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ORIGINAL ARTICLE

Diverse forms of xylem-Like cells and strand formation in Xylogenic *Eucalyptus bosistoana* callus culture

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Abstract

In vitro xylem induction system is a basic tool in physiological, biochemical, and molecular studies of secondary cell wall formation, lignin biosynthesis and deposition associated with tracheary element formation. *Eucalyptus bosistoana* is a Class 1 durable hardwood tree species, selected by the New Zealand Dryland Forest Initiative for good quality wood and high adaptability to the NZ growing conditions. Xylogenic *E. bosistoana* callus culture was established and up to 40% of the callus cells were xylem-like cells (XLCs) which may have differentiated from small, cytoplasmically dense or compact dividing, and exhibited increased lignin contents during culture. The eucalyptus XLCs showed diverse sizes, patterns of secondary cell wall thickenings similar to the xylem cells in the young shoots and organized development including cell–cell connections of the XLCs to form xylem strands. This is the first report of the organized development of XLCs in *E. bosistoana* callus culture.

Key message

This is the first report of xylogensis in an eucalyptus callus culture and the xylem-like cells with cell wall thickenings resembling the xylem cells in the young shoots.

Keywords Secondary cell wall · Lignin · Tracheary elements · Xylem formation

Introduction

Xylem elements or vessel members are mainly found in the vascular tissues of angiosperms such as eucalypts and serve as a pipeline or conducting tube through which water and mineral nutrients are transported throughout the plant body (Déjardin et al. 2010). During primary growth of the plant body, vascular tissues including vessel members are differentiated from the procambium, while during secondary growth they arise from the vascular cambium (Watson 1976). Xylogenesis has also been reported in in vitro cultures of many plants, for example, *Zinnia elegans* L. (Fukuda 2015; Iakimova and Woltering 2017), poplar trees (Jin et al. 2011; Yamagishi et al. 2013), Bamboo (Ogita et al. 2012), Douglas-fir (Pillai et al. 2011), *Cryptomeria japonica* (Yamagishi et al. 2015, 2017), *Arabidopsis thaliana* (Hirakawa et al. 2008) and *Musa* species (Negi et al. 2015). There is, however, a paucity of information on xylem strand development in proliferating callus culture. The formation of strand formation must involve organized development of callus cells into xylem cells while still being connected. This kind of information is a pre-requisite to the potential use of callus culture to study the regulation of xylem strand formation and even making wood in vitro.

In in vitro xylogenesis, xylem-like cells (XLCs) may originate from different types of cells. Leaf mesophyll cells have been induced to undergo trans-differentiation in the *Zinnia* cell culture system (Fukuda 1997), while parenchyma cells may develop into XLCs in pine and poplar cell cultures (Möller et al. 2003; Yamagishi et al. 2013, 2015). XLCs can be induced in xylogenic cell cultures by manipulating nutrient medium with sucrose and plant growth regulator combinations and maintaining optimum culture conditions such as light intensity, photoperiod, and temperature (Devillard and Walter 2014).

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The XLCs formed in plant cell cultures are morphologically similar to the tracheary element cells (TEs) *in planta*. Both exhibit similar patterns of secondary cell wall (SCW), and undergo lignification and programmed cell death. Since it is relatively hard to get hold of large quantities of cambial cells to study wood formation, induction of XLCs in vitro are a useful alternative approach for physiological, biochemical and molecular studies on xylem formation (Ye 2002). There were relatively few studies on callus culture showing any organized development of the induced XLCs which might have implications for a better understanding of wood formation. Eucalypts are important hardwood species, but there is no prior detailed information on xylogenesis in Eucalyptus callus culture. The objective of the present study was to investigate xylogensis in *E. bosistoana* callus culture. In particular, we were able to show the formation of diverse types of XLCs. Moreover, this is the first report showing that some of the XLCs were joined together and organised as strands or clusters within the callus based on 3-dimensional confocal microscopic examination.

Materials and methods

Establishment of xylogenic cell culture

Nodal explants from 5-year-old *Eucalyptus bosistoana* plants maintained at the glasshouse of the University of Canterbury (Christchurch, New Zealand) were cultured on basal Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} of agar (Oxoid Ltd., UK), 20 g L^{-1} insoluble polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich, St. Louis, USA), 1.5 mg L^{-1} NAA (1-naphthalene acetic acid, Sigma-Aldrich) and 1 mg L^{-1} BA (benzyl adenine, Sigma-Aldrich) for callus initiation (Shwe and Leung 2020). Callus was formed after 14 days of culturing and was then sub-cultured on fresh callus induction medium at monthly intervals. After 4 subcultures on the same medium, a piece of callus (approximately 1 cm in diameter) was transferred to xylogenic culture medium (basal MS medium supplemented with 1.5 mg L^{-1} NAA, 0.5 mg L^{-1} BA and 100 µM sodium nitroprusside, a donor of nitric oxide, or NO).

