content were measured immediately after preparation of bud explants from frozen material. Equal letters for morphological parameters indicates non-significant differences at $P = 0.05$ between the time points using Student's *t*-test. Data are means from three replicates for every time point (± SE for biochemical measurements). For morphological characteristics 10 explants per replicate were measured. For sterility 20 explants per replicate were estimated.

	Time of storage [months]							
	0	1	2	3	4	5	6	10
Sterility [%]	43[a]	n.d.	n.d.	54[b]	n.d.	26[c]	10[d]	0
Callus-forming explants [%]	57[a]	n.d.	n.d.	100[b]	n.d.	67[c]	0	0
Needle-forming explants [%]	0	n.d.	n.d.	29[a]	n.d.	20[a]	0	0
Peroxidase activity [$\Delta A\ g^{-1}$(f.m.) min^{-1}]	95.6 ± 3.4	n.d.	n.d.	84.6 ± 2.6	n.d.	84.9 ± 3.3	98.4 ± 10.5	110.4 ± 5.8
Polyphenoloxidase activity [$\Delta A\ g^{-1}$ (f.m.) min^{-1}]	3.5 ± 0.9	n.d.	n.d.	6.5 ± 1.0	n.d.	8.7 ± 2.0	13.3 ± 0.2	19.2 ± 1.0
Sucrose [% d.m.]	7.50 ± 0.05	7.46 ± 0.10	7.64 ± 0.06	7.98 ± 0.09	6.76 ± 0.12	6.87 ± 0.03	6.13 ± 0.13	3.84 ± 0.14
Starch [% d.m.]	1.92 ± 0.05	1.61 ± 0.06	1.62 ± 0.03	1.36 ± 0.12	1.63 ± 0.05	1.58 ± 0.04	1.48 ± 0.05	1.38 ± 0.03
Glucose + fructose [% d.m.]	0.80 ± 0.05	0.63 ± 0.05	1.00 ± 0.02	1.21 ± 0.04	1.60 ± 0.06	1.65 ± 0.01	2.37 ± 0.07	4.16 ± 0.45

changes of the total content of non-structural sugars (data not shown).

However significant changes in the composition were found. The content of sucrose increased during first months of storage reaching maximum at 3 months followed by a decline (Table 1). In contrast, the content of reducing sugars (hexoses, glucose, and fructose) moderately increased up to 5 months with a sharp increase during the last part of the incubation period. The content of starch in pine buds slowly decreased during incubation. At the end of the experiment, the content of starch had decreased for about 31 %, the content of sucrose 58 %, but content of glucose and fructose together increased for 79 % (Table 1).

The results of the presented experiments with frozen storage of pine buds before introducing them *in vitro* suggest that such storage of detached material could be used for affecting metabolic processes in Scots pine tissues. Only limited information is available on biochemical changes during prolonged storage of frozen plant material. In contrast to widespread opinion that frozen state represents a situation of arrested metabolism, the present experiments clearly showed that during frozen storage of pine buds at -18 °C significant changes of sugar metabolism and oxidative enzyme activities occur.

Due to frozen storage, morphogenic capacity considerably increased in pine buds stored for 3 months and, to a lesser extent in buds stored for 5 months, in comparison with control buds introduced in a culture immediately after collecting. It was revealed by our previous experiments (Andersone and Ievinsh 2002) that lowered activity of oxidative enzymes in pine tissues might contribute to better morphogenic potential. In the present experiments, increased ability to form callus and needles coincided with the lowest peroxidase activity in frozen stored pine buds (Table 1). In addition, the period of highest morphogenic capacity coincided with a highest sucrose content.

In leaf buds of pines, extracellular freezing occurs in response to subfreezing temperatures, in contrast to extra-organ freezing in leaf buds of other conifers (Ide *et al.* 1998). This leads to extremely high freezing tolerance of pine bud tissues. In our experiments, Scots pine buds were collected in winter in a state of full adaptation to low temperature conditions. Therefore, biochemical changes in plant tissues related to cold acclimation should be taken into account for understanding the results of the present experiments. Increase of soluble sugars, mostly sucrose, usually occurs in plants during cold hardening in parallel with decline in the content of insoluble sugars (Oleksyn *et al.* 2000). It is thought that increase in sugar concentration is a result of the degradation of starch (Fischer and Höll 1991). Accumulation of soluble sugars can prevent membrane injuries during freezing (Fujikawa and Jitsuyama 2000). In addition, during low-temperature storage free radical mediated oxidative stress occurs (Hendry 1993).

Therefore, increase in antioxidant enzyme activities is essential for cold acclimation (Tao *et al*. 1998). However, increased oxidative activity in mature pine tissues is among factors leading to decreased morphogenic potential (Andersone and Ievinsh 2202).

It may be concluded that changes in composition of non-structural sugars in pine buds after 5 months of frozen storage are part of metabolic changes leading to loss of morphogenic capacity.

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6. Barotnes pH ietekme uz *Pinus sylvestris* audu kultūras reģenerācijas spējām un oksidatīvo fermentu aktivitāti

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Medium pH affects regeneration capacity and oxidative enzyme activity of *Pinus sylvestris* in tissue culture

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Abstract

Medium pH that is one of the important factors of the physico-chemical environment during development of plant tissues in conditions of *in vitro* culture which is modified during preparation of media, but also changes with developing explants. The present experiments were performed to monitor the changes of medium pH and to determine the effect of medium pH on physiological characteristics of mature *Pinus sylvestris* L. tissue cultivated *in vitro*. The final changes of medium pH after addition of agar and autoclaving were towards alkalinity in the range of initial pH 3.0 to 5.5. Results of the experiments proved that in general cultivation of pine buds causes acidification of the medium. The degree of acidification of medium during cultivation depends on initial pH of medium, morphogenesis of explants and duration of cultivation period without transplantation. Lowered activity of oxidative enzymes in buds cultivated on more acidic medium and less necessity for acidification to reach the equilibrium in medium pH suggest that a low initial pH is more suitable for successful pine bud morphogenesis.

Key words: medium pH, morphogenesis, peroxidase, *Pinus sylvestris*, polyphenol oxidase, Scots pine, tissue culture.

Introduction

Medium pH is one of the important factors of physico-chemical environment during development of plant tissues in conditions of *in vitro* culture (Williams et al. 1990). The optimal pH of *in vitro* medium for different phases of morphogenesis (successful *in vitro* culture establishment, intensive shoot proliferation, induction of the rooting) for woody species varies (von Arnold, Eriksson 1982; Williams et al. 1985; Saborio et al. 1997; Ostrolucka et al. 2004).

Suboptimal pH levels can cause abnormalities in the development of explants e.a. reduction in growth of the hypocotyls, stem and roots, changes in leaf coloring (Gurel, Gulsen 1998; Laukkanen et al. 2000b). Optimal pH of media *in vitro* can be different from that of soil *ex vitro* (Sen et al. 1994) and in *ex vitro* acclimatization in hydroponic culture (Ingestad 1979; Bozhkov, von Arnold 1998).

In tissue culture practice pH of medium is adjusted before autoclaving and changes of pH during autoclaving and cultivation of tissues are usually ignored. The changes during autoclaving depend upon the initial pH and characteristics of the gelling agent (Williams et al. 1990; Van Winkle, Pullman 2003). Contrary to common belief, the changes of medium

pH during cultivation are not a response to wounding. Although wounding stimulates the decrease in pH over the first few days, the effect is insignificant (Williams et al. 1990).

Plant tissues can maintain a relatively constant cytoplasmic pH across an external pH range of 4 to 9 (Caponetti et al. 1971). Plant cells can also modify the external pH – the explants raise or lower the pH, depending on which pH range they grow, until an equilibrium occurs (Mac AntSaoir, Damvoglou 1994). A localized change of pH takes place at the site of contact between the plant tissue and the medium in tissue culture as well as in soil (Constable 1963; Haussling et al. 1985).

For tissue culture of different *Pinus* species pH 5.5 to 6.0 is used (Durzan, Chalupa 1976; Sen et al. 1994; Saborio et al. 1997; Sul, Korban 2004; Tang et al. 2004). *Pinus sylvestris* L. usually is cultivated in media with pH 5.5 to 5.8 (Bornman, Jansson 1980; Hohtola 1988; Žel et al. 1988; Supriyanto, Rohr 1994; Laukkanen et al. 1997; Laukkanen at al. 1999; Lelu et al. 1999; Laukkanen et al. 2000b). Conifers can also change the media pH during cultivation. Embryogenic cultures of Norway spruce, loblolly pine and Douglas-fir tend to decrease the pH of growth medium (Van Winkle, Pullman 2003), but suspension culture of *Pinus banksiana* slightly increases pH of the medium during cultivation (Durzan, Chalupa 1976).

