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DEVELOPING A MODEL SYSTEM IN VITRO TO UNDERSTAND TRACHEARY ELEMENT DEVELOPMENT IN DOUGLAS-FIR (PSEUDOSTUGA MENSZIESII)

Karthik V. Pillai[1] , Armando G. McDonald[1], Francis G. Wagner[1]

ABSTRACT

Callus cells were initiated on cambial strips obtained from 4 to 8 y old Douglas-fir *(Pseudostuga menziesii)* trees, cultured on solidified Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyaceticacid (2,4-D) and benzylaminopurine (BA). The cultures could be maintained by sub-culturing on fresh medium every four weeks. When the callus cells were subsequently transferred to liquid MS medium supplemented with different phytohormones, suspension cultures could be initiated and maintained by periodic sub-culture. Approximately 65% of the callus cells cultured on liquid MS medium supplemented with 2,4-D, when maintained for 6-7 weeks without sub-culture, differentiated to tracheary element (TE) like cells. The formation of TE like cells was confirmed histochemically by staining with phloroglucinol-HCl. Secondary thickening of the cell walls were confirmed by polarized light microscopy, which showed strong birefringence of the cell wall due the presence of crystalline cellulose. The presence of lignin was determined by pyrolysis-GC-MS and FTIR spectroscopy. The lignin content in differentiated cell wall samples was quantified at 21% by the lignothioglycolic acid assay. Analysis of monosaccharide composition of cell wall samples after acid hydrolysis showed that the percentage of glucose, xylose and mannose had increased in the differentiated cell walls. These increases correspond to the formation of cellulose, glucomannan and xylan, primarily associated with secondary cell walls.

Keywords: Callus culture, cell differentiation, Douglas-fir, plant cell walls, tracheary elements

INTRODUCTION

Wood cells of softwoods are composed of 90-95 % longitudinal tracheids. Therefore, softwood properties are largely dependent on the chemical composition and structure of the cell wall of these tracheids (Sjöström 1993). The knowledge of the process of cell wall biogenesis will open immense opportunities for modifying wood, especially by using genetic engineering. Genetically modified trees could lead to a significant impact on forestry and production of pulp and paper (O'Connell *et al.* 1998). However, producing and assessing transgenic plants to find genes that alter wood properties in a desired way can be tedious and time consuming. An in vitro system would therefore be extremely useful for studying the effects of introduced genes on tracheid differentiation and cell-wall biosynthesis (Möller *et al.* 2003).

Tracheary elements (TE) are cells with localized thickenings of the secondary cell wall with a reticulate, spiral, annular or pitted pattern which is their most characteristic morphological feature (Fukuda 1992). The formation of TEs is a dramatic case of cytodifferentiation in higher plants and

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can be used as a model system for the physiological and biochemical study of cytodifferentiation (Fukuda and Komamine 1980). Induction of TE formation on callus cells grown on agar medium has been reported on a wide range of plants (Fukuda 1992). Many plants, both angiosperms, and gymnosperms have been used as systems for studying TE formation *in vitro.* Though many model systems of TE differentiation are available, most of the work has been focused on angiosperms and detailed information on factors affecting *in vitro* TE formation is lacking for gymnosperms. However, from the studies done so far it is known that various growth factors and medium components influence differentiation of gymnosperms (Havel *et al.* 1997, Möller *et al.* 2003, Washer *et al.* 1977). To our knowledge, no report(s) on in vitro TE differentiation of Douglas-fir have been observed.

Genetic studies associated with secondary cell wall formation are now mostly carried out on herbaceous species like *Zinnia elegans, Arabidopsis thaliana* (L.) Heynh. (Arabidopsis) and *Nicotiana tabacum L.* (tobacco) (Chaffey 1999a, Chaffey 1999b). Relatively small genome size and ease of genetic transformations using *in vitro* systems make it advantageous to use Arabidopsis and tobacco as model plant systems. The *Arabidopsis* genome has been completely sequenced, and it is the best genetically characterized plant with a genome size of 1.25 x 10^8 base pairs (The Arabidopsis Genome Initiative 2000). Though the above mentioned herbaceous species have significantly contributed to the understanding of xylogenesis, it is ideal to have trees species as model plants to study xylogenesis and wood formation (Chaffey 1999a). Angiospermic tree species belonging to the genus *Populus* have been used as a model system for genetic studies on xylogenesis (Mellerowicz *et al.* 2001). The major advantage of using *Populus* as a model system is its relatively small genomic size of 5.5 x 10^8 base pairs, which is only about 5 times that of *Arabidopsis thaliana.* Another important advantage of *Populus* is its amenability to be transformed readily with *Agrobacterium*-mediated gene transfer systems (Kim *et al.* 1997).

Though the above-mentioned systems have clearly improved our understanding of the TE formation process, these systems are exclusively based on angiosperms. Although *Populus* is a promising system to study xylogenesis, considering its distant relationship with gymnosperms, it is highly desirable to have a system involving a gymnosperm to explain TE formation. This study aims to establish an *in vitro* tissue culture system to study high yield TE formation in the gymnosperm Douglas-fir *(Pseudostuga menziesii)*, an important softwood timber species in the Pacific North West.