Cell maceration and histochemistry

Cell maceration was carried out according to Chatelet et al. (2008) with some modifications. In vitro shoots (2–3 cm length) or pieces of callus were incubated for 24 h at 100 °C with an acid maceration solution (1 part of 30% H_2O_2:4 parts of deionized water:1 part of acetic acid) to isolate xylem vessel cells or XLCs, respectively, for a closer examination of their cell wall thickening patterns. After incubation, the cells were washed three times with deionised water and then stored in 70% (v/v) ethanol at 4–6 °C until further analysis. Before acid maceration, lignified cells in the xylogenic callus were stained using phloroglucinol-HCL (Davidson et al. 1995), and after maceration, the cells were stained with toluidine blue-O or TBO (O'Brien et al. 1964).

XLCs were categorised according to their secondary cell wall thickening patterns and colour reaction with different stains. The staining of cells red in the presence of phloroglucinol HCL would suggest the presence of cinnamyl aldehyde residues in lignin polymers (Davidson et al. 1995). After acid maceration, the cells were then stained with TBO (O'Brien et al. 1964), the lignified cells would exhibit blue to greenish blue colour in the secondary cell wall while the staining of the cells purple would suggest the presence of pectin in the primary cell wall.

The percentages of lignified cells and XLCs were calculated in relation to the total number of cells counted after maceration using a hemocytometer under a light microscope. Since the XLCs were highly variable in shapes and sizes, they were divided into three groups according to their length and diameter: the XLCs < 25 µm in length were defined as short, those between 30 and 50 µm in length were defined as of medium length and over 70 µm in length as long XLCs. However, irregularly shaped XLCs were omitted in this observation. In total, thirty TEs from the shoot and each of the XLC groups by length were included in this investigation.

Microtome sectioning

Pieces of xylogenic callus cultures and stem pieces excised from shoot tips (6 cm long) of 5-year-old *E. bosistoana* plants were fixed in formaldehyde/acetic acid/alcohol (FAA) solution under vacuum. The plant materials were then dehydrated sequentially using different concentrations of tertiary butyl alcohol and ethanol. Infiltration and embedding of the plant materials were then carried out in molten paraffin. The wax-embedded section (10 µm thick) were cut on a microtome and were then stained with 1% (w/v) safranin and 0.05% (w/v) fast green for microscopic observations.

Confocal laser scanning microscopy

Confocal laser scanning microscopy was carried out according to Collings and Harper (2008) and Verhertbruggen et al. (2017) with a slight modification. Pieces of callus were fixed in a fixative solution (formaldehyde 3.7%, glutaraldehyde 0.5%, dimethyl sulphate 0.1% and Triton X-100 0.1% (v/v)) for 1 h and were then washed in PME buffer (50 mM Pipes, 2 mM $MgSO_4$ and 2 mM EGTA, pH 7.0 (K^+) for 3 times. After this, the callus samples were covered in PME + 1% Triton X-100 and kept on an orbital shaker for 1 h before permeahydrolisation using

100% methanol at − 20 °C for 15 min and rehydrated in PBS (phosphate saline buffer) solution for 20 min. The callus pieces were finally embedded in 3% agar in PME buffer and the blocks were kept in a fridge at 4 °C inside an air-tight container for later vibratome cutting and laser scanning microscopy. Section (100 µm thick) were cut with a razor blade on a vibratome machine (Leica VT 1000s). The sections were stained in 0.01% (w/v) safranin for 30 min and washed in PME buffer for 3 times. Finally, the sections were imaged on a confocal laser scanning microscope (Leica SP2 system) with an optical series collected at 1 µm intervals by laser at 488 nm argon and 590 nm fluorescence.

Thioglycolic acid lignin (TGAL) assay

Thioglycolic acid lignin (TGAL) assay was carried out according to the method described in Möller et al. (2006). Ten xylogenic callus pieces from each treatment were collected and thioglycolic acid lignin content was separately quantified at 20, 40 and 60 days after transfer to the xylogeneis induction medium. The callus pieces were air dried over 48 h and 20 mg of the dry cell powder obtained was used for the extraction of cell wall biomass (SBM) through sequential washing with 0.1 M potassium phosphate buffer (pH- 7.4) + 0.5% Triton X-100 and 100% methanol. SBM (2 mg) was digested in 1.5 mL of 2 N HCL + 0.3 mL of TGA (1%, w/v) for 4 h at 95 °C using a water bath. After centrifuging at 14,000×*g* for 10 min at 23 °C, the pellet was washed in deionised water for 3 times and dissolved in 0.5 M NaOH for 18 h by shaking on a shaker at 30 °C. After centrifugation, 0.3 mL of conc. HCL was added to the supernatant and the mixture was kept at 4 °C for 4 h to precipitate phenylpropanoid derivatives. The precipitate was collected after centrifuging at 14,000×*g* for 15 min at 23 °C. The pellet of lignin thioglycolate compounds was dissolved again in 1 mL of 0.5 M NaOH before absorbance was measured at 280 nm. The relative absorbance of TGAL per dry cell mass was calculated form non-xylogenic (control) and xylogenic calli after different culture durations.

