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Somatická embryogeneze smrku ztepilého (*Picea abies* (L.) Karst.)

(disertační práce)

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Lucie Fischerová

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SEZNAM POUŽÍVANÝCH ZKRATEK

2,4-D	kyselina 2,4 dichlorphenoxyoctová
ABA	kyselina abscisová
BAP	benzylaminopurin
DMSO	dimethylsulfoxid
LCO	lipochitooligosaccharidy
MES	kyselina 2-(N-morpholino) ethansulfonová
PEG	polyethylenglykol
RFO	sacharidy rafinosové řady

Zkratky názvů proteinů:

ABI3	abscisic acid insensitive 3
ABI4	abscisic acid insensitive 4
ABI5	abscisic acid insensitive 5
LEA	late embryogenesis abundant proteins
PaHB1	*Picea abies* homeobox 1
PaVP1	*Picea abies* viviparous1
SCR	SCARECROW
VP1	viviparous1

OBSAH

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1. ÚVOD

Convice me that you have a seed there
and I am prepared to expect wonders...

Henry David Thoreau (1860)

V roce 1985 publikovaly nezávisle na sobě tři laboratoře první práce popisující somatickou embryogenezi jehličnanů. Na počátku, jako primární explantát, bylo zralé či nezralé zygotické embryo, jehož buňky pod vlivem auxinu a cytokininů v médiu prokázaly totipotenci a daly vznik diferencované embryogenní kultuře. Spuštěný proces somatické embryogeneze pak pokračoval, opět řízen přidaným fytohormonem, kyselinou abscisovou. Somatická embrya morfologicky připomínala své zygotické protějšky, měla schopnost klíčit, semenáčky se dále vyvíjely.

Kdyby měl ctihodný Henry D. Thoreau možnost listovat těmito pracemi, nejspíš by se potěšeně usmíval. Schopnost totipotence buněk semene jehličnanů (kromě zygotického embrya i megagametofytu) by pravděpodobně jen utvrdila jeho celoživotní fascinaci semeny. Další práce navíc ukázaly, že tato schopnost semen je víceméně unikátní, že u jehličnanů lze jen velmi obtížně odvodit embryogenní kulturu z vegetativních materiálů.

Následovaly četné další práce. Množství rodů a druhů jehličnanů, u kterých byla popsána somatická embryogenese, se rozšiřovalo. Jak výzkum pokračoval, bylo stále více zřejmé proč uplynulo více než čtvrtstoletí od objevu somatické embryogeneze u krytosemenných rostlin, než se analogický fenomén podařilo popsat u nahosemenných. Rozdíly v somatické embryogenezi ve srovnáni s krytosemennými rostlinami byly totiž více než značné. Fylogenetické stáří jehličnanů a odlišné vývojové cesty se navíc projevily ve výrazných rozdílech mezi jednotlivými rody jehličnanů.

Více než dvacet let výzkumu somatické embryogeneze jehličnanů bylo dosud možno jen v několika málo případech přetavit v patentované mikropropagační postupy, které jsou komerčně úspěšně využívány pro produkci semenáčků (*Pseudotsuga*, *Picea glauca*, některé druhy *Pinus*). U početné skupiny jehličnanů byla sice somatická embryogeneze popsána, ale proces je stále zatížen problémy, které brání uplatnění v praxi. To je nesporné rozčarování, publikace konce osmdesátých a začátku devadesátých let predikovaly využití nové biotechnologie s podstatně větším optimismem. Přitom kombinace účinné mikropropagační metody

pracující s vybraným elitním genotypem či s cíleně geneticky modifikovaným materiálem nabízí značné využití zejména při výsadbě porostů s předem určeným hospodářským využitím (tzv. crop trees).

Je několik hlavních problémů, které limitují praktické využití. Pro komerčně úspěšnou produkci semenáčků je potřeba, aby proces byl co nejvíce automatizován. To předpokládá využití bioreaktorů, a tedy tekutých živných médií. Vývoj somatických embryí a zejména jejich zrání v tekutém médiu je však u většiny druhů problematický a neuspokojivý. Náklady zvyšují i komplikace při převádění klíčících semenáčků *ex vitro* (mykorrhiza). Značné omezení je skryto na samotném začátku, kde výběr primárního explantátu pro indukci je prakticky omezen na nezralé či zralé zygotické embryo. Prolomit tuto bariéru, tedy indukovat embryogenní kulturu z vegetativních materiálů, by mělo naprosto zásadní význam pro mikropropagaci stromů s konkrétní požadovanou vlastností, danou genotypem.

Byly tedy smělé perspektivy užití somatické embryogeneze pro produkci jehličnanů jen nafouklou bublinou či floskulí vhodnou do příslušných kolonek grantových přihlášek? Zdá se, že ne, jen pionýrské období, kdy se vědci snažili nalézt kultivační postupy pro bezproblémové druhy jehličnanů s cílem vytvořit v praxi okamžitě využitelný mikropropagační systém, skončilo. Nastal čas návratu a pátrání po fyziologických, biochemických a genetických závislostech procesu.

Smrk ztepilý patří mezi druhy, u kterých proces somatické embryogeneze probíhá vcelku uspokojivě. S vysokou procentuální úspěšností lze indukovat embryogenní kulturu, která má ve většině případů dostatečný růstový potenciál. Embryogenní kultura je poměrně stabilní a až na výjimky si roky drží své vlastnosti. Proembrya citlivě reagují na kyselinu abscisovou a jejich vývoj bývá poměrně synchronní. Somatická embrya významného podílu genotypů jsou schopna po desikaci vyklíčit. Embryogenní kulturu je možno úspěšně kryoprezervovat. Na druhou stranu tento příznivý průběh není charakteristický pro všechny embryogenní kultury smrku ztepilého, v míře úspěšnosti somatické embryogeneze existují značné rozdíly mezi genotypy. Právě tyto vlastnosti pasují smrk ztepilý do role ideálního modelu pro studium.

V průběhu disertační práce jsem se věnovala anatomické a biochemické charakterizaci vývoje somatických embryí, a to od fáze proliferace až po fázi klíčení. Ve vztahu ke struktuře embryí popsané na anatomické úrovni jsem sledovala obsah endogenních sacharidů a polyaminů u linie s vysokou embryogenní kapacitou a pro

fáze desikace a klíčení jsem navíc použila jako vztažný systém zygotickou embryogenezi. Desikovaná a klíčící semena jsem podrobila anatomické analýze a stanovila v nich obsah polyaminů. Kromě modelové, vysoce embryogenní linie, jsem ke studiu využila i linie s nižší schopností tvořit embrya, a to pro sledování vlivu různých typů kultivací a pro molekulární analýzu exprese transkripčního faktoru PaVP1, který zprostředkovává přenos signálu kyseliny abscisové. Takto široce pojatá práce měla umožnit co nejlepší pochopení procesu somatické embryogeneze smrku ztepilého. Výsledky studia obsahu polyaminů v somatických a zygotických embryích jsou však toho času připravovány k publikování. Jelikož je předkládaná práce podávána formou svázaných publikací, nemohly být v práci zahrnuty.

Henry David Thoreau: Faith in a seed: The dispersion of seeds & other late natural history writings

2. CÍLE

Získáním podrobnějších poznatků o procesu somatické embryogeneze smrku ztepilého přispět k optimalizaci kultivačních podmínek, a to zejména ve fázi maturace, kdy dochází k vývoji proembryí ve zralá embrya.

Dílčí cíle:

- Popsat anatomické změny v průběhu vývoje somatických embryí na základním maturačním médiu a po přidání nepenetrujícího osmotika PEG 4000 do média.

- Charakterizovat dynamiku změn obsahu nestrukturních sacharidů v průběhu vývoje embryogenní kultury na maturačním médiu a její ovlivnění osmotikem PEG 4000.

- Porovnat úspěšnost různých kultivačních postupů u širokého spektra embryogenních linií (zařazení pre-matuační fáze, užití osmotika v maturaci, kultivace na prámcích).

- Na základě vlastních i převzatých výsledků vytvořit vzorový protokol pro práci s embryogenními kulturami smrku ztepilého.

- Charakterizovat expresi genu pro transkripční faktor PaVP1 v embryogenních liniích s různou schopností tvořit somatická embrya.

3. LITERÁRNÍ PŘEHLED

3.1. Somatická embryogeneze

Životní cyklus kvetoucích rostlin je charakterizován střídáním dvou generací, haploidní (gametofytické) a diploidní (sporofytické). Sporofyt krytosemenných rostlin vzniká dvojím oplodněním mateřské buňky megaspory. V průběhu oplození proniká pylová láčka do zárodečného vaku a uvolní se z ní dvě spermatické buňky. Splynutím jedné z nich s vaječnou buňkou vzniká zygota, zatímco druhá spermatická buňka splývá s centrální buňkou zárodečného vaku a vzniká triploidní endosperm, který plní převážně vyživující funkci pro vyvíjející se embryo a pro jeho pozdější klíčení (West a Harada 1993). U nahosemenných rostlin k dvojímu oplození nedochází, jejich endosperm je založen již před oplozením a je haploidní (např. Owens a Molder 1979).

Embryogeneze (vývoj embrya) však může probíhat také alternativně bez oplození či fúze gamet. Původ takových nepohlavních embryí je různý. Apomiktická embrya vznikají z neoplozené vaječné buňky, či z jiné buňky zárodečného vaku. V *in vitro* podmínkách lze získat embrya také z nezralých mikrospor a pylových zrn (Maraschin a kol. 2005), či embrya somatická z buněk somatických (Zimmerman 1993).

Somatická embryogeneze je tedy proces, kdy za indukčních podmínek vznikají ze somatických buněk buňky embryogenní, které jsou dále schopny vývoje v somatická embrya. Ta vznikají řadou morfologických a biochemických změn, které ve velké míře odpovídají změnám probíhajícím při vývoji embrya zygotického v oplodněném vajíčku (Quiroz-Figueroa a kol. 2006).

Somatická embryogeneze je ideálním modelovým systémem pro studium vývoje rostlin, neboť vývoj somatických embryí je na rozdíl od embryí zygotických snadno pozorovatelný, může být cíleně ovlivňován složením média či transformací somatických embryí a velkou výhodou je i produkce dostatečného množství experimentálního materiálu (Quiroz-Figueroa a kol. 2006). Praktické využití somatické embryogeneze spočívá v produkci velkého množství geneticky shodných embryí, která mohou být dále použita pro rozmnožování rostlin s výhodnými či geneticky upravenými vlastnostmi (Tautorus a kol. 1991).

3.2. Somatická embryogeneze jehličnanů

Přestože byla somatická embryogeneze popsána u *Daucus carota* již v roce 1958 (Reinert 1958; Steward a kol. 1958), embryogenní kultury jehličnanů byly získány až v roce 1985, a to díky velmi specifickým nárokům na indukci. Poprvé byla popsána třemi na sobě nezávislými skupinami: Hakman a kol. (1985) a Chalupa (1985) indukovali vznik embryogenní kultury ze zygotických embryí *Picea abies*, Nagmani a Bonga (1985) indukovali vznik embryogenní kultury *Larix decidua* z pletiva megagametofytu.

Proces somatické embryogeneze se skládá z několika fází: založení (indukce) embryogenní kultury, její udržování (proliferace), vývoj a zrání somatických embryí (maturace), desikace embryí (u některých druhů rostlin, či u některých genotypů není nutná) a vlastní klíčení.

3.2.1. Zakládání (indukce) embryogenních kultur

Úspěšnost indukce embryogenní kultury se udává počtem (resp. podílem - %) explantátů, na nichž se vytvářejí raná somatická embrya. Je dána výběrem materiálu, typem primárního explantátu, složením média a fyzikálními podmínkami.

Embryogenní kultury mohou vznikat z různých explantátů, úspěšnost indukce je však na typu použitého explantátu velmi závislá. Použití různých výchozích explantátů je různě vhodné pro jednotlivé rostlinné druhy a dokonce i pro jednotlivé genotypy. Nejčastěji jsou embryogenní kultury získávány ze zygotických embryí, a to jak zralých tak nezralých (Atree a Fowke 1993). Jako nezralá jsou označována embrya, u kterých se ještě nevyvinuly dělohy. K indukci somatické embryogeneze lze použít i megagametofyt. Úspěšnost indukce bývá nejvyšší při použití nezralých embryí (Salajová a kol. 1996, Tautorus a kol. 1990). Von Arnold a kol. (1995) dospěli k obecnému závěru, že úspěšnost indukce se snižuje se zvyšujícím se stářím explantátu. Podobně spolu se stárnutím spojeným se zvyšováním obsahu zásobních proteinů v zygotických embryích hybridního smrku *Picea glauca* × *Picea engelmanii* se snižuje jejich schopnost vytvořit embryogenní kulturu (Roberts a kol. 1989). Velmi vzácné jsou případy, kdy se povedlo embryogenní kulturu indukovat i z děložních lístků semenáčků, či jehlic dospělých stromů (Ruaud 1992, Lelu a kol. 1994).

Úspěšnost indukce embryogenní kultury je dále ovlivněna složením indukčního média. Optimální hormonální složení indukčního média je však druhově či dokonce

genotypově závislé. Pro druhy rodu *Picea* je nutná přítomnost cytokininu i auxinu; cytokinin (nejčastěji N^6-benzyladenin) bývá přidáván v koncentracích pohybujících se okolo 5 $\mu mol.l^{-1}$, auxin (nejčastěji 2,4-D) v 5-10μM koncentracích (např. Mo a von Arnold 1991). Stejně tak u druhů rodu *Pinus* bývají používána média obsahující auxin i cytokinin (např. Bozhkov a kol. 1997), některé genotypy však vyžadují pouze auxin (Becwar a kol. 1990). U druhů rodu *Abies* je vznik embryogenních kultur vázán na použití média obsahujícího pouze cytokinin (Salajová a kol. 1996; Vooková a kol. 1998). Tato zobecnění však neplatí absolutně, například Lelu a kol. (1999) indukovali embryogenní kultury *Pinus sylvestris* a *Pinus pinaster* i na médiu bez růstových regulátorů.

Také u *Daucus carota* (modelového systému somatické embryogeneze krytosemenných rostlin) lze vznik embryogenní kultury indukovat i na médiu bez růstových regulátorů, a to změnami osmotického potenciálu média (vysokou koncentrací sacharosy, NaCl) či přidáním chloridů těžkých kovů do indukčního média (Kiyosue a kol. 1993). Zdá se proto, že vznik embryogenní kultury *Daucus carota* je indukován spíše stresem než přímým působením růstových regulátorů (Nishiwaki 2000). Zda totéž platí i pro indukci embryogenních kultur jehličnanů nebylo zatím ověřeno.

Indukce somatické embryogeneze jehličnanů může být kromě uvedených růstových regulátorů ovlivněna i dalšími modifikacemi ve složení média. Pro indukci embryogenní kultury *Pinus taeda* bylo výhodné obohatit médium o $AgNO_3$, inhibitor biosyntézy etylénu (Pullman a kol. 2003a). Kombinace MES pufru, biotinu a kyseliny listové dále zvyšovala množství založených embryogenních kultur (Pullman a kol. 2005). Další látkou výrazně ovlivňující úspěšnost indukce je brassinolid, který byl účinný u *Pinus taeda*, *Pseudotsuga menziesii* a *Picea abies* (Pullman a kol. 2003b).

3.2.2. Udržování (proliferace) embryogenních kultur

Proliferující embryogenní kultury jehličnanů jsou tvořeny velkým množstvím nevyvinutých somatických embryí, která se skládají z meristematické části a suspenzorového systému (von Arnold a kol. 1995). Někteří autoři (například Bozkov 1995) nazývají tuto kulturu embryonální suspenzorová hmota; embryogenní kultury též bývají označovány jako kultury raných somatických embryí (Hakman 1993). Na základě morfologie a vývojového schématu rozdělila Filonova a kol. (2000a) vývoj

proliferující embryogenní kultury *Picea abies* na tři fáze a označila je jako proembryogenní hmota I, II a III.

Médium, na kterém jsou embryogenní kultury jehličnanů udržovány, mívá obvykle stejné složení jako médium indukční, obsahuje tedy auxin, cytokinin a sacharózu v nízké koncentraci. V některých případech jsou však embryogenní kultury udržovány na médiu se sníženým obsahem růstových regulátorů oproti médiu indukčnímu (např. Krogstrup a kol. 1988). Embryogenní kultury některých druhů jehličnanů lze v této fázi embryogeneze pěstovat i v tekutých médiích. Výhodou této metody je rychlejší růst kultury a nižší riziko poškození při manipulaci s kulturou (Silveira a kol. 2004).

Subkultivační interval embryogenních kultur bývá 1-4 týdny v závislosti na druhu a genotypu. Stejně tak světelné a teplotní podmínky, za jakých jsou kultury udržovány, jsou založeny na zkušenostech s jednotlivými druhy či klony a nelze je zobecnit. Například suspenzní kultura *Picea glauca* byla udržována za stálé ozářenosti 3-5 μmol $m^{-2}s^{-1}$ při 25°C (Hakman a von Arnold 1988), suspenzní kultura *Pinus taeda* prospívala nejlépe ve tmě při 25°C (Silveira a kol. 2004) stejně jako kultura *Pinus nigra* pěstovaná na zpevněném médiu (Salajová a kol. 1995).

V této fázi vývoje lze udržovat embryogenní kultury po dlouhou dobu, popř. napěstovat dostatečné množství materiálu pro další použití. Kultury ve fázi proliferace je možné také zamrazit a uchovávat v nízkých teplotách (v tekutém dusíku). Tato technika je označována jako kryoprezervace, tj. proces řízeného zamrazování. Je běžně používána u řady jehličnanů mikropropagovaných cestou somatické embryogeneze (Cyr 1999). Jednotlivé druhy i genotypy se však liší v míře tolerance k nízkým teplotám. Zatím nejsou zcela jasné příčiny těchto odlišností. Mezi tolerantní patří např. *Pseudotsuga menziessii*, naopak *Abies alba* a *Picea abies* patří spíše k méně tolerantním druhům (Norgaard a kol. 1993, Cyr a kol. 1994, Park a kol. 1994). Důležitou roli zde nejspíš hraje regenerační schopnost embryogenní kultury, která se uplatňuje v době po rozmražení. V tomto období může dojít i k selekci buněk, která ovlivní embryogenní kapacitu kultury pozitivně (Galerne a kol. 1992). Předpokladem je, že se zvýší synchronizace vývoje embryí. Řada autorů však tento efekt popírá (např. Norgaard a kol. 1993, Cyr a kol. 1994, von Arnold a kol. 1995).

Použitím kryoprezervace se také může snížit riziko somaklonální variability, neboť lze na minimum omezit trvání proliferační fáze. U embryogenních kultur jehličnanů však byla popsána jen nízká míra somaklonální variability (Fourre a kol.

1997), případně nebyla vůbec prokázána (Cyr a kol. 1994, Isabel a kol. 1993, Nkongolo a Klimaszewska 1994, 1995).

Proliferující embryogenní kultury řady druhů je možné transformovat, a to buď biolistickou metodou či prostřednictvím *Agrobacterium tumefaciens*. Transformace pomocí *Agrobacterium tumefaciens* byla výrazně úspěšnější při použití kmenů se zvýšeným počtem kopií genů virulence. Takto byly transformovány reportérovým genem pro β-glukuronidázu například *Picea abies* a *Pinus taeda* (Wenck a kol. 1999) či *Picea glauca* (Le a kol. 2001). Transformace je v posledních letech velmi využívanou metodou studia somatické embryogeneze jehličnanů (např. Sabala a kol. 2000, Ingouff a kol. 2001, Ciavata a kol. 2002, Mathieu a kol. 2006).

3.2.3. Zrání (maturace) somatických embryí

Další vývoj raných somatických embryí je vázán na přenos embryogenní kultury na maturační médium. To se v původních pracích od indukčního lišilo pouze sníženou hladinou auxinů a cytokininů nebo jejich úplným vynecháním (Hakman a Fowke 1987; Chalupa 1985). Vznikající embrya však vykazovala řadu vývojových poruch a nebyla schopna klíčení. Pozdější studie ukázaly, že úspěšný vývoj somatických embryí jehličnanů je podmíněn přenosem na maturační médium bez auxinů a cytokininů, které však obsahuje kyselinu abscisovou (ABA). O ABA je známo, že pozitivně ovlivňuje i zrání somatických embryí krytosemených rostlin (Kiyosue a kol. 1993), a to zejména synchronizací celého procesu. Práce (především skupiny Sary von Arnold) z posledních let ukazují, že optimálního zrání somatických embryí lze dosáhnout přechodným pěstováním embryogenních kultur na médiu bez růstových regulátorů a posléze jejich přenesením na médium obsahující ABA. Podrobnější popis působení ABA na vývoj somatických embryí je uveden v kapitole 3.3.2.

Dalšího zlepšení vývoje somatických embryí jehličnanů lze dosáhnout obohacením maturačního média o osmotikum. Ovlivnění vývoje somatických embryí však závisí na typu a koncentraci použitého osmotika (sacharidy, polyethylenglykol, dextrany). Většina autorů (Johnson a kol. 1997; Carrier a kol. 1997) předpokládá, že sacharidy přidávané do maturačního média mají několikerou roli. Slouží jako zdroj energie a stavebních prvků buněčné stěny, přímo ovlivňují genovou expresi a zvyšují osmotický potenciál média. Osmotické působení sacharosy při zrání somatických embryí prokázali např. Finer a kol. (1989), Tremblay a Tremblay (1991, 1995).

Jednoduché cukry však nejsou tak účinné jako osmotika s vysokou molekulovou hmotností (větší než 1000 Da, označovaná jako nepenetrující), např. polyethylenglykol (PEG) nebo dextrany. Je to způsobeno tím, že osmotika s malou molekulovou hmotností procházejí do symplastu rostlinného pletiva a dochází k postupnému vyrovnání osmotických potenciálů. Naproti tomu látky s vysokou molekulovou hmotností buněčnou stěnou a plasmatickou membránou neprocházejí a osmotický potenciál je vyrovnáván vlastní tvorbou kompatibilních solutů (Attree a Fowke 1993). Tak například růst embryogenní kultury *Picea glauca* byl pozitivně ovlivněn přidáním PEG 1000 a 4000 a dextranů 6000 a 8000 do maturačního média, nedocházelo k předčasnému zrání embryí. Nejlepších výsledků bylo dosaženo při použití PEG 4000 (Atree a kol. 1995). Vliv PEG 4000 na vývoj somatických embryí *Picea abies* popsal Find (1997). Při pěstování této kultury na médiu obsahujícím 5% PEG 4000, se zvýšil počet zralých somatických embryí o 50% oproti embryogenní kultuře rostoucí bez PEG.

Zrání somatických embryí je ovlivněno také typem a koncentrací gelu použitého při přípravě zpevněných médií (Klimaszewska a Smith 1997), koncentrací minerálních látek, zvláště nitrátu (Barrett a kol. 1997), typem a koncentrací sacharidu a pH média (Tremblay a Tremblay 1991). Také přidání $AgNO_3$, inhibitoru biosyntézy etylénu, do maturačního média vede ke zlepšení dalšího vývoje somatických embryí (Kong a Yeung 1995).

Úspěšnost maturace je dána počtem zralých somatických embryí. Embryogenní kultury se vyvíjejí nesynchronně, jen část embryí je schopná dokončit maturaci. Podmínky maturace však mohou měnit poměr mezi celkovým počtem embryí na počátku a zralých embryí na konci maturace (Bozhkov a kol. 2002). Kritériem úspěšného průběhu maturace je nejen množství, ale i kvalita embryí. O ní vypovídá jednak jejich anatomická stavba, jednak úroveň metabolické aktivity a následné ukládání zásobních látek, charakteristické především pro pozdější fáze vývoje somatického embrya. Z hlediska ukládání látek lze embryo jehličnanů rozdělit do dvou zón: hypokotyl a dělohy jsou místa ukládání zásobních látek (lipidy, škrob, proteiny), zatímco bazální část akumuluje polyfenoly, tedy látky sekundárního metabolismu (Gutman a kol. 1996).

Ukládání zásobních látek je v somatických embryích jehličnanů stimulováno ABA a především osmotikem. ABA stimulovala akumulaci lipidů u embryogenní kultury *Picea abies* (von Arnold a Hakman 1988). V porovnání s embryi

zygotickými však somatická embrya *Picea glauca* pěstovaná na maturačním médiu obsahujícím ABA obsahovala dvakrát méně zásobních lipidů. Tento rozdíl byl smazán přidáním PEG 4000 do maturačního média (Atree a kol. 1992). V pozdější studii Atree a kol. (1995) dokonce po aplikaci PEG 4000 pozorovali až 5x vyšší obsah lipidů v somatických embryích oproti embryím zygotickým. Osmotický stres indukoval také akumulaci zásobních proteinů a jejich transkriptů v somatických embryích *Picea glauca* × *Picea engelmanii* (Flinn a kol. 1993). Spektrum zásobních proteinů této kultury se nelišilo od spektra zásobních proteinů v zygotických embryích, avšak jejich absolutní množství se u jednotlivých linií lišilo (Flinn a kol. 1991). Škrob je další významnou zásobní látkou v somatických embryích jehličnanů. Jeho ukládání předchází tvorbě zásobních lipidů a proteinů (Joy a kol. 1991). U kultur pěstovaných bez osmotika však byla škrobová zrna pozorována až do stádia s vyvinutými dělohami, což dokládá, že změnu v ukládání zásobních látek ve prospěch lipidů a proteinů opravdu způsobuje stres z nedostatku vody (Attree a Fowke 1993).

3.2.4. Desikace a klíčení somatických embryí

Hodnocení úspěšnosti procesu somatické embryogeneze je dáno počtem somatických embryí schopných klíčit a vyvíjet se dále v rostliny. Významnou etapou somatické embryogeneze, která klíčení ovlivňuje, je desikace, tj. doba, kdy jsou již plně vyvinutá somatická embrya vystavena podmínkám, za kterých dochází k postupnému úbytku vody v embryích. Desikace somatických embryí je paralelou vysýchání, ke kterému dochází v pozdních fázích vývoje zygotického embrya v semeni. Desikace však není charakteristická pouze úbytkem vody v embryích, ale především jejich biochemickým dozráváním. Vnější morfologie desikovaných somatických embryí *Picea glauca* pozorovaná rastrovacím elektronovým mikroskopem se velmi podobala zralým embryím zygotickým (Fowke a kol. 1994).

Pro úspěšné klíčení byla desikace nezbytná například u somatických embryí *Picea sitchensis* a *Picea glauca* (Roberts a kol. 1991, Find 1997). Na druhou stranu však Bomal a Tremblay (1999) nenalezli rozdíly v úspěšnosti klíčení somatických embryí *Picea mariana*, která byla a nebyla vystavena desikačním podmínkám. Dá se tedy říci, že fáze desikace není nutná pro všechny druhy, případně genotypy, embryogenních kultur jehličnanů.

Tolerance somatických embryí k desikaci záleží na jejich kvalitě a také na podmínkách desikace. Lze ji zvýšit působením ABA (Beardmore a Charest 1995) a osmotika (Attree a kol. 1995) v předchozích stádiích vývoje, případně působením nízkých teplot na zralá somatická embrya (Pond a kol. 2002). Další nutnou podmínkou úspěšného průběhu desikace je vysoká (97%) vzdušná vlhkost, při které proces probíhá (Bomal a Tremblay 1999, Högberg a kol. 2001).

Desikace ovlivňuje somatická embrya na několika úrovních. V průběhu desikace se v somatických embryích snižuje množství zásobních proteinů, u *Picea glauca x Picea engelmanii* docházelo ke stejnému poklesu i v průběhu desikace embryí zygotických (Flinn a kol. 1993). U somatických embryí *Picea glauca* bylo v průběhu desikace pozorováno hromadění nukleotidů, pravděpodobně jako příprava na pozdější klíčení (Stasolla a kol. 2001a). Desikace také snižuje hladinu ABA v somatických embryích (Find 1997). Tím přímo ovlivňuje úspěšnost klíčení, neboť endogenní ABA v somatických embryích klíčení inhibuje (Lelu a Label 1994), podobně jako je tomu u semen mnoha rostlinných druhů (Holdsworth a kol. 1999). Zvýšení počtu klíčících somatických embryí lze dosáhnout i přidáním aktivního uhlí do média, na kterém embrya klíčí (např. Vooková a kol. 1998); aktivní uhlí zde slouží k odstranění fytohormonů z embryí.

Médium, na kterém probíhá klíčení, obvykle obsahuje také nízkou hladinu sacharózy. Carrier a kol. (1997) zjistili, že přidání 1 % sacharózy do média zpomalilo pokles obsahu lipidů, ke kterému docházelo při klíčení somatických embryí *Picea glauca x Picea engelmanii*. U embryí pěstovaných v přítomnosti sacharosy se také postupně zvyšoval obsah kyseliny linolenové, která je hlavní složkou chloroplastových membrán a je podle autorů možným markerem úspěšnosti klíčení. Růst kořene lze u somatických embryí podpořit přidáním indolyl-3-máselné kyseliny (Vooková a Kormuťák 2001), naopak růst nadzemní části a zakládání nových růstových primordií lze ovlivnit přidáním kyseliny askorbové (Stasolla a kol. 1999). V menší míře kyselina askorbová pozitivně ovlivňuje i růst kořenů. Vývoj nadzemní části ovlivňuje kyselina askorbová pravděpodobně tím, že indukuje organizované buněčné dělení v apikálním meristému. Dalším efektem přidání kyseliny askorbové do média, na kterém probíhá klíčení, je zvýšená schopnost somatických embryí využívat adenin a adenosin, prekurzory purinů (Stasolla a kol. 2001b).