MATERIALS AND METHODS

Callus Initiation: Cambial tissue isolated from young Douglas-fir trees (4–8 year old) were used to initiate callus formation in a suitable plant tissue culture medium. The explants were isolated from branches cut into small segments of 5-6 cm followed by surface sterilization. The outer bark of the surface sterilized branch segments were peeled off and thin strips of inner bark or phloem with vascular cambium tissue (2.0 cm x 0.5 cm) including the cambial cell layers (termed here as cambial strips) were excised using a sterile scalpel. The cambial strips were further inoculated in an upright position on MS medium (Murashige and Skoog 1962) (MP biomedicals) solidified with 7 g/l agar, containing 3 mg/l 2,4-D and 3 mg/l BA (Acros organics). The cultures were incubated in sealed Petri-plates in darkness at 24 ± 2° C. After 7-8 weeks the callus formed on the cambial strips were scraped off using a sterile scalpel and suspended in liquid MS medium without any phytohormones for 5 min. Small pieces of callus from the suspension were transferred to sterile nylon fabric (pore size – 36 µm; 2 cm x 2 cm), excess liquid removed, and transferred to fresh solidified MS medium supplemented with 3 mg/l 2,4-D and 3 mg/l BA in Petri-plates. The Petri-plates were then sealed and incubated in darkness at 24° C. After 4 weeks, small pieces of proliferating callus on nylon-discs were directly transferred to fresh medium. The sub-culturing process was carried out every 4 w to maintain callus growth.

Initiation of TE differentiation in suspension cultures: Fresh callus maintained on solidified media were used for initiating suspension cultures. Callus (3-4 g) was transferred to 125 ml Erlenmeyer flasks containing 40 ml of liquid MS medium supplemented with phytohormone combinations (2 mg/l 2,4-D, 2 mg/l 2,4-D + 2 mg/l BA and 2 mg/l naphthaleneacetic acid (NAA) and shaken on a rotary shaker at 70 rpm. Subculturing was carried out every 2 weeks. In the first subculture, 20 ml of the culture was transferred to 125 ml Erlenmeyer flasks with 20 ml fresh medium. For the second subculture, 10 ml of the first subculture was added to 30 ml of fresh medium. From the third subculture onwards, 5ml of the previous subcultures were transferred to 35 ml of fresh medium. All subcultures were carried out in aseptic conditions in 125 ml Erlenmeyer flasks and shaken on an orbital shaker at 70 rpm. To induce TE formation in suspension cultures, the rapidly dividing cells grown in liquid MS medium supplemented with growth regulators were maintained in the same medium without further sub-culture for an extended period of time (6-7 weeks)

Initiation of TE differentiation in solid medium: Activated carbon (AC) (Sigma-Aldrich) was used to induce TE formation in callus cells maintained on solidified medium. AC was used at concentrations of 2, 4, 6, 8 and 10 g/l with basal MS medium (4.4 g/l MS formulation and 30 g/l sucrose) without the use of any plant growth regulators. Callus grown on sustenance medium were transferred to Petri plates with the differentiation medium, and the cultures were incubated in darkness at 24°C for 6-7 weeks.

Microscopy: The callus and differentiated cells were examined with an Olympus BX51 microscope with polarizing and fluorescence capability and an Olympus DP70 digital camera for imaging. The illumination for light and polarized light microscopy was provided by either a halogen or a mercury lamp (model UL-H100HGAPO). An UV excitation filter cube (Olympus U-MWU2) was used for fluorescence microscopy.

Three types of stains were used: (i) phloroglucinol (1%w/v) in aqueous ethanol (95%) and few drops of 12 M HCl, (ii) *O*-toluidine blue (0.05% w/v) in 20 mM sodium benzoate buffer (pH 4.4), and (iii) safranin (1% w/v). Squash preparations of cells were made by careful homogenization of callus with a Trenbroek ground glass grinder to segregate clumps of cells. However, care was taken in order to avoid the rupture of cells. The cells were filtered onto a nylon mesh (pore size 36 µm, 5 cm x 5 cm) and stained.

Isolation of cell walls and preparation of wood samples for analysis: Cell walls of callus, differentiated cells and inner bark were isolated following the technique of Möller *et al.* (2003). The cells (2-3 g) were homogenized in 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (pH 6.8) containing 20 mM sodium metabisulfite using a Tenbroeck ground-glass tissue grinder. The cytoplasmic proteins were removed by treating the homogenized cells with a mixture of phenol-acetic acid-water mixture (2:1:1 w/v/v) and the removal of proteins was monitored with Ponceau 2R stain. Cytoplasmic starch contamination present in the cell wall preparations was removed as described by Carnachan and Harris (2000) using porcine pancreatic α-amylase in TRIZMA® buffer solution. Small wood flakes were excised from cross sections of the stems of 4-8 year old Douglas-fir trees. The flakes were cut into small pieces and further reduced in dimension by grinding with glass beads. Since starch could not be detected in the samples by iodine staining, starch removal step was not carried out. The ground wood samples were extracted with 80% aqueous ethanol for 48 h at 4º C. Following the extraction, samples were centrifuged and the supernatants discarded. The samples were washed three times with 80% aqueous ethanol by centrifugation. The pellets were then resuspended in 95% aqueous ethanol, centrifuged and the supernatant discarded. The pellets thus obtained were vacuum dried and stored for further analysis.

Analysis of cell wall polysaccharides: The isolated and purified cell wall samples were hydrolyzed, and the monosaccharides released were reduced and acetylated to alditol acetates (AA). The volatile AA were analyzed and quantified by GC-MS (Polaris Q, Thermofinnigan). The hydrolytic method used in this study was a 2 M trifluoroacetic acid (TFA) technique (Albersheim *et al.* 1967). TFA mainly hydrolyzes the amorphous hemicellulosic polysaccharides. The AA from the cell walls were separated on a BPX-70 capillary column (SGE, 30 m, 0.25 mm Ø) and a temperature program of 190ºC (1 min) ramped to 250ºC (20 min) at a rate of 2ºC/min.