Statistical analysis

Cell length, width, shapes, SCW thickening, and wall patterns were recorded at every 10 days intervals. The data were analysed using the SAS software version 9.1. Turkey test was used to determined means difference at 5% level of significant difference.

Results and discussion

After 10 days of culture on xylem induction medium, active cell division was observed in the xylogenic callus and there were small dividing cells that appeared to be cytoplasmically dense or compact (Fig. 1A). After 20 days of induction, formation of XLCs was evident based on their distinctive SCW thickening patterns. Some of the xylogenic callus were suspended in deionised water and were then stained with phloroglucinol HCL or toluidine blue-O dye. Following phloroglucinol HCL staining (compare Fig. 1A and B), some of the cells were stained red suggesting the presence of cinnamylaldehyde residues in lignin (Davidson et al. 1995). After staining with toluidine blue-O dye (Fig. 1C and D), the primary cell wall of the parenchyma cells exhibited purple colour, while some cells exhibited blue to greenish blue color indicating the presence of lignin (O'Brien et al. 1964). Based on these two histochemical staining results, it appeared that there were many lignified cells formed in *Eucalyptus* callus after culturing on the XLC induction medium.

Secondary cell wall (SCW) thickening patterns

After tissue maceration, single XLCs can be distinguished from other callus cells based on their distinctive SCW thickening patterns, which resembled the secondary cell wall thickening patterns of xylem cells in the plant body. These include the annular and spiral cell wall patterns in

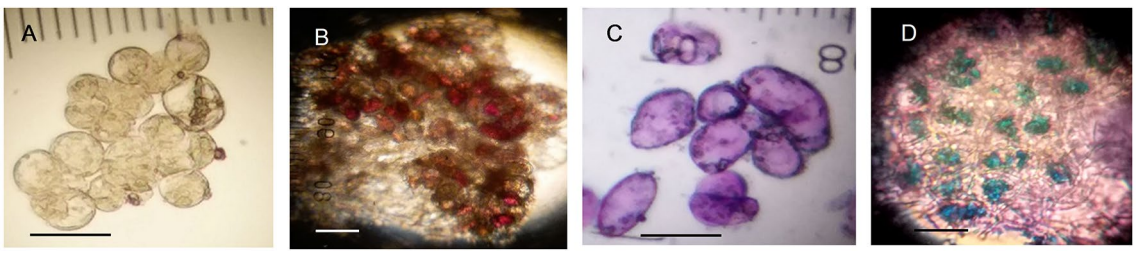

Fig. 1 **A** Non-lignified cells and **B** red color reaction in callus after staining for lignin with phloroglucinol HCL test; **C** non-lignified parenchyma in callus stained with toluidine blue-O staining and **D** lignin positive blue green color reaction among purple colored parenchyma cells in the *E. bosistoana* callus cells before maceration, observed under a light microscope. Bar = 50 µm

the protoxylems, and the reticulated and pitted patterns in the metaxylems. At the beginning stage of SCW formation, microtubules are transversely oriented in relation to the primary cell wall and provide a framework for lignin deposition (Fukuda 2016; Růžička et al. 2015). Lignin monomers are synthesised and transported to the apoplast where plasma-membrane localised peroxidase and laccase enzymes catalysed oxidation leading to polymerisation of lignin monomers (Schuetz et al. 2014; Tobimatsu and Schuetz 2019). Lignin deposition creates annual or helical bands of SCW thickening where microtubules depletion areas show only primary thin walls called pits (Sun et al. 2006). Pit pairs between adjacent xylem vessel cells allow water movement across the neighboring xylem cells (Taiz et al. 2015).

During 20–30 days after induction (DAI) on the xylogenesis induction medium, most of the XLCs (82%) showed spiral cell wall and 16.8% with the annular or ring SCW patterns. A mere 3.2% of the XLCs showed reticulated thickening pattern. Interestingly, the total number of the XLCs with reticulated cell wall increased to 31.4% at 40–60 DAI, and a massive 90.6% at 70–90 DAI. While the percentage of the XLCs with spiral SCW decreased from 82% at 20–30 DAI to 66% at 40–60 DAI, and only a few (4.1%) the XLCs with spiral SCW were found at 70–90 DAI (Fig. 2). Xylem maturation and SCW pattern formation might depend on the culture period on the same xylogenesis induction medium. Based on the stains used, more lignin deposition appeared to be present in the later XLC maturation stages such as the metaxylems with reticulated or pitted cell wall patterns, compared to the early protoxylem stage with spiral or ring patterns. Purple color indicated the presence of pectin in the primary cell wall while blue to blue-green color indicated higher lignin deposition (Fig. 3).