Po fázi klíčení jsou klíční rostliny, u kterých se již vyvinul primární kořen a u kterých z apikálního meristému vyrostly první lístky, přenášeny do podmínek *ex vitro* (Högberg a kol. 2001). Následný růst a vývoj těchto rostlin již není zahrnován do procesu somatické embryogeneze, proto jsem se mu v přehledu literatury nevěnovala.

3.3. Vybrané kapitoly somatické embryogeneze jehličnanů

3.3.1. Vývoj somatických embryí

Názvosloví používané při popisu vývoje somatického embrya je odvozené od názvosloví klasické embryologie nahosemenných rostlin, proto se nejprve krátce zmíním o zygotické embryogenezi.

3.3.1.1. Vývoj zygotického embrya

Vývoj zygotického embrya bývá popisován jako dvoufázový proces: V první morfogenetické fázi vznikají základní struktury embrya, zatímco druhá metabolická fáze je charakterizovaná biochemickou přípravou embrya na klidové stádium (Harada 1999). Je však třeba mít na mysli, že jde pouze o formální rozdělení, neboť obě fáze se překrývají. Morfogenetická fáze vývoje zygotického embrya může být dále rozdělena na několik na sebe navazujících stádií, u nahosemenných rostlin označovaných jako proembryogeneze, raná embryogeneze a pozdní embryogeneze. Jako proembryogeneze jsou označována stádia vývoje embrya před prodlužováním suspenzoru, raná embryogeneze pak trvá od prodlužování suspenzoru po ustavení primárního kořenového meristému a pozdní embryogeneze je charakterizována intenzivní tvorbou pletiv včetně založení primárního kořene a apikálního meristému (Singh 1978).

Pro nahosemenné rostliny je charakteristická polyembryonie, což je vývoj více než jednoho embrya v nezralém semeni. Dochází k ní buď oplozením více než jednoho vajíčka a vznikem několika zygot (jednoduchá polyembryonie), anebo častěji rozdělením jedné zygoty na několik částí, které jsou schopné vývoje jako jednotlivá proembrya (štěpná polyembryonie). Většinou však pouze jedno proembryo pokračuje ve vývoji ve zralé embryo (Owens a Molder 1979).

3.3.1.2. Vlastní vznik somatických embryí

U jehličnanů byly popsány tři způsoby vzniku somatických embryí:

1) Somatická embrya primárně vznikají asymetrickým dělením somatické buňky, které vymezí oblast budoucího embrya a suspenzoru. Vzniká buňka s hustou cytoplasmou, která je připojená k vysoce vakuolizované buňce označované jako primární suspenzor. Tato dvojice buněk vytváří bipolární systém. Bipolární charakter si embrya zachovávají po celou dobu svého vývoje. Po vytvoření dvoubuněčného stádia dochází k opakovanému dělení buňky s hustou cytoplasmou, jehož výsledkem je řada morfologicky podobných buněk. Pod nimi je stále primární suspenzor (Tautorus a kol. 1991, Jasik a kol. 1995). Toto stádium bývá též označováno jako proembryogenní hmota I (Filonova a kol. 2000a). Ta se dále vyvíjí v proembryogenní hmotu II, charakterizovanou přítomností více než jedné vakuolizované buňky. Pro obě tato stádia je typické polární uspořádání. Dalším růstem proembryogenní hmoty II vzniká proembryogenní hmota III, která obsahuje jednotlivé shluky meristematických buněk volně propojených vakuolizovanými buňkami. Tyto shluky již nemají polární uspořádání.

2) Somatická embrya mohou vzniknout také sekundárně z malých meristematických buněk v suspenzoru. Tautorus a kol. (1991) se domnívají, že tyto iniciály mají původ v asymetrickém dělení jedné z buněk suspenzoru či meristematické buňky, která se během vývoje raného somatického embrya náhodně oddělila a ponořila mezi buňky suspenzoru. Filonova a kol. (2000a) usuzují, že tento proces se shoduje s jejich pozorováním, při kterém vznikala proembryogenní hmota I z více vyvinutých stádií (proembryogenní hmoty II a III).

3) Somatická embrya mohou vzniknout také mechanismem shodným se štěpnou polyembryonií. Výsledkem je několik somatických embryí, která mají společný suspenzor (Jasik a kol. 1995, Korlach a Zoglauer 1995).

Výše uvedenými způsoby vznikají somatická embrya na indukčním, případně proliferačním médiu, za přítomnosti auxinů a cytokininů. Další vývoj somatických embryí probíhá fázemi srovnatelnými s ranou a pozdní zygotickou embryogenezí, a to buď přímo na maturačním médiu (s kyselinou abscisovou) nebo bývá mezi fázi proliferace a maturace vřazena přechodná fáze kultivace bez růstových regulátorů (Bozhkov a kol. 2002). Na médiu bez růstových regulátorů vznikají raná somatická embrya, která již mají vyvinutou vrstvu protodermálních buněk a obsahují embryonální trubicové buňky (embryonal tube cells), ze kterých později vzniká

sekundární suspenzor (Filonova a kol. 2000a). Tento vývojový krok je spojen s programovanou buněčnou smrtí zbývající proembryogenní hmoty. V buňkách proembryogenní hmoty dochází k postupné autolýze a tvorbě velké centrální vakuoly, jaderná DNA se rozpadá. Programovanou buněčnou smrtí zanikají také buňky suspenzoru v průběhu rané embryogeneze (Filonova a kol. 2000b).

Prvním diferencovaným pletivem zygotického i somatického embrya je protoderm. U somatických embryí *Picea abies* byla jeho diferenciace spojena s lokální expresí genu *PaHB1* (*Picea abies* homeobox 1). Ektopická exprese *PaHB1* vedla k brzkému zablokování vývoje embryí (Ingouff a kol. 2001). Další gen, jehož exprese je lokalizovaná do buněk protodermu, kóduje nespecifický přenašeč lipidických látek (lipid transfer protein Pa18). Transgenní embrya se zvýšenou či sníženou hladinou tohoto proteinu se sice vyvíjela, avšak buňky jejich vnější vrstvy byly protáhlé a vakuolizované (Sabala a kol. 2000). V obou těchto studiích autoři dále ukázali, že při regulaci vývoje protodermu se u jehličnanů a krytosemenných rostlin uplatňují podobné mechanismy.

Další vývoj ranného somatického embrya je již vázán na přítomnost ABA v médiu (Bozhkov a kol. 2002). V průběhu pozdní embryogeneze probíhá diferenciace pletiv. V bazální části embrya se zakládá kořenový meristém, střední část embrya je tvořena kortexem, prokambiem a dření. Zároveň se vyvíjí i apikální meristém a nakonec jsou založena děložní primordia. Z nich posléze prorůstají dělohy (Filonova a kol. 2000a).

Pro popis vývoje somatického embrya bylo vytvořeno několik systémů morfologických klasifikací. Hakman a von Arnold (1988) dělí vývoj somatických embryí na 4 stádia: stádium 1 - embrya se skládají pouze z oblasti malých meristematických buněk s hustou cytoplasmou, která je spojena s dlouhými suspenzorovými buňkami; stádium 2 - embrya vyčnívají nad povrch embryogenní hmoty, jsou neprůhledná s hladkým a lesklým povrchem a s embryogenní hmotou jsou spojena dlouhými, vysoce vakuolizovanými buňkami suspenzoru; stádium 3 - somatická embrya s vyvinutými dělohami; stádium 4 - mladé zelené rostliny. Stádium 3 strukturálně odpovídá zygotickému embryu ve zralém semeni. Vyvíjející se embrya byla klasifikována na základě vnější morfologie, jejich vnitřní struktura nebyla do této klasifikace zahrnuta. Nagmani a kol. (1995) aplikovali na celý proces somatické embryogeneze klasifikaci používanou pro popis vývoje zygotických embryí. Tato klasifikace rozděluje vývoj zygotických embryí do 13 kroků, zabývá se

však především fází proembryogeneze. Další klasifikační systém vytvořili Pullman a Webb (1994), kteří rozdělili vývoj zygotického embrya do 9 stádií. Tuto klasifikaci pak použili i k popisu somatické embryogeneze *Picea abies, Pinus taeda* a *Pseudotsuga menziesii.*

3.3.1.3. Rozdíly mezi embryogenními kulturami

Schopnost jednotlivých embryogenních kultur produkovat zralá embrya je různá. Embryogenní buněčné linie *Picea abies* lze podle morfologie a podle jejich schopnosti tvořit somatická embrya rozdělit do dvou skupin (Egertsdotter a von Arnold 1993, Mo a kol. 1996): do skupiny A se řadí kultury, jejichž proembrya mají dobře rozlišitelnou meristematickou oblast a suspenzorovou část, a která jsou v přítomnosti ABA schopná vývoje ve zralé somatické embryo (Filonova a kol. 2000a); do skupiny B se pak řadí kultury obsahující proembrya s malými meristematickými oblastmi, které jsou obklopeny vysoce vakuolizovanými buňkami. Některé z těchto linií vůbec nereagují na přítomnost ABA v médiu a nikdy neprodukují embrya. Některé linie sice embrya produkují, ovšem tato embrya vykazují řadu vývojových poruch a nejsou schopna klíčit. V porovnání s embryi z linií A je u těchto embryí zpožděna diferenciace pletiv a posléze dochází i k jejich rozpadu (Filonova a kol. 2000a).

Egertsdotter a kol. (1993) rozdělili embryogenní linie podle produkovaných extracelulárních proteinů opět do dvou skupin (A a B), které se lišily i morfologií embryí. Toto rozdělení odpovídá výše popsanému rozdělení embryogenních linií, neboť skupina A byla charakteristická embryi s hustě nahloučenými meristematickými buňkami a skupina B embryi s nekompaktní oblastí meristematických buněk. Embrya skupiny A vykazovala lepší pozdější vývoj. Přidání extracelulárních proteinů produkovaných embryogenní buněčnou linií ze skupiny A k embryogenní linii ze skupiny B vedlo ke změně morfologie embryí, která se poté podobala embryím ze skupiny A. Zdá se tedy, že embryogenní buněčné linie skupiny A vylučují proteiny nutné pro normální vývoj somatických embryí.

Pozdější studie Egertsdotter a von Arnold (1995) prokázala, že aktivní složkou extracelulárních proteinů zodpovědnou za normální vývoj somatických embryí jsou arabinogalaktany. V proliferující proembryogenní hmotě *Picea abies* byly nalezeny arabinogalaktany reagující s protilátkou proti JIM13 epitopu arabinogalaktanů izolovaných z embryogenní kultury *Daucus carota* (Filonova a kol. 2000a). V raných

somatických embryích však tyto arabinogalaktany nalezeny nebyly. Arabinogalaktany byly nalezeny jak v extracelulárních proteinech somatických embryí skupiny A, tak v extraktu ze semen *Picea abies* (Egertsdotter a von Arnold 1995). Arabinogalaktany tedy byly aktivní složkou kondicionovaného média. Arabinogalaktany jsou proteoglykany, které jsou připojené k plasmatické membráně či k buněčné stěně. Z 90 % jsou tvořeny cukernými zbytky a obsahují také lipidickou složku. Předpokládá se, že jejich štěpením jsou uvolňovány oligosacharidy se signální funkcí (von Arnold a kol. 2002). Jejich přesná chemická struktura zatím nebyla popsána, ale zdá se, že by mohlo jít o lipochitooligosaccharidy (LCO).

LCO jsou signální molekuly způsobující dělení buněk, patří mezi ně i Nod-faktory produkované bakteriemi rodu *Rhizobium*. Dyachok a kol. (2000) prokázali, že Nod-faktory podporují vývoj proembryogenní hmoty, nikoliv však následný vývoj embryí, a že jsou schopny nahradit auxin a cytokinin při stimulaci dělení embryogenních buněk. V následující práci Dyachok a kol. (2002) izolovali z embryogenních kultur *Picea abies* endogenní LCO, které měly stejný efekt jako Nod-faktory a které nebyly nalezeny v neembryogenních liniích. Množství endogenních LCO bylo vývojově regulováno, nejvyšší hladiny byly nalezeny v proembryogenní hmotě, v průběhu následujícího vývoje embryí jejich množství klesalo. Vysoké hladiny endogenních i exogenně přidaných LCO potlačovaly buněčnou smrt nutnou pro vývoj embryí z proembryogenní hmoty. Stejný efekt mělo také přidání chitinázy ze *Streptomyces griseus*. To je v souladu s prací Egertsdotter a von Arnold (1998), kde endochitináza z cukrové řepy také stimulovala proliferaci proembryogenní hmoty. Chitinázy jsou enzymy štěpící jako primární substrát chitin. Ten se v rostlinách nevyskytuje, van Hengel a kol. (2001) však prokázali, že chitinázy jsou schopny štěpit i arabinogalaktany. V průběhu růstu embryogenní kultury *Picea abies* na médiu bez růstových regulátorů docházelo ke zvýšení transkripce i translace genů pro *Chia4-Pa* chitinázy (Wiweger a kol. 2003). Další analýzy prokázaly, že k expresi *Chia4-Pa* dochází nejen v proliferujících buňkách, ale také v buňkách bazální části raných somatických embryí, což jsou místa, kde dochází k programované buněčné smrti.

Shrnutí výše popsaného potvrzuje teorii (von Arnold a kol. 2002), že působením endochitináz jsou z arabinogalaktanů uvolňovány endogenní LCO, které jsou strukturně analogické Nod-faktorům z *Rhizobium*, a které fungují jako signální molekuly stimulující vývoj somatických embryí.

3.3.2. Role ABA v somatické embryogenezi jehličnanů

I když není známo přesné působení ABA, je jisté, že u jehličnanů výrazně ovlivňuje průběh maturace somatických embryí. Její přítomnost v médiu spouští vývoj dalších stádií z raných somatických embryí, zvyšuje jejich počet a celý proces synchronizuje. Dále snižuje množství špatně vyvinutých embryí, brání předčasnému klíčení embryí a stimuluje ukládání zásobních látek. Všechny úrovně působení ABA popsali například Gutman a kol. (1996), kteří porovnávali zrání somatických embryí hybridního modřínu *Larix* × *leptoeuropaea* na médiích s přidáním a bez přidání ABA.

Po prvním týdnu kultivace na maturačních médiích nebyl mezi variantami pozorován žádný rozdíl. Embrya byla globulární, v oblasti budoucí kořenové čepičky se nacházely vakuolizované buňky obsahující polyfenoly. Po dvou týdnech na médiu s ABA se vyvíjela embrya cylindrického tvaru, byla pozorována častá dělení buněk a buňky obsahující polyfenolické látky byly rozmístěny náhodně v bazální části embrya. Na médiu bez přídavku ABA zůstávala embrya v globulární fázi vývoje, buňky střední části embrya byly vysoce vakuolizované, častější byl i výskyt buněk s polyfenoly. Po třech týdnech se u embryí pěstovaných na médiu s ABA vyvíjely dělohy a kořenový pól. Polyfenoly byly nalezeny pouze v bazální části embrya. Naproti tomu embrya rostoucí bez ABA se pouze prodloužila (díky růstu buněk, ne jejich dělení), bazální část těchto embryí byla pokryta vrstvou buněk kořenové čepičky, které akumulovaly polyfenoly. Během čtvrtého týdne se v embryích rostoucích na médiu obsahujícím ABA začaly ukládat zásobní proteiny, a to v podobě granulí či krystalů. U embryí rostoucích bez ABA nebyly zásobní proteiny nalezeny. Roberts a kol. (1990) navíc prokázali, že záleží také na koncentraci exogenní ABA v médiu. Teprve při koncentracích 30-40 μM vznikala u embryogenní kultury *Picea glauca* × *Picea engelmanii* embrya, která obsahovala stejné zásobní proteiny, jako byly nalezeny v zygotických embryích. Při nižších koncentracích ABA vznikala embrya zavalitá či embrya předčasně zralá, která zásobní proteiny neobsahovala. Akumulace zásobních látek během pěstování na médiu obsahujícím ABA byla pozorována i u *Picea glauca* (Hakman a von Arnold 1988). Zde se somatická embrya bez ABA vůbec nevyvíjela. Někteří autoři (např. Attree a Fowke 1993) se domnívají, že pozitivní vliv ABA na vývoj somatických embryí je dán především ovlivněním ukládání zásobních látek. Tuto myšlenku

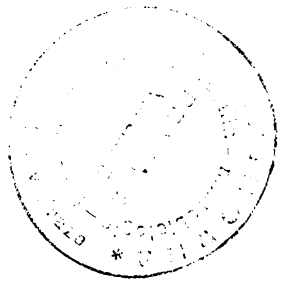
dokládají srovnáním s embryogenezí zygotickou. Během pozdní fáze vývoje zygotického embrya v semeni totiž dochází k akumulaci kyseliny abscisové, která zabraňuje předčasné zralosti embryí. ABA zde aktivuje geny (*rab* geny), jejichž produkty kontrolují ukládání zásobních látek a LEA (late embryogenesis abundant) proteinů. Ty hrají důležitou roli v řízení tolerance k desikaci (Skriver a Mundy 1990).

Důležitá je doba, po kterou jsou embrya pěstována na médiu s ABA. Dlouhotrvající přítomnost ABA v maturačním médiu negativně ovlivňuje fázi klíčení somatických embryí. Vysoký obsah ABA v médiu během pozdních fází vývoje somatických embryí vedl k její akumulaci v embryích *Larix* x *leptoeuropaea*, což mělo za následek zpomalení nebo i inhibici klíčení (Gutman a kol. 1996). Lelu a Label (1994) pozorovali, že nejvíce embryí stejné kultury se vyvíjelo v rostliny po 3 týdnech maturace na médiu s ABA, delší kultivace klíčení neprospívala. Podobně Bozhkov a von Arnold (1998) uvádějí, že počet klíčících somatických embryí *Picea abies* byl vyšší u varianty pěstované na maturačním médiu pět týdnů ve srovnání s variantou rostoucí na maturačním médiu sedm týdnů.

Koncentrace endogenní ABA je u somatických embryí závislá na množství ABA v maturačním médiu. Po přenesení embryogenní kultury *Picea abies* na maturační médium obsahující ABA se množství endogenní ABA prudce zvýšilo, toto zvýšení korelovalo s koncentrací ABA v médiu. Po odstranění ABA z média hladina endogenní ABA poklesla, stále však zůstávala vysoká ve srovnání se stavem před přenesením na maturační médium (Vágner a kol. 1998). V embryích *Larix* x *leptoeuropaea* byla nejvyšší hladina endogenní ABA detekována po pátém týdnu růstu na médiu s 60 μM ABA (Lelu a Label 1994). ABA byla detekována v řádově nižší koncentraci i v embryích rostoucích bez jejího exogenního dodání, což svědčí o schopnosti embryí ABA syntetizovat. Tato syntéza však není pro řízení vývoje somatických embryí dostačující. Dunstan a kol. (1988) sledovali vliv ABA a jejích analogů na vývoj somatických embryí *Picea glauca*. Rostliny obecně jsou schopné analogy ABA přeměnit na ABA, zde však byly některé analogy ABA schopné pouze spustit vývoj embryí, ten se však posléze zastavil a u embryí se nevyvíjely dělohy.

Nutnost přítomnosti exogenní ABA pro úspěšnost somatické embryogeneze lze vysvětlit, podíváme-li se na situaci v embryích zygotických. V průběhu zrání semen většiny rostlin byly popsány dva vrcholy akumulace ABA. První, který bezprostředně předchází začátku zrání semen, je syntetizován mateřským pletivem a

teprve druhý vrchol akumulace ABA je syntetizován vlastním embryem (Karssen a kol. 1983). Tento druhý vrchol akumulace ABA je nezbytný pro indukci dormance, kterou si semena udrží i poté, co hladina ABA na konci zrání prudce klesá (Finkelstein a kol. 2002).



4. DISKUZE

4.1. Indukce a proliferace embryogenních kultur

Jako primární explantát byla k indukci embryogenních kultur použita nezralá i zralá zygotická embrya. Úspěšnost indukce byla vyšší při použití nezralých embryí, také riziko kontaminace bylo nižší. Na druhou stranu bylo nutné přesné načasování sběru šišek a jejich okamžité použití. Ze zralých zygotických embryí lze embryogenní kultury indukovat kdykoliv v průběhu roku, neboť lze použít skladovaná semena (Vágner a kol. 2005a).

Většina embryogenních kultur používaných v našich studiích náleží podle klasifikace Jalonen a von Arnold (1991) do skupiny A. Jsou tedy schopny produkovat zralá somatická embrya, která se pak dále vyvíjejí v rostliny. Pouze embryogenní linie C203 patří do skupiny B, charakterizované dobrým růstem v průběhu proliferace, avšak neschopností vytvářet embrya. Na mikroskopické úrovni jsme pozorovali struktury charakteristické pro embryogenní kultury jehličnanů. Embryogenní kultury se skládaly z oblastí meristematických buněk, pod kterými byly připojeny vysoce vakuolizované buňky suspenzoru (Svobodová a kol. 1999, Fischerová a kol., odesláno do tisku). Stejnou stavbu proliferující embryogenní kultury popisují i další autoři (např. Jasik a kol. 1995, Filonova a kol. 2000a).

Vybrané embryogenní kultury byly v průběhu proliferace úspěšně zamraženy (Vágner a kol. 2005a), což umožnilo jejich dlouhodobé skladování v tekutém dusíku. I když se díky kryoprezervaci zkracuje doba proliferace, a tedy i riziko somaklonální variability, je nutné si uvědomit, že v průběhu kryoprezervace je používán dimethylsulfoxid (DMSO) a to ve vysokých koncentracích. DMSO je mutagenní látka potenciálně schopná vyvolat po kryoprezervaci genetické změny v embryogenních kulturách (Aronen a kol. 1999).

Mezi fázi proliferace a maturace bývá někdy vřazována fáze kultivace embryogenní kultury na médiu bez růstových regulátorů (Bozhkov a kol. 2002). Týdenní kultivace vybraných embryogenních kultur na médiu bez růstových regulátorů výrazně zvýšila počet zralých somatických embryí (Vágner a kol. 2005b). Odstranění, či alespoň částečné snížení obsahu auxinů a cytokininů stimuluje programovanou buněčnou smrt v proembryogenní hmotě. Programovaná buněčná smrt je proces úzce spojený s následným vývojem raných somatických embryí (Filonova a kol. 2000b).

4.2. Maturace embryogenních kultur

Po přenesení embryogenní kultury z proliferačního na maturační médium dochází k vývoji embrya srovnatelnému s ranou a pozdní embryogenezí embrya zygotického (von Arnold a kol. 2002). Stejný průběh vývoje embrya jsme pozorovali i u embryogenní kultury AFO 541, typického zástupce embryogenních linií skupiny A (Svobodová a kol. 1999). Jako první se zakládal protoderm, posléze se začaly vyvíjet i vnitřní struktury embrya. Po šesti týdnech kultivace na maturačním médiu již byla embrya plně vyvinutá, měla zřetelný apikální meristém, dobře vyvinuté dělohy, prokambiální provazce, kořenový meristém a kořenovou čepičku. Stejný postup diferenciace pletiv somatických embryí *Picea abies* popsali i Filonova a kol. (2000a). Déle trvající maturace (7 a 8 týdnů) vedla pouze k rozvolnění povrchových vrstev embrya. To bylo spojeno s ukládáním fenolických látek a škrobových zrn (Svobodová a kol. 1999). Akumulace fenolických látek je u jehličnanů spojena se stresovými podmínkami (Gutmann a kol. 1996). K vývojovým poruchám docházelo především v místech kontaktu embryí s médiem, s jinými embryi, či v místech mechanického poškození embryí způsobeného při subkultivaci kultur (Svobodová a kol. 1999). Posledně jmenovanému riziku se lze vyhnout pěstováním embryogenních kultur na prámcích s polypropylenovou membránou, což je systém používaný u řady *in vitro* kultur (např. Watad a kol. 1995). Použití tohoto systému u embryogenních kultur *Picea abies* se ukázalo velmi výhodným. Počet zralých somatických embryí se zvýšil u 7 z 12 testovaných linií, celý proces byl lépe synchronizován a průměrně o 6 dní urychlen (Vágner a kol. 2005b). Zkrácení doby, po kterou jsou embrya vystavena působení vysokých koncentrací ABA v maturačním médiu, je prospěšné, neboť ABA v embryích inhibuje klíčení (Bozhkov a von Arnold 1998).

Přidání polyethylenglykolu 4000 (PEG 4000) do maturačního média vedlo k urychlení celého procesu, tento efekt byl výraznější u varianty s nižší (3,75%) koncentrací PEG 4000. Obě použité koncentrace (3,75 % a 7,5 %) měly pozitivní vliv na vývoj kořenové čepičky embryí, nedocházelo k jejímu rozpadu, který byl častý u varianty bez PEG (Svobodová a kol. 1999). Hakman a von Arnold (1988) přičítají pomalý růst rostlin ze somatických embryí *Picea glauca* právě špatně vyvinutému kořenovému pólu embryí. Bozhkov a von Arnold (1998) pozorovali vývoj kořenové čepičky embryí *Picea abies* na médiu se 7,5 % PEG, kořenová

čepička těchto embryí byla masivní a obsahovala četné mezibuněčné prostory. V naší studii způsobil 7,5% PEG prodloužení kořenové čepičky až o 40 % ve srovnání s variantou s 3,75% PEG, žádné mezibuněčné prostory však nebyly pozorovány (Svobodová a kol. 1999). Stasolla a kol. (2003) popsali u embryogenní kultury *Picea glauca* ošetřené 7,5% PEG zvýšení exprese genu homologního *SCR* (*SCARECROW*). *SCR* je u *Arabidopsis thaliana* odpovědný za radiální růst kořene, neboť určuje identitu endodermis. Autoři se proto domnívají, že PEG může ovlivňovat i radiální symetrii kořenů somatických embryí a pozitivně tak působit na jejich následné klíčení.

Somatická embrya *Picea abies* pěstovaná na médiu s přídavkem 5% PEG 4000 byla znatelně menší a vyskytovaly se u nich některé strukturní abnormality jako dutiny pod apikálním meristémem (Find 1997). Tvorbu prasklin a řady mrtvých buněk jsme pozorovali na konci maturace v kortexu některých embryí vyvíjejících se v přítomnosti 7,5% PEG, žádné praskliny a řady mrtvých buněk jsme však nenalezli v embryích rostoucích na médiu s 3,75% PEG. Kvantitativní analýzou jsme prokázali, že embrya rostoucí na médiích obsahujících PEG v obou koncentracích byla v každém kroku vývoje delší než embrya rostoucí bez přítomnosti PEG (Svobodová a kol. 1999).

Úspěšný průběh maturace je charakterizován vysokým počtem zralých somatických embryí. O jejich kvalitě vypovídá jednak jejich anatomická stavba, jednak úroveň metabolické aktivity a následné ukládání zásobních látek, charakteristické především pro pozdější fáze vývoje somatického embrya (Gutman a kol. 1996). Akumulace zásobních látek je pro somatická embrya nezbytná, neboť jejich klíčení probíhá bez podpory megagametofytu, hlavního zásobního orgánu semen (Misra a Green 1990). Soustředili jsme se proto též na charakterizaci maturace jako procesu ukládání zásobních látek.

V průběhu maturace jsme sledovali obsah nestrukturních sacharidů v embryogenní kultuře AFO 541 (Lipavská a kol. 2000). Celková hladina rozpustných sacharidů se zvýšila s přenesením kultury na základní maturační médium, s pokračujícím růstem kultury však klesala. Tento pokles byl zapříčiněn snížením hladin hexos (glukosy a fruktosy), množství sacharosy se naopak v průběhu maturace zvyšovalo. Za tento posun ve prospěch sacharosy byla odpovědná vyvíjející se somatická embrya. Otázkou zůstává, zda se jednalo pouze o akumulaci sacharosy z média či o její *de novo* syntézu. Podle práce Konrádová a

kol. (2002) se zdá, že se jedná spíše o syntézu *de novo*, neboť buňky v blízkosti vyvíjejícího se embrya, které zprostředkovávají kontakt embrya s médiem, obsahovaly vysoké hladiny hexos a vykazovaly vysokou aktivitu invertázy. Ta byla vysoká též v somatických embryích na počátku maturace, zatímco aktivita sacharosa-syntázy byla v těchto embryích nízká. Pozdní fáze maturace somatických embryí byly naopak charakteristické téměř nedetekovatelnou aktivitou invertázy a zvyšující se aktivitou sacharosa-syntázy. Iraqi a Tremblay (2001) pozorovali, že pokles aktivity invertáz na konci maturace byl spojen se zvyšujícím se poměrem sacharosy ku hexosám a následné akumulaci škrobu a proteinů. Výše popsaná pozorování odpovídají představě, že vyvíjející se somatické embryo se z metabolického sinku stává sinkem zásobním (Lipavská a Konrádová 2004), tak, jako se tomu děje i ve vyvíjejícím se semeni (Weber a kol. 1997).