The present experiments were performed to study changes of medium pH and the effect of medium pH on physiological characteristics of mature *Pinus sylvestris* tissue cultivated *in vitro*. Oxidative enzyme activities (peroxidase and polyphenol oxidase) were used as indicators of morphogenic potential (Andersone, Ievinsh 2005).

Materials and methods

Plant material

Plant material was collected from mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Salaspils (Riga Region, Latvia). Buds were taken randomly from different trees from the lower part of the crown. Buds were surface sterilized with a half-diluted commercial bleach ACE (Procture and Gamble; containing 5 to 15 % sodium hypochlorite) for 20 min, rinsed for 10 min in sterile distilled water, sterilized again in 15 % hydrogen peroxide and rinsed three times for 10 min in sterile distilled water. The buds were peeled and dissected aseptically.

Culture conditions and media

Explants were cultivated in 40 × 100 mm glass tubes containing 20 ml agarized medium (for experiments with medium pH measurements after autoclaving) or in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium (for experiments with medium pH measurements before adding agar and autoclaving). Vessels were closed with cotton-wool plugs, and covered with polythene film fixed with a rubber band. Each vessel contained one explant. They were cultivated at temperature 20 ± 5 °C in natural light (if not indicated otherwise).

Explants were cultivated on Woody Plant Medium (WPM; Lloyd, McCown 1981) as modified by Andersone and Ievinsh (2002). 0.5 mM naphtyl acetic acid (NAA), 54 mM adenine, and 4.7mM kinetin were used as growth regulators. The medium was supplemented with 0.6 mM myo-inositol, 88.9 mM thiamine hydrochloride, 48.6 mM pyridoxine hydrochloride, 81.2 mM nicotinic acid and 131.4 mM sucrose. It contained

0.57 % of plant agar.

For experiments on the effect of tissues on medium pH, medium was adjusted to 5.8 before adding agar and autoclaving. For experiments of effect of medium pH on tissue development, pH was adjusted 3.0 to 7.0 with interval pH 0.5 before adding agar and autoclaving. The adjustment was performed either by 1N HCl or 1N KOH.

Measurement of pH

Measurements of pH were carried out with a pH meter pH 211, by immersion of the electrode HI 1131B (Hanna Instruments). For measurement of medium pH before autoclaving the electrode was stirred in the liquid medium before agar was added. For measurement of media after autoclaving the electrode was imbedded in it two days after autoclaving. To investigate medium pH changes after cultivation of mature tissue, pH was measured by placing the electrode into the hole from which the bud explant was removed.

Effect of media preparation

To determine changes of pH during media preparation the appropriate mineral salts and organic components of the medium were mixed and pH was adjusted (2.5 to 7.0 with 0.5 intervals). Agar was then added, dissolved in a half of the volume by heating, media were dispensed into cultivation vessels and autoclaved at 121 °C and 103 KPa for 20 min. The pH in each vessel was measured two days later.

Effect of in vitro *cultivated bud tissue on pH of the medium*

Buds were cultivated in vitro and placed on fresh medium (pH 5.8) weekly or after every 2, 3, 4 or 5 weeks. The third part of the explants were cultivated in a growth chamber with a 16 h photoperiod, where illumination was provided by fluorescent tubes OSRAM L 36/W77, the rest were cultivated near a window in laboratory under natural light with a brief period of direct sunlight or with a prolonged period of direct sunlight. Twenty sterile explants were taken for each variant. pH of the media was measured after autoclaving and after removal of bud explants weekly or after every 2, 3, 4 or 5 weeks. The changes of pH during cultivation were estimated for 6 to 8 weeks. The experiment was carried out for 10 months. The morphological condition of bud explants (development of brachyoblasts and needles, necroses) was recorded during cultivation and at the end of experiment.

Effect of medium on in vitro *cultivated bud tissue*

Buds were cultivated on media with different pH levels (pH 3.0 to 7.0 with intervals pH 0.5) adjusted before autoclaving.

To investigate the changes of medium pH during cultivation, buds were cultivated for two months, then transplanted on fresh medium with the same variant of pH and cultivated for 10 months. Media pH was measured after removal of cultured buds. To investigate the effect of different medium pH on bud morphogenesis, morphological characteristics (needle formation, amount of needles, length of needles, necroses) were recorded after 2, 4 and 8 months of cultivation.

To investigate the effect of different medium pH on activity of peroxidase and polyphenol oxidase, buds, cultivated in vitro for seven weeks, were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. The experiment was repeated twice.

Enzymes were extracted with 25 mmol l^{-1} HEPES/KOH buffer (pH 7.2) containing 1 mmol l^{-1} EDTA, 3 % (w/v) PVPP and 0.8 % (v/v) Triton X-100 for 15 min at 4 °C. The homogenate was centrifuged at 15 000 g for 20 min. The supernatant was used for assays.

Peroxidase activity was measured spectrophotometrically at 470 nm in reaction mixture containing 2 ml 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) with 10 mmol l^{-1} guaiacol, 0.5 ml 0.03 mol l^{-1} H_2O_2 and 0.01 ml enzymatic extract. The reaction mixture without H_2O_2 was used as a reference.

The activity of polyphenol oxidase was determined spectrophotometrically in a reaction mixture (3 ml) containing 20 mmol l^{-1} sodium phosphate (pH 6.5) with 25 mmol l^{-1} pyrocatechol and the enzymatic extract (0.01 ml). The change in absorbance was monitored at 410 nm.

Results

During medium preparation pH changed towards alkalinity. Strongly acidic (pH 2.5) or alkaline (6.0 to 7.0) media did not change significantly during autoclaving (Fig. 1). Results of both experiments proved that in general, cultivation of pine buds caused acidification of the medium (Fig. 1 to 4).

During bud cultivation on media with different initial pH the final pH was similar. Two- and 10-month long cultivation of buds on media with pH 3.5 to 7.0 resulted in almost the same acidity of medium for all variants of pH. After 2 months of cultivation the mean pH of medium was 5.26 ± 0.06, but in 10 months cultivation the mean medium pH declined to 4.18 ± 0.11 (Fig. 1). Buds, cultivated on medium with alkaline initial pH, acidified the medium more than buds cultivated on medium with initially acidic pH.

The rate of acidification depended on the duration of cultivation without transplantation.

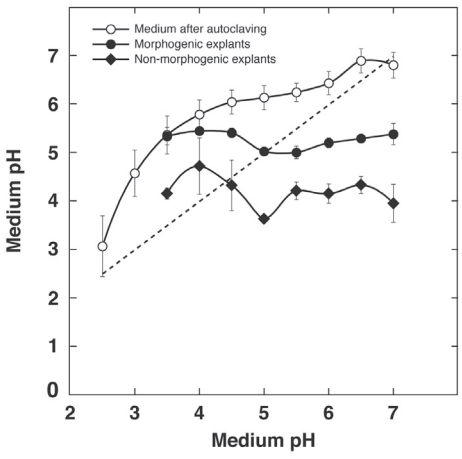

Fig. 1. Change of medium pH after autoclaving and with different types of pine bud explants during 10 months of cultivation without transplanting. Dashed line indicates no change in the pH values.

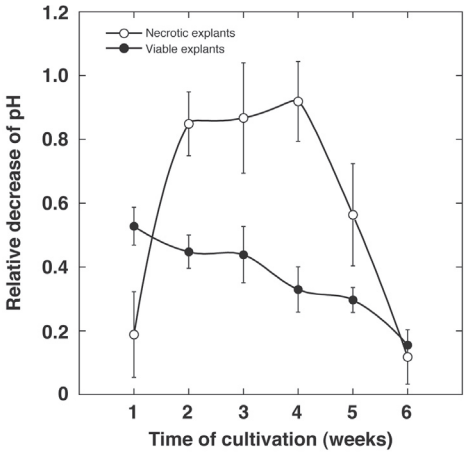

Fig. 2. Effect of time of cultivation and explant type on relative decrease of medium pH. Initial pH of the cultivation medium was 5.8 (6.35 after autoclaving). Explants were transplanted to the fresh medium with initial pH value every week.

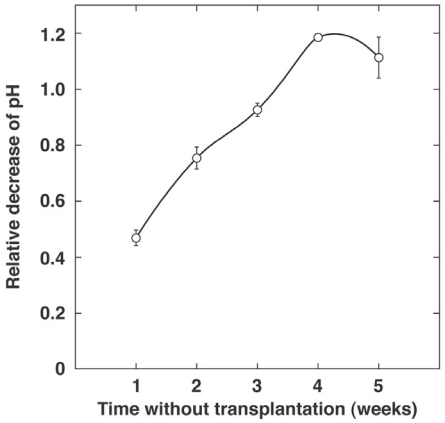

Fig. 3. Effect of time of cultivation before transplanting on relative decrease of medium pH. Initial pH of the cultivation medium was 5.8 (6.25 after autoclaving). Explants were transplanted on a fresh medium with initial pH after the indicated intervals of time and cultivated for 10 months.