During xylogenesis induction, lignin deposition had increased dramatically in *E. bosistoana* callus culture based on the thioglycolic acid lignin (TGAL) assay (Fig. 4). Although XLC initiation started at 20 DAI, the xylogenic callus exhibited significantly higher lignin deposition than control after 60 DAI. At 80 DAI, the amount of lignin in the xylogenic callus was five times higher than that at 20 DAI. This was correlated with an increasing number of XLCs exhibiting reticulated thicker cell wall at the metaxylem stage and the formation of more sclereid cells. Moreover, post-mortem lignification, a common event in angiosperms (Pesquet et al. 2013; Serk et al. 2015), could occur in the

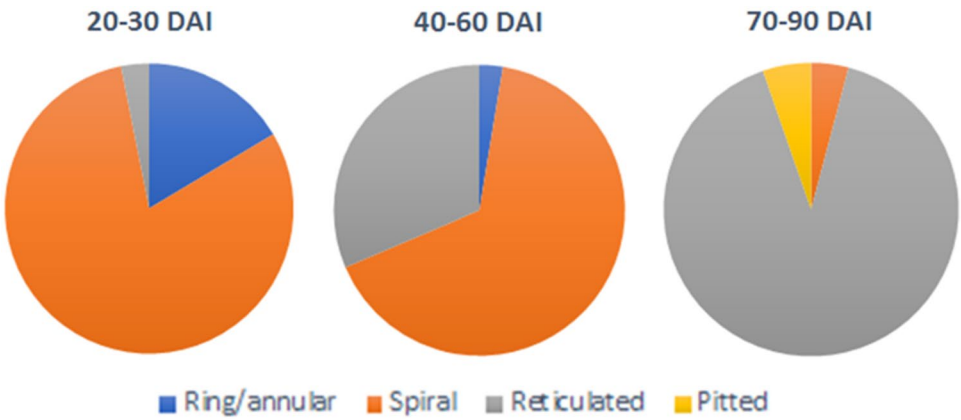

Fig. 2 Distribution (%) of xylem-like cells (XLCs) with different types of secondary cell wall thickening patterns (ring/angular, spiral, reticulated or pitted) in *Eucalyptus bosistoana* callus cultured on xylogenesis induction medium for 20–30 days (DAI), 40–60 DAI and 70–90 DAI. Number of XLCs in each group of culture period = 100

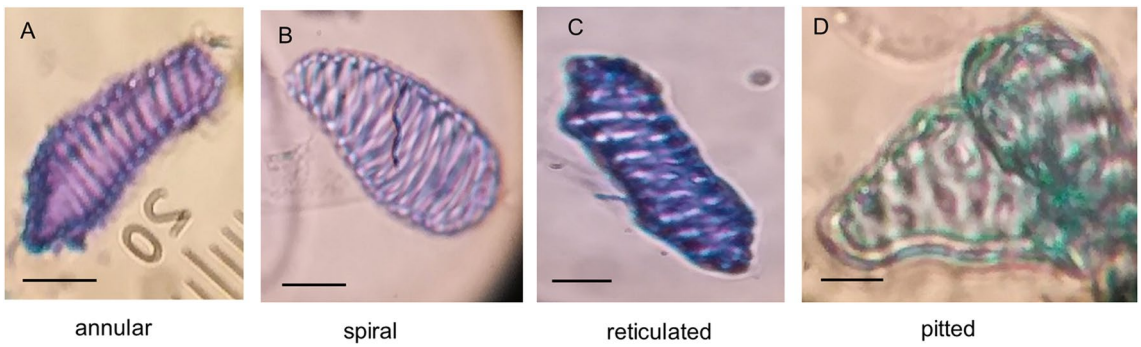

Fig. 3 Secondary cell wall thickening patterns of xylem-like cells in *Eucalyptus bosistoana* xylogenic callus after maceration in glacial acetic acid + H_2O_2 and staining with toluidine blue-O dye. Different patterns of secondary cell wall thickening; **A** annular, **B** spiral, **C** reticulate and **D** scalariform secondary cell wall thickenings observed under a light microscope; Bar = 10 µm

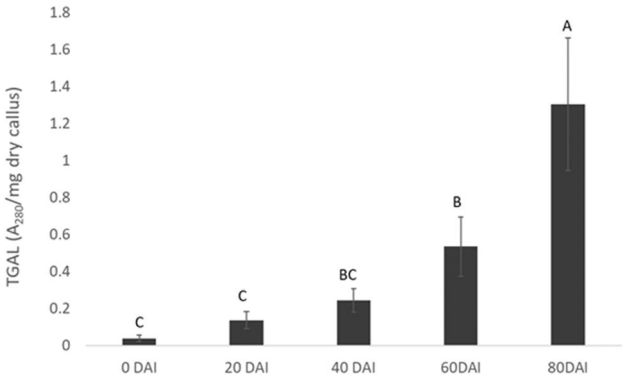

Fig. 4 Quantification of thioglycolic acid lignin (TGAL) per mg of dry callus at 280 nm from xylogenic callus on XLC induction medium from 0 day to 80 days after induction, n = ten pieces of calli, means ± standard deviation, bars with same letter was not significantly different at $MSD_{0.05}$

xylogenic eucalyptus callus with the help of neighboring living callus cells by providing monolignols and substrates such as H_2O_2/O_2 for lignin polymerization.