Další zásobní látkou v somatických embryích jehličnanů je škrob. Jeho ukládání předchází tvorbě zásobních lipidů a proteinů (Joy a kol. 1991). Pomocí histochemické detekce jsme popsali dynamiku ukládání škrobu v průběhu zrání somatických embryí (Lipavská a kol. 2000). V raných somatických embryích nebyl škrob nalezen, k jeho pomalé akumulaci docházelo po dvou týdnech kultivace na maturačním médiu. Škrobová zrna se po celou dobu zrání embryí preferenčně ukládala v bazální části a po jejím založení v kořenové čepičce embryí. Preferenční ukládání škrobu v bazální části embrya popsali i Joy a kol. (1991). Po pěti týdnech maturace se škrobová zrna nacházela i v buňkách kortexu. Následná kultivace embryí na maturačním médiu vedla ke snížení obsahu škrobu (Lipavská a kol. 2000). Ve zralých zygotických embryích *Picea glauca* nebyl škrob vůbec nalezen (Joy a kol. 1991). Histochemická detekce škrobu byla doplněna také jeho přímým stanovením v pozdních fázích maturace (5. – 8. týden), které potvrdilo pokles obsahu škrobu na konci maturace. Tento pokles však nebyl tak dramatický, jako při histochemické detekci. Tento nesoulad byl pravděpodobně vyvolán tím, že akumulace škrobu je spojena s vývojovými poškozeními (Svobodová a kol. 1999), které se na konci maturace vyskytovaly poměrně často. Proto je možné, že dobře vyvinutá nepoškozená embrya obsahují nižší množství škrobu, než bylo detekováno pomocí HPLC ve směsném vzorku embryí.

Studovali jsme také dynamiku obsahu nestrukturních sacharidů během maturace, kdy byl do média přidán PEG v koncentraci 3,75 %, neboť z předchozí studie (Svobodová a kol. 1999) bylo zřejmé, že toto ošetření má pozitivní vliv na vývoj

somatických embryí. Celkový obsah sacharidů byl nižší, zvýšil se však poměr sacharosy ku hexosám. Stasolla a kol. (2003) pozorovali po aplikaci PEG snížení hladin transkriptů mnoha enzymů účastnících se katabolismu sacharosy. U kontrolních embryí rostoucích bez přítomnosti PEG se naopak hladiny těchto transkriptů zvyšovaly. Autoři se domnívají, že proto tato embrya nedosáhla fyziologické zralosti. Inhibiční efekt PEG na předčasné klíčení (Attree a kol. 1991) pak připisují právě změnám v sacharidovém metabolismu.

Další zvýšení koncentrace PEG v maturačním médiu na 7,5 % vedlo k výrazným změnám v obsahu sacharidů, a to jak ve srovnání s kontrolní variantou, tak i ve srovnání s variantou s 3,75% PEG. V průběhu 4. a 5. týdne maturace došlo k výraznému zvýšení obsahu sacharosy i hexos (Lipavská a kol. 2000). To může být dáno výskytem vývojových poškození pozorovaných na anatomické úrovni v pozdních fázích maturace (Svobodová a kol. 1999). Jednotlivé embryogenní linie navíc reagují rozdílně na různé koncentrace PEG (Vágner a kol. 2005b), proto je těžké vyvodit konkrétní zobecnění.

Jednotlivé embryogenní kultury se liší i schopností reagovat na přítomnost ABA v médiu. Výše popisovaný genotyp AFO 541 (Svobodová a kol. 1999, Lipavská a kol. 2000) patří mezi kultury s vysokou embryogenní kapacitou, tj. s velkou schopností vytvářet embrya po aplikaci ABA. Další embryogenní kultury *Picea abies* odvozené v naší laboratoři podle protokolu popsaného v práci Vágner a kol. (2005a) se však ve schopnosti reagovat na exogenně dodanou ABA velmi lišily.

Obecný mechanismus působení ABA v rostlinách není jasný, neboť zatím nebyly popsány žádné receptory pro ABA (Finkelstein a kol. 2002). Bylo však popsáno mnoho mutantů se změněnou reakcí na ABA, většina z nich u *Arabidopsis thaliana*. Mezi ně patří též *abi* (abscisic acid insensitive) mutantní rostliny, které mají nezměněnou hladinu endogenní ABA, avšak na ABA nereagují (Giraudat 1995). Byly popsány jako rostliny klíčící na koncentracích ABA, které za normálních okolností klíčení inhibují (Koorneef a kol. 1984). Fenotypový projev *abi3*, *abi4* a *abi5* mutantů je vázán na semena (Holdsworth a kol. 1999). *ABI3*, *ABI4* a *ABI5* geny kódují transkripční faktory, které společně regulují odpověď semen na ABA (Söderman a kol. 2000). Různé studie prokázaly, že ABI3 transkripční faktor stojí na začátku signální dráhy přenosu ABA signálu (McCourt 1999, Lopez-Molina a kol. 2002). *ABI3* ortology byly izolovány z mnoha rostlinných druhů, např. z *Populus trichocarpa* (Rohde a kol. 1998), *Triticum aestivum* (Nakamura a Toyama

2001), *Chamaecyparis nootkatensis* (Lazarova a kol. 2002) a také z *Picea abies* (*PaVP1*; Footitt a kol. 2003).

Pro pochopení rozdílné senzitivity jednotlivých embryogenních linií na ABA jsme se rozhodli analyzovat expresi *PaVP1* genu v průběhu maturace vybraných kultur s kontrastní embryogenní kapacitou. Analýzu exprese *PaVP1* jsme doplnili anatomickou studií (Fischerová a kol., odesláno do tisku). Exprese *PaVP1* v neembryogenní kultuře nebyla detekována, a to ani po jejím přenesení na médium obsahující ABA. Tento přenos vedl k ukládání fenolických látek v kultuře a k jejímu postupnému odumírání. Ukládání fenolických látek je považováno za stresovou reakci (Gutmann a kol. 1996), jako stresový faktor u nediferencované neembryogenní kultury pravděpodobně působila vysoká koncentrace ABA v médiu.

Pro analýzu exprese *PaVP1* jsme dále vybrali dvě kontrastní embryogenní linie, lišící se schopností vytvářet embrya na maturačním médiu, a tedy možná i schopností reagovat na ABA. Zatímco AFO 541 je typickým zástupcem kultur s vysokou embryogenní kapacitou, embryogenní linie C203 má embryogenní kapacitu nízkou a embrya tvoří jen výjimečně (Vágner a kol. 2005b). Podle klasifikace Jalonen a von Arnold (1991) tedy C203 patří do skupiny B embryogenních kultur. Přenesení kultury C203 na maturační médium bylo spojeno se zvýšením exprese *PaVP1*, tato hladina zůstala nezměněna do 2. týdne maturace. Ve 3. týdnu klesla a dále již nemohla být detekována, neboť nebylo možné izolovat RNA z kultury. Embryogenní kultura C203 reagovala na přenos na maturační médium nejprve dělením buněk v meristematických oblastech a zvětšením meristematických částí, následná kultivace na maturačním médiu však vedla k postupnému rozpadu kultury. Kultura si zachovávala charakter proembryogenní hmoty i na maturačním médiu. Jako proembryogenní hmota bývají označovány embryogenní kultury ve fázi proliferace (Filonova a kol. 2000a), přenos proembryogenní hmoty na maturační médium bývá spojen s tvorbou raných somatických embryí. Ta se od proembryogenní hmoty liší ustavením protodermu (Sabala a kol. 2000). U kultury C203 se však protoderm nezakládal ani po 3 týdnech maturace, není tedy možné u této linie hovořit o vývoji raných somatických embryí. Naproti tomu u kultury AFO 541 docházelo k ustavení protodermu již 2 týdny po přenosu na maturační médium (Svobodová a kol. 1999, Fischerová a kol. odesláno do tisku). Přenos této kultury na médium obsahující ABA byl spojen s nárůstem exprese *PaVP1*, hladina exprese se s postupným vývojem embryí zvyšovala, a to až

do 5. týdne maturace. Embrya v té době dosáhla kotyledonárního stádia vývoje, tj. byly založeny všechny vnitřní struktury embrya a začalo prorůstání děložních primordií. Poté došlo k prudkému poklesu exprese *PaVP1*. Stejný průběh exprese *PaVP1* v průběhu maturace vysoce embryogenní kultury *Picea abies* popisují i Footitt a kol. (2003) a byl popsán i u *Daucus carota* (Shiota a kol. 1998). Expresi *PaVP1* jsme popsali také u linie C110, další vysoce embryogenní kultury odvozené v naší laboratoři podle protokolu Vágner a kol. (2005a). Důvodem bylo potvrzení průběhu exprese *PaVP1* u další vysoce embryogenní kultury. Průběh exprese *PaVP1* byl u embryogenní linie C110 pěstované na maturačním médiu stejný jako u linie AFO 541. Pokud však byla po dvou týdnech maturace odstraněna ABA z média, exprese *PaVP1* se rychle snížila a raná somatická embrya se začala rozpadat. Není však zřejmé, zda byl rozpad embryí primárně ovlivněn absencí ABA v médiu či snížením exprese *PaVP1*, neboť samo snížení exprese *PaVP1* mohlo souviset buďto s nepřítomností ABA v médiu či s rozpadem embryí, ve kterých k expresi dochází (Footitt a kol. 2003). Jelikož ke snížení exprese *PaVP1* docházelo i za přítomnosti ABA v médiu, a to u rozpadajících se embryí linie C203, je možné spekulovat, že exprese *PaVP1* je podmíněna jak přítomností ABA v médiu, tak přítomností vyvíjejících se meristematických center. Zvyšující se expresi *PaVP1* je tedy možno považovat za marker správného vývoje somatických embryí.

4.3. Desikace a klíčení

Desikace je významnou etapou somatické embryogeneze, která ovlivňuje úspěšnost klíčení. Ve všech experimentech jsme použili systém desikace popsaný v práci Vágner a kol. (2005a). Na anatomické úrovni jsme nenalezli žádné výrazné rozdíly mezi zralými embryi na konci maturace a embryi vystavenými desikaci, pouze došlo k zahuštění cytoplasmy buněk povrchových vrstev embrya. V průběhu desikace se však v somatických embryích začaly akumulovat sacharidy rafinosové řady (RFO: rafinosa, stachyosa a verbascosa; Kumstýřová a kol. 2000). Ve zralých zygotických embryích *Picea abies* také dochází k akumulaci RFO (Gösslová a kol. 2001). Předpokládá se, že RFO mohou bránit krystalizaci sacharosy, a tím zvyšovat odolnost embryí k vysoušení (Lipavská a Konrádová 2004). U zygotických embryí slouží RFO rovněž jako zdroj uhlíku a energie v raných fázích klíčení (Downie a Bewley 2000).

Vlastní klíčení somatických embryí je tedy výrazně ovlivněno předchozími fázemi somatické embryogeneze. Často diskutovanou otázkou je vliv použití osmotika v průběhu maturace na úspěšnost klíčení somatických embryí. Find (1997) ukázal, že 5% PEG 4000 negativně ovlivňoval prorůstání kořene a vývoj apikální části somatických embryí *Picea abies*. Ke stejnému závěru došli i Bozhkov a von Arnold (1998) při použití 7,5 a 2,5% PEG. V našich experimentech se však počet klíčících embryí po aplikaci 3,75% PEG v průběhu maturace zvýšil. Koncentrace 7,5 % naopak počet klíčících embryí snížila (Svobodová a kol. 1999). Možným vysvětlením je rozdílná citlivost jednotlivých embryogenních linií na různé koncentrace PEG (Vágner a kol. 2005b). Högberg a kol. (2001) prokázali, že negativní vliv PEG vymizel v průběhu růstu rostlin ze somatických embryí v *ex vitro* podmínkách.

5. ZÁVĚRY

- Vývoj somatických embryí probíhající na maturačním médiu lze na anatomické úrovni rozdělit do čtyř vývojových stádií lišících se různým stupněm vnitřní diferenciace: stádium raných somatických embryí, cylindrické stádium, stádium prekotyledonární a stádium kotyledonární.

- U embryí kultivovaných na maturačním médiu po dosažení kotyledonárního stádia dochází k rozpadu kořenové čepičky a vývojovým poruchám, spojeným s ukládáním polyfenolických látek a škrobu.

- Časová posloupnost jednotlivých vývojových stádií maturace somatických embryí je ovlivněna působením PEG 4000, při použití 3,75% PEG se celý proces maturace zrychlí až o dva týdny ve srovnání s maturací probíhající na médiu bez PEG. Zvýšená koncentrace PEG (7,5%) vede ke vzniku prasklin v hypokotylu somatických embryí.

- Obsah nestrukturních sacharidů v embryogenní kultuře rostoucí na maturačním médiu se s jejím vývojem zvyšuje, a to díky zvyšujícímu se obsahu sacharosy v embryích. Obsah hexos v embryích je nízký.

- PEG 4000 v koncentraci 3,75 % v maturačním médiu zvyšuje poměr sacharosy ku hexosám v somatických embryích, celkový obsah sacharidů v embryogenní kultuře mírně snižuje. Přidání 7,5 % PEG do maturačního média vede ke změnám v obsahu a dynamice ukládání nestrukturních sacharidů, embrya obsahují hexosy ve větší míře než embrya pěstovaná bez, či s 3,75% PEG.

- Celkový počet vyvinutých embryí se u většiny testovaných linií zvýší zařazením pre-maturační fáze (na médiu bez růstových regulátorů) mezi proliferaci a maturaci. Podobný efekt má kultivace embryogenních kultur na prámcích s polypropylenovou membránou, která zároveň zkracuje dobu nutnou k jejich vývoji.

- Byl vytvořen vzorový protokol pro práci s embryogenními kulturami smrku ztepilého od fáze indukce až po převod do *ex vitro* podmínek, včetně postupu kryoprezervace.

- Exprese genu pro transkripční faktor PaVP1 je detekovatelná jen v embryogenních liniích, bez ohledu na jejich schopnost tvořit zralá embrya. V neembryogenní kultuře není exprese *PaVP1* detekovatelná.

- Exprese *PaVP1* je u embryogenních linií indukována přenesením na maturační médium obsahující ABA. Vynechání ABA v průběhu maturace vede k rychlému vymizení exprese *PaVP1* a také k poškození vývoje embryí.

- U linie s nízkou embryogenní kapacitou je zvýšení exprese *PaVP1* po přenesení na maturační médium pouze přechodné. Následný pokles exprese *PaVP1* je spojen s rozpadem meristematických center.

- V liniích s vysokou embryogenní kapacitou se udržuje exprese *PaVP1* v průběhu maturace na vysoké hladině s maximem v kotyledonárním stádiu vývoje embryí.

- *PaVP1* sonda specificky hybridizuje se dvěma transkripty odlišné délky, jejichž vzájemný poměr se v průběhu vývoje embryogenních kultur mění. To indikuje možnost regulace syntézy PaVP1 proteinu alternativním sestřihem.

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Somatic embryogenesis in Norway spruce: Anatomical study of embryo development and influence of polyethylene glycol on maturation process

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Abstract — The sequence of the developmental stages during somatic embryo maturation in Norway spruce (*Picea abies* (L.) Karst.) was described for a maturation medium (containing abscisic acid) and for the same medium supplemented with 3.75 and 7.5 % polyethylene glycol (PEG-4000). Embryo maturation was accomplished after six weeks of cultivation on the PEG-free medium, and prolonged cultivation resulted in a higher frequency of irregularly developing embryos. The addition of 3.75 % PEG increased the speed of the maturation process by two weeks and caused changes in the morphometric characteristics, e.g. an increase in embryo length (approx. 30–40 %). PEG at a concentration of 7.5 % did not further speed up the process. In addition, the size of the embryos remained approximately the same, and only the root cap length increased by approx. 40 % as compared to the 3.75 % PEG variant. On the other hand, some morphological abnormalities occurred (e.g. rows of dead cells and ruptures in the center of embryos), which are unambiguously negative for further germination. As the developmental process improved, the number of subcultivations needed was lowered and thus the probability of damage due to mechanical irritation also decreased. This has been attributed to the undoubtedly positive effect of the presence of 3.75 % PEG in the medium. Based on all of the obtained results, we concluded that, of all the treatments tested, the medium supplied with 3.75 % PEG proved to be the most beneficial for embryo development of the studied genotype of Norway spruce. © Elsevier, Paris

Anatomy / histochemistry / morphology / osmotic stress / PEG-4000 / *Picea abies*

ABA, abscisic acid / BAP, benzylaminopurine / MM, maturation medium / PEG, polyethylene glycol / 2,4-D, 2,4-dichlorophenoxyacetic acid

1. INTRODUCTION

Norway spruce (*Picea abies* (L.) Karst.) is an important species within central and northern parts of Europe. The natural regeneration of *P. abies* is from seeds. When seed production fails (infrequent flowering, problematic and slow propagation of genetically improved material, etc.), vegetative propagation, e.g. somatic embryogenesis, can play an important role.

Somatic embryogenesis is the development of embryos from somatic cells. This is accomplished via a series of developmental stages, most of which are similar to zygotic embryogenesis [11]. The approach of introducing a sufficient amount of superior genotypes for planting cannot be completely used due to problems with the maturation, rooting and dormancy of cultures, although substantial progress has been made towards the development of systems for somatic embryogenesis in conifers. The efficiency of somatic embryo maturation and embryo conversion into emblings needs improvement [6, 15, 30]. Therefore, investigation into the basic physiology and morphogenic events of the embryogenic process of conifer in vitro cultures may help to clarify underlying problems.

Much attention has been paid to the optimization of conditions needed to establish embryogenic cultures of conifers (reviewed by Gupta and Grob [11]). After induction, embryogenic cultures multiply via cleavage of embryonal groups or from single cells in the suspensor region via so-called diploid parthenogenesis [7, 16]. Abscisic acid (ABA) promotes the transition of the culture from proliferation to maturation stage [13]. Generally, ABA and osmotic stress are implicated in the control of many processes during the

maturation and the germination of somatic embryos of conifers [11]. A combination of ABA and a non-penetrating osmoticum as supplement in the culture medium can help prevent precocious germination and allow embryo development to proceed [4, 21, 29, 30]. Attree et al. [4] showed that only non-penetrating osmotica are able to promote the maturation of spruce embryos. This kind of osmotica restricts water uptake and, hence, provides a natural drought stress during embryo development [1, 2]. The best results were obtained with polyethylene glycol 4000 (PEG-4000), a non-plasmolyzing osmoticum which cannot penetrate into the plant cell but can induce water stress. This type of stimulating effect by PEG in a maturation medium was first reported in *Picea glauca* [2]. The addition of 5–7.5 % (w/v) PEG-4000 increased the maturation frequency approximately two to three times and also increased desiccation tolerance. Each of the genotypes of white spruce (*P. glauca*), however, reacted in a different way and the timing of the transfer to the PEG-containing medium was also important. Subsequent studies showed that PEG-4000 and ABA promoted the accumulation of storage lipids [3] and proteins [24], and resulted in a polypeptide and fatty acids composition comparable to zygotic embryos and a decrease of embryo water content [4].

References to the structure of the different developmental stages of somatic embryos of conifers are less frequent [14, 15, 20]. The detailed description of the whole process especially at an anatomical level is missing. There are two main types of classification for conifer somatic embryo maturation. The first, introduced by von Arnold and Hakman [32], recognizes four stages and further substages. The developing embryos were classified according to their outer morphology while the inner structure was not taken into consideration. The second, introduced by Nagmani et al. [25], divides the whole process, including proembryo structure induction, development and maturation, into thirteen stages but the authors focused first of all on the free nuclear and the cellular somatic embryo phases. The maturation of embryos after ABA treatment was described using only four phases which are also based on the outer morphology. Some authors (e.g. [18]) gave a detailed description of the structure of embryogenic cultures and attempted to relate their morphology at the start of the embryo formation to the final yield (expressed as frequency of properly developed somatic embryos). Gutmann et al. [12] made a comparative structural and histochemical investigation of larch somatic embryos. Embryos, which were cultured for four weeks on the maturation medium with ABA, developed normally, but on a medium with no ABA supplement, they grew abnormally. The authors suppose that the morphogenetic abnormalities, such as the expanded and highly vacuolated cells, or the precocious germination of ABA-minus embryos were largely due to the abnormal timing of cell expansion. Joy et al. [20] followed the developmental sequence of white spruce somatic embryos from the early filamentous stage to the cotyledonary stage both histochemically and biochemically. A detailed analysis, however, of the morphogenetic events for *P. abies* during the development of the somatic embryos at an anatomical level is still missing, and an information gap exists regarding the correlation between a structure and its physiological state. The situation with somatic embryogenesis in conifers is even more complicated, because it is difficult to compare different cultivation conditions. According to literature, there is also heterogeneity in the timing of morphogenetic events between different cell lines and, of course, between different genotypes of conifer embryogenic tissues [22]. For example, the maximum effect of PEG on the maturation of *Abies nordmandiana* coincides with the period when precotyledonary embryos are formed. When PEG is applied during storage nutrient accumulation in the later stages, this effect is not exerted [26]. Information on the influence of PEG on somatic embryo structure is still sparse. According to Find [8], Norway spruce embryos grown on 5 % (w/v) PEG were remarkably smaller and they showed some critical structural abnormalities such as air spaces below the apical meristem. He attributed these morphological traits to the low germination frequency in the PEG-treated variants.

The present study aims to better understand the maturation process of Norway spruce embryos by characterizing particular developmental stages and by examining the influence of osmotic stress caused by the presence of different PEG concentrations on the sequence of the morphogenetic events of the developing embryos.

2. RESULTS

2.1. Structural characteristics of somatic embryo development

2.1.1. Embryogenic tissue on proliferation medium

The Norway spruce embryogenic tissue grown on the medium containing auxin and cytokinin was white, mucilaginous in texture and relatively consistent. The

density of embryo occurrence in the cell line under investigation was very high. Embryo morphology is also very reminiscent of young zygotic embryos of conifers. According to the classification of Jalonen and von Arnold [18], the embryogenic tissue studied in the present paper belongs to group A, which the authors consider to be capable of proper maturation and subsequent development into plantlets.

At a microscopical level, we were using a double-staining procedure which allows to distinguish meristematic areas not only by the typical morphology of meristematic cells but also by the intensive red coloring of active nuclei. Among highly vacuolated cells of the embryogenic tissue, small regions of densely cytoplasmic cells were present (*figure 1 A*). Those regions represented meristematic parts of early somatic embryos. The embryo structure was strictly bipolar and had a filamentous shape due to the attached suspensor composed of highly vacuolated and elongated cells. Often, we found somatic embryos with a very broad embryogenic region which eventually gives rise to several embryos by cleavage polyembryony. Those structures were observed not only on proliferation medium but also during the first two weeks of embryo development on maturation medium (*figure 1 C*). Cytoplasm-rich cells among the highly vacuolated suspensor cells were seen only very sporadically.

2.1.2. Embryo development on maturation medium

Further highly synchronized development of embryogenic tissue containing early somatic embryos was initiated by transfer onto maturation medium where auxin and cytokinins were replaced by abscisic acid. At all stages of embryogenesis, the embryos were bipolar.

In week 1 (*figure 1 B, C*), the basic architecture of embryos was similar to early somatic embryos on proliferation medium: the globular meristematic region was still attached to suspensor cells but cells on the basal pole of the meristematic region started to vacuolize. The apical, densely cytoplasmic meristematic region of the embryos underwent frequent cell divisions. This process showed the first signs of gradual histodifferentiation. We still called this developmental stage, the stage of early somatic embryo.

In week 2 (*figure 1 D*), the cylindrical shape of the embryos began to appear. Thus, this developmental stage was named the cylindrical stage, which was characterized at a microscopic level by the lack of any inner histological differentiation. Protodermis was already established and at a macroscopic level, the glossy and glabrous apical parts of the embryos were recognizable from the remaining tissue. The meristematic cells on the basal pole proceeded in their vacuolization, the nucleus size decreased. The suspensor cells with nuclei on the basal pole were still present.

In week 3 (*figure 1 E*), the embryos showed proceeding elongation of an embryo shape and the further establishment of embryonic inner structure appeared. The root cap with a well-established columella arose on the basal pole of the embryo. Due to proceeding inner histodifferentiation, this stage was named the precotyledonary stage. During week 4 (*figure 1 F*) of cultivation, the formation of the embryo continued, the root cap became more distinct as did the starting formation of procambial strains apparent in a rib meristem above the differentiating root cap. The former suspensor system at this point necrotized and remained attached to the embryo proper as a mass of withered cells.

Week 5 (*figure 1 G*) of cultivation was characterized by the formation of an apical dome and a root apical meristem cotyledon primordia began to grow and differentiate. Since the cotyledons were apparent, the developmental stage was named the cotyledonary stage. At this stage of development, all the embryonic structural parts were established and no other new organ primordia or inner structures appeared.

In week 6 (*figure 1 H*), the embryos continued to elongate, particularly the cotyledons, and the first signs of a root cap disintegration appeared. The mature somatic embryos had the distinct apical meristem, the procambial strains, the well-developed cotyledons and the root meristem, but disintegration of the root cap continued (older than week 6; *figure 1 I*). Prolonged cultivation did not lead to further elongation (length acquisition was less than 8 %) and no further progress in embryo structure was observed.

Furthermore, macroscopic browning and swelling of embryos were observed, especially in weeks 7 and 8 of cultivation. At a microscopic level, swelling and structural abnormalities ('callusing' of embryo surface) occurred, connected with phenolics and starch deposition (see section 2.4.). Phenolics were localized ectopically in both cotyledon and hypocotyl peripheral cell layers (former protodermis or epidermis where callusing began) and starch grains accumulated in a primary cortex of the embryo hypocotyl (*figure 2 A, C, D, F*).

2.2. The effect of PEG on the maturation process

The main difference between treatments growing on maturation medium (MM) and on maturation medium

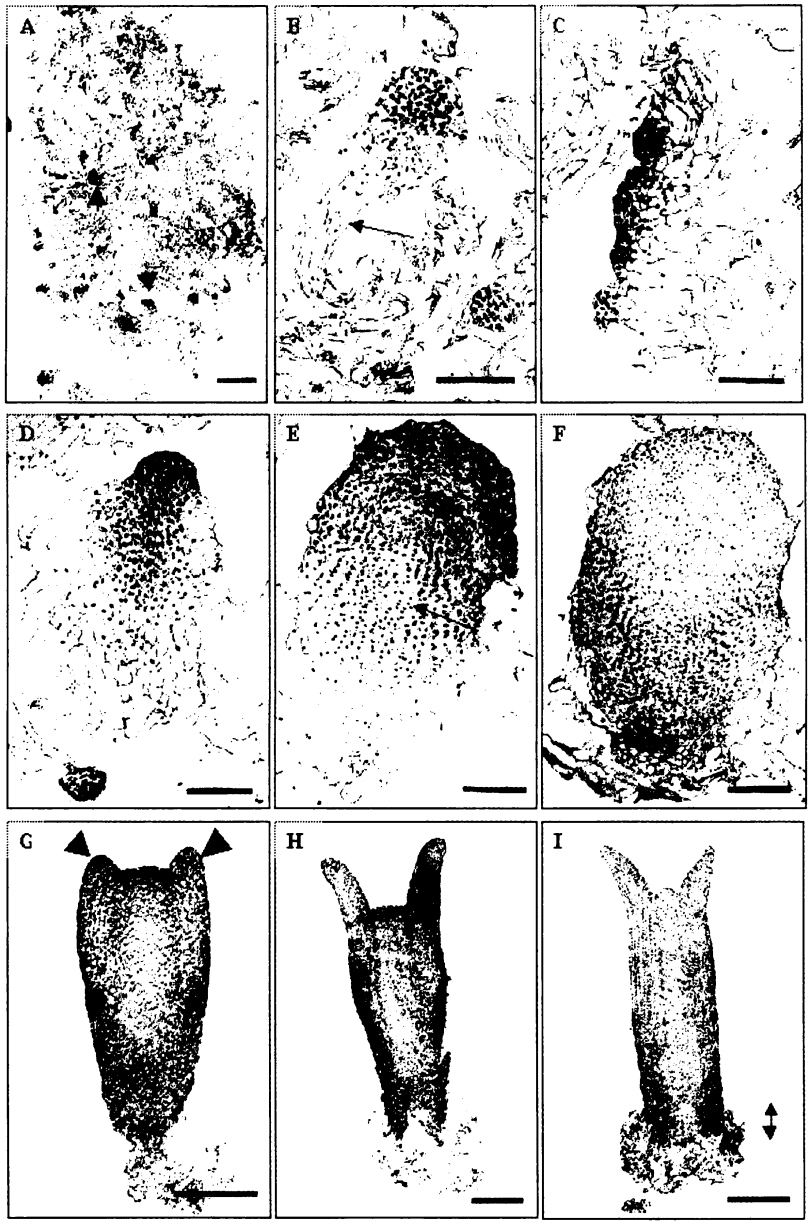

Figure 1. Development of somatic embryos. Longitudinal sections, staining – alcian blue and nuclear fast red; type of microscopy – bright field. **A**, Embryonal suspensor mass (ESM) during cultivation on proliferation medium. The ESM consists of embryogenic regions (red coloring; arrowheads) and the suspensor regions (blue coloring). **B**, Early somatic embryo at week 1 on maturation medium (MM). The meristematic part of the embryo is colored red, suspensor apparatus is attached (arrow). **C**, Polyembryony of early somatic embryos at week 1 on MM. The somatic embryo with a very broad embryogenic region eventually gives rise to several embryos by cleavage. **D**, Cylindrical stage of the developing embryo at week 2 on MM. **E**, Precotyledonary stage of the developing embryo at week 3 on MM. The columella of the root cap differentiates (arrow). **F**, Precotyledonary stage of the developing embryo at week 4 on MM. The root cap is formed. **G**, Cotyledonary stage of the developing embryo at week 5 on MM. Note the newly-initiated cotyledons (arrowheads). **H**, Cotyledonary stage of the developing embryo at week 6 on MM. Note the disintegration of the root cap. **I**, Cotyledonary stage of the developing embryo at week 8 on MM. Small root cap (double arrow) and signs of its disintegration are seen. **A**, **G**, **H** and **I**, Bar represents 500 μm; **B**–**F**, bar represents 200 μm.