Quantitative and qualitative analysis of lignin: For qualitative lignin analysis, the sample (50 µg) was packed into a quartz tube and pyrolyzed (SGE Pyrojector II, Ringwood, Australia) at 600ºC. The pyrolysis unit was coupled to a GC-MS (PolarisQ, Thermonfinnigan). Separation was achieved using a ZB-1 capillary column (Phenomenex, 30 m, 0.25 mm Ø) and a temperature program of 40ºC (2 min) ramped to 300ºC (10 min) at a rate of 5ºC/min. The compounds were identified by comparison with standards and their corresponding mass spectra. The chemical signatures of the functional groups in the cell wall samples (4 mg), especially for lignin, were analyzed using Fourier transform infrared (FTIR) spectroscopy (Avatar 370 FTIR spectrometer, ThermoNicolet) in the attenuated total reflectance (ATR, SmartPerformer cell) mode. The spectra were processed and analyzed with the Omnic software.

For quantitative lignin analysis, the method of Möller *et al.* (2003) was used. Sample (1.0 mg) was weighed into to a test tube and 1 M NaOH (1 ml) was added and incubated at 20ºC to dissolve the base soluble materials. To extract lignin from the cell wall samples, 2M HCl (900 µl) and thioglycolic acid (100 µl) (Acros organics) were added to the samples and heated at 100º C for 4 h. The thioglycolic acid lignin (TGAL) was dissolved from the cell wall using 1M NaOH and the absorbance of dissolved TGAL was measured at 280 nm against 1M NaOH background with a UV-VIS spectrophotometer (Beckman model DU640).

RESULTS AND DISCUSSION

Initiation of callus and suspension cultures: After 7 to10 days of inoculation, the Douglas-fir cambial strips started to swell, and after 3 to 4 weeks callus growth started to appear on the swollen strips. Of all the media used to initiate callus, the MS medium in combination with 3 mg/l 2,4-D and 3 mg/l BA induced the best response with 100% callus initiation on all inoculated cambial strips. The callus from cambial strips that were subcultured on to sustenance medium started to show profuse growth (Fig. 1a). The subculturing process had to be carried out every 4 weeks to sustain and multiply the callus. If the callus was not subcultured in 4 weeks, the nutrients were depleted, and the callus turned brown and gradually died. Therefore, timely subculture of callus was a prerequisite for maintaining cultures. After 3 to 4 subculture cycles, the callus started to show presence of lignin as indicated by phloroglucinol-HCl staining (Fig. 1b). However, secondary thickened cells of TEs were not observed. Callus cells could be maintained in liquid MS medium supplemented with plant growth regulators. Multiplication of callus was successful in liquid MS containing 2 mg/l 2,4-D; MS plus 2 mg/l 2,4-D and 2 mg/l BA; and MS + 2mg/l NAA. Subculturing the callus every 2 weeks was vital in maintaining the suspension cultures. The presence of lignin was detected in suspension-cultured cells after three subcultures, but secondary thickened cells were not detected. Cell morphology of suspension cultured cells was similar to that of cells found in callus maintained on solid medium (Fig. 3). Möller *et al.* (2003) reported that the ray parenchyma at the wound surface of the xylem strips from *Pinus radiata* swelled and underwent mitosis. The dividing ray parenchyma cells formed a friable layer of cells over the xylem strip and produced callus. Though the origin of callus was not studied in Douglas-fir, a process similar to that of *P. radiata* could be expected. The auxin to cytokinin ratio was always kept 1:1 (w/w) for callus initiation as

well as in the sustenance medium to facilitate callus growth. Intermediate ratios (close to 1:1) of auxin/cytokinin are known to support callus growth whereas, higher ratios of auxin/cytokinin are known to promote root formation and lower ratios are known to induce shoot formation (Banno *et al.* 2001). Exactly how the different auxin/cytokinin ratios induce such responses in plants is little understood (Banno *et al.* 2001).

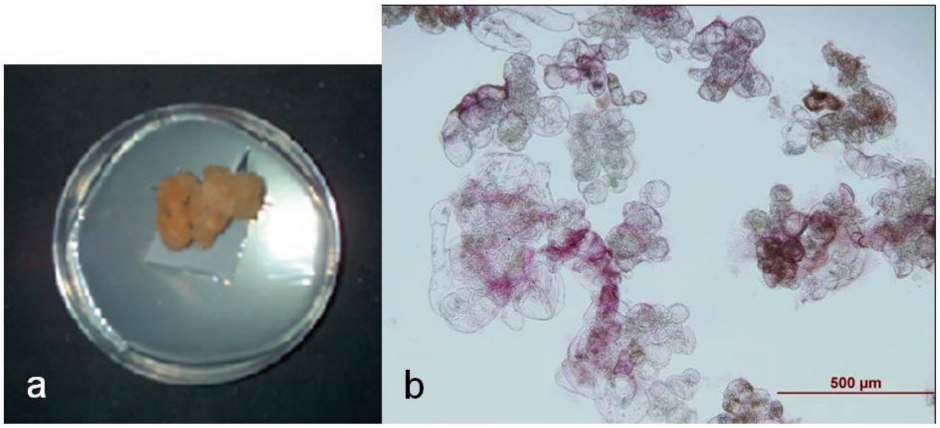

Figure 1. Subcultured cells showed profuse growth in solidified sustenance medium (a) and after several sub-cultures showed the presence of lignin as detected by pink coloration with phloroglucinol-HCl staining (b).

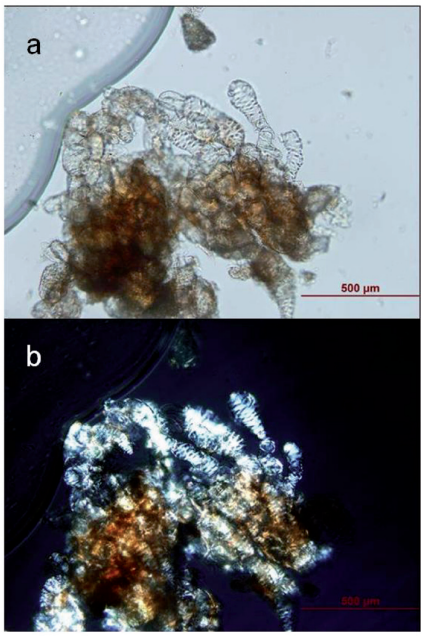

Figure 2. After about 6 to 7 weeks in culture on solidified MS medium 4 g/l AC, numerous TE like cells where found from regions of callus that turned pink on treatment with phloroglucinol-HCl stain. A group of cells with reticluate cell wall sculpturing can be seen (a). The same cells observed under polarized light showing birefringence (b).