Shapes and sizes of XLCs

Xylem vessel element or tracheary element (TE) cells in both the stem of the 5-year-old plants or in vitro plantlets were observed as long and straight wall/cylinder-like cells (Fig. 5B and C). However, the shapes of the XLCs formed in vitro in Eucalyptus callus culture were highly variable, and their walls were not always straight, but their shapes were mostly ovate, spatulate, fusiform or long cylinder cells, while some were of triangular or irregular in shape (Fig. 6). The variable shapes of XLCs are common in the xylogenic cultures reported by others (Devillard and Walter 2014; Fukuda 2016). Their sizes also varied ranging from 30 to 100 µm in length and 10–30 µm in width.

Xylem element cells (TEs) of the in vitro shoots showed uniform size and shape; all were observed as very long cylinder-like cells with an average diameter of 9.24 µm and almost 300 µm in length. They showed the same diameter throughout the long cell with spiral secondary wall patterns (Fig. 5C and Table 1). On the other hand, XLCs were of significantly shorter length and larger diameter than normal xylem element cells. In summary, the shortest, medium and longest XLCs were around 21.8 µm, 44.2 and 75.4 µm, respectively. On the other hand, the diameter of natural

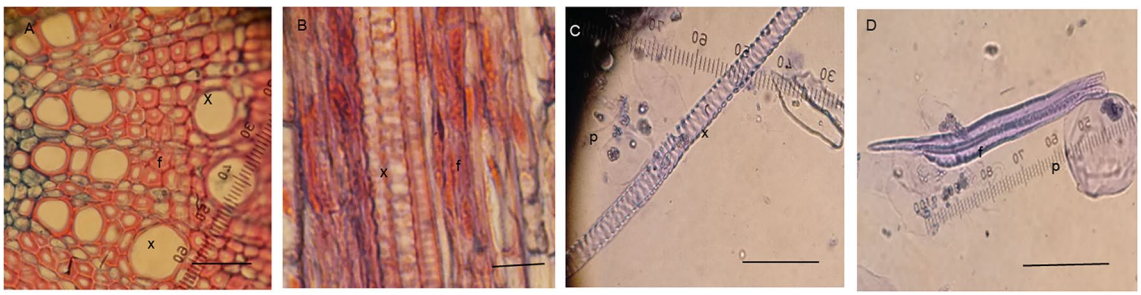

Fig. 5 **A** Transverse and **B** longitudinal sections of a stem taken at 6 cm from the shoot tip of 5-year-old *E. bosistoana*, **C** single xylem cell, and **D** fiber cell macerated from the stem of 2-month-old in vitro shoot of an *E. bosistoana* plantlet; bars = 50 µm; xylem vessel element cells = x, fiber cells = f, parenchyma cells = p; Xylem vessel element cells = x, fiber cells = f, parenchyma cells = p

Fig. 6 Asynchronised XLCs formation in *Eucalyptus bosistoana* xylogenic callus culture; a cluster of TE cells (**A**), and various shapes and sizes in TE cell mass (**B**) after staining with toluidine blue-O dye; arrow = callus parenchyma cells, Bar = 20 µm

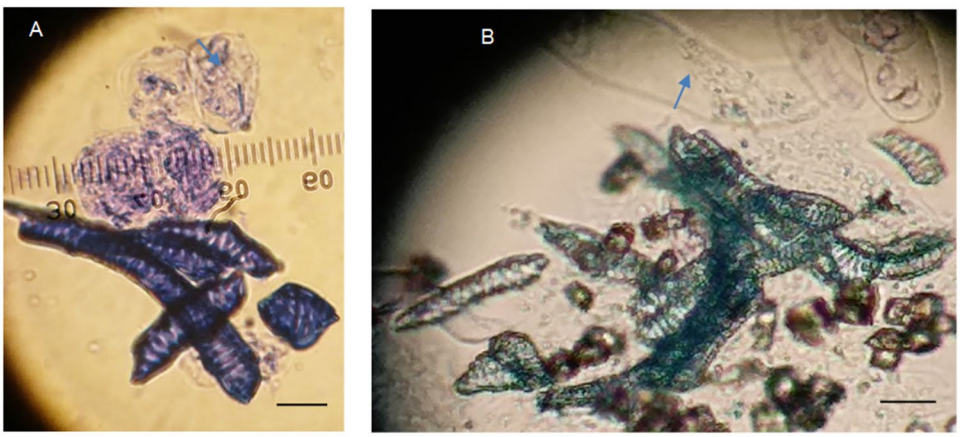

Table 1 Means comparison of size of xylem elements of in vitro shoots and xylem-like cells (XLCs) from xylogenic callus after complete maceration