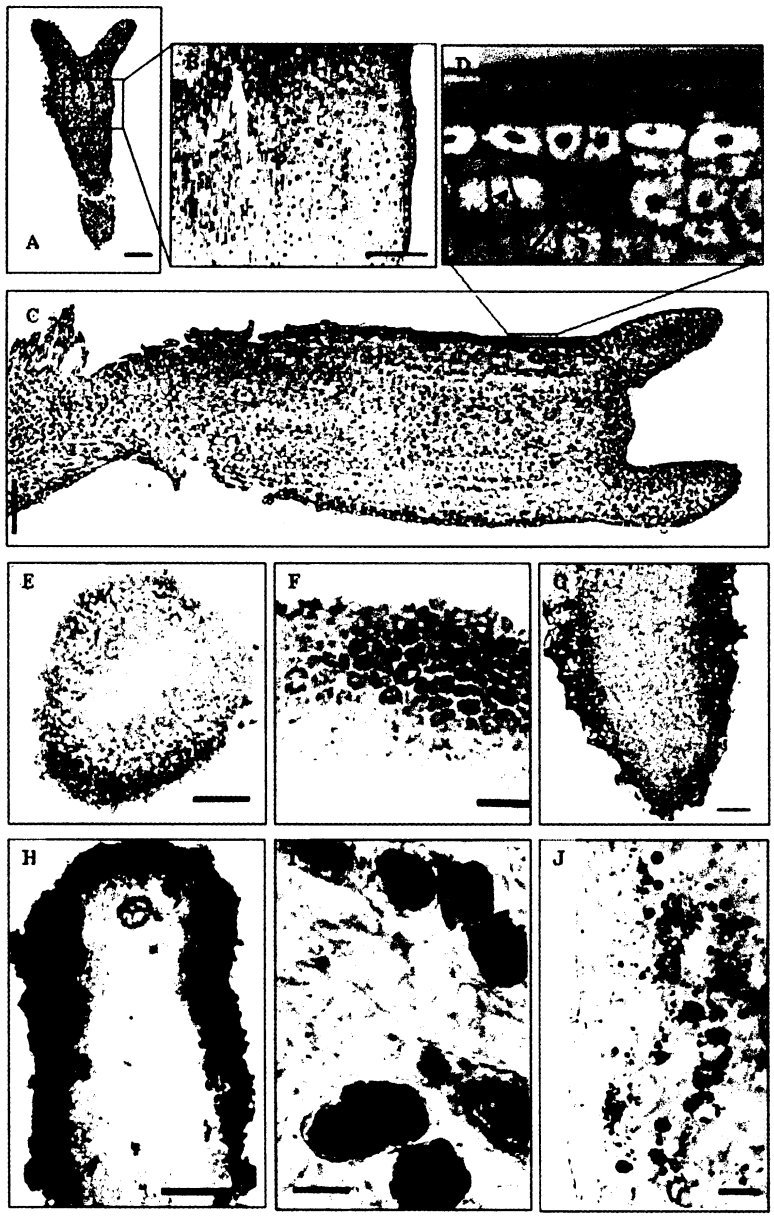

Figure 2. Developmental abnormalities and histochemical analysis. If not otherwise specified: longitudinal sections, staining – alcian blue and nuclear fast red; type of microscopy – bright field. **A**, **C**, Cotyledonary stage of the somatic embryo at week 7 of cultivation on MM + 7.5 % PEG. **A**, Note the presence of a rupture in the central part of the embryo. **B**, Rupture in the central part of the embryo (detail of **A**). **C**, Note the damage of the root cap. **D**, Rows of dead cells in the primary cortex of the hypocotyl (detail of **C**). For visualization of starch grains in cortex region (bright white grains), polarized light was used. Note the presence of dead cells with (thin arrow) or without (thick arrow) starch grains and big idioblast (star) below the epidermis. **E**, Callusing embryo at week 5 on MM. Note starch accumulation (purple-to-black grains) below the dedifferentiating surface layer. Transverse section, Lugol solution. **F**, Surface of the callusing embryo (detail of **E**). Note orange-to-brownish coloring of non-specific reaction for phenolic compounds. Transverse section, Lugol solution. **G**, Non-specific staining of phenolic substances with intracellular location in the surface of the callusing embryo. **H**, Histochemical detection on condensed tannins (red coloring) in the surface of the callusing embryo. Vanillin + HCl. **I**, Subcellular vacuolar deposition of condensed tannins in cells on the surface of the callusing embryo (detail of **H**). **J**, Subcellular deposition of lipidic substances (red-colored droplets) in the primary cortex of hypocotyl at week 6 on MM. Note that the epidermis is devoid of lipidic bodies. Sudan 7B. **A** and **C**, Bar represents 500 μm; **B**, **E** and **G**, bar represents 200 μm; **F** and **H**, bar represents 100 μm; **D**, bar represents 50 μm; **I** and **J**, bar represents 10 μm.

Table I. Timing of the developmental stages during somatic embryogenesis as affected by PEG. Treatments: MM, maturation medium; MM + 3.75 % PEG, maturation medium with 3.75 % PEG; MM + 7.5 % PEG, maturation medium with 7.5 % PEG. ESE, Stage of early somatic embryo; Cy, cylindrical stage; Pc, precotyledonary stage; Co, cotyledonary stage (see *figure 4*).

Weeks of cultivation	Stages of development		
	MM	MM + 3.75 % PEG	MM + 7.5 % PEG
1	ESE	(ESE) Cy	ESE
2	(ESE) Cy	Cy (Pc)	Cy
3	Cy (Pc)	(Pc) Co	Pc
4	Pc	Co	Pc, Co
5	Co	Co	Co
6	Co	Co	Co
7	Co	Co	Co
8	Co		

supplemented with PEG (MM + 3.75 % PEG; MM + 7.5 % PEG) was observed in the timing of developmental stages (*table I*). The most striking differences between MM and MM + 3.75 % PEG treatments occurred between weeks 1–4 of cultivation expressed as a speeding-up of maturation. The 3.75 % PEG treatment caused the establishment of the cylindrical stage to already occur in week 1 (*figure 3 A*) which was not observed for the PEG-free embryos till week 3 of cultivation. In week 2, the embryos started to exhibit distinct strains of columella-cells in the forming root cap (*figure 3 B*); the precotyledonary stage with differentiating procambial strains was rarely recorded. Three-week-old embryos (*figure 3 C*) showed proceeding elongation and further inner differentiation typical for the cotyledonary stage. Thus, the first appearance of cotyledons and formation of the apical dome were found at this stage of development, whilst in the case of PEG-free treatment, they were achieved two weeks later. Further steps in embryo maturation in the presence of 3.75 % PEG are shown in *figure 3 D–F*. The embryos were fully developed by week 5 of cultivation.

The variant grown on MM + 7.5 % PEG showed a timing of morphogenic processes similar to the variant grown on MM (PEG-free). No cotyledons were present at week 3 of cultivation; embryos only exhibited the precotyledonary stage of development (*figure 3 G*). The cotyledonary stage was first recorded at week 4 (*figure 3 H*), which corresponds to a 1-week speeding-up of the maturation process compared to the PEG-free treatment. *Figure 3 I* shows the 7.5 % PEG-treated embryo at week 6 which is fully developed without any sign of root cap disintegration.

Intercellular spaces were found to some extent in all embryos matured on MM + 7.5 % PEG in week 7 (*figure 2 A–D*). There were two types of ruptures observed: less frequent large ones found in the center of the embryo (*figure 2 A, B*); and more frequent smaller ones found as rows of dead cells in the cortex of the embryos (*figure 2 C, D*). The latter dead cells were with or without starch. The process of rupture-formation could be observed since week 5 of cultivation in this treatment. After examination of fifteen embryos matured on MM + 3.75 % PEG, only two embryos (i.e. approx. 13 %) with smaller intercellular spaces (spots or short rows of dead cells) were found and the spaces appeared at a lower frequency and were smaller. On PEG-free medium, neither ruptures nor rows of dead cells were observed.

2.3. Quantitative anatomy of the maturation process

To quantify both size and shape changes during the embryo maturation, we measured selected length characteristics: the length and the width of the meristematic part of the embryo (all developmental stages) and the length of the root cap (the precotyledonary and cotyledonary stages); both are shown in detail in *figure 4*.

The embryo development in all three treatments was characterized by the elongation of the length axis (*figure 5 A*). Almost no elongation was found later than week 6 (data shown only until week 7). The embryos growing in the presence of 3.75 % PEG were generally significantly larger than embryos from the PEG-free treatment which exhibited the lowest length increment from all three treatments since week 3. The speeding-up of the developmental process in the 3.75 % PEG treated variant, observed at a structural level as the appearance of the cotyledonary stage in week 3, corresponded to more than a doubled length increment when compared with the length measured at week 2. The embryos older than week 3 growing in the presence of 7.5 % PEG had lengths comparable to that of the 3.75 % PEG-treated embryos.

The development of the shape of the somatic embryos is partly characterized in *figure 5 B*, which shows the length to width ratio of the meristematic part of the embryos. The value of this ratio oscillated around 1 during the first two weeks of maturation for all treatments, indicating more or less the round shape of embryos. This pattern changed by week 3, when the PEG-free embryos kept their round shape whilst both PEG-treated variants elongated. While the PEG-free and the 3.75 % PEG-treated embryos exhibited

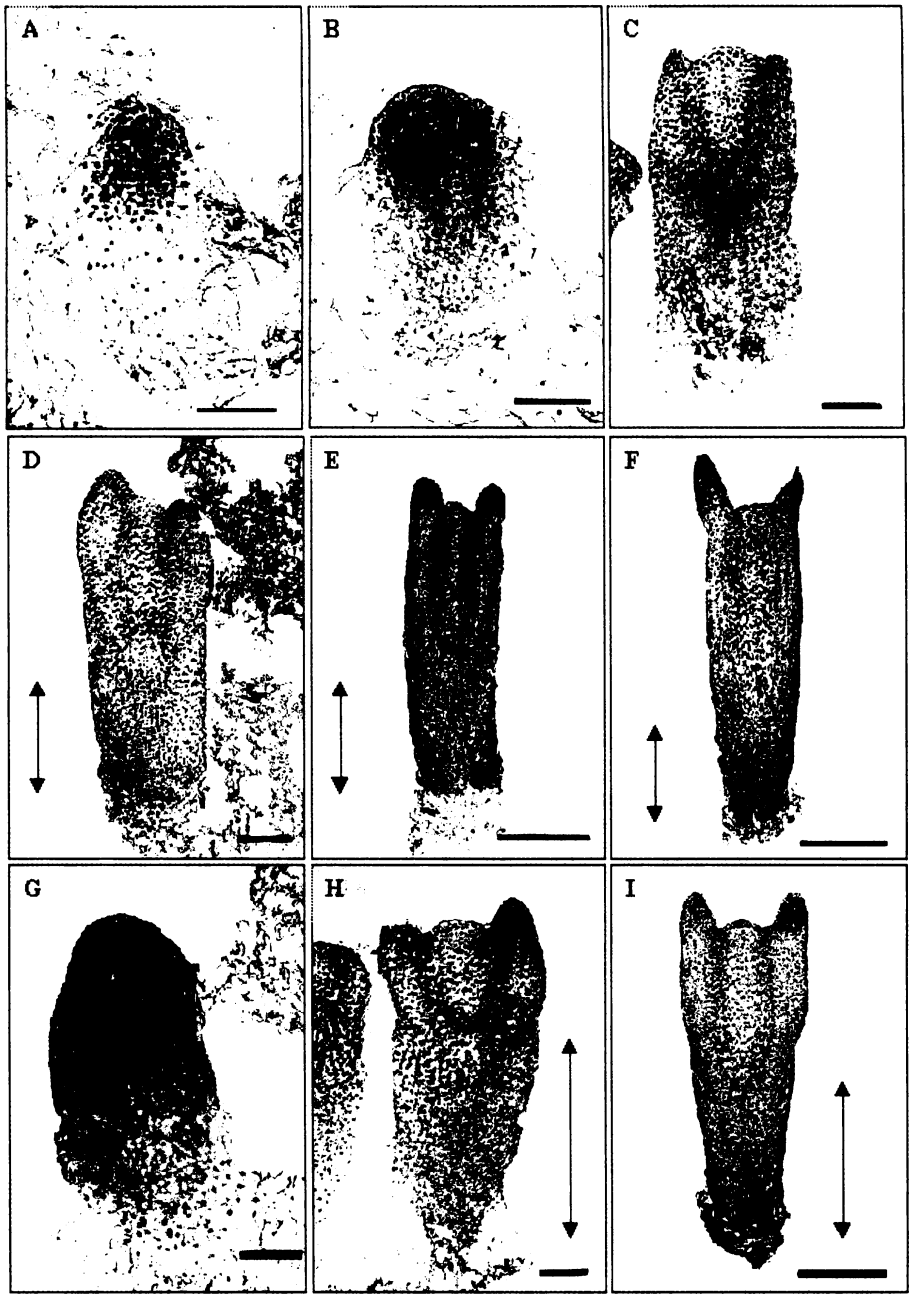

Figure 3. Development of somatic embryos in the presence of PEG. Longitudinal sections, staining – alcian blue and nuclear fast red; type of microscopy: bright field. **A**, Cylindrical stage of the developing embryo at week 1 of cultivation on maturation medium containing 3.75 % PEG (MM + 3.75 % PEG). **B**, Transition from the cylindrical stage to the precotyledonary stage of the developing embryo at week 2 of cultivation on MM + 3.75 % PEG. Note the formation of the columella in the root cap. Cotyledonary stage of the developing embryo at week 3 (**C**), 4 (**D**), 5 (**E**), 6 (**F**) of cultivation on MM + 3.75 % PEG. **C**, Note newly-initiated cotyledons. **D**, **E**, **F**, Proportionally longer root cap (double arrow), compare with *figure 1 1*. **G**, Precotyledonary stage of the developing embryo at week 3 of cultivation on MM + 7.5 % PEG. **H**, Cotyledonary stage of the developing embryo at week 4 of cultivation on MM + 7.5 % PEG. Note newly-initiated cotyledons. Proportionally longer root cap (double arrow), compare with *figure 1 1* and **D**. **I**, Cotyledonary stage of the developing embryo at week 6 of cultivation on MM + 7.5 % PEG. Proportionally longer root cap (double arrow), compare with *figure 1 1* and **F**. **A–D**, **G–I**, bar represents 200 μm, **E** and **F**, bar represents 500 μm.

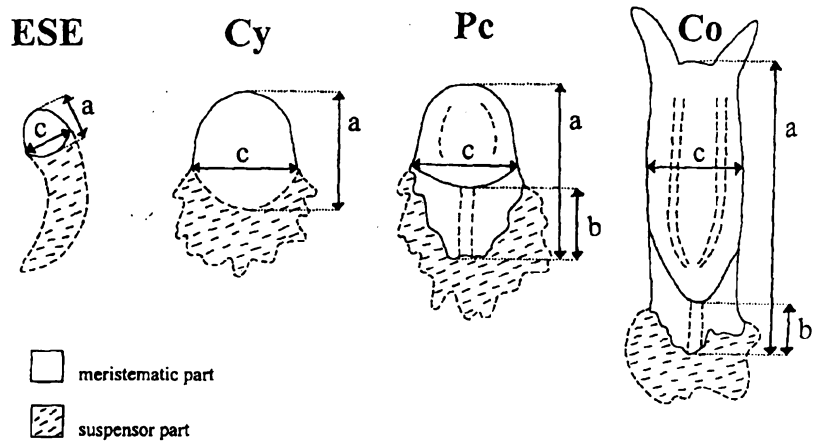

Figure 4. Length characteristics used for the quantitative description of embryo development. ESE, Stage of early somatic embryo; Cy, cylindrical stage; Pc, precotyledonary stage; Co, cotyledonary stage. a, Length of the meristematic part of an embryo; b, length of the root cap; c, width of the meristematic part of an embryo.

gradual change of their shape, the 7.5 % PEG-treated embryos underwent a dramatic shape change between weeks 2 and 3.

Figure 5 C compares the length of the root cap in developing embryos. The presence of PEG in a medium generally induced a significant elongation of the root cap. The length of the root cap represented 40–55 % of the total length of mature embryos in the MM + 7.5 % PEG treatment.

2.4. Histochemistry of the maturation process

The double staining procedure used for anatomical developmental study allowed the observation of substances, which often filled the vacuoles of cells (e.g. *figure 1 E*, the intensive blue color of necrotic cells of former suspensor cells). Also during the histochemical location of starch, we often found, in abnormal embryos, cells filled with substances which were non-specifically colored (brownish, *figure 2 E, F*). Such substances were found relatively often in surface layers of embryos especially during later stages of embryogenesis, when we observed the damaged embryos with altered morphology (*figure 2 E–G*). In conifers, products of secondary metabolism are common, so we hypothesized that disintegration of mainly surface layers of developing embryos, the so-called 'callusing' but also aging of the culture seemed to be connected with phenolics accumulation. After the application of the specific staining for phenolic compounds (Hoepfner-Vorsatz test and vanillin + HCl; both tests gave identical results), we confirmed phenolic compound accumulation in the cases previously observed. In both tests, condensed tannins (leucoantocyanins and catechins) were localized as a bright red-colored substance in cells of a root cap surface and in case of so-called 'callusing' also in other parts of the embryo (*figure 2 H*). The subcellular compartmentation of condensed tannins was in vacuolar deposits (*figure 2 I*). In regularly developing embryos, we did not find differences in location of phenolics for all three variants tested.

During histochemical location of starch, we observed granular or droplet structures in embryos which did not give positive reaction with IIK. After applying the histochemical test specific for lipidic substances, we identified a part of these structures as lipidic droplets. The nature of lipid accumulation is very unstable. Generally, only occasional lipid droplets could be found within early embryos of the embryogenic tissue. The lipid accumulation seemed to begin approximately three weeks after transfer onto maturation medium. The pattern of the lipid accumulation closely followed (both in time and place) the pattern of starch deposition in the suspensor end and the cortical region (*figure 2 J*) of the embryo, which contained the highest amount of lipidic bodies. The lipidic droplets were localized within the cytoplasm. The amount of lipidic bodies varied in the embryos. Some embryos,

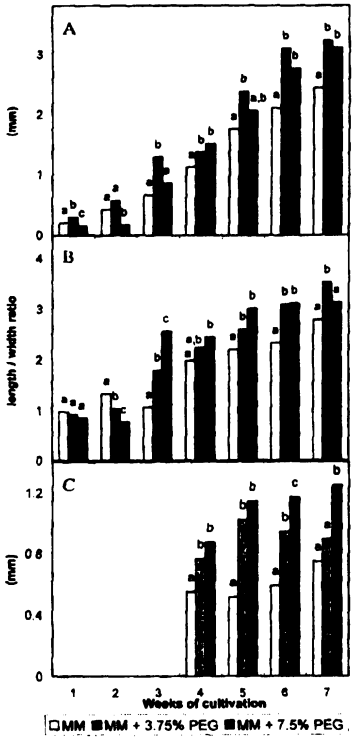

Figure 5. Length characteristics of maturating embryos. **A**, Total length; **B**, length to width ratio; **C**, length of the root cap during weeks 4–7. Treatments: MM, maturation medium; MM + 3.75 % PEG, maturation medium with 3.75 % PEG; MM + 7.5 % PEG, maturation medium with 7.5 % PEG. Data with common letters are not statistically different by Fisher's LSD Multiple-Comparison Test or Kruskal-Wallis Multiple-Comparison Z-Value Test (if data were not distributed normally); n = 8–15.

especially in the cotyledon region, were extremely abundant in lipids whereas others had little or almost none.

In all cases, the occurrence of callusing embryos coincided with high amounts of starch grains, especially in damaged parts of the embryo, i.e. parts where the integrity of the surface was affected (*figure 2 E, F*). This could coincide with the cessation of the morphogenic activity of a tissue.

In order to detect possible lignification, a specific histochemical test for lignin was applied. We did not find positive reaction for lignin at any stage of embryo development during our experiment.

2.5. The effect of PEG on the germination process

A preliminary germination experiment was carried out to reveal the ability of mature somatic embryos

Table II. Germination frequency of embryos as affected by maturation on different maturation media. Treatments: MM, maturation medium; MM + 3.75 % PEG, maturation medium with 3.75 % PEG; MM + 7.5 % PEG, maturation medium with 7.5 % PEG. The data represent the average germination frequency counted independently for eight Petri dishes per variant, every dish contained approx. 15–20 embryos. Germination was recorded after three weeks of germination at 24 °C in darkness.

Variant	Germination frequency (%)	Standard error
MM	93.5	1.7
MM + 3.75 % PEG	94.6	1.5
MM + 7.5 % PEG	75.2	3.8

matured on PEG-free (MM) and both 3.75 and 7.5 % PEG-containing media (*table II*) to germinate. The highest germination percentage (94.6 %) was achieved for embryos maturing for six weeks on MM + 3.75 % PEG. Approximately the same germination percentage (93.5 %) was achieved for the MM variant (PEG-free). The significantly lowest germination frequency (75.2 %) was achieved for the MM + 7.5 % variant.

3. DISCUSSION

The present structural and histochemical studies aim at characterizing the time sequence of morphogenic events in order to provide a basis for further experimentation for studying the effects of changes in the cultivation procedure. Therefore, in order to reach a quite clear and simple description of the process, we preferred to give a detailed characterization of embryo development for each week of cultivation. The embryo development we observed was similar to that seen by other authors with different conifer species [12, 15, 20]. Since from a structural point of view, the embryos of the MM treatment were fully developed and mature at week 6 and prolonged cultivation caused no further progress in embryo structure, the question rises as to when to stop the cultivation on maturation media.

Moreover, the last two weeks of embryo cultivation were characterized by a higher frequency of embryo development irregularities. This was especially true when embryos were irritated by contact with a medium or with another embryo or if they were mechanically damaged during the subcultivation procedure. It is likely that these irregularities are fatal for successful conversion to plantlets. Whatever the particular reasons, there are a number of papers dealing with phenolics accumulation in conifers exposed to a variety of stresses [12, 17].

In our study, we described the increase in the rate of the maturation process from the addition of 3.75 % PEG into the maturation medium. Joy et al. [20] and Hakman and von Arnold [15] found a four-week cultivation sufficient for white spruce embryos to reach the mature cotyledonary stage irrespective of PEG treatment. In our study, the maturation process until the cotyledonary stage, which lasted five weeks in the MM treatment, lasted 3–4 weeks in the case of PEG-treated embryos. The hastening of the process described at an anatomical level corresponded also to physiological traits. In simultaneous study on the same material, we recorded [31] the same hastening of maximum endogenous levels of IAA in maturating embryos corresponding to cotyledon establishment. Especially in the latest weeks of cultivation (week 6 and 7), we often noticed the progressive disintegration of the root cap (*figure I H, I*) which may result in a poor protection of the root meristem during radicle protrusion. This shortcoming existed especially in the PEG-free variant where the proportion of the root cap to the whole embryo length was lower. Problems with poor root development in *P. glauca* (white spruce) were also described by Hakman and von Arnold [15] who proposed that the lack of a properly developed root pole was later responsible for the slow growth of the regenerated plantlets. The difficulties with root pole establishment seem to be more serious for *Picea* species in comparison with other conifer genera. Similar problems were not mentioned in papers dealing with larch or fir somatic embryo morphogenesis [12, 26].

At present, there is no doubt that the presence of osmoticum in the medium is a very important factor needed to support efficient somatic embryo development. The observed stimulation of maturation by the use of PEG as osmoticum is consistent with the reported results from the maturation of several conifer species, e.g. white spruce [1, 22] and Norway spruce [8]. Attree et al. [4] observed that PEG positively affected the germination of somatic embryos of white spruce. On the other hand, some authors emphasized the very low germination frequency of embryos previously matured on media containing PEG [22]. For example, on medium containing 5 % PEG, Find [8] found an increased maturation frequency but also a resulting negatively-influenced germination of embryos. Find [8] then related the low germination frequency of the PEG-treated embryos to morphological traits such as small embryo size and intercellular spaces below the apical meristem. However, he did not evaluate the size of the embryos by any quantification. Because of the major importance of germination in the whole process of somatic embryogenesis, we thus decided to evaluate the quantitative traits describing embryo size. The embryos growing in the presence of 3.75 and 7.5 % PEG were significantly longer than embryos from the PEG-free variant at all stages of the process studied. We recorded that the presence of PEG in the maturation medium affected not only the length of the embryo but also other morphological traits (length of root cap, ratio of length:width characteristics). The question remains, however, as to which quantitative parameters to use in order to estimate changes in embryo size. Although we are aware that the parameters used in our study are not the only that exist, in contrast to Find's findings, they definitely indicate, that the PEG-treated embryos were either the same size or larger than the PEG-free ones. As our 7.5 % PEG-treated embryos germinated with a lower frequency than the PEG-free embryos, we cannot, unlike Find, relate this problem to the small size of embryos. However, our preliminary results show that embryos previously grown in the presence of 3.75 % PEG germinate with approximately the same frequency as PEG-free embryos (*table II*). The lowest germination frequency of MM + 7.5 % PEG variant might be ascribed to the negative nature of ruptures and rows of dead cells in the embryo proper. However, it would be necessary to check whether the desiccation optimum is the same for all variants tested.

Regarding the macroscopically observed abnormalities, the frequency of embryo browning and callusing did not differ in the treatments. As was mentioned above, we suppose that subcultivation is the source of mechanical irritation which causes the higher frequency of browning and callusing. Thus, lowering the number of subcultivations needed to obtain mature embryos by adding PEG must therefore be advantageous.

Most authors used higher PEG concentrations, usually in the range of 5–10 % (w/v), and most frequently 7.5 % [1, 3, 4, 24]. Therefore, we tried to intensify the observed changes in the rate of the maturation process by increasing the PEG concentration to 7.5 %. Unfortunately, the speed of maturation did not increase and, in contrast to the findings of Find [8], the embryo length was significantly higher in comparison to that of PEG-free embryos. The only possible positive effect of 7.5 % PEG is the increase in the length of the root cap by approx. 40 % in comparison with MM + 3.75 % PEG treated embryos (week 7). We have already mentioned that this trait might be important for reaching a higher frequency of properlygerminating

embryos. On the other hand, the negative effect observed was the appearance of ruptures and rows of dead cells. As these abnormalities are ubiquitously disadvantageous for subsequent embryo development, we can hardly expect a compensation of these negative changes by the positive effect of the root cap enlargement.

Our study verified the necessity of the presence of osmotic stress induced by non-penetrating osmoticum for successful embryo maturation. On the other hand, our results indicate that the optimum concentration of non-penetrating osmoticum in the medium is genotype-specific and probably lower than the frequently recommended 7–8 % PEG.

4. METHODS

4.1. Plant material and cultivation protocol

The embryogenic culture of *Picea abies* (L.) Karst., genotype AFO 541, was a generous gift from Dr Bercetche (AFOCEL, France). The cultures were grown on media according to Gupta and Durzan [10], solidified with 0.75 % (w/v) agar (Sigma), and pH was adjusted to 5.8 ± 0.05 prior to autoclaving. Proliferation (maintenance) medium was supplemented with 5 μM 2,4-D, 2 μM kinetin, 2 μM BAP (all Sigma), and 30 $g \cdot L^{-1}$ sucrose (Lachema, CZ). Under weekly subcultivation, the culture can be maintained for several years without a loss of regeneration capacity. The medium for development and maturation of somatic embryos (maturation medium – MM) lacked auxin and cytokinins, whilst 20 μM ABA (Sigma) was added to trigger the development of embryos. To induce osmotic stress, the maturation medium was supplemented either with 3.75 or 7.5 % (w/v) polyethylene glycol (PEG-4000). The PEG solution was autoclaved separately and mixed with the remaining part of the medium after autoclaving. The embryogenic cultures were maintained by weekly subculturing to fresh media in Magenta vessels (Sigma) or Drigalski plates (diameter 100 mm and height 35 mm), containing 40 mL of a medium. Cultures were kept in darkness at 24 ± 1 °C. The experiment was repeated twice. The embryogenic cultures were sampled according to the time elapsed after the transfer on the maturation medium. Samples for all analyses were taken between the 5th and 6th day of a 1-week subcultivation period. Embryogenic mass or embryos isolated later were collected from randomly chosen 1–2 vessels. The selection was then governed by the requirement to cover the representative culture state for the sampling day. Embryos were dissected from remaining mass starting at week 5 (in case of PEG-treated embryos at week 4) when cotyledons appeared.

