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Development of *Pinus radiata* suspension cultures from xylogenic callus

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Abstract

Background: The ability to grow xylogenic *Pinus radiata* D.Don in a liquid medium rather than on a solid one would produce a more homogeneous culture, and this in turn would improve cell and gene studies. We report the development of a liquid culture system for two xylogenic *P. radiata* cell lines and compare the subsequent formation of tracheary elements induced on the conventional solid media.

Findings: The cell viability (fluorescein diacetate staining) in liquid cultures and subsequent tracheary element (TE) differentiation was as high as, or higher than, that observed with conventional callus cultures on solid media. The growth of cells in liquid culture was confirmed by comparing organic carbon consumption and dry weight increase. Conditions for optimal growth were determined by measuring substrate consumption and cell dry weight with two different cell lines, flask volumes, and starting inoculum densities. Changes to flask volume and cell line were observed to modify substrate carbon consumption within the cell culture, whilst having no significant impact on overall cellular yield. Inoculum density and cell line were the most significant factors affecting the percentage of TE produced.

Conclusion: Overall, these preliminary findings confirm that *P. radiata* xylogenic cells were able to be grown in liquid cultures and did produce TE when induced on solid medium. Therefore, liquid culture has the potential to replace the current standard solid medium system for xylogenic culture of *P. radiata*.

Keywords: Xylogenic, *Pinus radiata*, Suspension culture, Tracheary element

Introduction

A unique in vitro technique developed at Scion (Wagner et al. 2013) induces *Pinus radiata* D.Don xylogenic callus to differentiate, forming cells with lignified secondary cell walls similar to tracheary elements (TE) (Möller et al. 2003). This system enables candidate genes to be tested in vitro on solid media in order to achieve further genetic gains in *P. radiata* production. However, the heterogeneous characteristics of cells grown on solid media introduce difficulty when testing for functional genes. Callus cultures on solid media have differences in spatial location of cells relative to the base media, other cells, and the air-callus interface. Liquid-phase media-based systems have many potential advantages over solid systems, in particular because plant cells cultured in suspension are relatively homogenous. Therefore, their biochemistry and modifications to their biochemistry can be studied easily (Blee et al. 2001). These attributes make high-throughput assessments possible.

Work with *P. radiata* cells in liquid culture has been reported (Ishii and Teasdale 1997; Campbell et al. 1992; Teasdale and Richards 1991), but there are no published results on cell suspension cultures of xylogenic lines from *P. radiata* (Devillard and Walter 2014).

Liquid culture has been used successfully for tracheary element differentiation in a number of other species, including *Zinnia elegans* L. (Fukuda and Komamine 1980), *Arabidopsis thaliana* (L.) Heynh. (Oda et al. 2005), and *Phyllostachys nigra* (Lodd. ex Lindl.) Munro (Ogita et al. 2012). Induction of TE directly within the liquid phase has been achieved in the *Z. elegans* and *A. thaliana* systems, but converting the entire *P. radiata* xylogenic system to a liquid phase has some technological issues to be overcome (such as

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the need for activated charcoal during the induction phase) that are not examined in this study. The aims of this study were (i) to determine whether or not primary *P. radiata* xylogenic cells could be cultured in liquid medium and, if so, (ii) test the efficacy of cultured cells in inducing secondary cell walls thereby demonstrating the potential for enhanced biotechnological development of these cell lines. The following hypothesis was tested: that liquid media can be as effective as callus culture in growing primary cell cultures of *P. radiata* amenable to subsequent induction of TE on solid medium.

Material and methods

Plant/callus starting material

Callus cultures were initiated from xylem strips of *P. radiata* as described by Möller et al. (2003). Two xylogenic cultures were used, Xy8 and Xy14. These cell lines are able to form xylogenic (Xy) cells and were labelled numerically from when they were first isolated. The callus cultures were maintained in the dark at 21.5 ± 0.5 °C on standard solid P6-SHv medium (Möller et al. 2003). These cultures were sub-cultured every 14 days as described in Möller et al. (2003).

Culture on solid medium Calli were grown either on standard solid P6-SHv medium (which contains 30 g L^{-1} of sucrose) or P6-SHvSu6, which contained an increased level of sucrose (60 g L^{-1}). Cells were collected every second day for 14 days.