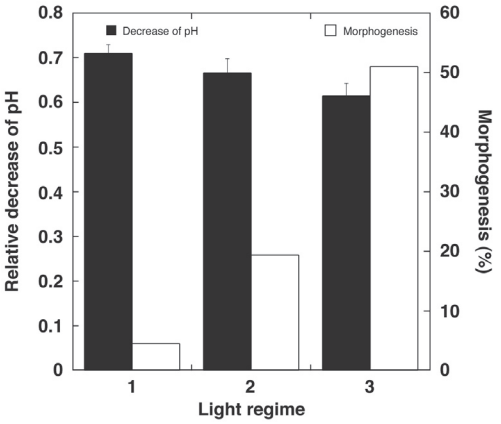

Fig. 4. Effect of illumination conditions on relative decrease of medium pH and percentage of morphogenesis in pine bud explants. Artificial light only (1), artificial light with short periods of sunlight (2), artificial light with long periods of direct sunlight (3). Initial pH of the cultivation medium was 5.8 (6.25 after autoclaving). Explants were cultivated for 10 months without transplanting.

The maximal rate of acidification was observed during the first four weeks of cultivation, then decreased, but slower acidification continued when explants were not transplanted for 10 months (Fig. 1, 3). In the first cultivation week acidification was from pH 6.25 to 5.76 (acidification rate 0.49), but in 10 months time from pH 6.25 to 4.18 (acidification rate 0.048 weekly).

The results of our experiments showed that the degree of acidification depended on the physiological condition of explants. To evaluate if morphogenesis affected the ability of pine buds to change medium pH buds were divided into groups according to different developmental events or lack of development. If constructive processes in buds dominated over destructive, medium acidification was less marked than for buds with dominating destructive processes. This relationship was characteristic for buds in the first experiment, transplanted every week (Fig. 2), for buds transplanted every 2, 3, 4 or 5 weeks (data not shown) or grown for 10 months without transplantation in the second experiment, when average medium pH with needle forming buds was 4.59 ± 0.15, but for buds without needles it was 3.91 ± 0.08.

The same relationship was characteristic also for buds, cultivated in different light intensities. Change of medium pH for different light intensities in the first experiment was lowest for explants cultivated in natural light with a prolonged period of direct sunlight (greater percentage of viable buds with enlarged brachioblasts and needle-forming buds), and highest for explants cultivated in artificial light that did not form needles and became necrotic sooner (Fig. 4).

The second experiment was performed to test if the initial pH of the medium affects pine tissue development. The morphological characteristics of buds (average percentage of needle forming buds, average amount of needle pairs on each needle forming bud,

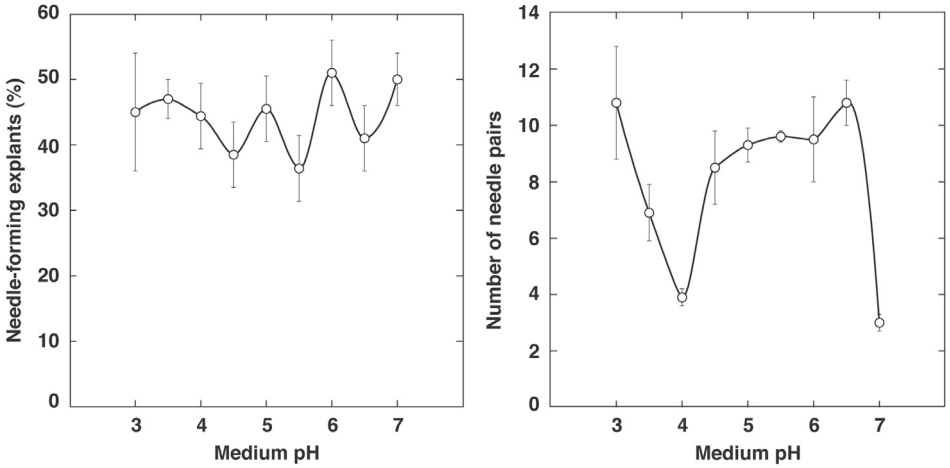

Fig. 5. Effect of initial medium pH on percentage of needle-forming explants (A) and number of needle pairs per needle-forming explant (B) within 10 months of cultivation. Explants were cultivated without transplanting.

length of needles) varied (Fig. 5, 6), but on the whole the results were not convincingly affected by initial pH of medium.

The percentage of necrotic tissue in buds cultivated *in vitro* for four months (second experiment) was lowest on media with the most acidic and most alcaline initial pH (Fig. 6B).

The activity of peroxidase in buds cultivated *in vitro* for 7 weeks on media with different pH was significantly lower for buds cultivated on pH 3.0, increased for higher pH values up to 5.0, but lowered again and stopped decreasing at pH 6.5 (Fig. 7A). The activity of polyphenol oxidase in the same buds was also lowest for pH 3.0 and increased up to pH 6.5 (Fig. 7B).

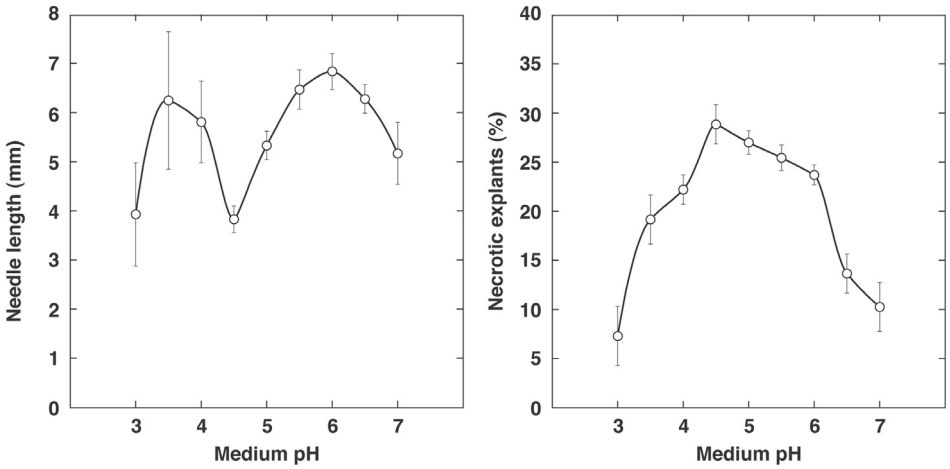

Fig. 6. Effect of initial medium pH on percentage of needle length (A) and percentage of necrotic pine bud explants (B) within 10 months of cultivation. Explants were cultivated without transplanting.

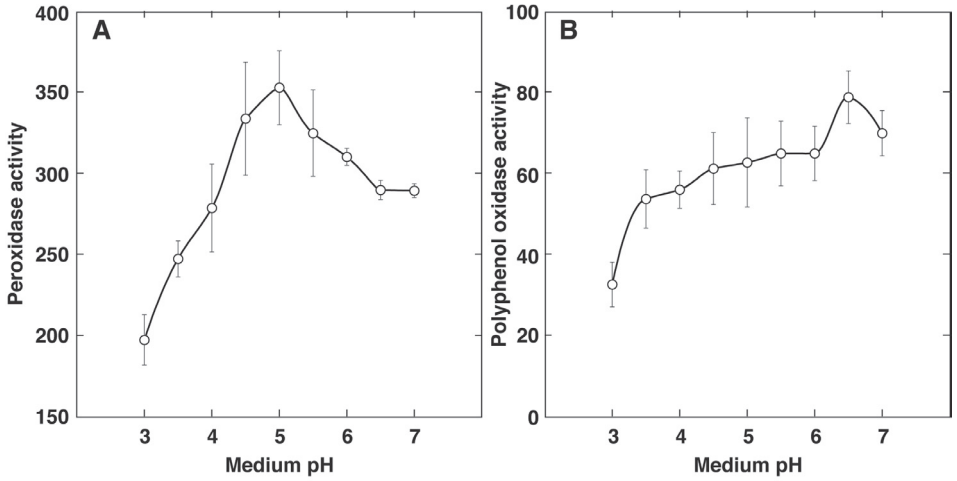

Fig. 7. Effect of initial medium pH on peroxidase (A) and polyphenol oxidase (B) activity in pine bud explants after 10 months of cultivation. Explants were cultivated without transplanting.

Discussion

Changes of medium pH should be taken in to the account when using plant tissue culture both for practical purposes as well as for research. Changes of pH can occur from preparation of medium until *ex vitro* transplantation and are affected both by interactions between chemical constituents as well as plant tissues and medium constituents.

Adjustment of medium pH is usually performed before adding agar, but the agar itself and subsequent autoclaving change medium pH. The addition of agar to the nutrient solution raised the pH when initial pH was below 5.5, but autoclaving decreased pH, except when pH was lower than 4.0 (Williams at al. 1990). In our investigation we recorded only the final medium pH after addition of agar and autoclaving which were towards alkalinity (Fig. 1). Differences in pH may arise from different types of agar, other medium components or medium preparation features.