	Length (µm)	Width/diameter (µm)	%	Shape	SCW thickening patterns
Xylem (in shoot)	285 ± 43.42 A	9.24 ± 0.85 C	100	cylinder	Spiral
XLCs (short and broad)	21.8 ± 3.04 D	26.6 ± 2.63 A	32	Ovate, triangular, rectangular	Ring, spiral, reticulated
XLCs (medium)	44.2 ± 7.68 C	17.8 ± 2.39 B	44	Spatulated, spindle, fusiform	Spiral, reticulated, pitted
XLCs (long)	75.4 ± 12.89 B	10.26 ± 1.29 C	24	Cylinder or fusiform with tapering ends	Spiral, reticulated
Pr > F	**	**			
$LSD_{0.05}$	19.99	5.29			

Means ± standard deviation followed by different letter in the same column are significantly different at 95% level with Turkey's test, n = 30 XLCs

** denotes minimum significant difference at Turkey 0.05

xylem element cells was about 9.24 µm along the vessel of an in vitro shoot. The diameters of XLCs were highly variable. Generally, the diameters of the widest, medium and narrowest XLCs were 26.6 µm, 17.8 µm and about 10.26 µm, respectively (Table 1). Natural xylem members maintain straight wall cylinder shapes probably due to the pressure from their accompany cells and tissues such as fiber cells, xylem parenchyma and other vascular bundle tissues such as sieve tubes and companion cells in the vascular bundle of a stem. In comparison, the XLCs were produced from friable callus with pockets of airspaces and were surrounded by unorganized callus (parenchyma) cells with thin primary cell walls which would be expected to less of physical barriers and/or constraints to the growth of the XLCs in friable *E. bosistoana* calli.

Organised development of XLCs

After acid macerating the *Eucalyptus* callus, some XLCs were still found to be attached or bound together as organised clusters. At least pairs of XLCs were found to be attached but usually 3 to 12 XLCs were found to be joined in clusters. The XLCs were mainly attached to each in parallel, but some were also found to connected end to end to form xylem strand-like structures (Fig. 6). Interestingly, the XLCs in the xylem strand-like structures were mostly uniform in shape and size, although they were significantly broader and shorter than natural xylem vessel elements. They were assumed to be connected at the end walls with the perforation plates as in a natural xylem strand or vessel (Fig. 7). In some XLC strands, the XLCs were not only connected with end walls but there seemed to be pit pairs between parallel adjacent XLCs (Fig. 7B). Some XLCs strands had a branch-like structure, resembling a well-connected and branched water pipeline (Fig. 7C). It is tempting to suggest that there might be certain polarity or a gradient of xylogenesis signaling molecules among callus cells. This remains to investigated in further studies.

Some (2 to 6) XLCs attached side by side were also observed. On careful microscopic examination, pit pairs were visible between two adjacent XLCs (Fig. 8). Overall, the XLCs and natural xylem cells appear to be similar in morphology and cell wall chemistry. Nevertheless, the XLCs were of significantly shorter length and of greater diameter compared to the natural xylem vessel members.

Organized development of XLCs and XLC strands

Xylem vessels are well organized and aligned in the vascular bundle with other complementary tissues such as xylem fibers, xylem parenchyma, and other accompany cells (Taiz et al. 2015). However, the XLCs in the xylogenic *E.*

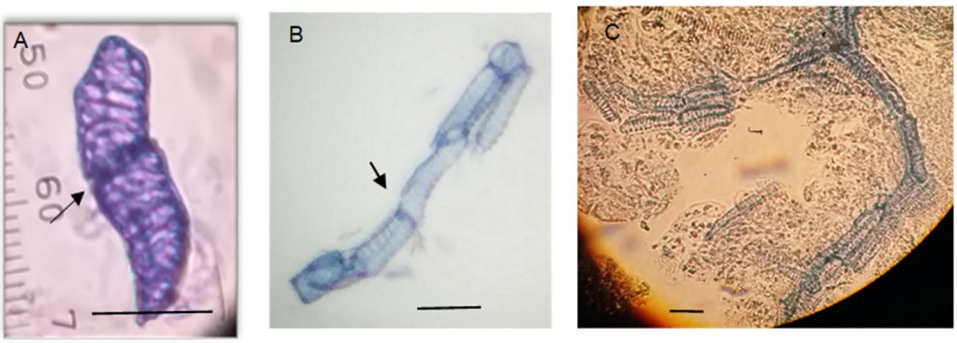

Fig. 7 End to end connection of xylem-like cells (XLCs), and arrows show region resembling perforation plates. Xylem strand-like structures with 2 XLCs (**A**), with 6 XLCs (**B**), more than 12 XLCs among callus cells (**C**), arrows = location resembling a perforation plate, bars = 20 µm