4.2. Anatomical analysis

For light microscopy, paraffin sections of embryogenic cultures and somatic embryos were prepared essentially according to Johansen [19]. Briefly, samples were fixed with 50 % FAA (formaldehyde/acetic acid/ethanol/water, 1/1/9/9, v/v/v/v) for at least 24 h. After washing with 50 % ethanol, the samples were dehydrated gradually in the ethanol-butanol series, infiltrated with paraffin. Longitudinal sections were cut on a Leitz microtome; section thickness was 12 μm. Sections were stained by a two-step staining procedure using alcian blue and nuclear fast red [27]. Observation and measurements of selected quantitative length characteristics (*figure 4*) were made using a binocular microscope with a calibrated ocular ruler. The anatomical examinations were repeated on 10–15 embryos per each sampling and treatment. The obtained data were checked for normal distribution. Then data were analyzed by analysis of variance (ANOVA) and Fisher's LSD Multiple-Comparison Test (normally distributed data) or Kruskal-Wallis Multiple-Comparison Z-Value Test (if data were not distributed normally). The embryogenic tissue was also studied according to the classification of Jalonen and von Arnold [18]. Embryogenic cell-lines grown on solid medium could be divided into groups A and B (according to embryo morphology and growth habit). Cell lines belonging to group A generally consist of embryos with comparatively large compact meristematic heads attached to the typical filamentous suspensors. Cell lines belonging to group B consist of somatic embryos comprised of only a few loosely aggregated cells in their embryogenic regions.

4.3. Histochemical analysis

For starch grain location, paraffin sections were used (see above). The sections were stained using IIK (Lugol solution) [19] and verification of the staining specificity was done using polarized light. Fresh material (non-fixed) was used for the following histochemical tests (free hand sections): lipidic bodies were localized using Sudan Red 7B staining [5]; lignin was identified by staining with phloroglucinol according to the method of McKenzie and Peterson [23]. Two tests were employed to detect condensed tannins (phenolics): the Hoepfner-Vorsatz test [28] and vanillin in HCl [9].

4.4. Germination experiment

Prior to germination, somatic embryos after six weeks of maturation (120–150 embryos per variant harvested from eight Magenta vessels) were isolated and placed in a sterile dish and desiccated for three weeks in darkness at 24 °C and a relative humidity of 95 %. Then the embryos were germinated on the medium according to Gupta and Durzan [10], without growth regulators, containing 1.5 % sucrose, 0.5 % active charcoal and solidified with 0.75 % agar (Sigma). The embryos with a radicle were considered germinated and were counted after three weeks of germination. One preliminary experiment was carried out.

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CARBOHYDRATE STATUS DURING SOMATIC EMBRYO MATURATION IN NORWAY SPRUCE

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SUMMARY

The development of Norway spruce (*Picea abies* (L.) Karst.) somatic embryos on a maturation medium was accompanied by changes in nonstructural carbohydrate status. During embryo maturation, the content of total soluble sugars in the embryonal suspensor mass decreased and the partitioning between sucrose and hexoses changed considerably in favor of sucrose. Developing somatic embryos were mainly responsible for these changes. Osmotic stress caused by the presence of 3.75% polyethylene glycol (PEG) in the maturation medium (decrease in osmotic potential by 52.5 kPa) resulted in dramatic changes in the content of endogenous saccharides. There was a lower total carbohydrate content in the embryonal suspensor mass grown on the medium containing PEG in comparison with the untreated control. Isolated embryos from later stages of embryo development contained mainly sucrose with a small amount (20%) of fructose and nearly no glucose. A further increase in PEG concentration in the medium (7.5%; decrease in osmotic potential by 112.5 kPa compared to the maturation medium) led to a large increase in the total endogenous sugar content. This increase in sugars was a result of the enhanced content of sucrose, fructose, and glucose. The increased glucose content was in contrast to embryos grown on the medium with lower or no PEG content.

Key words: conifers; embryo development; osmotic stress; *Picea abies*; polyethylene glycol; starch; sugars.

INTRODUCTION

Our understanding of the stimuli and conditions necessary for the induction and control of conifer somatic embryo (SE) development is limited; however some factors are known to play important roles. For example, the majority of conifer embryogenic cultures require abscisic acid (ABA) for proper maturation (Durzan and Gupta, 1987; Hakman and von Arnold, 1988; Becwar et al., 1990). There is no doubt that carbohydrate supply is also an important factor. Some authors (Carrier et al., 1997; Johnson et al., 1997) propose that carbohydrates might play multiple roles including the provision of a source of carbon and energy, causing an osmotic effect and also influencing the embryo maturation process by direct regulation of gene expression. The information available, however, varies greatly depending on the genotype used for the experiments. Although adequate reserves of lipids and proteins, and also carbohydrates, are important for proper embryo development (Attree et al., 1992), there is surprisingly little information about changes in carbohydrate status during SE maturation.

There is a considerable amount of data available on the effect of different exogenous carbohydrate supplies on SE development. A low concentration of sucrose in the maturation medium has been shown to reduce SE development (Jain et al., 1988, *Picea abies*; Finer et al., 1989, *Pinus strobus*). For *Picea mariana* and *Picea rubens*, the number of SE increased with increasing sucrose concentration to a maximum at 6% sucrose. A further increase to 12% sucrose had no promotive effect on embryo formation (Tremblay and Tremblay, 1991). Similar results were reported for *Picea glauca* (Lu and Thorpe, 1987; Hakman and von Arnold, 1988); *P. abies* (von Arnold and Hakman, 1988; Jain et al., 1988) and *P. strobus* (Finer et al., 1989). These results indicate that the effect of carbohydrates on maturation was at least partly osmotic.

Carbohydrates other than sucrose and glucose have been shown to support the growth of cultures to a similar extent (Verma and Dougall, 1977). Tremblay and Tremblay (1991) compared the effect of nine carbohydrates on the somatic embryogenesis of red and black spruce: sucrose, glucose, fructose, maltose and cellobiose were able to support embryo development, while arabinose, mannitol, myoinositol and sorbitol could not. For red spruce the 6% fructose level was found to be the best for SE development, while no such preference was observed for black spruce (Nørgaard, 1997). During embryogenesis, the carbohydrate requirements may change with the stage of the culture (Thompson and Thorpe, 1987). While sucrose is the preferred carbohydrate for both proliferation and embryo maturation in the majority of conifers, in *Abies alba* lactose was the only carbohydrate found to stimulate the formation of late-stage embryos (Schuller and Reuther, 1993). In the same species maltose proved to be superior to sucrose for embryo maturation. Maltose promoted the SE maturation in *Abies normandiana* and *Pinus taeda* (Nørgaard, 1997). Although the role of carbohydrate as a carbon and energy source is undoubtedly

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important, the osmotic effect of its presence in the media can not be neglected.

The maturation medium is often supplemented by an osmoticum to prevent precocious germination (e.g. Tremblay and Tremblay, 1991, 1995; Attree et al., 1995). Tremblay and Tremblay (1991) compared different sucrose concentrations, and sucrose combined with mannitol and sorbitol, in experiments with *P. mariana* and *P. rubens*. No significant difference was found in the number of embryos produced with 6% or with 3% sucrose with equimolar concentrations of either mannitol, sorbitol or myoinositol. Therefore the authors suggested that osmotic stress caused by the presence of carbohydrates in the medium is favorable for SE maturation.

The nature of the osmoticum added to the medium is an important factor influencing SE development. Attree et al. (1995) showed that only nonpenetrating osmotica are able to promote the maturation of spruce SE. The best results were obtained with polyethylene glycol 4000 (PEG 4000). Osmotica such as sugar alcohols cannot be considered ideal because of their fast uptake observed in cultures (Thompson and Thorpe, 1987), leading to osmotic recovery but no reduction in moisture content. Nonpenetrating osmotica, in contrast, restrict water uptake to provide a drought stress more similar to that observed in zygotic embryo development *in situ* (Attree et al., 1991, 1992). PEG treatment caused the moisture content to decrease by ~50% during white spruce SE maturation (Attree et al., 1995). The beneficial effect of PEG in a maturation medium was first reported in *P. glauca* (Attree et al., 1991), and it has been shown that different genotypes react in different ways (Nørgaard, 1997). For Norway spruce SE maturation, 3.75% PEG proved to be the most beneficial (Svobodová et al., 1999).

Although considerable data are available concerning the effects of exogenous saccharides and osmotica on the somatic embryogenic process, information is still lacking on the resulting levels of endogenous saccharide content during SE development. The aim of this study, therefore, was to determine the changes in carbohydrate status during Norway spruce SE maturation and to evaluate the effects of PEG treatments on this characteristic. The data were correlated with the findings of a study of embryo structure development performed on the same experimental material (Svobodová et al., 1999).

Materials and Methods

Plant material. Embryogenic culture of *Picea abies* L. (Karst.), genotype AFO 541, was a generous gift from Dr. Bercetche (AFOCEL, France). Cultures were grown on media according to Gupta and Durzan (1986), solidified with 0.75% (w/v) agar (Sigma), and adjusted to pH 5.8 ± 0.05 prior to autoclaving. Proliferation medium was supplemented with 5 μ*M* 2,4-dichlorophenoxyacetic acid, 2 μ*M* kinetin, 2 μ*M* benzylaminopurine (all Sigma), and 30 g l^{-1} sucrose (Lachema, Czech Republic).

Cultivation. Media for SE development and maturation (maturation medium) lacked auxins and cytokinins, and included 20 μ*M* ABA (Sigma) and 0, 3.75 or 7.5% (w/v) PEG 4000 (Sigma). PEG solution was autoclaved separately and mixed with the remaining medium after autoclaving. Embryogenic cultures were maintained by weekly subculturing (approximate fresh weight 1.5 g) to fresh media in Magenta vessels (Sigma) or Drigalski plates (Kavalier, Czech Republic; diameter 90 mm, height 35 mm) containing 40 ml medium. Cultures were kept in the dark at 24 ± 1°C.

Sampling of material. Embryogenic cultures were sampled according to the time elapsed following transfer to the maturation medium. In order to eliminate the effects of fluctuation in endogenous levels of different compounds during subcultivation, cultures were sampled between 5 and 7 d after subculture.

Nonstructural saccharides (NSS) content determination. Samples of whole embryonal suspensor mass (ESM) (control, weeks 1–4) and samples containing excised embryos (5–15 embryos per sample, depending on embryo developmental stage, to reach ~60 mg FW, weeks 5–8) were freeze-dried and dry weights were determined. The dried material was homogenized by shaking with glass beads (3 mm diameter) and boiled with 80% methanol (0.5 ml) at 75°C for 10 min. The solvent was evaporated and the residue was dissolved in double-distilled water in an ultrasonic bath for 10 min. The samples prepared for sugar determination were stored after filtration using 0.45 μm membrane filters (Whatman) at −18°C (Lipavská and Vreugdenhil, 1996). The content of extracted soluble NSS was detected using high-pressure liquid chromatography (HPLC) with refractometric detection (Spectra Physics; refractometer Shodex RI-71; integrator ChromJet; pre-column Hema-Bio 1000 Q + SB, Watrex, Czech Republic; column Hi Plex Ca^{2+}, Polymer Laboratories, U.K.; eluent ultrapure water MilliQ, Millipore; isocratic pump, Spectra Physics; flow rate: 0.5 ml min^{-1}, temperature 80°C; volume of injected sample 10 μl).

Starch content determination. The same types of samples were taken as for soluble NSS content determination. Samples frozen in liquid nitrogen were freeze-dried and dry weight was determined. The material was homogenized by shaking with glass beads (3 mm diameter) and boiled with 80% methanol (0.5 ml) at 75°C for 10 min. The methanol was evaporated and the residue was washed in double-distilled water in an ultrasonic bath for 10 min. Samples were centrifuged, the pellet was washed once more in the same way, then the water was evaporated. After addition of 0.5 ml of 0.1 *M* acetate buffer, pH 4.5, pellets were boiled for 10 min. Samples were incubated at 55°C for 4 h with 60 IU of amyloglucosidase (Rhizopus, Sigma), vacuum-dried, and solids resuspended in 0.5 ml double-distilled water in an ultrasonic bath for 10 min. The samples prepared for glucose content determination were stored after filtration using 0.45 μm membrane filters (Whatman) at −18°C. The extracted glucose content was detected using HPLC as above.

Starch localization. Paraffin sections of embryogenic cultures and somatic embryos were prepared essentially according to Johansen (1940) for starch grain location. Briefly, samples were fixed with 50% FAA (formaldehyde/acetic acid/ethanol/water 1/1/9/9, v/v/v/v) for at least 24 h. After washing with 50% ethanol, samples were dehydrated gradually in an ethanol–butanol series infiltrated with paraffin. Longitudinal sections (12 μm) were cut on a Leitz microtome. Sections were stained using I_2/KI solution (Lugol solution; Johansen, 1940) and verification of the staining specificity was carried out using polarized light.

Experimental design and statistics. For NSS content determination, each week 5–15 samples of whole ESM (control, weeks 1–4) and three to five samples containing excised embryos (5–15 embryos per sample, depending on the embryo developmental stage) were collected for every experimental variant. For starch histochemical detection, each week 8–10 specimens of whole ESM (control, weeks 1–4) and five excised embryos were evaluated. Figure legends contain information about the number of samples collected. The data obtained were checked for normal distribution, and data were analyzed by ANOVA and by Fisher's LSD multiple-comparison test (for normally distributed data) or the Kruskal–Wallis multiple-comparison *z*-value test (if data were not distributed normally). Differences were examined at $P \leq 0.05$ and $P \leq 0.01$ levels.

Results

Endogenous nonstructural carbohydrate status. To determine the changes in carbohydrate status during SE development on the maturation medium containing ABA, we measured the contents of soluble saccharides in the ESM (Fig. 1) and in later developmental stages (weeks 5, 6, 7, 8 after transfer to maturation medium) of isolated embryos (Fig. 3).

Total soluble saccharide content in the whole ESM increased significantly (Fig. 1) during the first week after transfer from proliferation to maturation medium, and a gradual decrease was observed as embryo development progressed. Decreasing contents

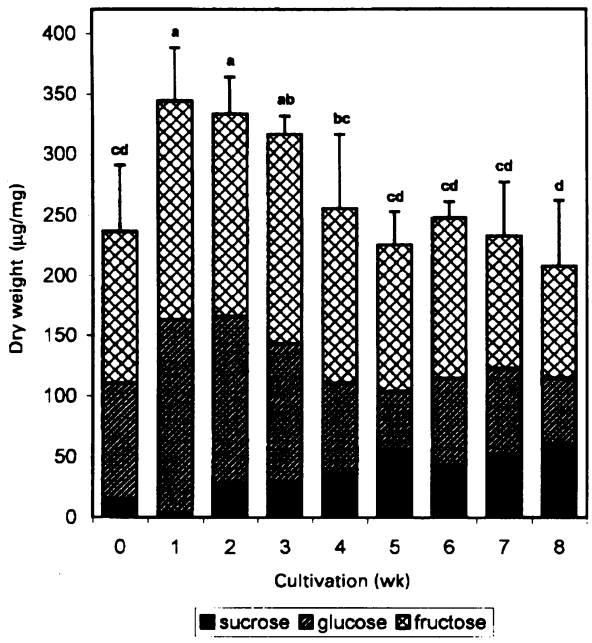

FIG. 1. Content of endogenous sugars in embryonal suspensor mass grown on the maturation medium. *Bars* indicate standard deviation (n = 5–15). Data on the content of total soluble saccharides for particular sampling dates were evaluated by ANOVA (factor: time). Data with common letters are not statistically different ($P \leq 0.05$) by the Kruskal–Wallis multiple-comparison z-value test (non-normal distribution).

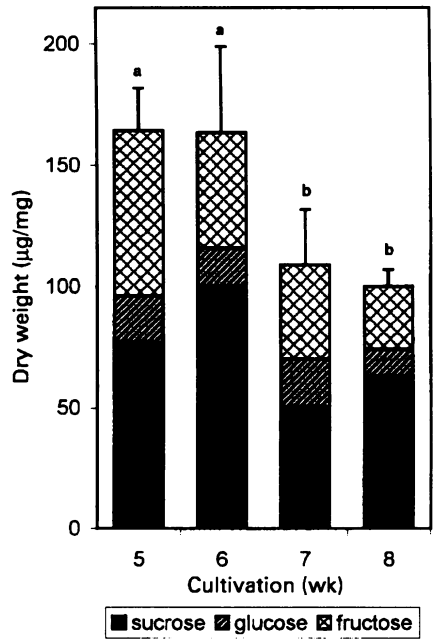

FIG. 3. Content of endogenous sugars in isolated embryos grown on the maturation medium. *Bars* indicate standard deviation (n = 3–5; each sample consisted of 5–15 embryos). Data of the content of total soluble saccharides for particular sampling dates were evaluated by ANOVA (factor: time). Data with common letters are not statistically different ($P \leq 0.05$) by the Kruskal–Wallis multiple-comparison z-value test (non-normal distribution).

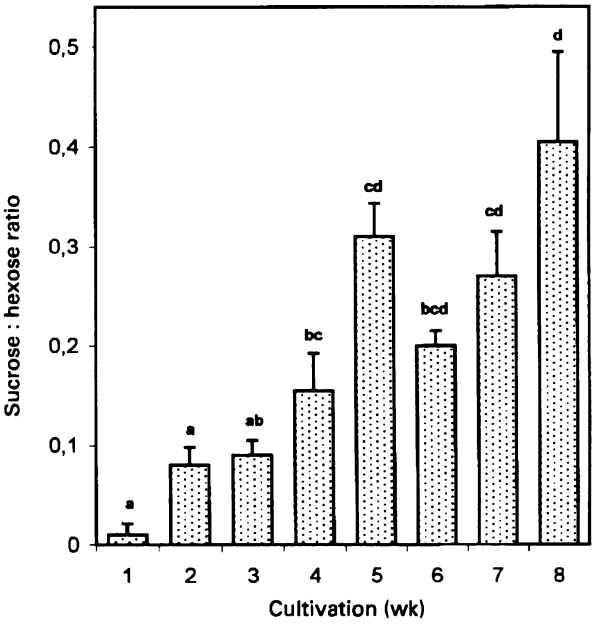

FIG. 2. Sucrose : hexose content ratio in embryonal suspensor mass cultivated on the maturation medium. *Bars* indicate standard deviation (n = 5–15). Data for particular sampling dates were evaluated by ANOVA (factor: time). Data with common letters are not statistically different ($P \leq 0.05$) by the Kruskal–Wallis multiple-comparison z-value test (non-normal distribution).

of glucose and, to a lesser extent, fructose were mainly responsible for the decline. The opposite trend was observed for sucrose content. There was almost no detectable sucrose in the samples from ESM 1 wk after transfer to maturation medium. Further SE development was characterized by the accumulation of sucrose so that its content at week 8 reached the values found for glucose; fructose content remained slightly higher at that stage of embryo development. The time course of the sucrose : hexose ratio indicates a significant change in the distribution between sucrose and hexoses (glucose plus fructose) during embryo maturation (Fig. 2).

Separate analyses of isolated embryos and the remaining mass revealed that changes in embryo saccharide content contributed a great deal to changes in the ESM analyzed as a whole (Fig. 3). There was a significant decrease in total saccharide content. The embryos contained comparable amounts of sucrose and fructose, while the glucose content was much lower at week 5. Statistical evaluation showed that the total saccharide content in isolated embryos was significantly ($P \leq 0.05$) lower compared to ESM analyzed as a whole. A further decrease in total saccharide content was observed in the embryos during the last 2 wk of development. At that time, isolated embryos contained mainly sucrose and a lesser amount of fructose. The sucrose : hexose ratio was found to be significantly shifted in favor of sucrose compared to the whole embryonic mass ($P \leq 0.05$). Glucose content represented less than 10% of total saccharide content.

Figure 4 shows data for starch content in ESM cultivated on proliferation medium compared to values for isolated embryos in weeks 5–8, and average values of starch content in the mass

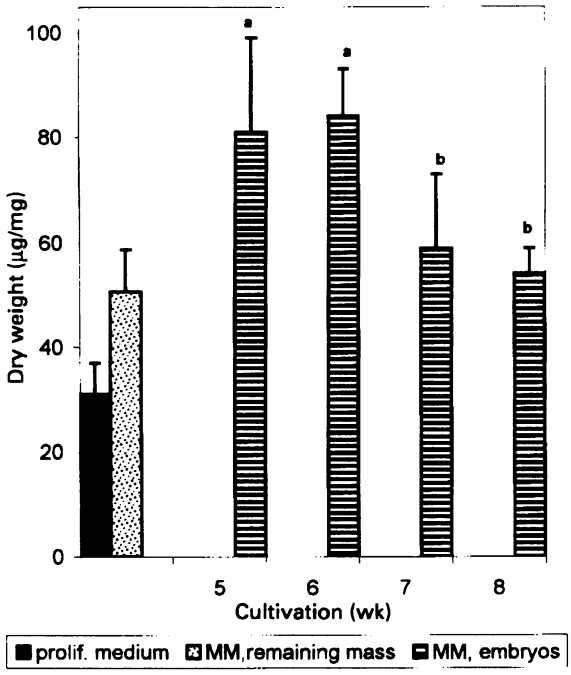

FIG. 4. Starch content in the embryonal suspensor mass grown on the proliferation medium ($n = 15$), content in isolated embryos (MM, embryos; $n = 5$; each sample consisted of 5–15 embryos) and average value of content in mass remaining after embryo isolation (MM, remaining mass; $n = 5$). MM, maturation medium. *Bars* indicate SD. Data for sampling dates 5–8 were evaluated by ANOVA (factor: time). Data with common letters are not statistically different ($P \leq 0.05$) by Fisher's LSD multiple-comparison test (normal distribution).

remaining after embryo isolation. Starch content increased in isolated embryos and was 33% higher compared to the remaining mass. The last 2 wk of cultivation were characterized by a significant decrease ($P \leq 0.05$) in starch content in isolated embryos. These findings formed the basis for the histochemical localization of starch in developing embryos (Fig. 5).

Generally, only a low number of starch grains were present within vacuolated suspensor cells of ESM. Meristematic, densely cytoplasmic cells of early SE were devoid of starch grains (Fig. 5A). It was rare to find isolated cells in the remaining mass, which contained many large starch grains. When ESM was transferred onto maturation medium, slow accumulation of starch started 2 wk later (Fig. 5B and C). Cells at the suspensor end of embryos were rich in starch grains, but cells at the apical pole of embryos contained fewer starch grains. The greatest accumulation of large starch grains was within the amyloplasts in cells of the root cap region between weeks 3 and 8. Figs. 5F and 5H show the root cap region in weeks 7 and 8, respectively. Large numbers of starch grains were clearly visible within the cortical cells of the lower part of the embryo in week 5 (Fig. 5D); however, the most abundant grains were still detected in the root cap region. Starch, in lower amounts than in the root cap region, also accumulated in cotyledons, reaching its maximum in weeks 5–7 (Fig. 5E). A subsequent decrease resulted in no detectable starch in cotyledons in week 8 (Fig. 5G).

Effect of PEG in maturation medium on carbohydrate status. Changes in endogenous sugar content in embryogenic cultures grown under osmotic stress, caused by addition of 3.75% PEG to the maturation medium, were determined. The presence of 3.75% PEG caused the osmotic potential of the media to decrease by 52.5 kPa. Under the imposed osmotic stress, sugar accumulation changed dramatically (compare Fig. 6 with Figs. 1, 3 and 4). Saccharide content in the whole ESM was determined until the 3rd wk of cultivation (Fig. 6A). Starting with the 4th wk of cultivation, the carbohydrate contents were analyzed in isolated embryos (Fig. 6B) as well as in the mass remaining after embryo isolation (Fig. 6C). Samples contained mainly fructose, and slightly less glucose and sucrose (Fig. 6C). In contrast, embryos contained mainly sucrose, much less fructose (20% of total sugars), and nearly no glucose (Fig. 6B). There was a significant difference ($P \leq 0.01$) in saccharide composition expressed as sucrose : hexose ratio comparing isolated embryos (Fig. 6B) to the remaining mass (Fig. 6C). Neither the total carbohydrate content nor the content of the particular sugar changed significantly during cultivation.

A comparison of the sucrose : hexose ratio in isolated embryos under osmotic stress with the control embryos grown on PEG-free medium is presented in Fig. 7. A much larger portion of total saccharides was found in sucrose compared to the hexoses in the samples from PEG-treated cultures. The process of SE maturation was speeded up by at least 1 wk in the PEG-treated cultures, allowing for embryo isolation 1 wk earlier, so the value for wk 4 can be presented. The moisture content of SE tended to decrease during cultivation on a medium with 3.5% PEG, from 92.3% in the control without PEG to 85, 83, 79, 79, and 78% for weeks 4, 5, 6, 7 and 8, respectively.

Both HPLC and histochemical analyses did not reveal any substantial difference in starch content and localization in PEG-treated embryos compared with controls (data not shown).

An increase in PEG concentration to 7.5% led to further changes in carbohydrate content. The changes were of a different nature compared with changes caused by 3.75% PEG. The content of total saccharides was lower in comparison to the control (PEG-free) at the beginning of cultivation on the maturation medium, but there was a dramatic increase in saccharide content at wk 4 and 5 (Fig. 8). The total saccharide level in 7.5% PEG-treated cultures exceeded the control level by 50%. HPLC analysis revealed that increases in all three sugars (glucose, fructose, and sucrose) were responsible (Fig. 9). During the 6th and 7th wk of cultivation the total content of sugars tended to decline, but an increase in sucrose content continued.