Medium pH changed during cultivation of tissues usually towards acidity. The degree of acidification of the medium during cultivation is influenced by several factors, including the source of nitrogen, physiological status of cultivated tissues, duration of cultivation, frequency of transplanting etc.

The source of nitrogen in the medium and its utilization by cells can play an important role in determining the shift in the pH value of the medium (Sathyanarayana, Blake 1994). Plant growth *in vitro* can occur regardless of initial pH as the medium contains both N forms (Mac AntSaoir 1994). Nitrogen uptake as nitrate is higher at an acidic pH and shifts the medium pH towards alkalinity, while ammonium utilization rate increases with increasing pH and shifts the medium pH towards acidity (Gamborg, Shyluk 1970; Martin, Rose 1976; Wetherell, Dougall 1976; Dougall, Verma 1978). In *Pinus sylvestris* callus cultures the medium became acidic when it contained NH_4NO_3 (from pH 5.7 to 4.3), but the pH of the medium containing KNO_3 remained close to the initial value (from pH 5.7 to 5.4; Laukkanen et al. 1997). Seedlings of a number of forest tree species, including *Pinus sylvestris* grow better when nitrogen is supplied as ammonium or as ammonium

plus nitrate, and when ammonium is preferred as the nitrogen source, the rhizosphere will be acidified by induced H^+ extrusion (Arnold 1992). In our second experiment where medium contained ammonium pine bud explants changed the medium pH to a constant value for the whole range of initial pH of medium (Fig. 1).

The degree of acidification in our experiments depended on the morphogenesis of explants. Needle formation on buds was associated with less acidified medium. From the results of the experiment on light intensity and changes of medium pH we can also conclude that pH change was less for natural illumination conditions, which stimulated morphogenesis, and promoted enlarged brachyoblast development and needle formation (Fig. 4). The degree of acidification of medium during cultivation depended also on the duration of cultivation period before transplantation. The observed change of medium pH for different cultivation periods before transplantation shows that it is not necessary to transplant pine buds to fresh medium more often than every two months or even less frequently.

The initial pH of medium also affects the degree of acidification during cultivation. If the initial pH is more alkaline, explants consume more energy to acidify the medium than explants cultivated on medium with initially acidic pH. Moreover production of acidic compounds by the mycobacterium living in pine buds may also cause acidification of the growth medium (Laukkanen et al. 2000).

Medium pH was changing due to release of some compounds from the plant or as a result of uptake of specific ions from the medium. Tissue secretes phytosiderophores, organic acids and proteins, which may react with metal ions present and change chemical dissociation kinetics, in turn altering their bioavailability (Friborg et al. 1978; Egertsdotter et al. 1993; Zhang 1993; Van Winkle, Pullman 2003). It is possible that buds becoming necrotic secrete into the medium compounds different from those of needle forming buds, which may result in different effects onmedium pH.

Although the difference in initial pH may have a significant effect on the culture system even though the pH difference is eliminated after some time of cultivation (Williams et al 1990), the obtained morphological data in our experiment do not show any simple trend of relationship between initial pH of medium and development of explants (Fig. 5, 6). In the acidic part of medium pH range the morphological development occurred during longer cultivation periods and the morphological parameters were better and buds less necrotic. For *Pinus sylvestris* callus cultures a lower pH in the presence of ammonium in culture medium correlated with better growth (Laukkanen et al. 1997). However, differences between pH variants may be caused also by biological diversity of the material used for experiments.

In our study peroxidase and polyphenol oxidase activity was lowest in the acidic end of medium pH range (Fig. 7). A low peroxidase and polyphenol oxidase activity corellated with increased morphogenic potential of mature *Pinus sylvestris* buds *in vitro* (Andersone, Ievinsh 2002; Andersone, Ievinsh 2005). Buds cultivated on initially more acidic medium need less acidification to reach equilibrium. These results may confirm the morphological results of the experiment with different initial pH of medium, e.g. that mature *Pinus sylvestris* buds *in vitro* need acidic initial medium pH (pH 3.0 to 3.5 before adding agar and autoclaving). The presence of ammonium in culture medium might have an effect on polyphenol oxidase and peroxidase by decreasing the pH and altering the water potential of the cells. However, increased browning in the presence of only nitrate

may result in significantly higher pH, because at higher pH levels phenolics will oxidize to form quinones, which will bind irreversibly to proteins in a non-enzymatic fashion (Laukkanen et al. 1997).

In conclusion the degree of acidification of medium during cultivation of mature *Pinus sylvestris* buds depends not only on the source of nitrogen, products of mycobacteria living in pine buds as described previously, but also on initial pH of medium, morphogenesis of explants and duration of cultivation period without transplantation. Lowered activity of oxidative enzymes in buds cultivated on more acidic medium, and that needed less acidification to reach equilibrium, suggests that low initial pH is more suitable for successful pine bud morphogenesis.

Acknowledgements

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Barotnes pH ietekmē reģenerācijas spējas un oksidatīvo fermentu aktivitāti *Pinus sylvestris* audu kultūrā

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Kopsavilkums

Barotnes pH ir viens no svarīgākajiem fizikālķīmiskās vides faktoriem augu audu attīstības laikā *in vitro* kultūras apstākļos. To ietekmē barotnes sagatavošana un tas mainās arī līdz ar eksplantu attīstību. Dotos ekesperimentus veica, lai novērotu barotnes pH izmaiņas un noteiktu barotnes pH ietekmi uz *in vitro* kultivētu nobriedušu *Pinus sylvestris* L. audu fizioloģiskajām īpašībām. Pēc agara pievienošanas un autoklavēšanas barotnes ar sākuma pH no 3.0 līdz 5.5 kļuva sārmainākas. Pētījumu rezultāti apstiprināja, ka priežu pumpuru kultivēšana izraisa barotnes paskābināšanos. Barotnes paskābināšanās pakāpe bija atkarīga no sākotnējā barotnes pH, eksplantu morfoģenēzes un kultivēšanas ilguma bez pārstādīšanas. Pazemināta oksidatīvo fermentu aktivitāte pumpuros uz skābākas barotnes un īsāks šādu kultūru pH stabilizācijai nepieciešamais laika periods liecina, ka veiksmīgai priežu pumpuru morfoģenēzei nepieciešams zemāks sākotnējais barotnes pH.

7. Priedes audu proliferācija *in vitro* ar aukstumapstrādi panāktās kultivēto audu citokinīnu kompetences pieauguma rezultātā

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Regulation of cytokinin response-competence by cold treatment of mature *Pinus sylvestris* tissues *in vitro*

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Abstract

One of the possible reasons for lack of morphogenesis during tissue culture of mature tissues is the inability to respond to cytokinin. In the present experiments, cold incubation of pine (*Pinus sylvestris* L.) bud explants was used as a means to affect cytokinin response competence. Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C did not result in increased morphogenesis. New bud and shoot formation was achieved on pine bud explants which were pulse-treated with BAP in combination with cold incubation. It is concluded that, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

Key words: cold incubation, cytokinin response competence, morphogenesis *in vitro*, *Pinus sylvestris*.

Introduction

Micropropagation is the most effective vegetative reproduction technique permitting rapid propagation and distribution of elite genotypes. Micropropagation of woody species with mature tissues could be very valuable, but it is often difficult, especially, with conifers. Mature tissues of *Pinus sylvestris* L. have a very low morphogenic potential *in vitro* (Bonga 1987; Hohtola 1988).

Several methods of pretreatment for the rejuvenation of mature conifer tissues or the increase of their morphogenic potential have been described – serial grafting (Huang et al. 1992; Ewald, Kretzschmar 1996; Centeno et al. 1998), cold or frozen storage of detached resting buds (Andersone, Ievinsh 2002), spraying of donor plants with cytokinin (Salonen 1991). The timing of collections is also a critical factor (Bonga 1987; Andersone, Ievinsh 2002). However, only brachioblast and needle formation is achieved on mature Scots pine bud explants (Andersone, Ievinsh 2002). Cultivation of the explants on media containing cytokinin did not result in new bud or shoot formation. One of the reasons for that could be very low cytokinin response-competence of the mature *P. sylvestris* tissue.

It is well known that bud production or adventitious branching is usually achieved by increasing the cytokinin level in the tissues. However, extremely rapid multiplication

in tissue culture due to high levels of cytokinin may lead to genetic changes (Peschke, Phillips 1992). It is also known that a high concentration of cytokinin, while stimulating organ induction, inhits their elongation and development (Salonen 1991). Therefore, pulse treatment with cytokinin between subcultures can be used to achieve a high level of multiplication without undesirable genetic effects. In experiments with mature intact conifer trees, repetitive cytokinin treatment has been used for juvenilization leading to development of new shoots (Krikorian 1982).