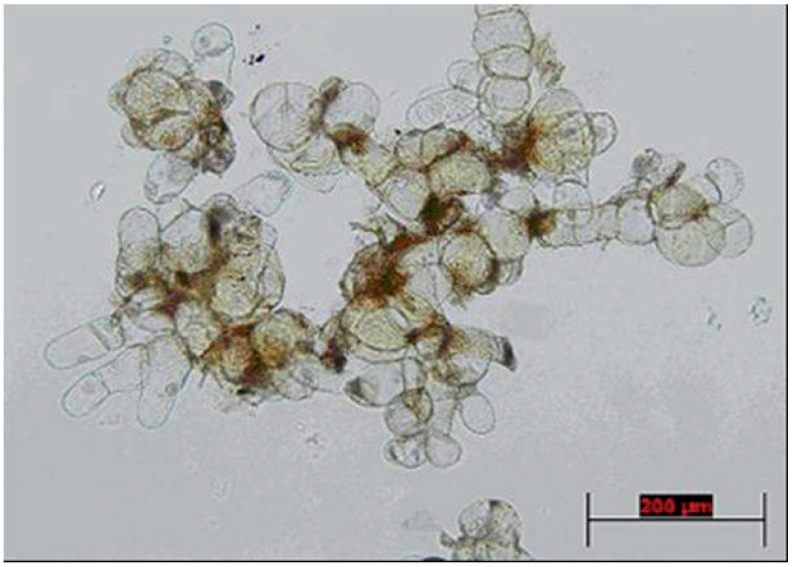

Figure 3. Non-differentiated cells from suspension cultures of Douglas-fir grown on MS medium supplemented with 2mg/l 2, 4-D and 2mg/l BA.

Cell differentiation: When callus cells were cultured on solidified MS medium supplemented with AC, callus growth slowed down and eventually ceased growing after about 2 weeks in the medium. The callus started producing lignin as observed by phloroglucinol-HCl staining. The presence of TE like cells were detected (very sporadically) within 4 weeks in solidified MS medium supplemented with 4 g/l AC. However, higher numbers of TEs started to appear within the callus after 6-7 weeks in culture. The TEs could be distinguished from normal cells by the presence of reticulate sculptured cell walls (Figure 2a). This was further confirmed by the strong birefringence shown by these cells under polarized light (Figure 2b). The cells in suspension cultures when sub-cultured frequently remained non-differentiated and continued to proliferate. However, after three subcultures, when the cells cultured on liquid MS medium supplemented with 2 mg/l 2,4-D were maintained in the same medium without further subculture for 6-7 weeks, the cells differentiated into TE like cells. About 65% of the cells observed were differentiated. Unlike the TEs formed on solidified medium, most of the cells in suspension cultures divided end to end forming strands of about 5 to 6 or more cells (Figure 4a-i). The formation of secondary cell wall was confirmed by the strong birefringence of the secondary cell wall under polarized light (Figure 4b,e,h) and due to the presence of reticulate cell wall sculpturing under fluorescence microscopy (Figure 4c,f,i). Cells cultured on liquid MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BA or 2 mg/l NAA failed to differentiate into TEs, but did show a strong presence of lignin as detected by phloroglucinol-HCl staining. The effect of phytohormones on *in vitro* TE formation of gymnosperms has not been studied in great detail. Nonetheless, a handful of authors have reported some details on gymnospermic species (Eberhardt *et al.* 1993, Möller *et al.* 2003, Ramsden and Northcote 1987, Savidge 1996, Webb 1981). In the present study with Douglas-fir, approximately 65% of suspension cultured cells could be differentiated into TEs in liquid MS medium supplemented with 2 mg/l 2,4-D as the lone phytohormone in the medium. None of the other treatments under the same conditions induced differentiation of callus cells to form TEs, indicating that 2,4-D is critical for *in-vitro* TE differentiation of Douglas-fir suspension cultured cells. Prolonged culture of the cells in the liquid medium for 6-7 weeks without subculture should result in the depletion of sucrose,

and a reduction in the concentration of 2,4-D. This depletion in the energy source and reduction of the auxin content may have contributed to the triggering of TE formation, however a detailed exploration of this effect was not done in this study. Ramsden and Northcote (1987) found that when suspension cultures of *Pinus sylvestris* were transferred from PRL-4 medium containing NAA and 2,4-D to a medium containing NAA and kinetin, TEs could be induced. Eberhardt *et al.* (1993) were also able to induce TE formation in *Pinus taeda* suspension cultures, when cells maintained in modified Brown and Lawrence medium (Brown and Lawrence 1968) supplemented with 11.0 µM 2,4-D were transferred to the same medium supplemented with 11.0 µM NAA. Therefore, swapping the auxin 2,4-D with NAA seemed to trigger TE formation in *P. sylvestris*, but the author failed to mention if TEs formed as a result of the replacement of 2,4-D with NAA or if it was due to the addition of kinetin in the medium. The common factor for both *P. sylvestris* and *P. taeda* is that, TEs formed when 2,4-D was removed from the medium. It is possible that high concentrations of 2,4-D may inhibit TE formation (Savidge 1996). On the contrary, Webb (1981) observed that TEs formed in suspension cultures of *Pinus contorta* in a medium supplemented with 4.5 µM 2,4-D and 4.6 µM kinetin. In the present study TEs could also be induced in Douglas-fir callus, when the callus was subcultured several times on sustenance medium and further transferred on to solidified MS medium supplemented with 4 g/l AC. The direct role of AC on tracheid differentiation or lignin production is unknown, but AC is known to absorb phenolic compounds and phytohormones, lower pH of the medium while autoclaving, and enhance sucrose hydrolysis (Fridborg *et al.* 1978, von Aderkas *et al.* 2002). Möller *et al.* (2003) found that callus cells maintained on P6-SHv medium supplemented with 4.5 µM 2,4-D and 4.4 µM BA when transferred to P6-SHv medium supplemented with 2 g/l activated charcoal differentiated into TEs (15-19 %) which were seen in nodules within the callus and on the surface of the callus. Up to 50% of the cells in the nodules were found to be differentiated cells.