DISCUSSION

We studied the contents of saccharides that can directly meet the metabolic requirements of SE, as well as those that play a role in reserve material. Starch was a minor part of the carbohydrate spectrum in developing SE. It was localized mainly in the root cap region throughout the culture period, and to some extent in cotyledons until wk 7. Negligible numbers of starch grains were detected histochemically in the ESM, but some starch was detected by HPLC quantification. This may have been caused by the presence of nonembryonic cells in the samples that did contain large starch grains. These cells may have enhanced the total starch content in whole samples. Starch localization and quantitative

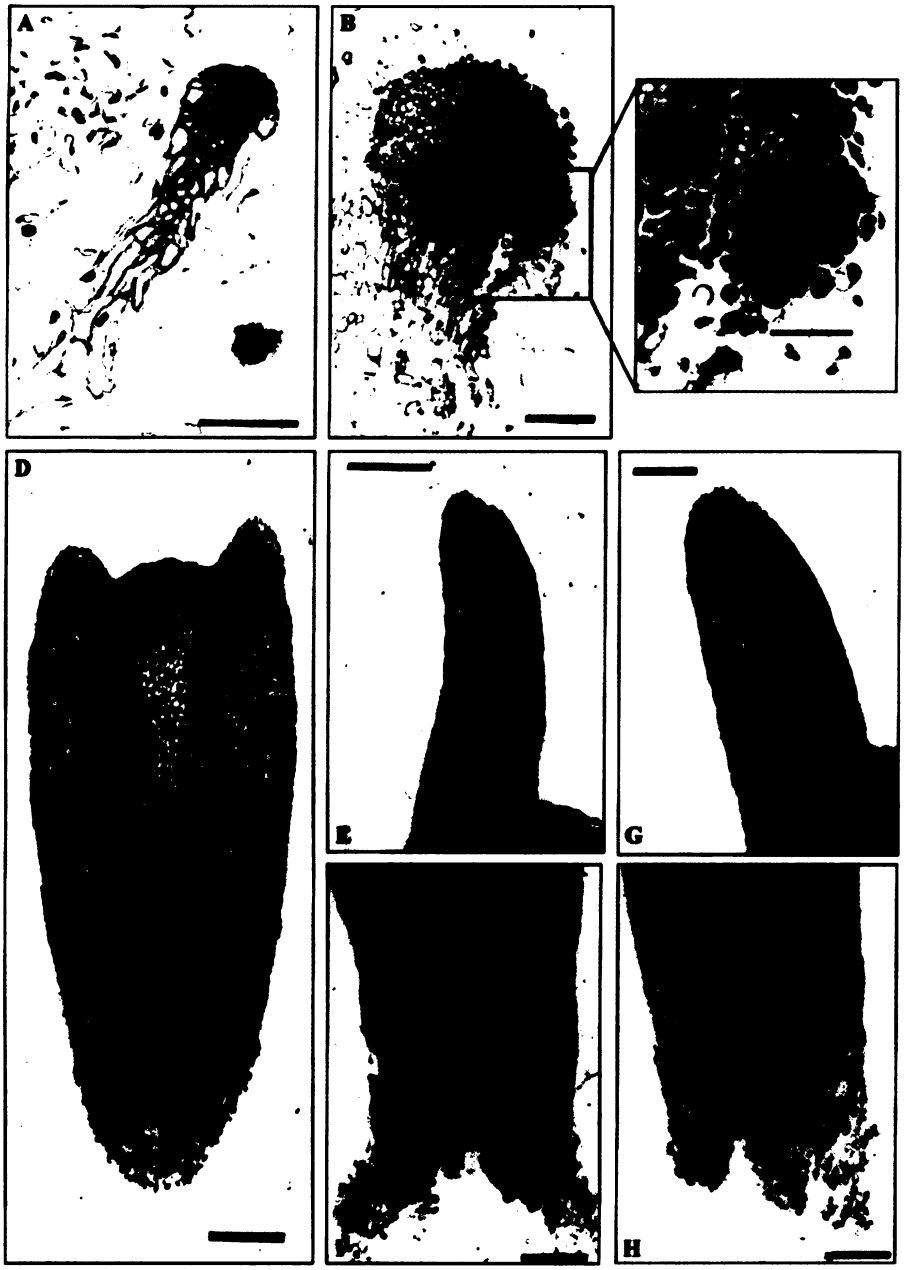

FIG. 5. Location of starch deposition (black), IIK detection (Lugol solution). (A) Early somatic embryo during cultivation on the proliferation medium. *Bar*, 200 μm. (B) Developing embryo at wk 3 on the maturation medium – beginning of starch accumulation. *Bar*, 200 μm. (C) Detail of starch containing part of the embryo shown in B. *Bar*, 100 μm. (D) Developing embryo at wk 5 on the maturation medium. Pronounced starch accumulation in cortical and root cap regions of the embryo. *Bar*, 500 μm. (E) Developing cotyledon of the embryo at wk 7 on the maturation medium. Remarkable starch accumulation in basal meristem. *Bar*, 200 μm. (F) Developing root pole of the embryo at wk 7 on the maturation medium. Striking starch accumulation in cortical and root cap regions. *Bar*, 200 μm. (G) Developing cotyledon of the embryo at wk 8 on the maturation medium. Absence of starch in all tissues. *Bar*, 200 μm. (H) Developing root pole of the embryo at wk 8 on the maturation medium. Gradual disappearance of starch. *Bar*, 200 μm.

measurements of starch content revealed that late-stage embryos have a reduced starch content. Starch-rich embryos with irregular shapes and with callus formation on the embryo surface occurred in the last 2 wk of cultivation. These irregularities may have caused the well developed, undamaged embryos to contain lower amounts of starch than determined by HPLC analysis. Reduction of starch content during SE maturation is in agreement with the fact that no detectable starch was found in mature white spruce zygotic embryos (Joy et al., 1991).

Sucrose is the other saccharide known to be a temporary storage

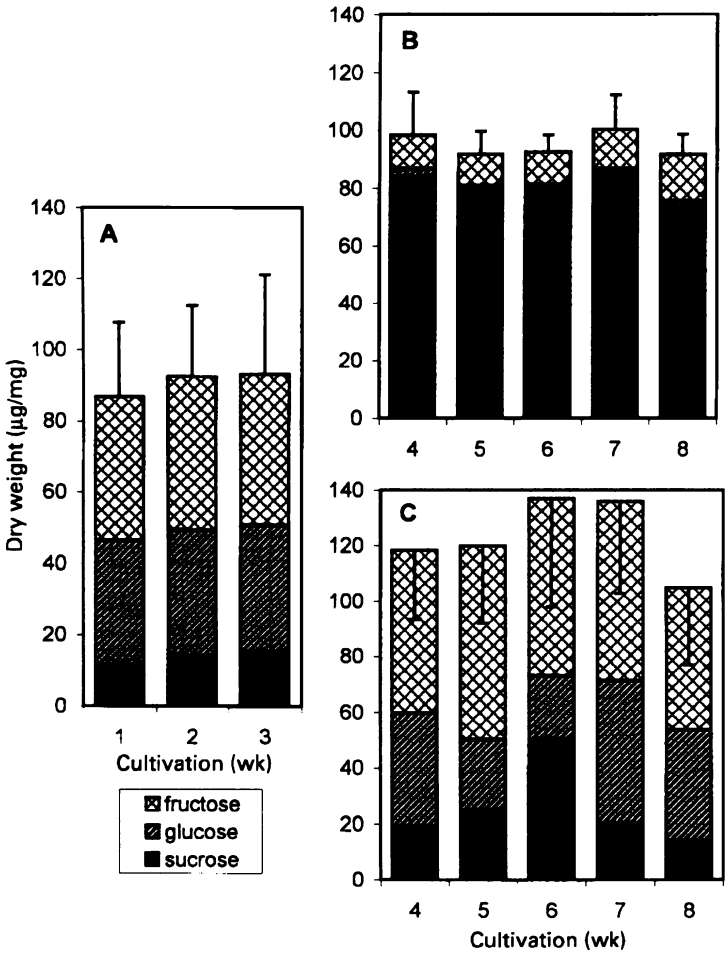

FIG. 6. Effect of 3.75% PEG in the maturation medium on the content of endogenous sugars in embryonal suspensor mass (A) and isolated embryos (B) ($n = 5$; each sample consisted of 5–15 embryos) and mass remaining after embryo isolation (C) ($n = 5$). *Bars* indicate SD.

compound in plant cells, but it was not present in embryos during the early stages of development. According to Nørgaard (1997) and our preliminary data, sucrose was split into glucose and fructose by exogenous enzymes before uptake. Glucose is probably preferentially used to meet metabolic demands (Treat et al., 1989). Sucrose content gradually increased during SE development. This is in agreement with the idea that maturation is connected with storage material accumulation (Roberts et al., 1990; Attree et al., 1992). Increasing cell vacuolization was observed in another study during the later stages of development (Svobodová et al., 1999). As sucrose is believed to be a vacuole-stored reserve material (Pollock and Kingston-Smith, 1997), it may be that vacuole formation is a necessary prerequisite for vacuole-compartmented sucrose accumulation. The decrease in glucose content during the embryogenic process can probably be ascribed to its role in the processes of embryo growth and development. These include being a source of carbon and energy, and a source for starch synthesis and later for sucrose synthesis.

There were no large saccharide content changes in ESM cells not directly involved in embryo formation. Thus it is likely that the developing embryos themselves are mainly responsible for the changes in saccharide status of ESM as a whole. From a structural point of view, embryos were fully developed by wk 6, although there were changes in NSS content after that time. The best growth extension and vigor of conifer somatic plantlets would be expected from SE that have the highest carbohydrate, lipid and protein reserves (Joy et al., 1991; Attree et al., 1992; Gutmann et al., 1996). The presence, location, and timing of the accumulation of adequate reserves may be vital to coniferous SE because they do not benefit from the haploid megagametophyte, which is a major storage organ within the conifer seed (Attree et al., 1992). Accumulation of sucrose and disappearance of starch during the last stages of cultivation might be important.

The total soluble saccharide content and partitioning between particular sugars were remarkably influenced by osmotic stress. The total amount of soluble sugars was lower in SE on maturation medium with 3.75% PEG. A large amount of sucrose, low amounts of fructose, nearly no glucose, and only slightly higher amounts of starch were the characteristic features of SE developing under osmotic stress caused by 3.75% PEG in the maturation medium.

When the PEG concentration in the maturation medium was increased to 7.5%, the expected intensification of osmotic stress effects, compared to 3.75% PEG, did not take place. Inclusion of 7.5% PEG stimulated quite a different pattern of saccharide accumulation. The contents of sucrose, glucose, and fructose were high, and increased during embryo maturation, especially in wk 4 and 5. This disorder may be related to irregularities found in a corresponding study on SE structure level (Svobodová et al., 1999).

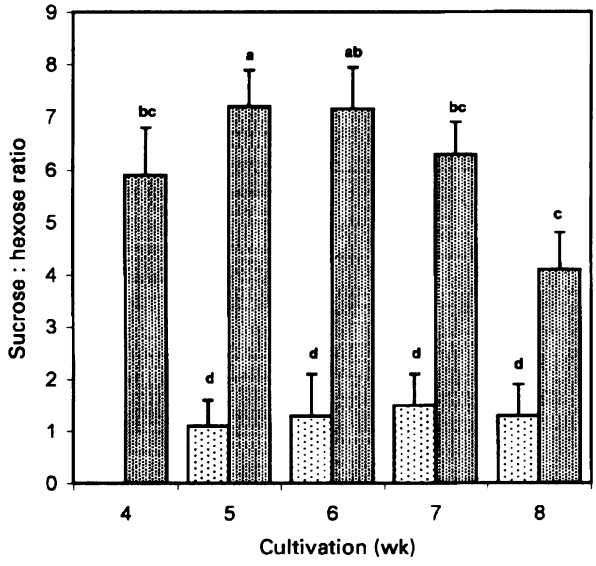

FIG. 7. Sucrose : hexose content ratio in isolated embryos cultivated on the maturation medium (MM; $n = 3–5$; each sample consisted of 5–15 embryos) and the maturation medium with 3.75% PEG ($n = 5$; each sample consisted of 5–15 embryos). *Bars* indicate SD. Data were evaluated by ANOVA tests (factors: time, treatment). Data with common letters are not statistically different ($P \leq 0.05$) by the Kruskal–Wallis multiple-comparison z-value test (non-normal distribution).

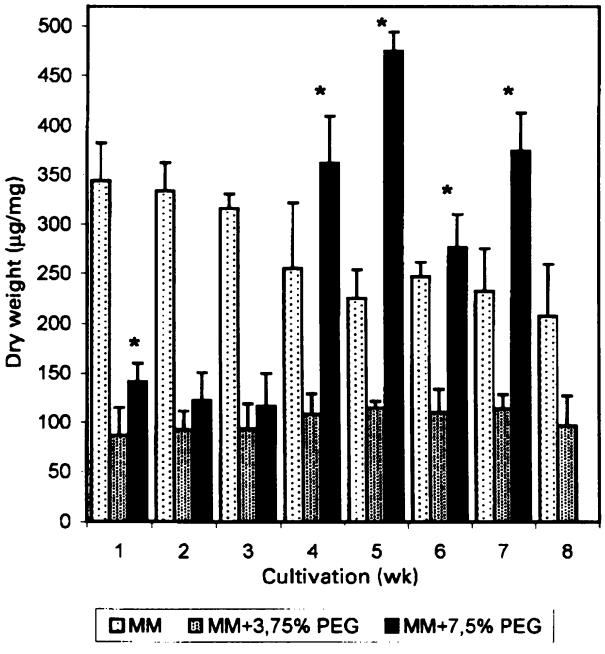

FIG. 8. Comparison of content of endogenous sugars in embryonal suspensor mass cultivated on the maturation medium (MM; $n = 5–15$) and MM + 3.75% PEG ($n = 5$) and MM + 7.5% PEG ($n = 3–5$). *Bars* indicate SD. Data for individual variants in particular sampling dates were evaluated by ANOVA (factor: treatment). As the data were normally distributed, Fisher's LSD multiple-comparison test was used. For each sampling date the data for PEG-free (MM) medium were significantly different ($P \leq 0.05$) from PEG-containing media. *Asterisks* above columns indicate the statistically significant difference between PEG-containing media for the given sampling date.

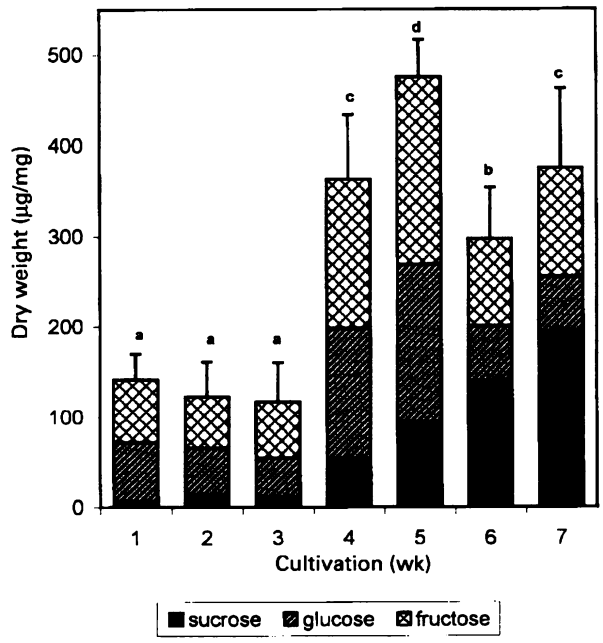

FIG. 9. Content of endogenous sugars in embryonal suspensor mass grown on the maturation medium with 7.5% PEG. *Bars* indicate SD ($n = 3–5$). Data on the content of total soluble saccharides for particular sampling dates were evaluated by ANOVA (factor: time). Data with common letters are not statistically different ($P \leq 0.05$) by Fisher's LSD multiple-comparsion test (normal distribution).

That study showed that 3.75% PEG provides a suitable osmotic stress, speeds up the process of maturation compared to control treatment, and leads to well developed embryos which are able to germinate. The present work showed that a PEG concentration of 7.5%, within the range frequently recommended for improvement of SE in other conifer species (e.g. Attree et al., 1992, 1995), is not favorable for embryo maturation of the Norway spruce genotype under study.

Preliminary germination experiments using the same plant material in another study revealed that SE that were matured on PEG-free (MM), and both 3.75 and 7.5% PEG-containing media, were able to germinate (Svobodová et al., 1999). In that paper we showed that the highest germination percentage (94.6%) was achieved for SE maturing for 6 wk on maturation medium + 3.75% PEG, with a slightly lower germination percentage (93.5%) using PEG-free treatment. A significantly lower germination frequency (75.2%) was achieved using maturation medium + 7.5% PEG.

A better understanding of the physiology of embryogenic culture growth is needed to improve productivity from somatic embryogenic systems. Plant growth and development are complex processes triggered by hormonal compounds and sustained by carbohydrate supply. The relationship between these two factors is not well-understood (Mehouachi et al., 1996). Therefore determining the nature of saccharide content changes during SE development may help to clarify factors that induce or promote embryo development. Efficient manipulation of endogenous carbohydrate and osmoticum in culture media may be facilitated through a comparison of the present results with the carbohydrate status of developing zygotic embryos.

Acknowledgments

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FORESTRY SCIENCES

Protocol for Somatic Embryogenesis in Woody Plants

S. Mohan Jain and Pramod K. Gupta
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SOMATIC EMBRYOGENESIS IN NORWAY SPRUCE

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1. INTRODUCTION

Norway spruce is most important conifer for wood production in central and northern Europe. Somatic embryogenesis of Norway spruce (*Picea abies* L. Karst.) was first reported in 1985 (Chalupa 1985, Hakman and von Arnold 1985). This method is a powerful tool for rapid *in vitro* micropropagation of desired genotypes. Almost two decades of research resulted in a relatively high degree of knowledge and graduated protocols for somatic embryogenesis. Norway spruce thus belongs to a few coniferous species, which are used as "model system" for studying of somatic embryogenesis.

2. CULTIVATION OF EMBRYOGENIC CULTURES

2.1. Preparation of the culture media

Somatic embryogenesis is regulated by changes of plant growth regulators exogenously supplied in the culture medium. Other changes include slight difference in the composition of nutrient media during specific phases of somatic embryogenesis. Several cultivation media are used for embryogenic cultures of Norway spruce in different laboratories (e.g. modified MS, LP, DCR, MSG, N6, NIII, for overview see Tautorus et al. 1991). The composition of slightly modified nutrient media, according to Gupta and Durzan (1986), including plant growth regulators and other compounds, is represented schematically in Table 1. Laboratory praxis, stock solutions in detail and protocol for preparation of nutrient media are summarized in Tables 2 and 3.

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2. 2. Initiation of embryogenic culture

Table 1. Overview of main changes in nutrient media during different phases of somatic embryogenesis of Norway spruce:

	induction	*proliferation*	*maturation*	*desiccation*	*germination*
medium	full GD			filter paper	½ GD
agar	0.8%			none	0.8%
pH	5.8			-	5.8
auxin	5 μM 2,4-D		none		
cytokinins	2 μM BA, 2 μM kinetin		none		
ABA	none		20 μM	none	
charcoal	none				0.5%
PEG	none		5 %	none	

Table 2: Media composition, stock solutions (A – macro salts, B – micro salts, C- Fe, D -organic compounds, E – auxin and cytokinins, F – abscisic acid

A: Macro salts:

	stock solution (20 x concentrated) (g/l)	**concentration in the medium**	
		(mg/l)	mM
KNO_3	46.8	2340	23.14
NH_4NO_3	5.5	275	3.44
$CaCl_2 . 2 H_2O$	4.4	220	1.50
$MgSO_4 . 7 H_2O$	3.7	185	0.75
KH_2PO_4	1.7	85	0.62

B: Micro salts:

	stock solution (100 x concentrated) (mg/l)	**concentration in the medium**	
		(mg/l)	μM
$MnSO_4 . H_2O$	1115	11.15	66
$ZnSO_4 . 5 H_2O$	430	4.30	17
H_3BO_3	310	3.10	50
KI	41.5	0.415	2.5
$CuSO_4 . 5 H_2O$	1.25	0.0125	0.05
$CoCl_2 . 6 H_2O$	1.25	0.0125	0.05
$Na_2MoO_4 . 2 H_2O$	12.5	0.125	0.52

Stock solutions of macro and micro salts are prepared from sterilized water in a semisterile way. After component dilution, solutions are kept refrigerated.

C: Fe:

	stock solution (100 x concentrated) (mg/l)	**concentration in the medium**	
		(mg/l)	µM
Na_2EDTA . 2 H_2O	1890	18.9	50
$FeSO_4$. 7 H_2O	1390	13.9	50

Both compounds are diluted in water and boiled (10 min). The volume is adjusted, the stock solution is left overnight and stored in refrigerator.

D: Organic compounds:

	stock solution (40 x concentrated) (g/l)	**concentration in the medium**	
		(mg/l)	µM
myo-inositol	40	1000	5550
caseinhydrolysate	20	500	-
L-glutamine	18	450	3080
glycine	0.04	1	13.32
thiamine HCl	0.02	0.50	1.48
nicotinic acid	0.01	0.25	2.03
pyridoxine HCl	0.01	0.25	1.22

Components are diluted in water, pH adjusted close to 5.8 and solution is sterilized through an 0.22 µm filter. Sterile stock solution is stored in freezer.

E : auxin and cytokinins:

	stock solution (40 x concentrated) (mg/100 ml)	**concentration in the medium**	
		(mg/l)	µM
2,4-D	4.4	0.110	5
BA	1.72	0.043	2
kinetin	1.72	0.043	2

All compounds are diluted separately (2,4-D in drop of ethanol, cytokinins in drop of 1 N KOH, after dilution add water, mix solutions together and adjust volume with water). Adjust pH to 6.0 (with HCl). Sterilize with an 0.22 µm filter and store in freezer.

F: abscisic acid:

	stock solution (40 x concentrated) (mg/100 ml)	**concentration in the medium**	
		(mg/l)	µM
ABA	26.4	0.66	20

Dilute ABA in a few drops of 1 N KOH; after dilution adjust volume with water. Adjust pH to 6.0 aproximately (with HCl). Sterilize with an 0.22 µm filter and store in freezer. Thawing of ABA stock solution should be done carefully as ABA is sensitive to high temperature and UV light.

***Table 3:** Preparation of the medium:*

	components of 400 ml medium for:		
	induction and proliferation	embryo development and maturation	germination
water	352 ml	354 ml	352 ml
A (macro salts)	20 ml	20 ml	10 ml
B (micro salts)	4 ml	4 ml	2 ml
C (Fe)	4 ml	4 ml	2 ml
sucrose	12 g	12 g	2 g
PEG 4000	-	15 g	-
active charcoal	-	-	2 g
agar*	3.2 g	3.2 g	3.2 g
pH	5.8	5.8	5.8
autoclaving of the media, after cooling add filter sterilized solutions:			
D (organics)	10 ml	10 ml	5 ml
E (2,4-D + cytokinins)	10 ml	-	-
F (ABA)	-	8 ml	-

* for solidified media only

2.2.1. Primary explant

Embryogenic cultures are mainly initiated from immature or mature zygotic embryos. The initiation rate of immature zygotic embryos changes only slightly during embryo ontogeny and can reach up to 100% (Hakman and von Arnold 1985). The initiation rate of mature zygotic embryos is somewhat lower. On the other hand, this way of induction is not seasonally dependent, sufficient initiation rate is reached if zygotic embryos from older seeds are

used as a primary explant. The initiation rate varies among different genotypes of primary explant (Jain et al. 1988).

Embryogenic culture is also initiated from somatic embryos and juvenile tissues of young plants (cotyledons, emblings). Their initiation rate is still markedly lower compared to zygotic embryos. Moreover, these ways still do not solve the major problem: induction of embryogenic culture from a mature tree, which is of great practical importance. However a few successful attempts were made (induction of embryogenic culture from needles, Ruaud et al. 1992, Harvengt et al. 2001), efficiency of these techniques is extremely low and the method is not still routinely used.

2.2.2. Initiation media

Both auxin and cytokinin(s) are necessary for the induction of embryogenic culture of Norway spruce. 2,4-D (2,4-dichlorophenoxyacetic acid) or NAA (naphtaleneacetic acid), and BA (N^6-benzyladenine) or/and kinetin are the preferred auxins and cytokinins for induction of embryogenic cultures.

Specific composition of media (composition of macro and micronutrients) is not a crucial factor for initiation of cultures. Embryogenic cultures are initiated from primary explants on media either solidified with agar or gelrite.

Immature Norway spruce cone could serve as a source of primary explant, zygotic embryo (Figure 1A), but it is difficult to determine an optimal harvest time. Initiation rate of early immature zygotic embryos is the highest, but it changes with the ontogeny of an embryo, and is usually sufficient during all stages of embryo development and maturation. Younger cones are more compact, which prevents mold infections. On the other hand, dissection of more developed immature seeds is more simple. Dissection of seeds and extraction of primary explants should follow immediately after a harvest of cones to decrease possible danger of mold and bacterial infection resulting from the storage of immature cones or seeds.

Wash the harvested Norway spruce cones in 70 % (v/v) ethanol (5 min), then rinse repeatedly with sterile distilled water. In a semisterile way, remove developing seeds. Then, in a flow-box, sterilize the seeds with 0.1% $HgCl_2$ (5 min, mix occasionally), and thoroughly wash the seeds at least three times with sterile distilled water. Dissect the seed (unarmed eye or under binocular microscope). The testa and endosperm are removed and the zygotic embryo is placed on induction medium.

If mature zygotic embryos are used as primary explant, surface of seeds should be sterilized as described above. In some cases, dissection of seeds and

extraction of zygotic embryo is difficult. Imbibition of sterilized seeds improves this.

Two types of cultivation are recommended: either in disposable petri dishes (60 or 90 mm, 10 – 15 embryos per dish) or in vials (1 embryo per vial, slanting medium). Slightly modified GD medium (Gupta and Durzan, 1986) solidified with 0.8 % agar (Sigma) is used (Table 1).

Induction of embryogenic tissue proceeds in dark plant growth room or thermostat (24 °C, darkness). Embryogenic tissue starts to differentiate on explant surface of within 3-4 weeks (Figure 1B). Developing embryogenic tissue is white, translucent and mucilaginous, and sharply contrasts either with brown turning tissue of primary explant or with developing nonembryogenic tissue (compact hard callus composed of small rounded cells, yellow, white or greenish with velvet surface). At this stage, a long subcultivation interval (4-6 w) is recommended. Do not separate developing embryogenic structures from primary explant prematurely; wait until embryogenic culture grows to 8 – 10 mm in diameter. Primary explant is able to generate embryogenic tissue for a relatively long time, usually for several months.

2.3. Proliferation of embryogenic culture

For a few weeks after induction of embryogenic culture, their appearance and growth become uniform. On the other hand, various cell lines differ markedly in growth parameters and structure of culture. Repeatedly passage on proliferation medium (Table 1), cultures could sustain their character for many years.

There are several ways to cultivate embryogenic cultures. The use of agar-solidified medium is most frequent. Cultures could be either proliferated in a liquid medium in flasks placed on a roller or on a gentle shaker. Growth rate of liquid grown cultures is usually higher compared to solidified media. Although some cell lines grow well even after long cultivations in liquid, others suffer from morphological aberrations (mainly shortening and disaggregating of suspensor cells) followed in decrease of growth rate and cell viability. Generally, cultivation in liquid medium could be used only for a shorter time and should be limited only to the proliferation phase. In practice, liquid media are used for cultivation of embryogenic cultures prior to cryopreservation.

Cultivation of cultures on support floating on the surface of the medium (*e.g.* membrane rafts, Osmotec) represents the third way of cultivation (Figure 1E). This method gives excellent results for the majority of cell lines, and is recommended mainly for the last proliferation passage(s) prior to maturation. The method saves labor costs and is ideal to the culture (whole rafts are

transferred to a fresh medium without touching of embryogenic tissue). Disadvantages of the method lie in the high cost of membrane rafts and frequent problems with optimal degree of wet ability of membrane.

For routine maintenance of cultures transfer small (up to 10 mm size) pieces of embryogenic tissue to the agar-solidified proliferation medium (Table 1). Manipulation of embryogenic cultures should be done carefully and in consideration to the tissue (nor cut the tissue with scalpel neither transfer to a fresh media in upside down orientation). Cultivation in Magenta boxes (Sigma) is more convenient than plastic petri dishes due to greater volume of air inside. Cultivation should proceed in total darkness at 24 °C, cultures are relatively sensitive to temperature variation. Subculture intervals differ among cell lines and depend on growth rate of culture (usually 1-2 w).

2.3.1. Visualization of embryogenic cultures

For simple microscopic observation, embryogenic cultures during proliferation stage could be stained with trypan blue (0.04 %, diluted in water) which is added directly to a small piece of tissue (approx. 2 mm size) without previous fixation. After a few seconds, a drop of water is added and the excessive dye is wiped with cellulose. This method could be also applied to a early somatic embryo maturation stages (up to 2 w on maturation medium).

2.4. Development and maturation of somatic embryos

Development of somatic embryos in embryogenic tissue is triggered by changes in plant growth regulators in the culture medium. Auxin and cytokinins are removed and abscisic acid (ABA), in relatively high concentration, is added to the maturation medium. Non-penetrating polyethylene glycol (PEG 4000 or 6000) is added to the maturation medium in order to increase osmotic strength.

Embryogenic cultures could either be transferred from the proliferation medium to the maturation medium, or for a short period (1-10 d) pre-culture on the proliferation medium without plant growth regulators (Bozhkov et al. 2002). Pre-culture leads to decrease of endogenous IAA. Thereafter, majority of embryogenic cultures synchronize well in the maturation medium containing ABA and PEG.

Transfer small pieces (up to 10 mm size) of embryogenic tissue, grown on agar-solidified media in Magenta box (or petri dish), to a membrane raft floating on nutrient medium. From this point, whole rafts are transferred to a fresh medium without manipulation of embryogenic tissue. Allow one or two more passages of cultures on proliferation medium (depends on growth of

tissue). Then rafts should be transferred onto the proliferation GD medium without plant growth regulators for another week, and thereafter onto a maturation medium containing 20 μM ABA and 5 % PEG 4000. Developing somatic embryos are visible after 1 - 2 weeks. After 2 - 3 w, cotyledons start to develop in apical pole of somatic embryos. The time necessary to get mature somatic embryos varies among cell lines (4-7 weeks) (Figure 1C).

Alternatively, the development and maturation of somatic embryos could also be completed on a solidified medium. Pieces of tissue must be transferred weekly onto the fresh medium. The rapidity of somatic embryo development on solidified medium is somewhat lower when compared with membrane rafts. Although liquid cultures are routinely used for somatic embryo production of several conifer species (e.g. *Pseudotsuga*), cultivation of Norway spruce embryogenic cultures submersed in liquid maturation medium (roller, shaker, bioreactors) do not bring satisfactory results.

2.4.1. Staining of embryogenic cultures and embryos

Shortly after the start of embryo development on maturation medium, developing somatic embryos become too big to be studied under a microscope without further preparation. For that it is necessary to prepare paraffin sections (thickness 12 μm) (Johnson 1940). The sections are stained in a 2-step procedure using alcian blue and nuclear fast red (Poláčková and Beneš, 1975). Alcian blue (0.1%) stains cell walls, whereas nuclear fast red (1%) visualizes chromatin structures in the nucleus.

2.4.2. Analysis of embryogenic cultures

Embryogenic cultures are analysed by the system of computer image analysis, which allows counting and measurement of different structures. In order to be analysed, the embryogenic culture is recorded either directly by digital TV camera (magnification 1 – 10 x), or by microscope (recommended magnification 15 – 150 x) and digital camera.
Microscope paraffin sections are prepared as described above (2.4.1.), or pieces of tissue are vitally stained as in 2.3.1. Paraffin sections are suitable for analysis of developing tissues of somatic embryo, whereas the vital staining is more convenient for observation of ESM (somatic embryos and suspensor cells).