The aim of the present study was to investigate the possibility of using a pulse treatment with cytokinin as well as to affect cytokinin response competence in mature P. *sylvestris* tissues by means of cold treatment of established *in vitro* culture of bud explants.

Materials and methods

Plant material was collected from mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Salaspils (Riga Region, Latvia). Buds were taken randomly from different trees from the lower part of the crown. Collection was carried out from the second half of July till the first half of August. Newly formed buds were used. They were surface sterilized with a half-diluted commercial bleach ACE (Procture and Gamble; containing 5 - 15 % sodium hypochlorite) for 20 min, rinsed for 10 min in sterile distilled water, sterilized again in 15 % hydrogen peroxide and rinsed three times for 10 min in sterile distilled water. The buds were peeled and dissected aseptically. Explants were cultivated in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium. Tubes were closed with cotton-wool

Table 1. Media used for cultivation of *Pinus sylvestris* bud explants

	Basal medium (BM)	Medium No. 1 (M1)	Medium No. 2 (M2)	Medium No. 3 (M3)
Woody Plant Medium mineral salts	+	+	+	+
Myo-inositol	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}
Thiamine hydrochloride	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}
Pyridoxine hydrochloride	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Nicotinic acid	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Glycine	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}
Benzylaminopurine	–	–	100 mg l^{-1}	–
Naphthylacetic acid	–	0.1 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}
Adenin	–	10 mg l^{-1}	–	250 mg l^{-1}
Kinetin	–	1 mg l^{-1}	–	25 mg l^{-1}
Sucrose	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}
Agar	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}
pH	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7

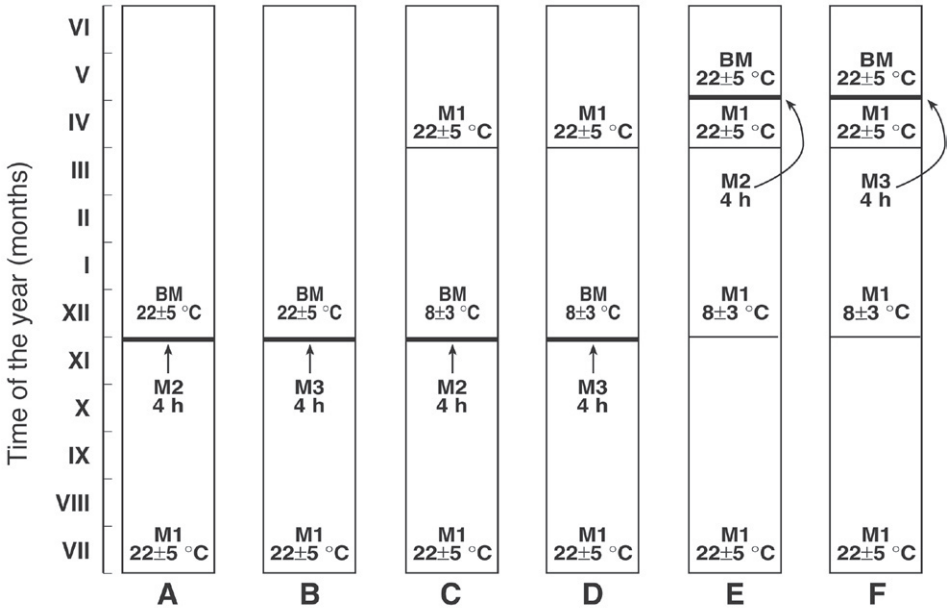

Fig. 1. A diagram outlining the experimental procedure. Time sequence, cultivation media used, as well as temperature regimes are shown for experimental variants A to F.

plugs, and covered also with polythene film fixed with a rubber band. Each tube contained one explant. During the entire time they were cultivated in natural lighting.

For culture establishment medium M1 was used (Table 1). Pulse treatment with high concentrations of cytokinins was carried out on media M2 (containing benzylaminopurine, BAP) or M3 (containing kinetin and adenin) for 4 h. After the cytokinin treatment, basal medium (BM) without growth regulators was used. During cold storage, explants were cultivated at 8 ± 3 °C; control explants were maintained at 22 ± 5 °C.

Six different variants of cytokinin treatment in combination with or without cold storage were tested (Fig. 1). For each variant three replicates of 10 bud explants per treatment were used.

Results

Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C (variants A and B) did not result in increased morphogenesis (Table 2). New bud and shoot formation was achieved on pine bud explants (variants C and E) which were pulse-treated with BAP in combination with cold incubation (Table 2). The effect was more pronounced when the treatment was performed after the cold incubation (40 %) in comparison with the treatment performed before the cold incubation (20 %). Also, the average amount of microshoots per one shoot-forming explant was higher in variant E than in variant C (Table 2). Kinetin in combination with adenin did not result in bud and shoot formation (Table 2, variants D and F).

In both variants of BAP treatment in combination with cold incubation (variants C

Table 2. The effect of cold storage on cytokinin response-competence of mature *Pinus sylvestris* tissues *in vitro*. Data are means from 3 replicates (10 explants per treatment) ±SE

	Treatment					
	A BAP	B Kinetin + adenin	C BAP followed by cold	D Kinetin + adenin followed by cold	E Cold followed by BAP	F Cold followed by kinetin + adenin
Bud explants, forming microshoots (%)	0	0	20 ± 2	0	40 ± 3	0
Microshoots per explant	0	0	5.5 ± 0.8	0	6.8 ± 1.2	0

and E), buds and microshoots developed directly on the lower part of the explants (Fig. 2A, D). Elongation of these buds occurred only in variant E (Fig. 2D). In variants C and E, also brachioblasts with short needles were formed on upper and middle part of explants (Fig. 2A, D). In variant C, axillary shoots developed between these needles and subsequentially elongated (Fig. 2B). The longest of these elongated axillary microshoots were isolated and transferred to a fresh BM medium (Fig. 2C).

Discussion

In previous experiments with *P. sylvestris* it was found that about 15 % of mature pine buds introduced *in vitro* in the second half of summer form brachioblasts with needles (Andersone, Ievinsh 2002). However, further development of these explants was not obtained: buds with needles as well as buds without needles did not survive longer than eight months *in vitro*. No further multiplication was achieved even on media with high concentrations of cytokinins. This situation is similar to the observation that micropropagated plants of mature origin may retain their physiological maturity during tissue culture (Nas et al. 2003). Consequently, inability to respond to growth regulators, e.g. cytokinins, is one of the characteristics of the "out of hormonal response competence" state of mature tissues during *in vitro* cultivation.

It has been suggested that accumulation of competence to induction is important before a particular induction phase during shoot organogenesis (Christianson, Warnick 1988). Thus, to deal with a lack of induction competence in mature pine explants reflected in lack of cytokinin-response competence, certain conditions should be met. As a possible candidate for such conditions, cold treatment can be used, as it was shown for intact juvenile *P. sylvestris* that cold exposure is necessary for elongation of cytokinin treatment-induced buds (Salonen 1991). In order to improve cytokinin response-competence of pine bud tissues, cold treatment of established *in vitro* cultures at 8 °C was used in the present experiments.

Due to a relatively slow transport of exogenous cytokinins, the concentration of BAP in pine bud tissues obviously decreased in the direction towards the apical part of the bud, explaining why the largest amount of shoots formed at the lower part of the explants. In