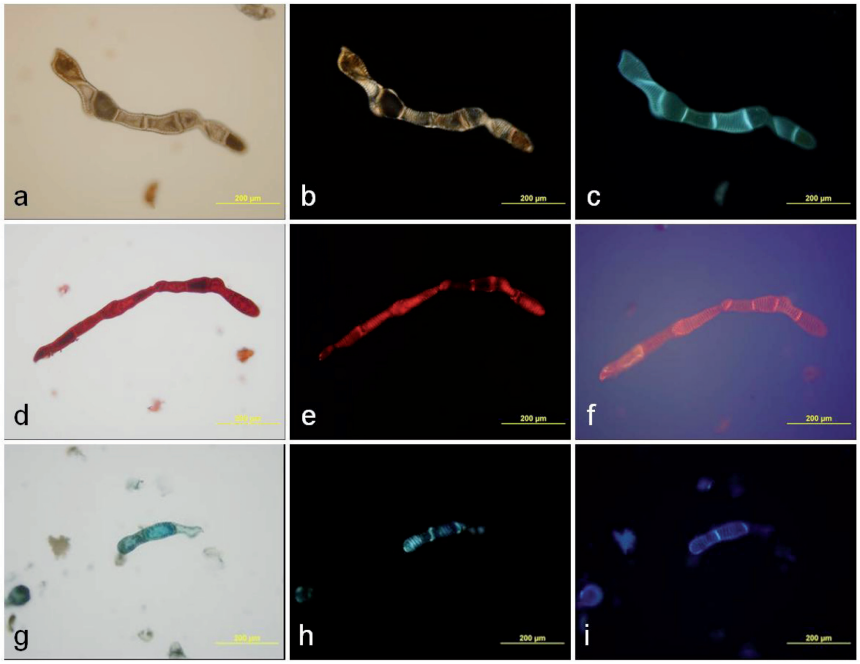

Figure 4. TE like cells (or longitudinal parenchyma cells) formed in suspension cultures grown in liquid MS medium supplemented with 2 mg/l 2, 4-D. (a), (b), and (c) are cells stained with phloroglucinol-HCl; (d), (e) and (f) cells stained with safranin; and (g), (h) and (i) cells stained with tolouidine blue. (a), (d) and (g) were observed under bright field mode, (b), (e) and (h) were observed under polarized light and (c), (f) and (i) were observed under epi-fluorescence light.

TFA hydrolysis: Carbohydrate analysis of TFA hydrolyzates of non-differentiated cells from solidified medium (MS supplemented with 2 mg/l 2,4-D and 2 mg/l BA) and suspension culture (liquid MS supplemented with 2 mg/l 2,4-D and 2 mg/l BA) respectively showed the presence of arabinose (48% and 43%) and galactose (27% and 32%) (Table 1). The glucose content was found to be low at 5.1% and 5.7% respectively, which was expected, since TFA is known to primarily hydrolyze hemicellulosic polysaccharides (Albersheim *et al.* 1967). Xylose (37%) was found to be the most abundant monosaccharide in TFA hydrolyzate from cell walls of differentiated cells from the suspension culture (liquid MS supplemented with 2 mg/l 2,4-D). Arabinose (30%) was found to be the second most abundant followed by mannose (13%), galactose (10%), glucose (6%), rhamnose (2.3%) and fucose (0.7%). The most abundant monosaccharide in the TFA hydrolyzates from cell walls of differentiated callus cultured on solidified MS medium supplemented with 4 g/l AC was arabinose (55%) followed by galactose (18%), xylose (17%), glucose (2.1%), mannose (1.8%), rhamnose (4.2%) and fucose (1.2%). Mannose (33%) was most abundant in Douglas-fir wood followed by xylose (23%), glucose (20%), galactose (14%), arabinose (7.8%), rhamnose (0.9%) and fucose (0.1%). In contrast to wood, arabinose (32%) was the most abundant in the TFA hydrolyzate from the inner bark (from strips), followed by galactose (22%), glucose (18%), mannose (13%), xylose (8.5%), rhamnose (5.5%) and fucose (1.4%). The TFA hydrolysis of the non-differentiated cells from both solid medium and suspension cultures were similar and showed a trend similar to that observed by Burke et al. (1974), who analyzed the primary cell walls of suspension cultured cells of Douglas-fir obtained from bud meristems. Moeller *et al.* (2003) had also observed a similar monosaccharide composition of the primary cell walls from callus cultures of *P. radiata.* In both the studies mentioned above, as in this study, the most abundant non-cellulosic polysaccharide was an arabinan. From the glycosyl linkage analysis, the preponderance of 5-linked and the occurrence of 3,5- and 2,3,5–linked arabinosyl residues were found to be due to the presence of a highly branched arabinan (Burke *et al.* 1974). A detailed study on the structure and composition of polysaccharides and the glycosyl linkage composition of the primary cell wall of suspension cultured Douglas-fir was done by Thomas *et al.* (1987). They arrived at the same conclusion as Burke *et al.* (1974), that the primary cell wall of Douglas-fir was more closely related to dicots than to graminaceous monocots. They also concluded that greater than 20% of Douglas-fir primary cell wall was composed of pectic polysaccharides (rhamnogalacturonan I, rhamnogalacturonan II and homogalacturonan) and the predominant hemicellulose is xyloglucan (Thomas *et al.* 1987). In this study, a low percentage of glucose was seen with TFA analysis which could be attributed to the fact that TFA preferentially hydrolyzes the amorphous hemicellulosic polysaccharides (Albersheim *et al.* 1967). Analysis of the TFA hydrolyzates of differentiated cell walls from suspension cultured Douglas-fir cells showed a decrease in arabinose and corresponding increase in xylose and mannose contents. The increase in mannose and xylose could be attributed to the formation of O-acetyl-glucomannan and xylan, which are known to be present in the secondary cell wall of softwoods (Fengel and Wegener 1989). The FTIR spectral peak at 1724 cm^{-1} corresponding to acetyl group, which is characteristic of O-acetyl-galactoglcomannan supports the fact that glucomannan was formed (Carpita *et al.* 2001). The glucomannans and xylans are known to account for approximately 20 to 25%, and 7%, respectively in softwoods (Fengel and Wegener 1989). An increase in glucose, mannose and xylose contents following cell differentiation to TEs have been previously reported in P. sylvestris and *P. radiata* (Möller *et al.* 2003). The increase in mannose content in *P. sylvestris* was attributed to the formation of a secondary cell wall glucomannan (Ramsden and Northcote 1987). In this study, monosaccharide composition of the cell walls from differentiated cells obtained from solidified medium supplemented with activated charcoal was similar to that of the non-differentiated primary cell walls. This could be due to the fact that the number of differentiated cells in solidified medium was much lower than that of the suspension culture. Therefore the overall cell wall sample is more representative of the primary cell wall.