In lower magnification, embryogenic tissue is recorded directly by a digital TV camera. The culture is placed in open petri dish against contrast backround, lightened by several cold light sources to avoid shadows, and recorded. Program of computer image analysis (Lucia, ver. 4.61, Laboratory Imaging, Czech Republic) enables to record series of images of a more spatial object, focusation continually changes from the top to the base of the object.

The final image with high depth of sharpness is then combined of the individual pictures. For the determination of the embryogenic capacity of the culture, a known amount of embryogenic culture is gently mixed with water in petri dish and recorded by the camera. The image is further processed by a computer image-analysing software. A number of macros were created with the aim to distinguish particular structures (e.g. embryos), count them and measure additional parameters.

2.5. Desiccation of somatic embryos

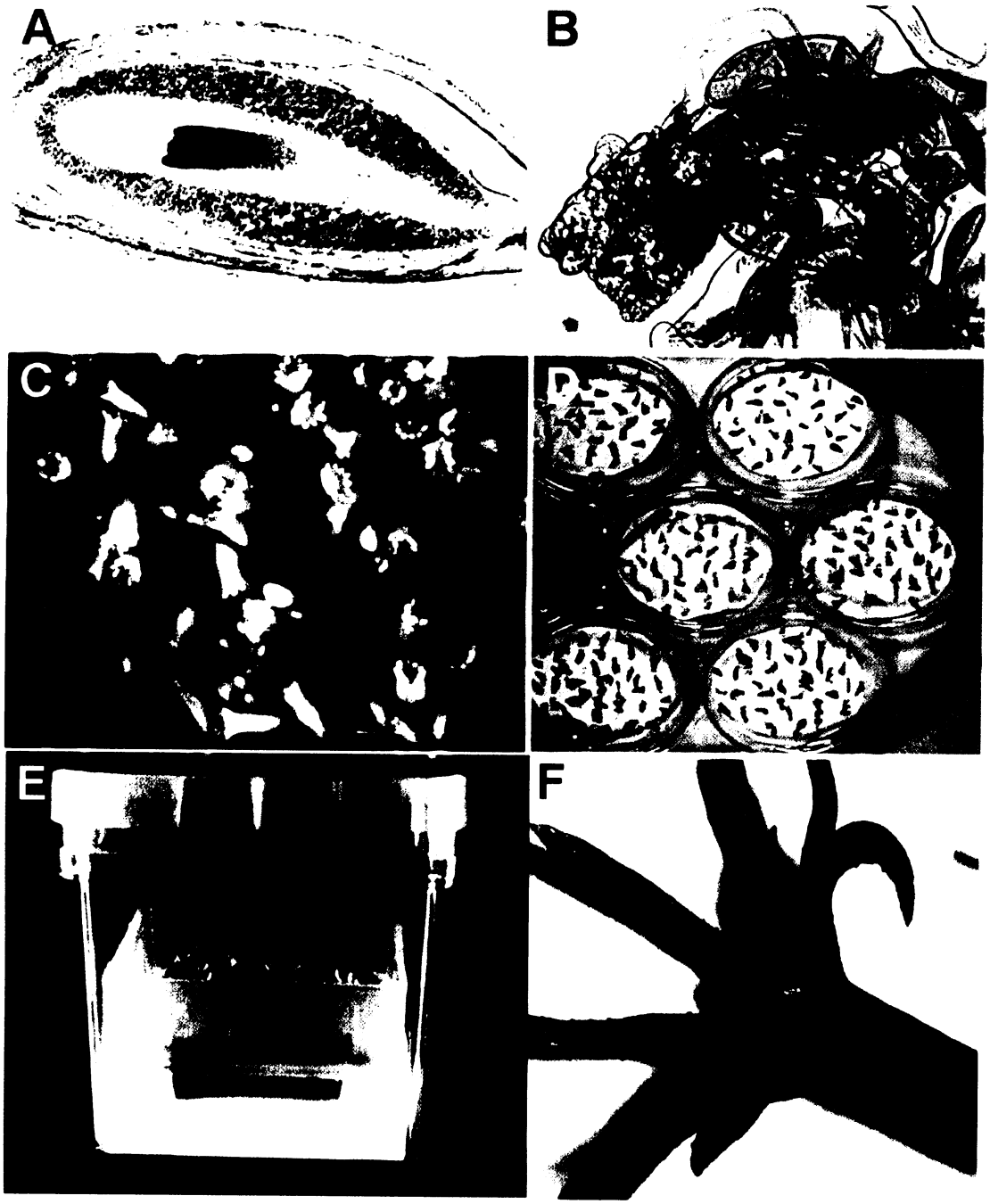
The germination rate of mature somatic embryos is usually improved by desiccation before the transfer of somatic embryos to the germination medium. However, the growth and anatomical development of somatic embryos has been already completed, whereas storage compounds still continue to accumulate; and the levels of endogenous ABA and water content decrease during this stage. The somatic embryos are in the process of germination. The necessity of this step is variable among different cell lines. A number of desiccation protocols is used. Generally somatic embryos are exposed to high relative humidity (>98 %) at the same or slightly decreased temperature (+ 18 °C) for 2-5 w.

Uniform mature somatic embryos are harvested by forceps and aseptically placed on a dry filter paper in a petri dish. Several small petri dishes are placed into a big sterile one on wet filter paper (sterile distilled water) and sealed with parafilm tape (Figure 1D). Sealed dishes should be placed at 20 - 24 °C. The role of light is not clear up to now. Slightly better germination is achieved if somatic embryos were exposed to dim light during desiccation compared to desiccation in total darkness. Desiccating somatic embryos could be kept in dim light/dark regime 12h/12h, intensity of light should not exceed 18 $\mu mol.m^{-2}s^{-1}$. Optimal time needed for desiccation varies from 2-5 weeks and also depends on cell line and previous procedure of maturation. If the desiccation lasts longer than optimal period, malformations in apical pole of emblings are visible, if the desiccation is too short, protruding primary root tends to stop growth and occur recallusing. Filter paper in lower petri dish should remain wet during the desiccation procedure and add additional sterile water if necessary.

2.6. Germination of somatic embryos

Desiccated somatic embryos are fully prepared to germinate. Germination medium consists of solidified half-strength GD medium without plant growth regulators. Usage of different gelling agents (agar, gelrite, transfer agar, phytoagar) gives similar results. At this stage, PEG is replaced by 0.5 %

Figure 1: A - primary explant: developing zygotic embryo, B - embryogenic culture composed of meristematic heads and long suspensor cells, C - mature somatic embryos, D - desiccation, E - cultivation on membrane raft, F - germinated somatic embryo with apical bud



active charcoal. It is recommended to fill Magenta boxes (or petri dishes) with an autoclaved medium, which is cooled sufficiently (temperature bellow 50 °C) to prevent sedimentation of active charcoal in the boxes. Desiccated somatic embryos are placed on the surface of the medium. Cultivation temperature should be set to around 20 °C; temperature in dark phase could be set somewhat lower. Germination is not as dependent on optimal temperature as it is an proliferation of embryogenic culture and maturation of somatic embryos. Mild variations of temperature from optimal do not seriously affect germination. Germination of

embryos starts immediately, visible growth could be seen after few days. At first primary root rapidly extrudes. Growth of apical pole is much slower and cells of apical meristem start to divide and form apical bud; hypocotyl slowly elongates along longitudinal axis during first weeks. Role of light is crucial during the germination. The most important for that is the photoperiod, but the intensity of light has minor effect. The effect of light quality is negligible and it has not been sufficiently studied so far. Short day conditions lead to formation of apical bud, long day conditions promote growth of the shoot. In this first phase, lasting 6 – 10 w after start of germination, photoperiod composed of shorter light phase (8 - 10 h) and longer dark phase (14-16 h) are recommended. After apical bud formation light phase of photoperiod should be prolonged to 16h light/8h darkness. Intensity of light should gradually increase during this period (to 80 – 20 $\mu mol.m^{-2}s$) (Figure 1F).

After 3–4 weeks, germinating somatic embryos should be transferred to a fresh medium (the same as for germination except active charcoal is omitted). Primary roots of small plantlets penetrate the solidified medium and secondary roots emerge. Long day conditions trigger longitudinal growth of apical buds.

2.7. Transfer of emblings *ex vitro*

Small seedlings with developed or growing apical bud and root are transferred to nonsterile substrate comprised of peat and sand (1:1). Alternatively a fresh natural substrate originating from a spruce forest is used. Seedlings are kept in a glasshouse in shadow. For the first few weeks after the transfer *ex vitro*, it is necessary to avoid high cultivation temperatures (over 35 °C) and keep the relative humidity high (more than 80 %). The humidity later gradually decreases. After plantlets grow to several centimeters and root systems are well developed; acclimatization to low temperatures and drought could start. Well acclimatized plants could be transferred to the field.

There is a distinct gap in the rate of growth after the *ex vitro* transfer. Even then, for several months, the growth of seedlings is slower than their zygotic counterparts. No significant differences in growth were found in trees 3 – 7 years of age grown naturally or through somatic embryogenesis.

3. CRYOPRESERVATION OF EMBRYOGENIC TISSUE

Cryopreservation of embryogenic cultures is a unique way for long-term storage of germplasm. Cultures should be kept genetically unchanged for a long time until clones are evaluated in the field test. Cryopreservation is also a potent tool for routine preservation of large numbers of cell lines in the laboratory.

Successful cryopreservation of embryogenic cultures of Norway spruce was first published in 1987 (Gupta et al. 1987). Bercetche et al. (1990) reported that cryopreservation enhances embryogenic capacity. Different cell lines showed different cryotolerance, however, no conclusions could be reached on the relationships of cryo-tolerance and distinct morphological or biochemical characteristic of cell lines (Nørgaard et al. 1993).

3.1. Pretreatment of cultures

The cryopreservation protocol of Bozhkov is used and slightly adapted (Bozhkov, pers. comm., 2002). Embryogenic cultures grown on solidified medium are passed to roller flasks (3 g of fresh weight per 30 ml liquid medium) and cultivated for 1 w. Cultures are then treated with sorbitol (0,16 ml of 4 M sorbitol is added to the flask ten times during 30 min, resulting concentration of sorbitol was 0.2 M) and return to the roller till the second day, when this procedure is repeated (concentration of sorbitol is then 0.4 M). On the third day the cultures in flasks are placed on ice and treated with DMSO (0,175 ml DMSO ten times during 30 min, final concentration of DMSO is 5 %), left for another 15 min and then filter through the sieve. Ice-cooled cryotubes are filled with cell suspension and then closed.

3.2. Cooling program

Two different cooling systems are used. Cryotubes are either put in simple cryobox (Mr. Frosty, Nalgene) filled with isopropanol and cool in laboratory freezer, or cool in the programmed cryomachine (Glacier, Sy-Lab) (Figure 2). Temperature in the first system is monitored directly by thermometer inserted in one separate cryotube. After the end of cooling, the cryotubes are transferred to the liquid nitrogen.

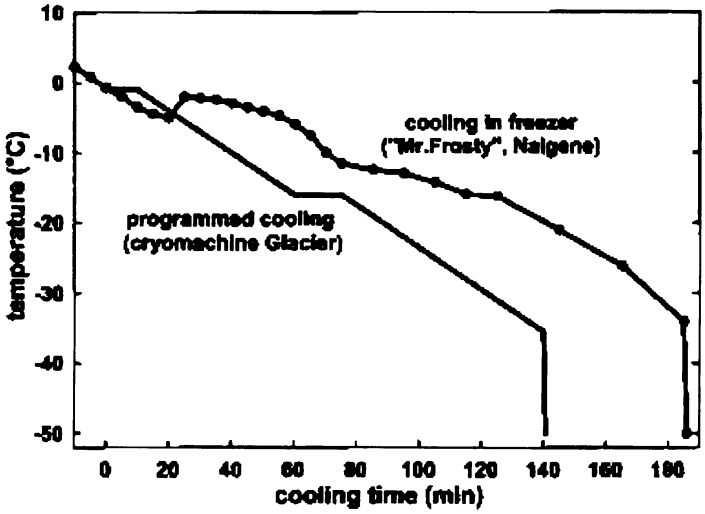


Figure 2: Temperature gradient of both two cooling systems.

3.3. Thawing

Cryotubes are transferred to sterile water (45 °C) for approx. 1 min. At this time the contents of tubes melt, the cryotubes are placed in sterile water (+ 4 °C) for a couple of minutes. After surface sterilization of vials in 70 % (v/v) ethanol, the cultures are layered on filter paper placed on proliferation medium in petri dish. Filter paper with culture is further transferred onto fresh medium after 1 h, and once more after 1 d. Cultures layered on filter paper are further transferred to fresh proliferation medium weekly. After tissue growth started, the filter paper is removed.

Cryopreservation using both cooling system yield in regrowth of embryogenic cultures. After thawing, the growth of embryogenic cultures is visible in 1-2 w. Cells of meristematic heads (the only part of culture which remained alive) start to divide. Osmotic sensitive suspensor cells, damaged by cryopreservation, recover. The growth rate of cultures is changed during the period of lag-phase. This period is variable for different cell lines. There is no doubt that cryopreservation represents a strong selection pressure which eventually could change the embryogenic capacity of the culture.

Chance for successful regrowth of cultures is slightly lower using an inexpensive cooling system (Mr. Frosty, Nalgene) compared to a sofisticated, programmed cryomachine due to unpredictable deviations from optimal cooling rates. On the other hand, even this simple system give satisfactory results.

4. CONCLUSION AND PROSPECTS

During almost twenty years, which passed since the first report on Norway spruce somatic embryogenesis, reliable cultivation protocols were elaborated. Norway spruce became one of the most studied species among other conifers in the aspect of somatic embryogenesis. These protocols comprise the induction of embryogenic tissue, development and maturation of somatic embryos, germination and transfer of plantlets *ex vitro*, cryopreservation of embryogenic tissue and construction of transgenic trees. On the other hand, at least two big issues still remain to be solved: 1) induction of embryogenic cultures from vegetative organs of mature tree, 2) elaborating of successful protocols in liquid media and bioreactors which are the must for automation and decrease of labour costs. Our knowledge on regulation of the somatic embryogenesis (signal pathways, the role and action of phytohormones, gene expression) is still low but gradually increases.

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Liquid Culture Systems for *in vitro* Plant Propagation

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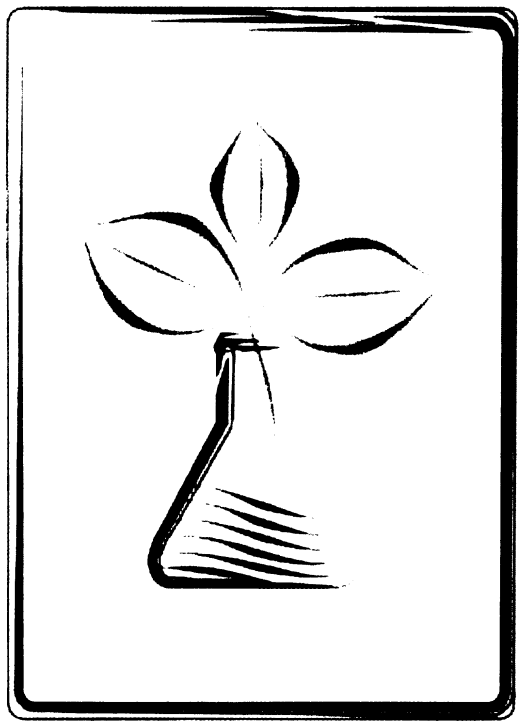

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Chapter 21

Norway spruce somatic embryogenesis: membrane rafts as a compromise between liquid and solidified media

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Abstract: Embryogenic cultures of Norway spruce (*Picea abies*) were cultivated either on solidified media, in liquid media, or on polypropylene membrane rafts (LifeRaft). The cultivation on rafts was found to be the most successful way: the number of developed somatic embryos increased, synchronization of the development was enhanced, and the time necessary for embryo development and maturation was shortened. It was shown that the process could be further improved by insertion of a pre-maturation phase on PGR-free medium between proliferation and maturation steps. Germination frequency remained unchanged. PEG 4000 added to the maturation medium increased the number of developed somatic embryos. PEG in lower concentration (1.87 % (w/v)) still had the significant beneficial effects on embryo numbers and development, but a decrease of germination frequency or increased aberrations of developing root and shoot were not found. On the other hand, PEG in the concentration 5 and 7.5 % (w/v) had a negative effect on the germination of somatic embryos and, in sensitive cell line, 7.5 % (w/v) PEG decreased somatic embryo yield.

Key words: conifer, membrane raft, Norway spruce, PEG, *Picea abies*, somatic embryogenesis

Abbreviations: ABA-abscisic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - 6-benzylaminopurine, N6-benzyl-adenine; IAA - indole-3-acetic acid; PEG - polyethylene glycol; PGR - plant growth regulator

1. Introduction

The majority of cultivation protocols for somatic embryogenesis of different coniferous species use solidified media, at least for part of the process. The quality of somatic embryos grown on solidified medium is still higher than that of their counterparts cultivated in liquid medium; germination frequencies thus differ considerably (Tautorus and Dunstan,

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1995). In liquid proliferation medium, cultures usually grow even more rapidly than on agar-solidified medium. Maturation of somatic embryos is considered as the more problematic step. The ability to produce mature somatic embryos in liquid medium differs widely across species and even across cell lines. On the other hand, the use of somatic embryogenesis as a large-scale micropropagation tool in commercial applications depends mainly on reduction of labour costs through the automation of the process. Automated techniques clearly demand protocols based on liquid media (Paques et al., 1992).

There are a few examples of successful use of liquid cultures for somatic embryo production of Norway spruce (Paques et al., 1992, Gorbatenko and Hakman, 2001). The use of liquid medium is usually limited to the proliferation phase, maturation cannot be easily achieved in the liquid medium (Paques et al., 1995). Many trials thus have been conducted to combine proliferation in liquid medium with maturation on a solidified one. Although the production of mature embryos and emblings was successful in a few cases, these protocols are rather complicated to be automated.

Successful use of the polypropylene membrane rafts (LifeRafts, Osmotec, Israel) was published for a number of tissue cultures (Luckett et al., 1991, Watad et al., 1995, Paek et al., 2001). Cultivation on membrane rafts floating on liquid medium could have a great potential to improve the development of coniferous somatic embryos. Beside the numerous problems with the cultivation of Norway spruce embryogenic cultures in liquid media, long vacuolated suspensor cells of embryogenic cultures are sensitive to the damage made by forceps during transfer to fresh medium. Therefore, as entire rafts with cultures are transferred to fresh liquid medium, embryogenic cultures are not touched from the proliferation stage untill the stage of mature embryos. Moreover, membrane rafts could combine the advantages both of solidified and liquid media: the cultures are sufficiently aerated, the exchange of compounds on the tissue-medium interface is enhanced, and medium can be rapidly replenished or replaced with minimal disturbance of embryogenic tissue.

Firstly, this work focused on the possibility of the use of polypropylene membrane rafts for the cultivation of Norway spruce embryogenic cultures and the production of mature somatic embryos. Different cultivation techniques were compared. The cultures grown on membrane rafts were further used for the evaluation of benefits and drawbacks of the insertion of a pre-maturation phase on PGR-free medium. Finally, we studied the effects of PEG in maturation medium on the embryo yield, quality and germination frequency.

2. Material and methods

Embryogenic cultures of Norway spruce (*Picea abies* L. [Karst.]) were obtained as a gift from laboratories in France (AFOCEL, Nangis, Dr. Paques) and Austria (ÖF Seibersdorf, Dr. Wilhelm), or induced from immature or mature zygotic embryo in our lab. The embryogenic cultures proliferated at 24 °C in total darkness on agar-solidified GD medium (Gupta and Durzan, 1986), containing 5 µmol 2,4-D, 2 µmol kinetin, and 2 µmol BAP. Twelve cell lines were selected for this study that differed in their embryogenic capacity (the ability to develop mature somatic embryos able to germinate). Both the age of embryogenic cultures (0.5 to 12 years since induction), and the geographical origin of primary explant (lowlands to alpine region) varied.

Cultures induced and maintained on agar-solidified media were transferred onto membrane rafts (Sigma), agar-solidified and liquid media (Vágner et al. 1998, 2000, 2001). Development and maturation of somatic embryos were started either with ABA alone, or with ABA and 1.87 – 5 % (w/v) polyethylene glycol (PEG 4000). This phase followed directly after the proliferation phase (during which the medium contained 2,4-D and cytokinins), or after the pre-maturation phase (1 week, no plant growth regulators), which was inserted between proliferation and maturation stages. Development and maturation of somatic embryos depended on the maturation treatment and cell line used, and took 4 – 7 weeks. Mature somatic embryos then were desiccated for 3 weeks in high relative humidity (HRH > 97 %, 18 °C, 12h/12h dark/light). Desiccated somatic embryos germinated on ¼-strength agar-solidified GD medium with 0.5 % (w/v) sucrose and 0.5 % (w/v) activated charcoal.

Development of somatic embryos (number, speed of development, and quality expressed as a germination ratio), and germinating plantlets was evaluated with the use of computer image analysis (Lucia, var. 4.61, Laboratory Imaging, Czech Republic).

3. Results and discussion

3.1 Membrane rafts compared to other cultivation techniques

The use of membrane rafts compared to solidified media brought a number of benefits. A number of mature embryos significantly increased in 7 of 12 tested cell lines (Figure 1). Synchronization of the process was enhanced, and the time necessary to reach the stage of mature embryo was shortened (the difference was 6 days on average). Just the shortening of the

maturation period, during which the cultures are exposed to high exogenous ABA concentration, is supposed to be crucial for further 'normal' seedling development. No difference in germination frequency was observed in cell lines with higher embryogenic capacity (Figure 2). Germination frequency of cell lines with low embryogenic capacity increased. Probably, this phenomenon could be ascribed to the better quality of somatic embryos grown on membrane rafts.

Compared to solidified media, routine passage of the cultures grown on membrane rafts is less time consuming, as the cultures are not transferred to fresh media individually, but with the whole raft. Moreover, there is probably a better exchange at the medium – embryogenic tissue interface in this system, because the cultures could be transferred at slightly longer subcultivation intervals. The relatively high price of membrane rafts together with their low durability remained the only drawback of this system.

The ability of different cell lines to proliferate in liquid medium varied markedly; a few of them did not change morphological appearance even after a long cultivation in liquid medium. On the other hand, cultivation of all embryogenic cell lines in liquid maturation medium resulted in severe decrease of the number of developed somatic embryos, which, after desiccation, were able to germinate.

3.2 Pre-maturation (PGR-free) phase

Insertion of a pre-maturation phase (no PGR in the medium) (Bozhkov et al., 2002) between proliferation (+ 2,4-D, BAP, and kinetin) and maturation phase (+ ABA) resulted in further improvement of synchronization, the enhancement of speed of somatic embryo development, and an increase in the number of developed embryos (Figure 3). This improvement was more pronounced in cultures grown on membrane rafts compared to solidified media. We suppose that after pre-maturation phase the endogenous levels of auxins (2,4-D, IAA) could be lower in cultures grown on rafts due to facilitated flow from the culture to the liquid medium. In a similar way after pre-maturation stage, the endogenous levels of cytokinins in embryogenic tissue grown on membrane rafts were found to be lower compared to cultures grown on solidified medium (data not shown). The ABA is thus supplied to the embryogenic cultures with lower levels of endogenous cytokinins and IAA in the moment of ABA application. These endogenous hormonal levels (high level of ABA, low levels of auxins and cytokinins) seem to be beneficial for embryo development.

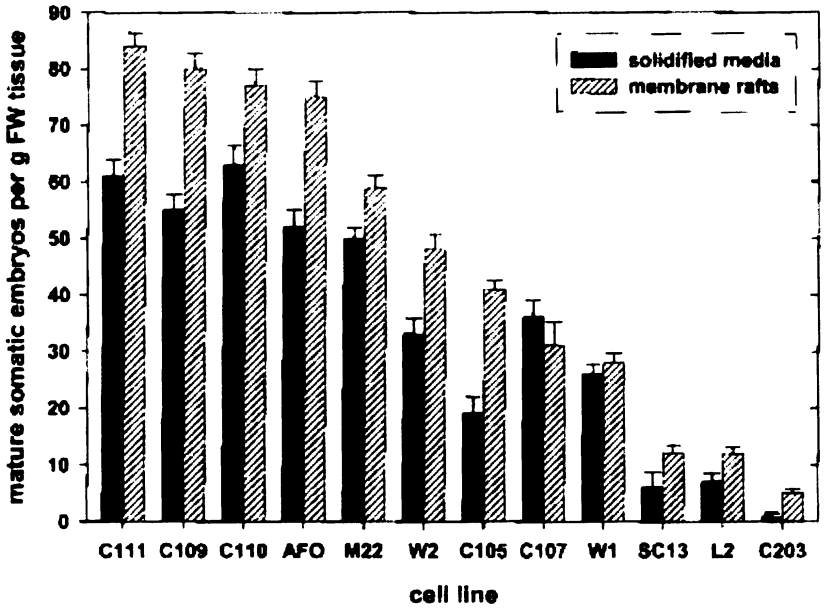

Figure 1: Comparison of yield of mature Norway spruce somatic embryos grown on solidified medium and on membrane rafts. 12 cell lines were used for the experiment. Bars indicate S_E.

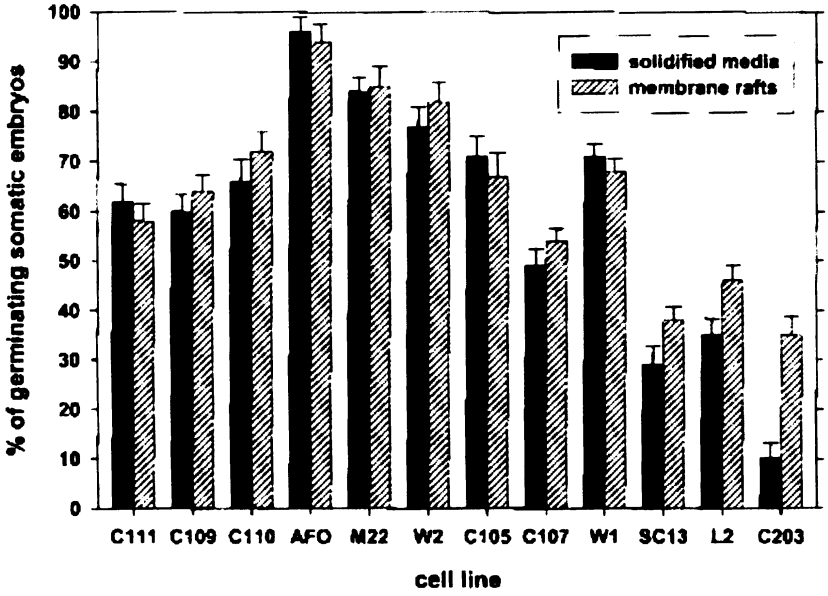

Figure 2: Comparison of germination frequencies of desiccated Norway spruce somatic embryos previously grown on solidified medium and on membrane rafts. The same cell lines were used as in figure 1. Bars indicate S_E.

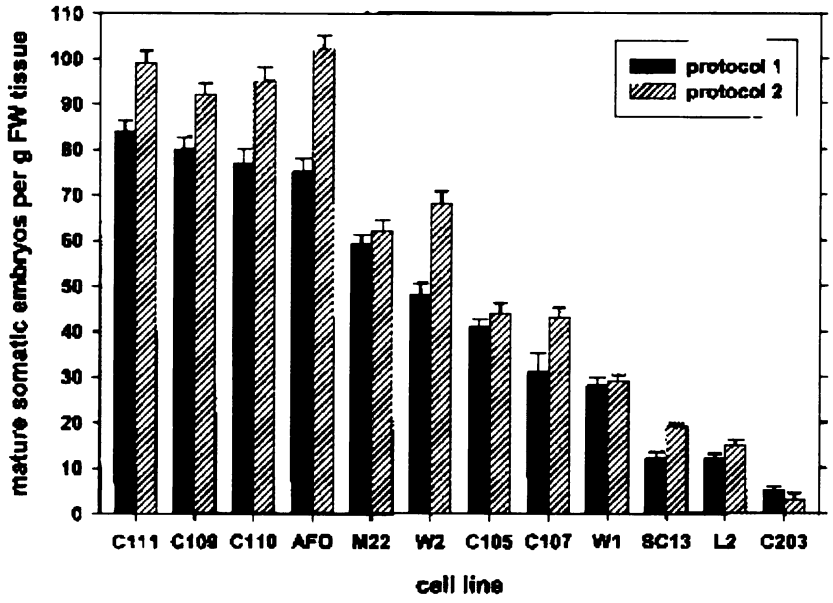

Figure 3: The yield of mature Norway spruce somatic embryos grown on membrane rafts. Protocol 1: Cultures transferred from proliferation medium (containing 5 µmol 2,4-D, 2 µmol kinetin and 2 µmol BAP) directly to maturation medium (20 µmol ABA).
Protocol 2: Cultures were transferred from proliferation medium to pre-maturation medium (no PGR, 1 week), and then to maturation medium. Bars indicate S_E.