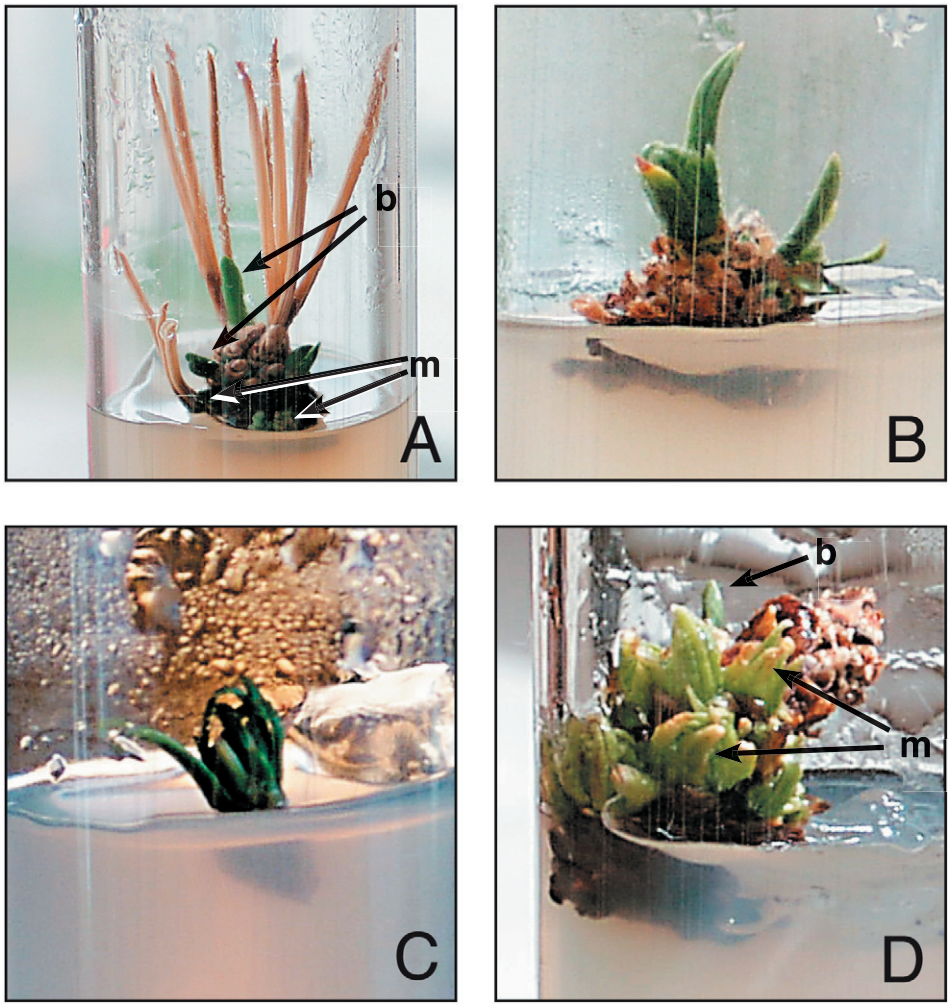

Fig. 2. Development of mature *Pinus sylvestris* bud explants *in vitro* under the effect of cold and cytokinin treatment. A, experimental variant C (according to Fig. 1, pulse treatment with BAP before cold incubation), 4 weeks after the end of the cold incubation; B, variant C, 6 weeks after the end of the cold treatment; C, variant C, isolated axillary shoot on BM medium, 2 weeks after isolation; D, variant E (pulse treatment with BAP after cold incubation), 6 weeks after the BAP treatment. m, microshoots; b, brachioblast with needles.

contrast, secondary needles were formed only in the central or upper part of the pine bud explants, which is in accordance to the observation that a high concentration of cytokinin prevents secondary needle formation (Zhang et al. 2003). Our results were similar to those obtained with *Actinidia deliciosa* explants, where gradient of exogenously applied BAP was responsible for different developmental patterns (Feito et al. 2001).

The effect of cytokinin was more pronounced when the pulse treatment with BAP was performed after the cold incubation (variant E), in comparison with the pulse treatment

before the cold incubation (variant C). Consequently, in the latter variant, during the development of competence in conditions of cold treatment, exogenously applied BAP was considerably decomposed in pine tissues leading to less pronounced cytokinin response. Decomposition of exogenously applied citokinin-like substances in plant tissues is a well known phenomenon (Harrison, Kaufman 1984).

In conclusion, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

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8. Diskusija

Pirms pētījumu uzsākšanas ar pieaugušu parasto priežu audu kultivēšanu veica eksperimentus ar juveniliem audiem, adaptējot un papildinot literatūrā aprakstītās metodes, lai iegūtu pieredzi darbā ar konkrēto sugu *in vitro*. Darbā aprakstītie eksperimenti ar juveniliem audiem bija pirmais pētnieciskais solis ceļā uz parastās priedes mikropavairošanas fizioloģiskā pamata izstrādi. To rezultāti pierāda, ka mikrodzinumu indukciju uz jauno dīgstu epikotila spraudeņiem var izmantot priedes mikropavairošanai no juveniliem audiem un tālākai veiksmīgai mikrodzinumu veidošanās indukcijai ir nepieciešams noteikts par eksplantiem izmantoto mikrodzinumu garums – vislabāko pavairošanu panāca, izmantojot mikrodzinumus ar garumu vismaz 10 mm (Andersone, Ieviņš 2000), pretstatā apgalvojumam, ka jau 8 mm gari eksplanti ir piemēroti tālākai parastās priedes pavairošanai *in vitro* apstākļos (Supriyanto, Rohr 1994).

Citu autoru rezultāti ir parādījuši, ka priežu hipokotilu vecums pirms tālākas pavairošanas uzsākšanas ir īpaši svarīgs tās norisei (Toribio, Pardos 1987). Mūsu pētījumos visveiksmīgakā pavairošanās bija novērojama piecus mēnešus veciem sekundārajiem eksplantiem (Andersone, Ieviņš 2000).

Domājams, ka no dažādiem priežu dīgstiem iegūto sekundāro eksplantu dzīvotspējas atšķirības atspoguļo no genotipa atkarīgās dažādu mātes koku īpašības. Priežu somatiskās embrioģenēzes iniciācijas atšķirīgo biežumu arī izraisa ģenētiskā heterogenitāte (Keinonen-Mettala *et al*. 1996). Tātad, šādos pētījumos jāņem vērā vecāku genotipa ietekme uz priedes veiksmīgu mikropavairošanu (Andersone, Ieviņš 2000).

Aprakstītie rezultāti par veiksmīgu parastās priedes mikropavairošanu, panākot mikrodzinumu attīstību uz epikotila spraudeņiem, veidoja metodisko pamatu tālākajiem pētījumiem, lai panāktu nobriedušu priežu audu pavairošanu audu kultūrā.

Kallusa audos, kas iegūti no nobriedušām priedēm, novērojama izteikti augsta peroksidāzes aktivitāte. Tā izraisa agru un ātru audu nobrūnēšanu un sekojošu šūnu bojāeju (Laukkanen *et al*., 1999). Ir zināms, ka skujukoku audi ir īpaši bagāti ar dažādiem fenolu dabas savienojumiem (Nyman 1985). Jādomā, ka kultivējamā augu materiāla sagatavošanas laikā izdarītie mehāniskie bojājumi izraisa šūnu kompartmentu ķīmisko komponentu sajaukšanos, izsaucot dažādu fenolu savienojumu oksidāciju ar peroksidāžu un polifenolu oksidāžu starpniecību. Parastajai priedei ir īpaši izteiktas ievainojuma atbildes reakcijas, tai skaitā, oksidatīvā metabolisma fermentu palielināta aktivitāte, kas, savukārt, inhibē diferenciāciju un augšanu (Hohtola 1995). Tas norāda, ka samazināta vai stabila peroksidāzes un polifenoloksidāzes aktivitāte ir saistīta ar palielinātu audu spēju sākt augšanu *in vitro* apstākļos.

Tomēr, ir arī iespēja, ka vēl citi faktori bez peroksidāzes un polifenoloksidāzes izraisa morfoģenēzes kompetences zudumu nobriedušos priedes audos. Relatīvi augstā audu infekcijas pakāpe no mātesauga

atdalīto pumpuru aukstumuzglabāšanas laikā apstiprina domu par to, ka parastās priedes pumpuri satur endofītiskos mikroorganismus, kas ir potenciāls aizsardzības reakciju induktors (Pirttilä *et al.* 2002).

Tā kā literatūrā nebija atrodami dati par pieaugušu priežu audu morfoģenēzi audu kultūrā, tika izvirzīta hipotēze, ka nepieciešami kādi īpaši, ar barotnes sastāvu nesaistīti faktori, kuri varētu ietekmēt metaboliskos procesus un stimulēt šādu audu organizētu augšanu *in vitro*.

Eksperimentu rezultāti parāda, ka viens no šādiem faktoriem ir audu metabolisko procesu sezonālās izmaiņas. *In vitro* ievadīto pieaugušo priežu pumpuru organizēta augšana un attīstība notika tikai vasaras otrajā pusē un rudenī noņemtajiem pumpuriem, t. i. jaunizveidotiem un miera periodā vēl neieslīgušiem audiem. Oksidatīvo fermentu aktivitāte šajā laikā bija samazināta vai relatīvi stabila (Andersone, Ievinsh 2002).

Lai panāktu miera periodā esošu pieaugušu priežu pumpuru attīstību *in vitro*, veicām eksperimentus ar audu ilgstošu uzglabāšanu aukstumā vai sasaldētā stāvoklī, lai tādējādi ietekmētu to morfogēno kompetenci.

Eksperimentu rezultāti par nobriedušu priežu pumpuru uzglabāšanu aukstumā pirms ievadīšanas *in vitro* norāda, ka ilgstošu no mātesauga atdalītu audu uzglabāšanu var izmantot, lai ietekmētu priežu audu metaboliskos procesus. Peroksidāzes un polifenoloksidāzes aktivitātes samazināšanās priedes audos laikā, kad tos uzglabā aukstumā, norāda uz iespējami paaugstinātu morfoģenēzes kapacitāti pazeminātas oksidatīvā metabolisma aktivitātes rezultātā (Andersone, Ievinsh 2002).