Table 1. Monosaccharide composition (% of TIC) of cell wall preparations from non-differentiated and differentiated cells grown on solidified and liquid medium, Douglas-fir wood and inner bark analyzed by GC-MS following hydrolysis with triflouroacetic acid. TFA mainly hydrolyses the non-cellulosic/amorphous polysaccharides. The hydrolysed sugars were acetylated to corresponding alditol acetates for analysis by GC-MS.

Source of cell wall	Glucose (mol%)	Galactose (mol%)	Mannose (mol%)	Xylose (mol%)	Arabinose (mol%)	Fucose (mol%)	Rhamnose (mol%)
Non-differentiated (solidified medium)	5.1	27.1	1.1	8.3	48.0	3.4	7.0
Non-differentiated (suspension culture)	5.7	31.8	1.7	6.8	42.8	0.1	9.1
Differentiated cells (solidified medium with AC)	2.1	18.3	1.8	17.2	55.2	1.2	4.2
Differentiated cells (liquid medium with 2 mg/l 2,4-D)	6.0	10.2	13.2	37.1	30.4	0.7	2.3
Douglas-fir wood	20.2	14.4	33.4	23.1	7.8	0.1	0.9
Douglas-fir inner bark	17.7	22.1	12.8	8.5	32.0	1.4	5.5
Douglas-fir cell wall of Burke et al. (1974).	21.0 (non-cellulosic)	20.0	1.3	13.0	34.0	2.7	6.7

Qualitative and quantitative analysis of lignin: Pyrolysis GC-MS was used to establish the presence of lignin in samples (Figure 5 and Table 2). A total of 15 compounds corresponding to pyrolysis products of softwood lignin could be identified from the different samples tested. Ten compounds were detected at very low levels (each peak < 1% of total ion current (TIC) from non-differentiated cells and xylem strips of Douglas-fir and listed in Table 2. In addition to the ten compounds mentioned above, six additional compounds (vanillin (11), cis-isoeugenol (12), *trans*-isoeugenol (13), homovanillin (14), guaiacyl-CH=C=CH_2 (15) and guaiacyl acetone (16)) were all detected in high levels (each peak between 2-8% of TIC) in the differentiated cells obtained from suspension culture (Figure 5) and solidified medium.

Table 2. Compounds identified as lignin degradation products from the cell wall samples by pyrolysis GC-MS, and its percentages determined from the % TIC of the corresponding signals

Peak No.	Retention time (min)	Compound	Non-differentiated callus cells (%)	Differentiated cells suspension culture (%)	Differentiated cells solid culture (%)
1	14.52	Phenol	2.0	6.0	4.1
2	17.48	2-methyl phenol	0.9	1.9	1.4
3	18.30	4-methyl phenol	0.8	1.3	3.0
4	18.59	Guaiacol	0.9	3.9	3.3
5	21.36	Dimethyl phenol	Trace	0.7	0.6
6	22.81	1-methyl guaiacol	Trace	4.1	3.1
7	23.79	vinyl phenol	Trace	1.0	1.0
8	26.09	1 ethyl phenol	Trace	1.2	1.5
9	27.20	Vinyl guaiacol	0.9	6.2	5.6
10	28.78	Eugenol	Trace	1.8	0.9
11	29.54	Vanillin	-	1.2	0.5
12	30.45	Cis-isoeugenol	-	1.0	0.6
13	31.77	Trans-isoeugenol	-	5.1	3.4
14	32.44	Homovanillin	-	1.0	0.6
15	32.77	G-CH=C=CH_2	-	0.3	0.2
16	33.82	Guaiacyl acetone	-	0.5	0.4