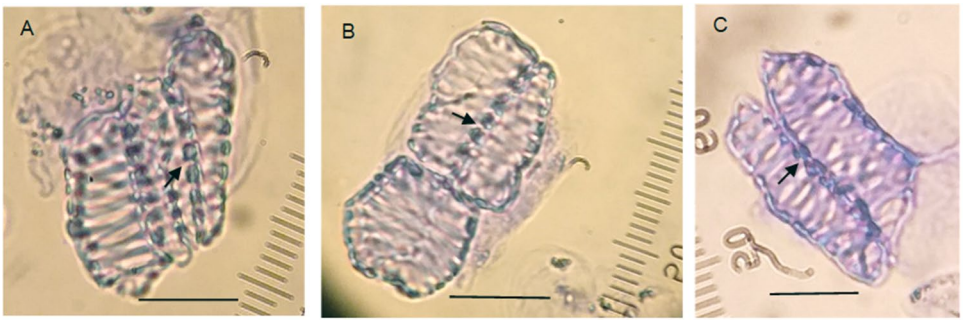

Fig. 8 Parallel arrangement of XLCs; arrows pointing to the location of apparent pit pairs; bars = 20 µm

bosistana callus cultures did not always follow the rule of organized xylem in the nature as in a vascular bundle. Some were scattered while some were organized in a variety of ways, even within the same piece of callus. Many XLCs had proliferated in different direction. However, in some cases, XLCs were observed as in a spherical arrangement while others looked like a file with many parallel XLC strands. In the spherical arrangements, some XLC strands were in a compact arrangement, but some seemed to be loosely associated. Some XLCs looked like a folding string (Fig. 9).

Vibratome sectioning of the xylogenic *E. bosistoana* calli and then confocal laser scanning microscopy showed the organized patterns of XLCs in the calli. Mass aggregation of XLCs were observed among callus parenchyma cells with both parallel and end to end connections. However, the polarity of the XLCs were not clear as they appeared in different directions. Moreover, their shapes were also irregular, and sizes were also variable. However, they exhibited very prominent SCW thickenings, and most of which were of the reticulated patterns. Especially, thicker SCW deposition was found at the cell corners (Fig. 10). XLCs of different sizes were found in a single type of organised XLCs. It could be assumed that the developing XLCs might stimulate other neighboring cells in the callus to initiate XLC differentiation. This might be mediated through the signaling involving NO or H_2O_2 because cell to cell communication during xylem differentiation (Pesquet et al. 2013; Serk et al. 2015) and production of nitric oxide (NO) and hydrogen peroxide (H_2O_2) were observed in the developing xylem vessel cells (Gabaldon et al. 2004; Gómez et al. 2006).

XLC strands were comprised of XLCs of different sizes and shapes. Although they were well connected through their end walls, presumably with perforation plates, the

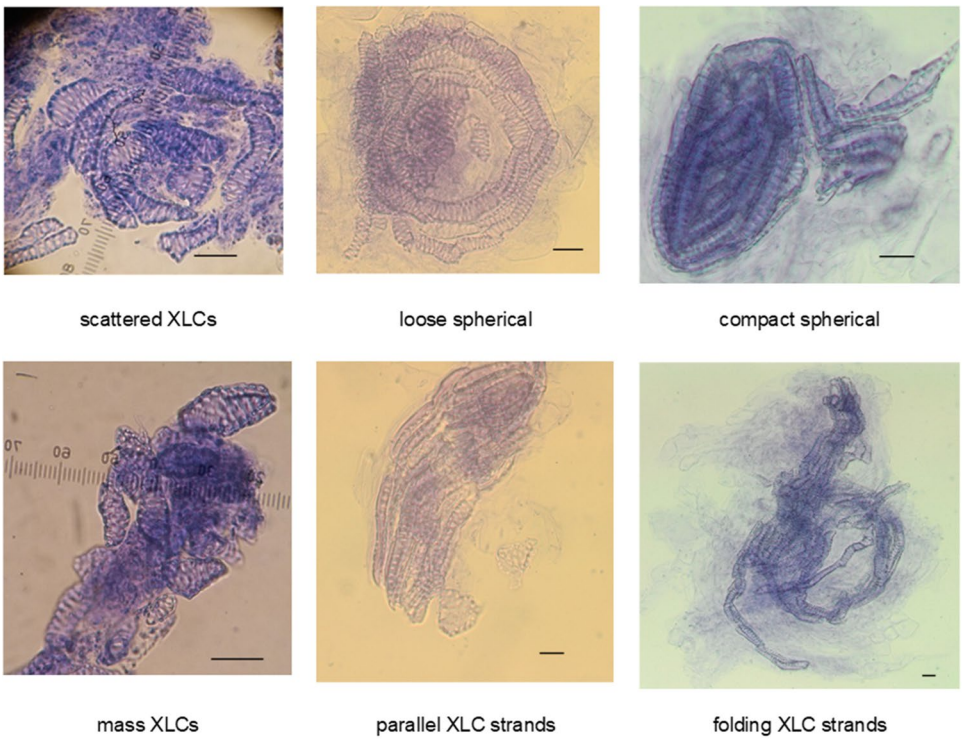

Fig. 9 Organized patterns of XLCs and XLC strands in the xylogenic *E. biosistoana* callus after maceration and staining with toluidine blue O dye. Bars = 20 µm