3.3 Use of PEG in maturation medium

PEG is often added to the media for conifer somatic embryo maturation as a nonpermeating osmoticum, which increases embryo yield and enhances embryo development and maturation. On the other hand, the use of PEG in the maturation medium is sometimes criticized. Find (1997) observed PEG-related intercellular spaces below apical meristems of somatic embryos. Bozhkov and von Arnold (1998) reported a PEG-related decrease of germination frequency and inhibition of post-germinative root growth. In our experiments, PEG 4000 at low concentration (1.87 % (w/v) increased embryo yield and shortened maturation phase: higher PEG concentrations (up to 5 % (w/v)) had even slightly more beneficial effect in these aspects (Figure 4). Germination frequency of somatic embryos grown on 3.75 % (w/v) PEG remained unchanged, whereas 5 and 7.5 % (w/v) PEG 4000 slightly inhibited germination (Figure 5). A marked difference in sensitivity to PEG treatment was observed between different cell lines. The study of microscopic sections revealed no morphological aberrations of developing somatic embryos, which were grown on medium containing 3.75 % (w/v) PEG, and the resultant germinating plantlets. However, we did not study development of plantlets after 8 weeks of germination. Thus we cannot

exclude the possibility that higher concentration of PEG could interfere with further plantlet development. On the other hand, our results suggest that low concentration of PEG can improve Norway spruce somatic embryogenesis without negative, or with minimal impact, on embling performance.

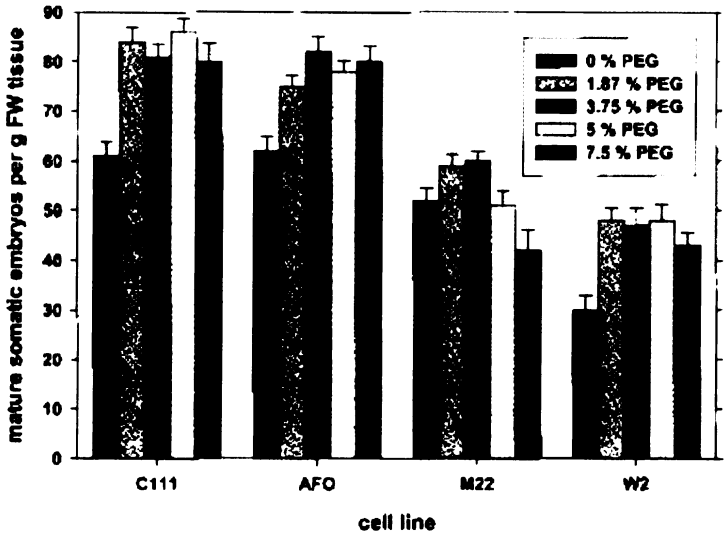

Figure 4: The effect of PEG on the yield of mature Norway spruce somatic embryos grown on membrane rafts. Maturation medium was supplemented with 0 – 7.5 % (w/v) PEG 4000. Bars indicate S_E.

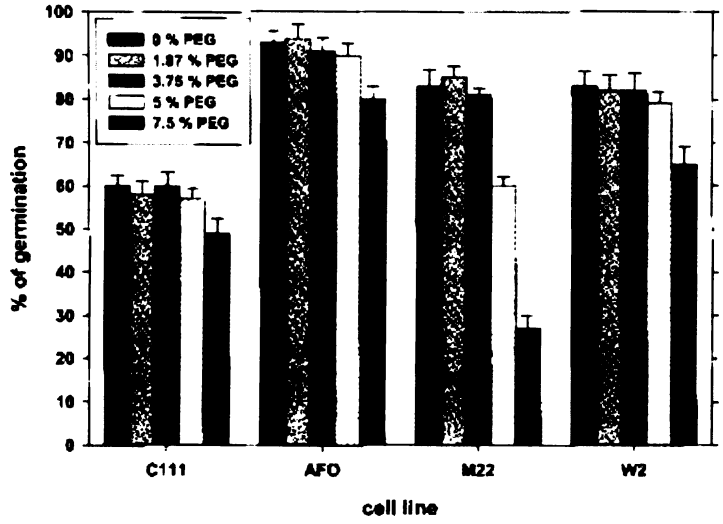

Figure 5: The effect of PEG on the germination of desiccated Norway spruce somatic embryos grown on membrane rafts. Maturation medium was supplemented with 0 – 7.5 % (w/v) PEG 4000. Bars indicate S_E.

Acknowledgement

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Expression of the gene encoding transcription factor PaVP1 differs in Norway spruce embryogenic lines depending on their ability to develop somatic embryos

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Abstract

Picea abies lines with contrast embryogenic capacities were characterized on anatomical level during proliferation and subsequent cultivation on maturation media containing abscisic acid (ABA). Expression of *PaVP1*, encoding an embryogenesis related transcription factor, determined by Northern hybridization, strongly differed among the lines. In non-embryogenic line, lacking any differentiated structures, the transcript level was undetectable on both proliferation and maturation media. Lines with low and high embryogenic capacity, containing meristematic centers connected with suspensor cells, were characterized by almost undetectable levels of the *PaVP1* transcript on proliferation media. After the transition onto maturation media containing ABA, the expression quickly and strongly increased. The *PaVP1* transcript level remained on a high level during five weeks of embryo development in the lines with high embryogenic capacity, while it dropped down in the line with low embryogenic capacity, in connection with advanced disintegration of meristematic centers. Removal of ABA from highly embryogenic culture after two weeks of maturation resulted in aberrant embryo development and rapid decrease in *PaVP1* expression, indicating the impact of exogenously supplemented ABA on both maintenance of *PaVP1* expression and continuation of embryo development. Since proper embryo development was accompanied with stable or increasing *PaVP1* expression in all experiments, it could be regarded as a good marker of this process. *PaVP1* probe hybridization with two separate transcripts is discussed in the respect of possible involvement of alternative splicing in regulation of PaVP1 protein synthesis.

Keywords: ABI3/VP1 transcription factors, alternative splicing, anatomy, embryogenic capacity, PaVP1, *Picea abies*

Abbreviations: PEG polyethyleneglycol, DIG digoxygenin

Introduction

Somatic embryogenesis of Norway spruce (*Picea abies* (L.) Karst.) belongs to the best-characterized systems in conifers. Embryogenic cultures, predominantly induced from immature zygotic embryos, are maintained on proliferation media containing growth regulators, auxins and cytokinins. Proliferating culture consists of embryogenic-suspensor mass, i.e. proembryos attached to the suspensor cells. Further development of early proembryos into fully developed embryos is induced after the transfer onto the maturation media containing abscisic acid (ABA; Tautorus et al. 1991). Significant portion of primarily induced embryogenic cultures is not able to produce mature somatic embryos; the majority of embryos stop the development before reaching the cotyledonary stage. These cultures are called developmentally arrested (Filonova et al. 2000).

In the process of somatic embryo maturation, ABA plays a key role. It corresponds to the situation in zygotic embryos, where beginning of seed maturation correlates with an increase in seed ABA content. This first peak of ABA is maternally derived, while the second peak of ABA accumulation (as documented in wild-type *Arabidopsis thaliana* seeds) depends on synthesis in the embryo itself (Karssen et al. 1983). This second peak is essential for the induction of dormancy, which is maintained despite a substantial decrease in ABA by seed maturity (Finkelstein et al. 2002). In embryogenic cultures of *Picea abies* ABA blocks proliferation of embryogenic-suspensor mass and induces further development of early proembryos (Bozhkov et al. 2002). ABA also stimulates deposition of storage compounds within the somatic embryo and induces embryo dormancy (Gutman et al. 1996).

Though the precise mechanism of ABA action in embryo/seed development remains unknown, it is clear that ABA regulates directly or indirectly expression of many genes. There are several genes in *Arabidopsis thaliana*; *ABI3*, *ABI4*, and *ABI5*, which were proven to be involved in ABA signal transduction pathway during seed development. *ABI3* encodes a transcription factor of B3 domain (Gazzarrini and McCourt, 2001), an orthologue of maize *VIVIPAROUS1* (*VP1*; Giraudat et al. 1992). ABI3 transcription factor acts in combination with ABI4 and ABI5 to regulate seed ABA response and late embryogenesis in *Arabidopsis* (Söderman et al. 2000). *PaVP1*, an orthologue of *ABI3* gene in *Picea abies*, was isolated by Footitt et al. (2003). They demonstrated its expression by semi-quantitative RT-PCR in highly embryogenic line. Relatively high levels of the *PaVP1* expression were documented already on the proliferation media without ABA, reaching about 30 % of the maximal expression, which was detected in early cotyledonary embryos (Footitt et al. 2003).

In this study, the expression of *PaVP1* was determined in four *Picea abies* lines with contrast embryogenic capacities to evaluate the impact of *PaVP1* expression on embryo maturation, and to assess a potential use of *PaVP1* as a marker of embryogenic capacity. The expression of *PaVP1*, determined by Northern hybridization, was supplemented with anatomical analysis of the cultures. The changes of *PaVP1* transcript level during maturation of individual cultures and the effect of ABA removal on both morphology of the culture and *PaVP1* expression are discussed in the respect of PaVP1 role in embryo development.

Results

Morphological characterization of lines with contrast embryogenic capacity

Non-embryogenic line C101N

Callus-like culture of C101N line propagated on the proliferation medium was white-yellow and consisted of uniform, more or less isodiametric cells, which did not form organized structures (Fig. 1A). After the transfer to the maturation medium no structural changes could be observed in the culture, whose proliferation gradually ceased. Prolonged cultivation under maturation conditions induced browning connected with accumulation of phenolic compounds, followed by extensive cell death (not shown).

Line C203 with low embryogenic capacity

Proliferating C203 culture was characterized by the presence of properly developed somatic proembryos attached to the suspensor cells (Fig. 1B). More than 99 % of these proembryos failed to undergo further development under maturation conditions. During three weeks of maturation, the majority of proembryonic regions expanded into meristematic clusters (fig. 1C), which further disintegrated during prolonged cultivation. The cells in the clusters successively lost their meristematic activity.

Line AFO 541 with high embryogenic capacity

Proliferating culture AFO 541 consisted of meristematic centers connected with suspensor cells (Fig. 1D). The majority of proembryos developed into mature cotyledonary somatic embryos, which were capable to germinate. Typical stages of zygotic embryo development, from proembryos to early cotyledonary embryos, could be observed during five weeks under maturation conditions (Fig. 1E, F). During the sixth week of maturation, only the elongation of cotyledons was observed with no further changes in embryo anatomy (data not shown).

Expression of *PaVP1*

The probe prepared from the sequence encoding the B3 domain of PaVP1 hybridized on Northern blots with two distinct bands in the majority of samples (Fig. 2). The changes in intensity of these two signals had an equal trend with more pronounced differences/extremes in the case of the longer (upper) one. The size of this longer band corresponded to the *PaVP1* transcript (2760 nucleotides long; Footitt et al. 2003) being situated between the band of 18S and 25S rRNAs (1808 and 3374 nucleotides long, respectively; Fig. 2A). Therefore, in the following text, the upper band is referred as *PaVP1* transcript, and the "lower band" is commented separately.

Non-embryogenic line C101N

The level of *PaVP1* transcript in non-embryogenic line C101N was under the detection limit in all samples (Fig. 2B). The *PaVP1* probe weakly hybridized with the "lower band" with no pronounced changes in response to the transfer onto the maturation medium containing ABA (Fig. 2B).

Line C203 with low embryogenic capacity

The level of both *PaVP1* transcript and the "lower band" quickly increased after the transition of the culture onto the maturation medium (Fig. 2D). The signal intensity detected after three days remained more or less unchanged for two weeks and then

substantially decreased during the third week on the maturation medium. Later on, isolation of intact RNA from the culture was not successful.

Line AFO 541 with high embryogenic capacity

Very low, almost undetectable levels of *PaVP1* transcript in proliferating culture gradually increased by about two orders of magnitude during standard five weeks of maturation, and than the expression almost completely disappeared when cultivation continued for the sixth week (Fig. 2C). The "lower band" intensity was significantly higher on the proliferation medium as compared to the *PaVP1* transcript. In contrast, the increase in signal intensity during maturation was more pronounced in case of the *PaVP1* transcript. Gradual increase in intensity of the lower band during cultivation on the maturation medium was followed by a drop in the sixth week as in the case of the *PaVP1* transcript, but only to the level detected in the first week of maturation (Fig. 2C).

The effects of ABA removal on embryo development and expression of *PaVP1*

In order to compare *PaVP1* expression in another line with high embryogenic capacity and to study the impact of ABA on *PaVP1* expression and embryo development, the line C110 was cultured on the maturation medium with ABA for five weeks, and in parallel the cultures were transferred onto the maturation medium without ABA after two weeks of maturation on the ABA-containing medium. Five weeks long maturation of C110 line on the ABA medium resulted in fully developed embryos comparable with that of AFO 541 line (Fig. 1G). Developing embryos transferred onto the ABA-free medium became vitrified and the majority of them underwent callus formation (Fig. 1H). The remaining, partially developed embryos were not capable to germinate (data not shown).

The expression of both *PaVP1* and the "lower band" in the C110 line kept on a high level from the first week on the maturation medium containing ABA, reaching its maximum after five weeks (Fig. 2E, F). Removal of ABA resulted in almost complete disappearing of *PaVP1* transcript in both the whole culture (not shown) and separated embryos (Fig. 2G). The "lower band" remained on relatively high level.

Figure 1

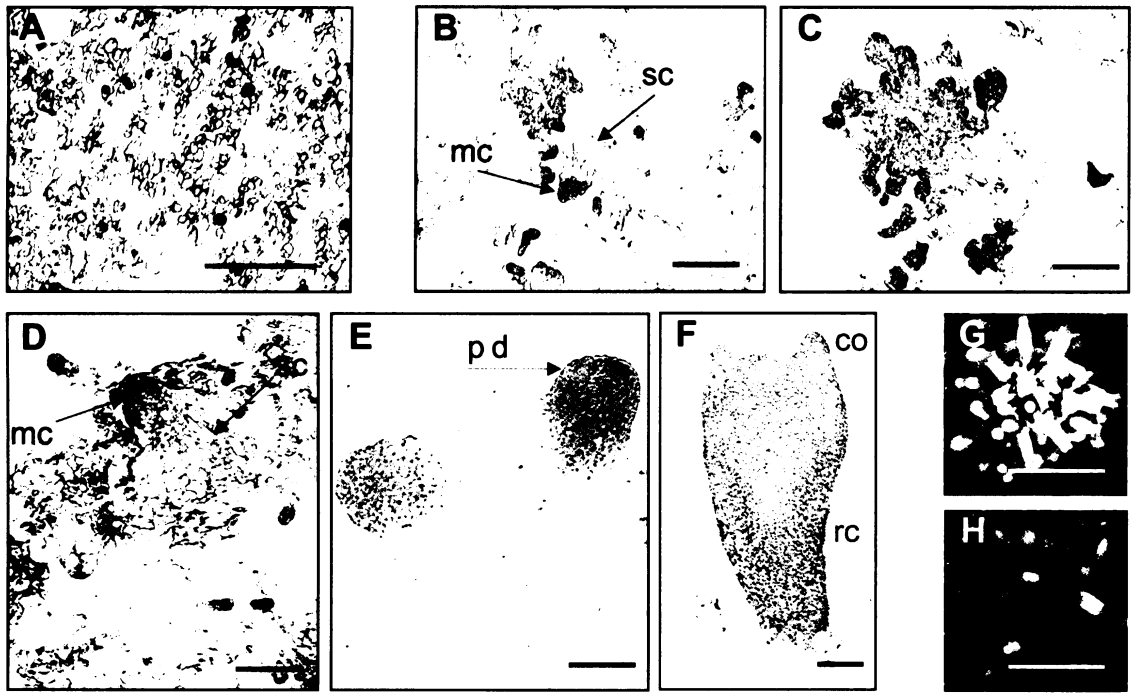

Figure 1. Anatomy and macroscopical view of Norway spruce lines growing on the proliferation and maturation media. Staining with alcian blue and nuclear fast red; microscopy in bright field; proembryos and embryos in longitudinal sections. **A**. Non-embryogenic line C101N during cultivation on proliferation media. **B**. Line C203 with low embryogenic capacity during cultivation on proliferation media; mc - meristematic cells (red stained), sc - suspensor cells (blue stained). **C**. Line C203 with low embryogenic capacity after three weeks on maturation media. **D**. Line AFO 541 with high embryogenic capacity during cultivation on proliferation media; mc - meristematic cells (red stained), sc - suspensor cells (blue stained) **E**. Developing somatic embryos of AFO 541 after three weeks on maturation media – early somatic embryo; pd – protoderm. **F**. Early cotyledonary somatic embryo of AFO 541 after five weeks on maturation media; co – cotyledons, rc – root cap. **G**. Macroscopical view of line C110 with high embryogenic capacity after five weeks on maturation media. **H**. Macroscopical view of line C110 with high embryogenic capacity after 2 weeks on maturation media with ABA followed by three weeks on maturation media without ABA. **A-F**, bar represents 200 μm; **G**, **H**, bar represents 5 mm.

Figure 2

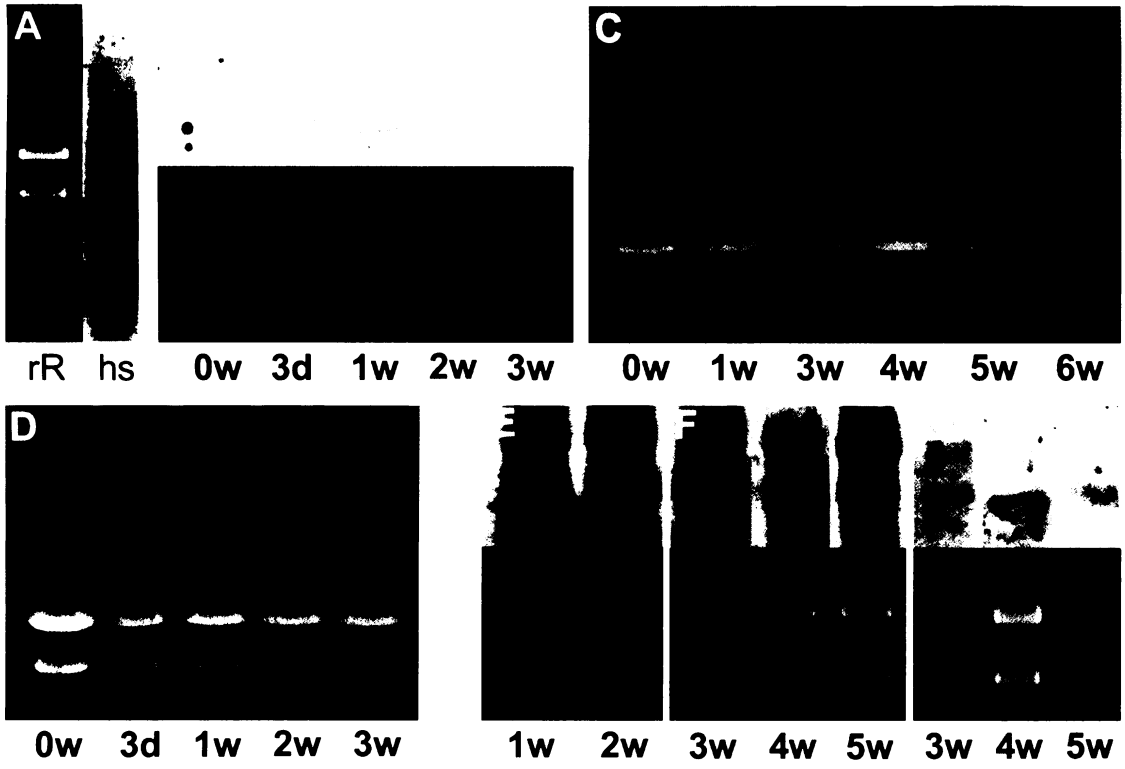

Figure 2. Expression of *PaVP1* in Norway spruce lines growing on the proliferation and maturation medium. Northern blots with total RNA (20 μg per line) isolated from non-fractionated culture (**B-F**) were hybridized with DIG labelled fragment (450 bp) of *PaVP1* gene. RTG films (the upper part of the pictures on bright background) were exposed to reach equal intensity of internal hybridization standard present on all membranes (not shown). Comparable loading of samples was confirmed by fluorescence of ethidium bromide stained rRNA (the bottom part of the pictures on the dark background) **A**. Comparison of the position of hybridizing signals (hs) with the ribosomal RNA bands (rR); 25S rRNA (the upper band; 3374 nucleotides) and 18S rRNA (the lower band;1808 nucleotides). **B**. Non-embryogenic line C101N. **C**. Line C203 with low embryogenic capacity. **D**. Line AFO 541 with high embryogenic capacity. **E-G**. Line C110 with high embryogenic capacity. After initial two weeks of maturation with ABA (**E**) the culture either continued cultivation on ABA containing medium (**F**) or was further cultivated on the medium without ABA (**G**; RNA was isolated from separated embryos). Days (**d**) or weeks (**w**) of cultivation on the maturation medium are indicated.

Discussion

Is the transcript of *PaVP1* alternatively spliced?

Expression of *PaVP1* increased during maturation in both lines with high embryogenic capacity, C110 and AFO541, culminating after five weeks, at the time when the embryos developed into the early cotyledonary stage. Similar trend in *PaVP1* expression was also demonstrated by Footitt et al. (2003) in 95:88:22 line, which developed embryos by about two weeks earlier, but the peak in *PaVP1* expression also correlated with the early cotyledonary embryo stage. Despite the similar trend, the relative transcript levels detected in our study by Northern blot hybridization strongly differed from that determined by semi-quantitative RT-PCR by Footitt et al. (2003). In our study, the maximum level of *PaVP1* transcript in cotyledonary embryos was by about two orders of magnitude higher as compared to the level on the proliferation media. In contrast, Footitt et al. (2003) demonstrated relatively high levels of *PaVP1* transcript already in the proliferation phase, and the expression increased only approximately three times towards the peak at the end of maturation in their study. Though the discrepancy might be explained by different character of the lines, used in the two studies, and differences in the cultivation conditions, there is another possibility related to the nature of *PaVP1* probe hybridization on Northern blots. Since the method of RT-PCR could not distinguish between transcripts of variable length, both RNAs hybridizing with *PaVP1* probe might be amplified by RT-PCR at once. Moreover, the "lower band" levels resembled those detected by Footitt et al. (2003), much better than the levels of the longer *PaVP1* band.

The shorter hybridizing band could be either a product of alternatively spliced mRNA of *PaVP1* gene or might represent a transcript of some other B3 domain transcription factor. Since no sequences homologous to B3 domain of *PaVP1* were found in angiosperm species, we tested the hypothesis of cross-hybridization among genes encoding different B3 domain-containing proteins in *Arabidopsis thaliana.* Nucleotide sequence encoding B3 domain of ABI3 (acc. no. AT3G24650.1), a PaVP1 ortologue, was used for Blastn search (Altschul et al. 1997) in *Arabidopsis* transcripts database (TAIR; www.arabidopsis.org). The best matches with genes encoding transcription factors LEC2 (acc. no. AT1G28300.1), and FUS3 (acc. no. AT3G26790.1) shared maximal 85% and 83% identity with the *ABI3* gene in a short region of 65 and 56 nucleotides, respectively (data not shown). Since the whole length of *PaVP1* probe used for hybridization was about 450 nucleotides, cross-hybridization could be hardly expected with these genes. Therefore, the "lower band" more likely represents a product of alternative splicing, that was also demonstrated by the group of M. J. Holdsworth in wheat embryos, where the majority of *VP1* orthologue transcripts were spliced incorrectly, contained insertions of intron sequences or deletions of coding region, and did not have the capacity to encode full-length proteins (McKibbin et al. 2002). In contrast to the situation in wheat, the ratio between the *PaVP1* transcript and the "lower band" significantly changed depending on the culture stage, suggesting that the supposed alternative splicing might participate in regulation of full-length PaVP1 protein synthesis.

PaVP1 expression during maturation of embryogenic cultures

ABA failed to induce *PaVP1* expression in the non-embryogenic line C101N, while in any embryogenic line, application of ABA resulted in strong increase in *PaVP1* transcript level, in agreement with predominantly embryonic tissue specific

expression of *VP1* orthologues (Holdsworth et al. 1999). Increased levels of *PaVP1* transcript, detected in C203 line prior to disintegration of proembryonic culture on the maturation medium, indicated that the incapability of C203 culture to develop mature embryos was not probably connected with ABA/*PaVP1* signal transduction pathway. Moreover, we can deduce that increased expression of *PaVP1* was not sufficient condition to provide embryos maturation in the line with low embryogenic capacity, though expression of the gene encoding ABI3 (VP1 orthologue) was demonstrated to be intimately connected with the complex regulatory network controlling embryo development (To et al. 2006). Differences in *PaVP1* expression between the lines with high and low embryogenic capacity started to be significant as late as at the time, when the low embryogenic line already exhibited macroscopical and anatomical developmental alterations compared to the line with high embryogenic capacity. Therefore, the expression of *PaVP1* cannot be used as a marker for evaluation of the embryogenic capacity of the lines.

Strongly impaired embryo development, resulting from removal of ABA from the maturation medium was accompanied by dramatic decrease in *PaVP1* expression in the C110 line with high embryogenic capacity. The *PaVP1* transcript was detected neither in the whole culture nor in selected malformed embryos on the medium without ABA. Therefore, exogenously supplemented ABA was necessary for both maintenance of *PaVP1* expression and proper embryo development. That is in agreement with the role of maternaly-derived ABA in the induction of embryo maturation during seed development, first described in *Arabidopsis thaliana* (Karssen et al. 1983). Malformations of embryos developed after ABA removal, and their inability to germinate might be connected with the drop in *PaVP1* expression. However, maize or *Arabidopsis thaliana* plants with mutated *VP1/ABI3* gene could develop embryos capable to germinate (precociously, without the period of dormancy; Hoecker et al. 1995, Koorneef et al. 1984). Therefore, development of impaired embryos was more likely the direct effect of ABA removal than the consequence of decreased *PaVP1* expression. On the other hand stable or increasing expression of *PaVP1* accompanied proper maturation of somatic embryos in all experiments, so it could be regarded as a good marker of this process.

Materials and Methods

Plant material. Embryogenic line of Norway spruce (*Picea abies* (L.) Karst.) AFO 541 was obtained as a gift from AFOCEL (France). The embryogenic lines C203 and C110 and non-embryogenic line C101N were induced from mature zygotic embryos as described by Vágner et al. (2005).

Cultivation. The cultures were grown on media according to Gupta and Durzan (1986), solidified with 0.75% (w/v) agar, and adjusted to pH 5.8 prior to autoclaving. Cultures were kept in darkness at 24°C. Proliferation medium was supplemented with 5μM 2,4-dichlorophenoxyacetic acid, 2μM kinetin, 2μM benzylaminopurine, and 30 g/l sucrose. The maturation medium for somatic embryo development lacked auxins and cytokinins, and contained 20μM ABA and 3.75% (w/v) polyethylene glycol (PEG) 4000. PEG solution was autoclaved separately and mixed with the remaining medium after autoclaving. The cultures were subcultivated weekly onto fresh media in Magenta vessels. From the proliferation medium the cultures were transferred directly to the maturation medium.

Anatomical analysis. Paraffin sections (12 μm thickness) of maturing cultures were prepared according to Johansen (1950). The sections were stained by the two-step procedure using alcian blue and nuclear fast red as described by Svobodová et al. (1999) Alcian blue is specific to polysaccharides of the cell wall and nuclear fast red counterstains chromatin structures.

PaVP1 expression analysis. RNA was isolated from 150 mg of culture fresh weight according to Stiekema et al. (1988). The total RNA (20 μg per line) was separated on agarose gel electrophoresis (Logemann et al. 1987), blotted onto the Nylon+ membrane (Roche), and crosslinked by UV radiation (1200 kJ/cm^2). The whole hybridization and detection procedure was done according to The DIG System Users Guide for Filter Hybridization (Roche/Boehringer Mannheim). The membranes were hybridized with digoxygenin (DIG) labeled probe overnight in high salt buffer and extensively washed to reach maximum stringency. Thereafter the blots were incubated with anti-DIG antibody (Fab fragments; Roche) conjugated with alkaline phosphatase diluted 1:15000 in blocking solution. After extensive washes of nonspecifically bound antibody, the blots were equilibrated in detection buffer and incubated with chemiluminiscent substrate (CDP-Star CL-AP Substrate, Novagen; 1ml per 100 cm^2 of membrane area) in a plastic wrap. RTG films attached to the wrapped blots were exposed for 3 to 15 minutes to reach equal intensity of internal hybridization standard present on all membranes.

For probe preparation, the fragment of *PaVP1* gene (containing the sequence encoding B3 conservative domain) was amplified with specific primers VP12US and VP12DS (Footitt et al. 2003) from cDNA prepared with anchored oligo-T_{23} primer and RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas). The identity of the fragment was confirmed by sequencing. The DIG labeled probe was prepared by PCR in the presence of dUTP-DIG according to the manufacturer instructions (Roche).

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