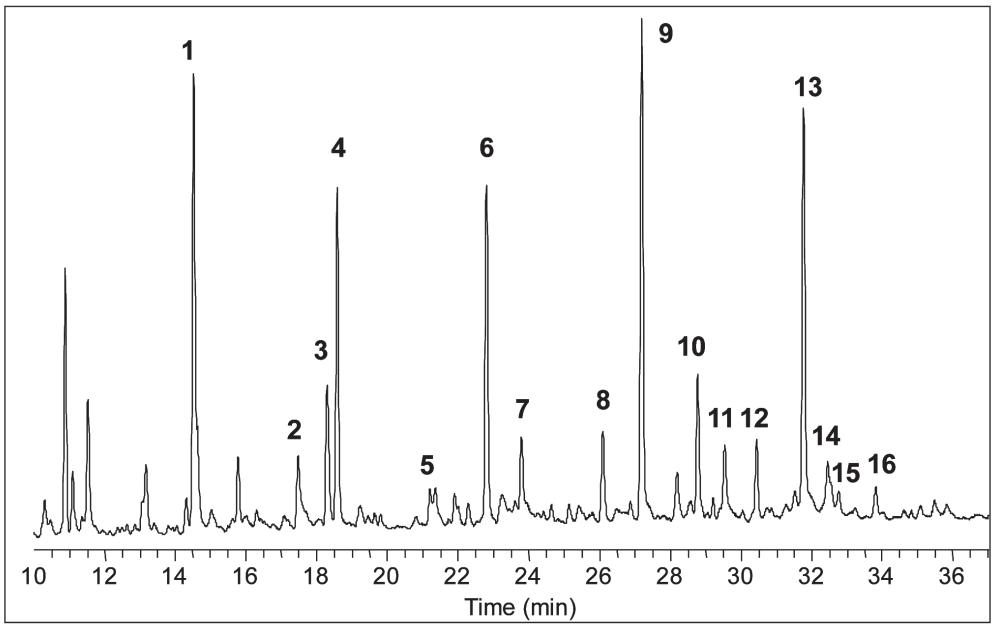

Figure 5. Total ion current chromatogram of pyrolyzed cell wall preparation from differentiated cells cultured in suspension culture (liquid MS medium supplemented with 2 mg/l 2,4-D).

The lignin content of the different cell wall samples was determined by TGAL method (Table 3). The lignin content of the cell wall from non-differentiated cells from suspension culture was 4%, whereas that from the differentiated cells was 21%. The lignin content of cell walls from non-differentiated cells cultured on solidified medium was 3%, whereas the lignin percent from the differentiated cells cultured on solidified medium supplemented with activated charcoal was 5%. The lignin content of differentiated cells of Douglas-fir from solidified medium was not very high compared to that of the differentiated cells from suspension culture. This difference could be attributed to the fact that fewer cells differentiated on solidified medium compared to suspension cultures. The TGAL content of differentiated cells from suspension culture was found to be high (21%), which could be expected because approximately 65% of the cells in suspension cultured cells grown in MS medium supplemented with 2 mg/l 2,4-D were found to be differentiated.

Table 3. The thioglycolic acid lignin (TGAL) content of the six cell wall samples calculated by measuring the UV (280 nm) absorbance against NaOH background.

Source of cell wall	Weight of cell walls (mg)	Absorbance (A_s - A_b)	Assay volume (mL)	TGAL %w/w
Non-differentiated cells from solidified sustenance medium	2.4	1.079	2	3.24
Non-diferentiated cells from suspension culture	2.7	1.476	2	4.05
Differentiated cells from solidified medium supplemented with AC	1.4	0.317	4	5.0
Differentiated cells from suspension culture supplemented with 2 mg/l 2,4-D	1.9	1.00	4	21.3
Wood	1.6	1.414	4	28.1
Inner bark	2.2	0.015	2	0.03

The presence of lignin was detected in both differentiated and non-differentiated cell walls by pyrolysis GC-MS, FTIR spectroscopy and TGAL assay. Presence of lignin was also confirmed histochemically by phloroglucinol-HCl staining. Even though the cells in non-differentiated callus from both solid medium and suspension cultures did not have secondary thickened cell walls, the presence of lignin was detected visually by staining with phloroglucinol-HCl and by spectroscopic methods. This could be due to the presence of lignin precursors (monolignols) in the cell wall. Another reason for this could be the presence of ester-linked ferulic acids, which has been previously detected in the primary cell walls of *P. radiata* (Carnachan and Harris 2000). The pyrolysis of ester linked ferulic acid breaks down into vinyl guaiacol, 4-methyl-guaiacol and guaiacol, which were detected in the pyrolysis gas chromotagrams of both differentiated and non-differentiated cells of Douglas-fir in this study. To isolate base soluble materials such as esterified ferulic acids from the cell wall, the samples were incubated with 1 M NaOH for 4 h prior to extraction of lignin with thioglycolic acid. However, significant amounts of lignin could be still detected from non-differentiated cells by TGAL assay even after this step, which strongly indicate that the compounds

detected by pyrolysis-GC-MS are from lignin degradation products rather than from esterified ferulic acid. Eberhardt *et al.* (1993) found cell wall thickening concurrent with the formation of S1 layer in the suspension cultures of *P. taeda,* but polarized light microscopy failed to show birefringence consistent with secondary thickened cell walls. They found that even though TEs failed to form, these cells were lignified which was confirmed histochemically by phloroglucinol HCl staining, ^{13}C NMR spectroscopy, TGAL assay, acetyl bromide assay, and measurement of induced enzyme activity