Izmantojot uzglabāšanu aukstumā kā līdzekli oksidatīvā metabolisma ietekmēšanai, bija iespējams panākt tiešu morfoģenēzi uz nobriedušu priežu pumpuru eksplantu audiem (Andersone, Ievinsh 2002). Šis ir pirmais zinātniski dokumentētais nobriedušas priedes audu veiksmīgas morfoģenēzes gadījums, līdz šim bija panākta vienīgi kallusa augšana un mikroskopiska izmēra jaunveidotu adventīvo dzinumu aizmetņu veidošanās, kuri tālāk nepieauga (Hohtola 1988).

Iegūtie rezultāti eksperimentos ar pieaugušu priežu pumpuru uzglabāšanu saldētā stāvoklī pirms to ievadīšanas audu kultūrā liecina, ka arī šāda veida uzglabāšanu var izmantot priežu audu metabolisma izmainīšanai (Andersone, Ievinsh 2005). Pretēji plaši izplatītajam viedoklim, ka saldētos audos metaboliskās reakcijas ir apturētas, atsevišķu gan fermentatīvo, gan nefermentatīvo ķīmisko reakciju ātrums var ievērojami palielināties. Šādu parādību sauc par temperatūras negatīvo efektu (Singh, Wang 1977). Mūsu eksperimenti nepārprotami parādīja, ka sasaldētu priežu pumpuru uzglabāšanas laikā –18 °C temperatūrā notiek ievērojamas ogļhidrātu metabolisma un oksidatīvo fermentu aktivitātes izmaiņas (Andersone, Ievinsh 2005). Saldēšana ietekmēja arī pieaugušu priežu audu morfogēno kapacitāti – tā ievērojami palielinājās trīs mēnešus uzglabātos pumpuros, un, mazākā mērā, arī piecus mēnešus uzglabātos pumpuros, salīdzinot ar tiem priežu pumpuriem, kurus ievadīja kultūrā tūlīt pēc ievākšanas. Paaugstināts saldēto pumpuru morfogēnais potenciāls, kas izpaudās kā spēja veidot kallusu un skujas, laikā sakrita ar zemāko peroksidāzes aktivitāti un visaugstāko saharozes saturu audos. Uzglabājot pumpurus sasaldētus ilgāk par pieciem mēnešiem, bija vērojams straujš audu morfogēnā potenciāla kritums, kas sakrita ar oksidatīvo fermentu aktivitātes pieaugumu un būtisku reducējošo cukuru daudzuma pieaugumu (Andersone, Ievinsh 2005). Saharozes hidrolīze par heksozi ar invertāzes satrpniecību var izmainīt šūnu osmotisko potenciālu, izraisot samazinātu audu

sala izturību. Tā rezultātā ir iespējama aktīvo skābekļa formu pastiprināta veidošanās, ko varbūtēji atspoguļo paaugstināta peroksidāzes aktivitāte. Reducējošo cukuru daudzuma pieaugums ir raksturīga atbildes reakcija uz dažādu abiotisko stresa faktoru iedarbību (Schubert *et al*. 1995; Prado *et al*. 2000).

Domājams, ka morfogēnā potenciāla pieaugums, pumpurus uzglabājot sasaldētus trīs līdz piecus mēnešus, nav tieši saistīts ar cukuru satura izmaiņām audos. Turpretī, audu morfogēnās kapacitātes samazināšanās un zudums, pumpurus uzglabājot sasaldētus ilgāk par pieciem mēnešiem, varētu būt saistīts ar noverotajām krasajām šķīstošo cukuru satura izmaiņām (Andersone, Ievinsh 2005).

No audu augšanu un attīstību potenciāli ietekmējošajiem faktoriem veicām pētījumus par barotnes pH ietekmi uz pieaugušu priežu audu morfogēno kapacitāti *in vitro* (Andersone, Ievinsh 2008).

Gan praktiskiem mērķiem, gan zinātniskiem nolūkiem izmantojot audu kultūru metodi, būtu jāņem vērā iespējamās barotnes pH izmaiņas. Šīs izmaiņas var notikt visa procesa laikā sākot no barotnes sagatavošanas līdz pat *in vitro* kultivēšanas beigām, un tās ietekmē gan ķīmisko sastāvdaļu savstarpējā mijiedarbība, gan augu audu un barotnes sastāvdaļu mijiedarbība.

Barotnes pH izmaiņas, kas rodas audu kultivēšanas rezultātā, parasti ir paskābināšanās virzienā. Tika analizēti barotnes pH izmaiņu pakāpi ietekmējošie faktori pieaugušu parasto priežu audu kultivēšanas laikā (Andersone, Ievinsh 2008). Pēc literatūrā pieejamiem datiem, barotnes pH maiņu parastās priedes audu kultivēšanas laikā nosaka slāpekļa avots barotnē (Laukkanen *et al*. 1997) un priežu pumpuros dzīvojošo mikobaktēriju saražotie skābie savienojumi (Laukkanen *et al*. 2000b). Pēc mūsu eksperimentu rezultātiem izrādījās, ka barotnes paskābināšanās pakāpe bija atkarīga arī no vairākiem citiem faktoriem: no eksplantu morfoģenēzes (brahioblastu palielināšnās un skuju veidošanās uz pumpuriem bija saistīta ar mazāk paskābinātu barotni), no kultivēšanas ilguma bez pārstādīšanas (no rezultātiem par barotnes pH izmaiņām dažāda ilguma vienlaidus kultivēšanas apstākļos var secināt, ka priežu pumpurus nav nepieciešams pārstādīt uz svaigas barotnes biežāk kā katrus divus mēnešus vai pat vēl retāk), no sākotnējā barotnes pH (ja sākotnējais pH ir bāziskāks, pH līdzsvara sasniegšanai eksplantiem vairāk jāpaskābina barotne nekā eksplantiem, kuri kultivēti barotnē ar skābu sākotnējo pH) (Andersone, Ievinsh 2008).

Barotnes pH izmaiņas varētu būt iespējamas kādu no auga izdalītu savienojumu dēļ, vai arī kā rezultāts specifisku jonu uzņemšanai no barotnes. Audi izdala barotnē fitosideroforus, organiskās skābes un olbaltumvielas, kuri var reaģēt ar klāt esošajiem metālu joniem un izmainīt ķīmiskās disociācijas kinētiku, savukārt mainot to biopieejamību (Friborg *et al*. 1978; Egertsdotter *et al*. 1993; Zhang 1993; Van Winkle, Pullman 2003). Iespējams, ka pumpuri, kuri nekrotizējas, izdala barotnē savienojumus, kuri atšķiras no tiem, kurus izdala skujas veidojoši pumpuri, un tas izraisa atšķirīgas barotnes pH izmaiņas (Andersone, Ievinsh 2008).

Lai arī atšķirībām sākotnējā barotnes pH ir būtiska ietekme uz *in vitro* kultūru, neskatoties uz to, ka pH atšķirības kultivēšanas laikā izlīdzinās (Williams *et al*. 1990), mūsu eksperimentā iegūtie morfoloģiskie dati neparāda vienkāršu tendenci sakarībai starp sākotnējo barotnes pH un eksplantu attīstību. Skābākajā barotnes pH intervāla daļā morfoloģiskā attīstība notika ilgākā kultivēšanas periodā, morfoloģiskie rādītāji bija nedaudz augstāki, un pumpuri mazāk nekrotizējās (Andersone, Ievinsh 2008). *Pinus sylvestris* kallusa kultūrām samazināts pH barotnē amonija jonu klātbūtnē korelēja ar labāku augšanu (Laukkanen *et al*. 1997). Mūsu pētījumā peroksidāzes un polifenoloksidāzes aktivitāte

bija zemāka skābākajā barotnes pH intervāla daļā. Zemā peroksidāzes un polifenoloksidāzes aktivitāte korelē ar palielinātu morfogēno potenciālu nobriedušu *Pinus sylvestris* pumpuriem *in vitro*. Pumpuriem, kuri kultivēti uz skābākas iniciālās barotnes, nepieciešama mazāka barotnes paskābināšana, lai panāktu tajā pH līdzsvaru. Šie rezultāti kopumā ļauj secināt, ka nobriedušu *Pinus sylvestris* pumpuriem *in vitro* nepieciešama skāba iniciālā barotne (pH 3.0 - 3.5 pirms agara pievienošanas un autoklavēšanas) (Andersone, Ievinsh 2008).