Culture in liquid medium Fourteen-day-old tissue grown on solid P6-SHv medium as described above was collected, pooled then added to liquid P6-SHv medium containing sucrose (60 g L^{-1}), and mixed thoroughly using a magnetic stirrer. Aliquots (10 or 100 mL) were then transferred to Erlenmeyer flasks (25- or 250-mL flasks). Flasks were wrapped in aluminium foil to exclude light and kept on an orbital shaker (throw 50 mm) at 180 rpm, at 23.7 ± 1 °C. Flasks were moved randomly around the shaker throughout the experiment to minimise effects of layout-related variables. Cells were collected every second day for 18 days.

Cell viability

Callus from two plates with two calli per plate were sampled. Four samples from each of three locations were taken. These cells were stained with fluorescein diacetate (FDA) to determine the percentage of live cells (see the 'Analytical methods' section). Aliquots (1 mL) of cells grown in liquid medium were also sampled and stained with FDA to determine the percentage of live cells.

Effects of cell line and culture environment

A separate experiment was conducted with each cell line independently. Cell line was included as a factor in the subsequent analysis, which contained the following factors: (i) cell line—Xy8 or Xy14; (ii) starting tissue concentration (50 or 100 g L^{-1}) in liquid P6-SHvSu6; and (iii) working volume, 10 mL (in a 25-mL flask) or 100 mL (in a 250-mL flask). Three flasks were replicated for each of the treatments and sampled at day 15. Day 15 was chosen for sampling based on cellular yield plateau seen in previous experiments (data not shown). In addition, a full set of replicate flasks containing the above suspensions were cultured for the 18 days, without sub-sampling for cell viability. Aliquots of suspensions were sub-cultured on to solid induction medium to form secondary cell walls after 10 days (see the 'Analytical methods' section).

Solid plates were also inoculated with the starting suspension and cultured in the dark at 21.5 ± 0.5 °C on standard solid P6-SHv medium. Sub-samples were collected every second day for up to 18 days and stained with FDA (see the 'Analytical methods' section). Organic carbon and dry weight were also measured (as described in the 'Analytical methods' section).

Analytical methods

Total and dissolved organic carbon (TOC and DOC, respectively), pH, and total suspended solid dry weight were determined according to the Standard Methods of the American Public Health Association (APHA) (Clesceri et al. 1998).

Fresh and dry cell weights were obtained by filtering each cell suspension under vacuum using a pre-weighed glass fibre filter then rinsing it with deionised H_2O before weighing. The cells were placed in an oven at approximately 100 °C for at least 24 h and then reweighed.

Filtrates were analysed for dissolved organic carbon and total organic carbon using an Elementar HiTOC machine (Elementar GmbH, Hanau, Germany) that was operated according to the manufacturer's recommendations. The method and machine complied with method 5310 B Standard Methods for the Examination of Water and Wastewater (Clesceri et al. 1998).

Cell viability determination (solid and liquid media) Fluorescein diacetate (FDA) was used to visually assess cell viability (Widholm 1972). Cell viability in liquid culture was assessed by adding FDA solution directly to a sub-sample of the liquid culture before counting the number of fluorescing cells. Fluorescing cells were deemed alive due to enzyme activity causing fluorescence. Cell viability on solid media was assessed by excising a small sample of callus and suspending it in distilled water to a final concentration of FDA of approximately 0.1 % (Razdan

1993). All samples were viewed under UV radiation using a Zeiss Axiovert inverted microscope (filter set 09, 450–490-nm excitation and 515-nm emission, Carl Zeiss, Jena, Germany) and the number of fluorescing cells recorded as a proportion of the total number of cells (two samples taken with a minimum of four fields of view counted).

Formation of tracheary elements Cells were collected from liquid media and sub-cultured, as during maintenance, but on to solid induction medium (Möller et al. 2006; Möller et al. 2003). Solid induction medium contained 5 g L^{-1} activated charcoal (Duchefa Biochemie B.V., The Netherlands). Cells were cultured using a 16-h photoperiod under cool-white fluorescent light (TLD58 W/33 cool-white fluorescent tubes; Philips, Thailand) with a photon flux density (PFD) of 90 μmol m^2 s^1 at 23.3 ± 2 °C, for 10 days. After this time, calli were collected and suspended in distilled water then viewed under bright field and polarised light using a Zeiss Axiovert inverted microscope (Carl Zeiss, Jena, Germany). Tracheary elements with birefringent secondary wall thickenings were counted as a proportion of the total number of cells (two samples taken with a minimum of four fields of view counted).