Fourier transform infrared (FTIR) spectroscopy of cell wall samples: Differentiated cells from suspension culture showed characteristic softwood lignin IR absorption peaks (Faix 1991; Faix 1992, Pandey 1999, Ucar *et al.* 2005) (Figure 6). The peaks identified in the differentiated cells confirmed the presence of lignin, namely: i) 1035 cm^{-1} due to the aromatic C-H in plane deformations of guaiacyl and syringyl components of lignin (Faix 1991, Faix 1992, Pandey 1999, Ucar *et al.* 2005). The contribution from guaiacyl portion is greater, since softwood lignin is predominantly composed of guaiacyl units; ii) 1234 cm^{-1} from condensed and etherified guaiacyl units, with a greater contribution from the condensed guaiacyl units (Faix 1991, Faix 1992); iii) 1265 cm^{-1} contributed from guaiacyl ring (Faix 1991, Faix 1992, Pandey 1999, Ucar et al. 2005) and iv) 1510 cm^{1} due to the aromatic skeletal vibrations from guaiacyl and syringyl units, with a higher contribution from the guaiacyl units (Faix 1991, Faix 1992, Pandey 1999, Ucar *et al.* 2005). The IR band at 810 cm^{-1} was assigned to the presence of mannan (Kotilainen, et al. 2000). This could be due to the presence of glucomannan in the differentiated secondary cell walls. The peak at 1370 cm^{-1} was identified to be from crystalline cellulose (Evans *et al.* 1995). The peak at 1730 cm^{-1} observed in differentiated cells could be assigned to the acetyl group from glucomannan (Carpita *et al.* 2001). The large peak in the region of between 900 and 1200 cm^{-1} was assigned to cellulosic polysaccharides. These characteristic peaks were also observed in xylem and differentiated cells grown in solid medium (data not shown). These results support the presence of differentiated cells with secondary cell wall characteristics similar to xylem. Non-differentiated cambium did not show the presence of lignin derived IR bands, namely the signal at 1510 cm^{-1} (Figure 7).

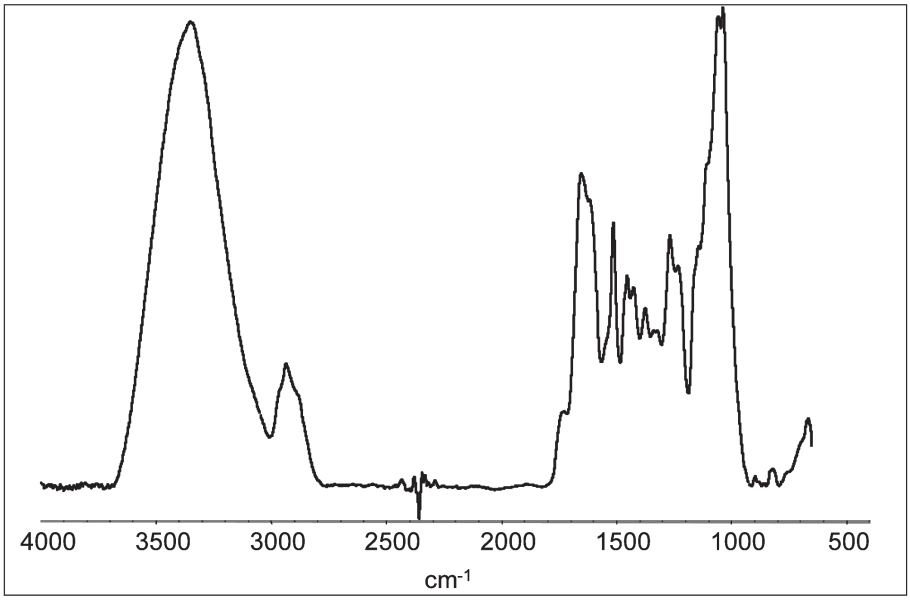

Figure 6. ATR-FTIR spectrum of a cell wall preparation from differentiated cells cultured in suspension culture (liquid MS medium supplemented with 2 mg/l 2,4-D).

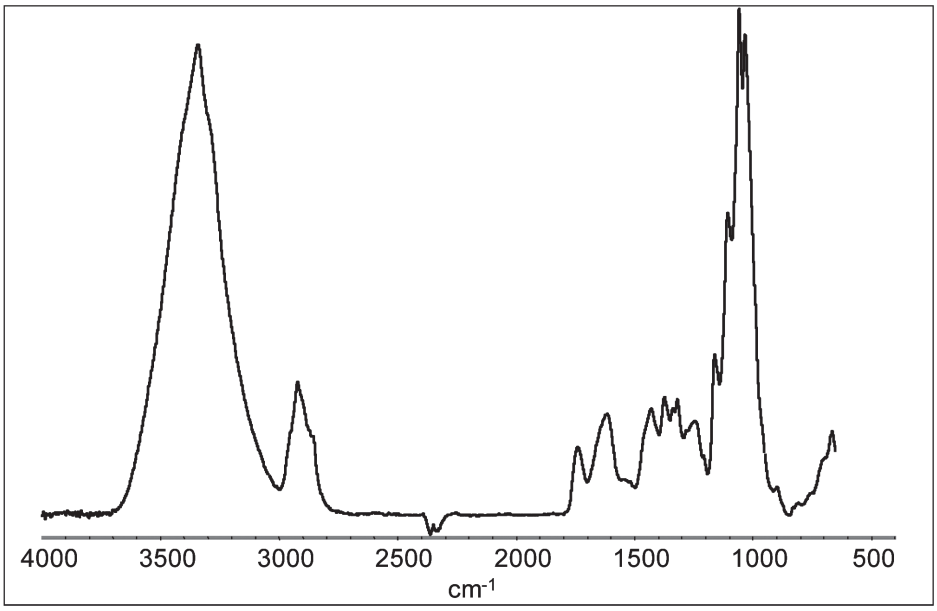

Figure 7. ATR-FTIR spectrum of a Douglas-fir inner-bark.

CONCLUSIONS

A Douglas-fir callus system has been developed and characterized and can be used as a model for examining the formation of primary and secondary cell walls of coniferous gymnosperms. As demonstrated here, the undifferentiated callus can be induced to differentiate at high yields (65%) and the effects on the cell walls monitored histochemically and chemically. This in-vitro system has the potential of providing a platform for proteomic analysis to examine enzymes involved in xylem formation and gene evaluation.

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