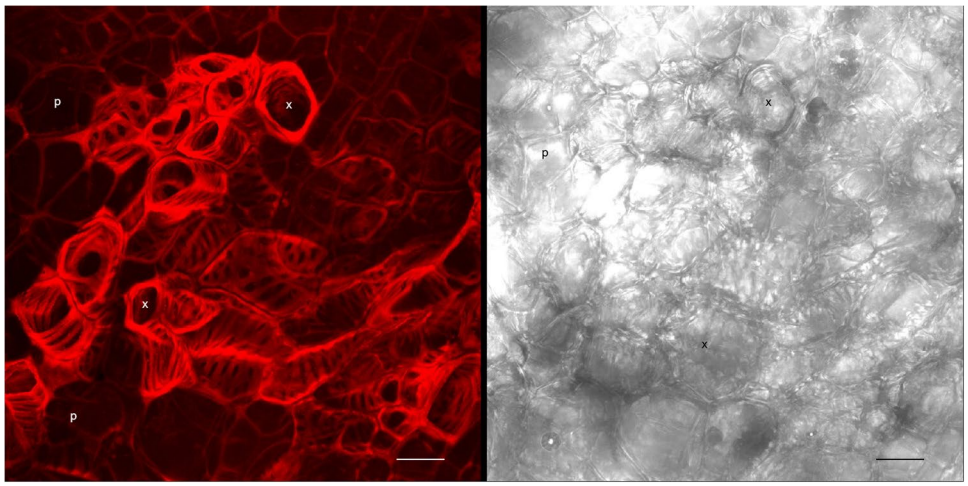

Fig. 10 Confocal laser microscopy and bright-field imaging on mass induction of xylem-like cells (XLCs) in the xylogenic *E. bosistoana* callus with safranin staining. P = parenchyma callus cells. X = XLCs. Bars = 20 µm

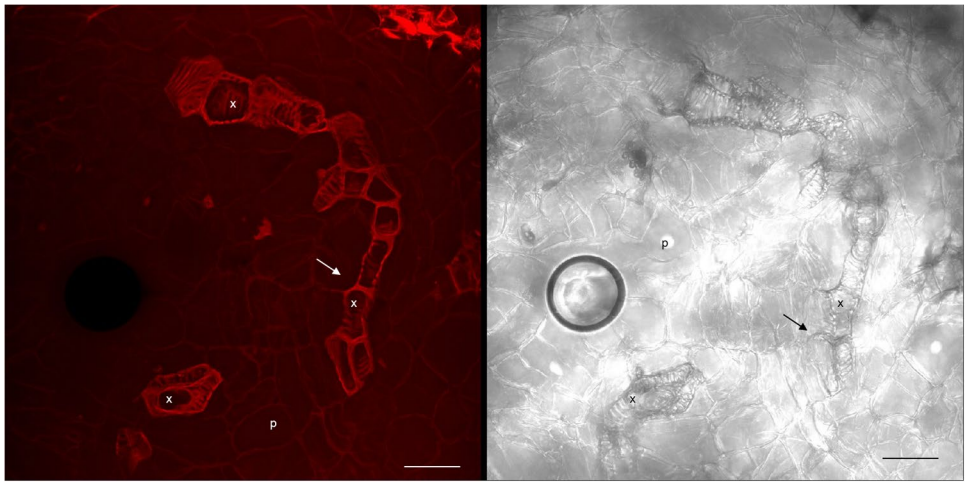

Fig. 11 Confocal laser scanning microscopy and bright-field imaging of a xylem-like cell (XLC) strand in the callus of *E. bosistoana* with safranin staining. P = parenchyma callus cells. X = XLCs. Arrows = perforation plates. Bars = 20 µm

irregular shapes and sizes made uneven diameter throughout the conducting tube (Fig. 11).

Conclusion

XLCs were successfully induced in *E. bosistoana* xylogenic callus culture. This system was different from a model system of *Zinnia* mesophyll cell culture in which a xylem tracheary element cell was directly trans-differentiated from a single mesophyll cell. The XLCs in xylogenic *E. bosistoana* calli appeared to have differentiated from small dividing cells that were cytoplamically dense or compact, and exhibited increased lignin contents during culture. Moreover, the eucalyptus XLCs showed diverse sizes, patterns of secondary cell wall thickenings and organized development including cell–cell connections of the XLCs to form xylem strands. This is the first report of the organized development of XLCs in *E. bosistoana* callus culture. Although the XLCs exhibited similar morphology and cell wall chemistry (lignin formation) associated with SCW formation to the natural xylem cells, their ability to perform the normal function of water transport remains to be clarified.

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Author contributions Sabai Saw Shwe carried out the experiments, collected data, prepared drafts and revision of the manuscript. Hossein Alizadeh helped Sabai with light microscopic measurements and Ayelen Tayagui contributed to the confocal microscopy. David W.M. Leung was responsible for the project conception, overall direction of the experiments, revision and finalisation of the manuscript.

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Declarations

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