Where factorial designs were utilised, statistical analysis was conducted using SAS statistical software (SAS Institute Inc 2011).

Findings

Cell viability

Aim (i) of this study was to determine whether or not *P. radiata* xylogenic cells could be cultured in liquid medium. Liquid cell cultures were indeed successful and maintained similar cell viability to cells of a similar age taken from callus culture on solid medium (Fig. 1).

Factorial experiment for effects of cell line and liquid culture environment

Cell line Xy8 consumed significantly more substrate carbon than cell line Xy14. Also, in most cases, substrate carbon consumption was larger in 250-mL flasks than in 25-mL flasks suggesting that working volume is important (Fig. 2).

Irrespective of shake flask mass transfer implications, the elevated substrate consumption did not generally lead to any enhancement of cellular yield or TE formation.

Aim (ii) of this study was to test the efficacy of cultured cells in inducing secondary cell walls thereby demonstrating the potential for enhanced biotechnological development of these cell lines. Inoculum density and cell line were the most significant factors affecting the percentage of TE produced (Fig. 3). Cell line Xy8 produced significantly greater proportion of TE than cell line Xy14. It also produced more TE from the liquid primary cultures than in the solid callus indicating that cells previously cultured in liquid medium were capable of inducing secondary cell walls.

The significance testing is summarised in Table 1. Details of the full statistical analysis are provided in Additional file 1.

Lower inoculum level increased the percentage of TE, which might be expected since lower inoculum level results in a lower oxygen demand and thus leads to a higher relative oxygen level in the liquid phase.

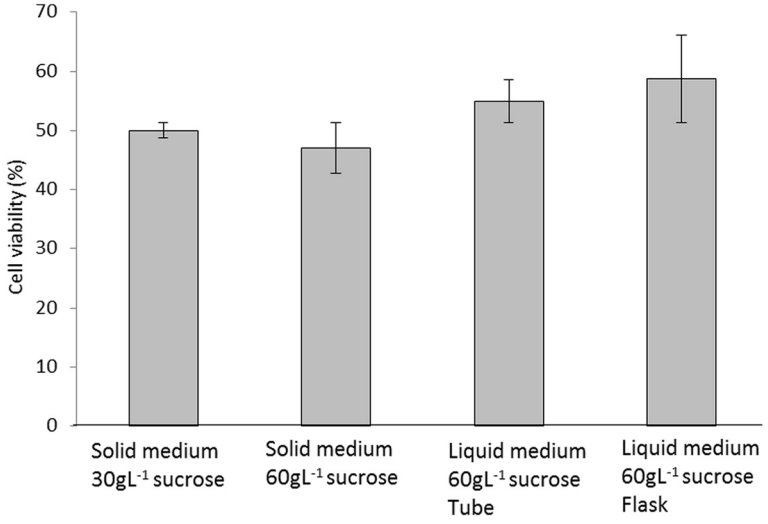

Fig. 1 Cell viability for various solid media and liquid cultures averaged over days 10, 12, 14, 16, and 18. *Error bars* indicate standard errors

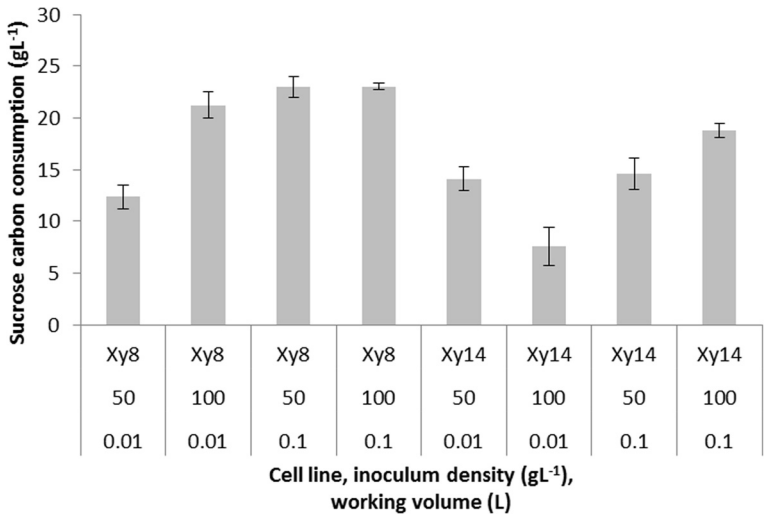

Fig. 2 Substrate carbon consumption in factorial experiment. Presentation of *x* axis datasets; cell line (*top*), inoculum density (g L^{-1}, *middle*), and working volume (L, *bottom*). *Error bars* represent standard error of replicate data ($n = 6$)