Iepriekš analizētajos eksperimentos ar nobriedušu parasto priedi audu kultūrā dažādu morfogēno kompetenci stimulējošu faktoru ietekmē panākts, ka daļa no pumpuru eksplantiem, kas ievadīti *in vitro*, veidoja brahioblastus ar skujām. Tomēr, tālāka šo eksplantu attīstība nenotika: gan pumpuri ar skujām, gan arī pumpuri bez skujām nebija spējīgi izdzīvot *in vitro* ilgāk par astoņiem - desmit mēnešiem (Andersone, Ievinsh 2002; Andersone, Ievinsh 2005; Andersone, Ievinsh 2008). Nebija iespējams panākt tālāku pavairošanu pat barotnēs ar augstām citokinīnu koncentrācijām. Šāds stāvoklis atgādina citu autoru novērojumus par to, ka no pieaugušiem augiem iegūti audi audu kultūras apstākļos var saglabāt t.s. "fizioloģiskā brieduma" stāvokli (Nas *et al*. 2003). Tātad, nespēja adekvāti atbildēt uz augšanas regulatoriem, piemēram, citokinīniem, ir viena no būtiskām pazīmēm t.s. "hormonālo signālu atbildes nekompetences" stāvoklim, kas raksturīgs nobriedušiem audiem to *in vitro* kultivēšanas laikā.

Uzskata, ka pirms dzinuma organoģenēzes indukcijas fāzes ir nepieciešama atbilstošas indukcijas kompetences uzkrāšanās (Christianson, Warnick 1988). Tātad, lai varētu pārvarēt nobriedušu priedes audu eksplantu kompetences iztrūkumu atbildēt uz citokinīnu, jāpanāk noteiktu, bet līdz šim neidentificētu priekšnosacījumu izpilde. Tika izvirzīta hipotēze, ka arī šajā kultivēšanas posmā nepieciešama kādu ar barotnes sastāvu nesaistītu, fizikālu faktoru iedarbība. Eksperimentos ar juvenīliem parastās priedes dīgstiem ir parādīta aukstuma ekspozīcijas nepieciešamība, lai panāktu ar citokinīna apstrādi inducētu pumpuru attīstību (Salonen 1991). Lai uzlabotu priežu pumpuru citokinīnu atbildes kompetenci, izmantojām nobriedušu priežu audu *in vitro* kultūru inkubāciju 8 °C temperatūrā, kā rezultātā notika intensīva jaunu dzinumu un skuju veidošanās uz eksplantiem. Citokinīna efekts bija vairāk izteikts, ja priežu eksplantu apstrādi ar benzilaminopurīnu veica pēc inkubēšanas aukstumā, salīdzinot ar apstrādi pirms aukstuma perioda. Tātad, apstrādājot ar citokinīnu pirms inkubēšanas aukstumā, laikā, kad attīstījās aukstuma inducētā kompetence atbildēt uz citokinīnu, ievērojams daudzums eksogēni pielietotā benzilaminopurīna pumpuru audos jau bija endogēni sadalījies, izraisot mazāk izteiktu citokinīna atbildes reakciju (Andersone, Ievinsh 2004). Eksogēni pielietotu citokinīnu dabas augšanas regulatoru sadalīšanās augu audos ir vispārzināma parādība (Harrison, Kaufman 1984).

Tā kā eksogēnie citokinīni eksplantos pārvietojas relatīvi lēni, jādomā, ka benzilaminopurīna koncentrācija priežu pumpuru audos samazinājās virzienā uz pumpura apikālo daļu. Tas izskaidro, kāpēc lielākais daudzums dzinumu veidojās tieši eksplantu apakšējā daļā. Pretēji tam, sekundārās skujas veidojās tikai eksplantu centrālajā vai augšējā daļā (Andersone, Ievinsh 2004), kas ir saskaņā ar novērojumu par to, ka augsta citokinīnu koncentrācija kavē sekundāro skuju attīstību (Zhang *et al*. 2003). Mūsu rezultāti ir līdzīgi tiem, kas iegūti eksperimentos ar *Actinidia deliciosa* eksplantiem, kur eksogēni pielietotā benzilaminopurīna gradients eksplantu audos bija saistīts ar dažādu attīstības programmu realizāciju dažādās eksplanta vietās (Feito *et al*. 2001).

Tātad, nobriedušu parasto priežu audu juvenilizācijai ir nepieciešama fizikālu un bioķīmisku faktoru mijiedarbība, tā veicinot to morfogēno kompetenci *in vitro* un nodrošinot eksplantu tālāku attīstību un proliferāciju audu kultūrā. Audu uzglabāšana un kultivēšana pazemінātā temperatūrā uzskatāma par piemērotu līdzekli šī mērķa sasniegšanai (Andersone, Ievinsh 2002; Andersone, Ievinsh 2004; Andersone, Ievinsh 2005).

9. Secinājumi

1. Citokinīnus (BAP 5 mg L^{-1} un 0.5 % kokosriekstu pienu vai 10 mg L^{-1} kopā ar IBA 1 mg L^{-1}) saturoša 1/2 MSBN pamatbarotne inducē proliferāciju uz juveniliem parastās priedes audiem *in vitro.* Inducēto dzinumu augšanai piemērota pamatbarotne ar samazinātu minerālvielu saturu (1/6 MS 1/2 BN) ar aktīvo ogli bez fitohormoniem.

2. Juvenilu *P. sylvestris* audu proliferācijas spēja *in vitro* ir būtiski atkarīga no genotipa. Eksistē sakarība starp juvenilu parastās priedes primāro eksplantu izveidoto jauno dzinumu izmēriem un iespēju tos tālāk pavairot *in vitro,* kas var palīdzēt atlasīt kultivēšanai piemērotākos genotipus. Veiksmīgai mikrodzinumu veidošanās indukcijai piemērotākais par eksplantiem izmantoto mikrodzinumu (sekundāro eksplantu) garums ir vismaz 10 mm.

3. Piemērotākais laiks pieaugušu *P. sylvestris* pumpuru audu ievadīšanai *in vitro* (augstākais to morfogēnais potenciāls) ir no jūlija līdz oktobra beigām, kad jaunie pumpuri ir nobrieduši, bet nav vēl sācies to fizioloģiskā miera periods.

4. Fizioloģiskā miera stāvoklī esošus, no mātesauga atdalītus pieaugušu parasto priežu audus ilgstoši uzglabājot aukstumā var ietekmēt metaboliskos procesus tajos un palielināt to morfogēno kompetenci.

5. Pazemināta oksidatīvā metabolisma aktivitāte pieaugušu parasto priežu pumpuru audos, ko raksturo samazināta vai stabila peroksidāzes un polifenolu oksidāzes aktivitāte, paaugstina morfoģenēzes kapacitāti, kas ir saistīta ar palielinātu audu spēju sākt augšanu *in vitro*.

6. Fizioloģiskā miera stāvoklī esošus, no mātesauga atdalītus pieaugušu parasto priežu pumpurus uzglabājot saldētā stāvoklī pirms to ievadīšanas audu kultūrā mainās gan audu oksidatīvo fermentu aktivitāte, gan ogļhidrātu metabolisms, un, uzglabājot 3-5 mēnešus, palielinās audu morfogēnais potenciāls.

7. Cietes un cukuru satura izmaiņas saldētu priežu pumpuru uzglabāšanas laikā nav tieši saistītas ar morfogēnā potenciāla pieaugumu, jo kopējais to daudzums, ko var izmantot augšanas procesu nodrošināšanai nepieciešamās enerģijas iegūšanai, priežu pumpuru uzglabāšanas laikā būtiski nemainās. Krasās saharozes, glikozes un fruktozes satura izmaiņas priežu pumpuros, tos uzglabājot saldētā stāvoklī ilgāk par pieciem mēnešiem, ir daļa no tām metaboliskajām izmaiņām, kas izraisa audu morfogēnās kapacitātes samazināšanos un zudumu.

8. Pieaugušu priežu pumpuru kultivēšana *in vitro* izraisa barotnes paskābināšanos, kuras pakāpi ietekmē sākotnējais barotnes pH, eksplantu morfoģenēze un kultivēšanas ilgums bez pārstādīšanas. Sākotnēji atšķirīgi barotņu pH līmeņi vismaz divu mēnešu ilgas priežu pumpuru vienlaidu kultivēšanas laikā izlīdzinās. Uz sākotnēji skābas barotnes kultivēti pumpuri pH līdzsvara sasniegšanai paskābina barotni mazāk, nekā uz sākotnēji bāziskas barotnes kultivētie. Pazemināta oksidatīvo fermentu aktivitāte pumpuros, kuri kultivēti skābākā barotnē, un kuriem nepieciešama mazāka barotnes paskābināšana, lai sasniegtu barotnes pH līdzsvara stāvokli, norāda, ka zems sākotnējais pH līmenis ir vispiemērotākais sekmīgai priežu pumpuru morfoģenēzei *in vitro.*

9. Nobriedušu priežu pumpuru juvenilizācijai ir nepieciešams apvienot fizikālus un bioķīmiskus pasākumus, lai panāktu morfogēnās kompetences veicināšanu un nodrošinātu eksplantu tālāku attīstību audu kultūrā. Lai pārvarētu nobriedušu priedes audu eksplantu kompetences iztrūkumu atbildēt uz citokinīnu apstrādi, pirms tās var izmantot audu *in vitro* kultūru inkubāciju pazeminātā temperatūrā.

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