The cells originate and had been maintained in aerobic conditions; therefore, oxygen level is likely to be an important factor. Inoculum density is also known to have an effect on enzymes involved in the phenylpropanoid pathway (Hahlbrock and Wellmann 1973). The phenylpropanoid pathway is involved in the formation of lignin in TE (Douglas 1996). Therefore, further optimisation of inoculum density is crucial.

Tested as one of the factors, cell line was found to be significant in both the growth and viability of the liquid cultures. Interestingly, the cellular yields between the two lines were not significantly different, despite significant differences in growth rate (data not shown) and substrate consumption. For optimisation, a greater number of cell lines would need to be tested, and an efficient cell-line screening protocol developed. Further investigation on this aspect of culturing is also warranted because of the limite knowledge on programmed cell death and autolysis in the TE of conifers.

Conclusion

Liquid cultures of *P. radiata* xylogenic cells maintained good viability and TE differentiation rates compared with the traditional callus culturing technique. Overall, these findings support our hypothesis that liquid media

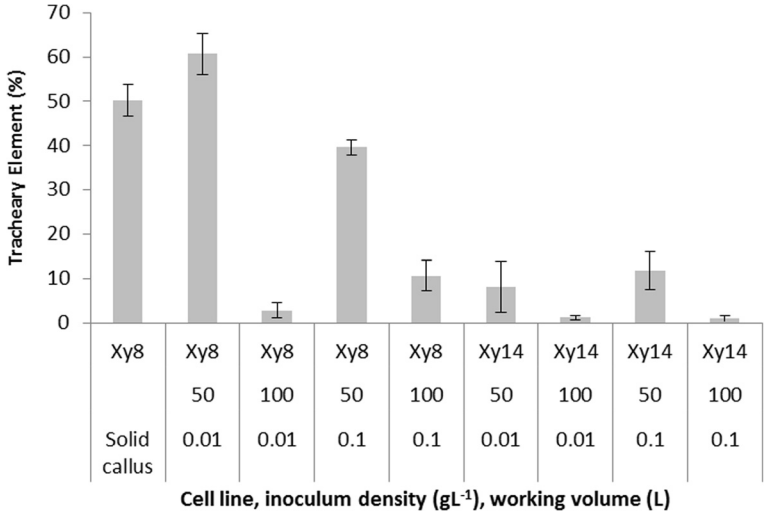

Fig. 3 Tracheary element percentage from factorial experiment. Presentation of *x* axis datasets; cell line (*top*), inoculum density (g L^{-1}, *middle*), and working volume (L, *bottom*). *Error bars* represent standard error of replicate data ($n = 6$)

Table 1 Significance of factors on effects from factorial flask experiment

Parameter	Experimental factor[a]						
	Inoculum density (I)	Working volume (W)	Cell line (C)	IxW interaction	IxC interaction	WxC interaction	IWC interaction
Substrate carbon consumption	++	++	++			+	+
Dry weight increase		++	++			+	+
Cellular yield	+						
% live cells	++			+	+		
% TE	++		++		++		++

[a]Type III sums of squares Pr > F; + significant, between 0.05 and 0.005; ++ highly significant <0.005; empty cells = not significant, >0.051

can be as effective as callus culture in growing primary cell cultures of *P. radiata* that are amenable to subsequent induction of TE. Whilst more development is required to optimise the system, the current work demonstrates that a liquid culture has the potential to replace the current standard solid medium system.

Additional file

Additional file 1: Details of three-way ANOVA of factorial design experiment for effect of cell line and growth environment.

Abbreviations
TE: tracheary elements; Xy: xylogenic.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AC was primary author and undertook tissue culture and data collection. GG contributed to the conception and technical design of the study and provided critical revisions of the manuscript. PD undertook carbon measurements and provided technical advice. CD Provided technical advice and revisions of the manuscript. CW contributed to technical design of the study, and revisions of the manuscript. DG contributed to the conception and technical design of the study and prepared manuscript. All authors read and approved the final manuscript.

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