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Research article

Immunolocalization of β-(1–4)-D-galactan, xyloglucans and xylans in the reaction xylem fibres of *Leucaena leucocephala* (Lam.) de Wit

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ABSTRACT

Cell wall architecture of tension wood fibres represents a suitable biological system to study the mechanism of growth and maintenance of posture of trees growing under various physical and physiological growth constraints. In the present study, we investigated the spatial distributions of β-(1–4)-D-galactan, xyloglucan and xylans (both less and highly substituted) in the opposite and tension wood fibres of bent *Leucaena leucocephala* by immunolabelling with monoclonal antibodies LM5, CCRCM1, LM10 and LM11 specific to these carbohydrate epitopes. The presence of non-lignified, tertiary wall layer is the typical tension wood characteristic associated with the reaction xylem fibres in *Leucaena*. LM5 labelling of opposite fibres showed weak labelling in the cell walls indicating less concentration of β-(1–4)-D-galactans while tension wood showed strong labelling in the tertiary wall layer suggesting the gelatinous layer (G-layer) has a strong cross linking with β-(1–4)-D-galactans. Xyloglucan distribution was more in the compound middle lamellae and the primary wall-S1 layer boundary of tension wood fibres as compared to that of opposite wood. A weak labelling was also evident near the boundary between the G-layer and the secondary wall of tension wood fibres. The secondary wall of opposite and tension wood fibres showed a strong distribution of both ls ACG Xs (LM10) and hs ACG Xs (LM11) while a weak labelling was noticed in the compound middle lamella. Tension wood fibres also showed strong xylan labelling mainly confined to the lignified secondary walls while the G-layer showed weak xylan labelling. In conclusion, our results suggest that β-(1–4)-D-galactans and xyloglucans could be implicated in the tensile stress generation within the G-layer of tension wood fibres of *Leucaena leucocephala*.

1. Introduction

The mechanical strength of trees growing under various physical and physiological constraints has been mainly attributed to the structural architecture and chemical composition of the cell wall in xylem fibres. In general, the fibre cell wall is a multi-layered composite composed of middle lamellae, primary and the secondary wall formed by cellulose, lignin and different matrix polymers. The source of strength in solid wood is wood fibre (Windsay and Rowell, 2009) and its mechanical properties are mainly due the thick secondary wall. Understanding the biology of cell wall formation, especially the complex interaction between wall polysaccharides and lignin is considered to be of paramount importance to unravel the science of cell wall assembly and also to modify the cell wall chemistry for the industrial applications.

Tension wood formation occurs in the tree trunk when its orientation is shifted from vertical since mechanical reinforcement is required to maintain the optimal architecture of dicotyledonous species (Mellerowicz et al., 2008; Almeras and Clair, 2016; Groover, 2016). The natural fibres in the tension wood could be an attractive experimental system for exploring the developmental and biochemical pathways of the secondary wall formation (Kwon, 2008). Tension wood plays an important role in the physiology of maintaining stems and branches with secondary growth in appropriate positions by allowing it to bend through generating tensional longitudinal stress (Washusen et al., 2003; Clair et al., 2013). Tension wood fibres are characterized by the presence of an additional gelatinous layer (G-layer) which is deposited on the lumen side of the cell wall. The chemical constitution of the G-layer mainly consists of highly crystalline cellulose (Daniel et al., 2006). Hemicelluloses, such as xyloglucan (Nishikubo et al., 2007), arabinogalactan protein (Lafarguette et al., 2004) or lignin (Joseleau et al., 2004) may also be present. A link between tension generation and lateral interaction of cellulose microfibrils with the matrix polysaccharides had been proposed (Mellerowicz et al., 2008). Therefore,

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non-cellullosic matrix polysaccharides are considered to be the most potential target molecules to study in relation to stress generation in tension wood. Due to their structural and chemical variations, opposite and tension wood fibres could be used as an ideal system to study the interactions among the different cell wall polymers. Therefore, studies on the cell wall structure and chemistry of the tension wood are necessary to understand adaptive growth in response of wall polymers in response to various physical and environmental forces such as wind and gravity (Groover, 2016). The cellulose microfibril angle is a reliable indicator for the tensile stress (Clair et al., 2011) while xyloglucan endotransglycosylase (Mellerowicz and Gorshkova, 2012) and fasciclin-like arabinogalactan-protein (MacMillan et al., 2010) are the key elements in force generation in poplar tension wood (Groover, 2016). However, the tension wood fibres in the tropical trees growing under different environmental and physical conditions have received very little attention on pattern and distribution of cell wall polymers and their possible role in growth stress-associated functions.

Leucaena leucocephala is an evergreen tree species adapted to different tropical eco-climatic zones, and the commercial value of its wood fibres is due to its suitability for production of paper and pulp. Recent studies on tension wood of *L. leucocephala* using anatomical, immunofluorescence and biochemical methods revealed a significant change in the cell wall chemistry compared to opposite wood (Pramod et al., 2013, 2017). Pramod et al. (2013) revealed that the G-layer in the tension wood fibre is devoid of lignin. Therefore, the present study is aimed to investigate the spatial distribution of matrix polysaccharides β-(1–4)-D-galactan, xyloglucans and xylans in the opposite and tension wood fibres of *Leucaena leucocephala* using immuno electron microscopy (IEM) methods. This study describes the distribution patterns of these matrix polysaccharides, their relation to the lignification status of cell wall layers and their possible role in tensile stress generation.

2. Materials and methods

2.1. Plant materials

Wood samples were harvested from two trees of *L. leucocephala* (5 years old) growing in the premises of Sardar Patel University, Gujarat, India. The wood discs were collected from bending region of the main trunk (located 2m above ground level) having 35 cm girth. The eccentric growth pattern found on the upper side of the disc is identified as tension wood and opposite wood found on the lower side of the wood disc.

2.2. Sample processing for LR white resin embedding

Samples were trimmed into 2 × 5 mm size pieces and fixed in modified karnovsky fixative (0.1% glutaraldehyde and 4% paraformaldehyde in 50 mM sodium cacodylate buffer) for 4 h at room temperature. Tissues were dehydrated in graded series of ethanol and infiltration with a mixture of LR White and ethanol followed by pure resin. Tissues were embedded in gelatin capsules filled with fresh LR White and were incubated for 2 days at 60 °C for polymerization.

2.3. Light microscopy

Transverse sections (1–2 μm thick) were taken with a diamond knife from the LR White embedded samples using an ultramicrotome (RMC Powertome model X, Boeckler Instruments., Inc., Tucson, AZ, USA) and they were stained with 0.05% toluidine blue O (Berlyn and Mikshe, 1976). Sections were examined and photographed using Leica microscope (DM2000) with a Canon digital camera (DM 150).

2.4. Immunogold labelling

Ultrathin transverse sections of 90 nm thickness were taken on a diamond knife using an ultramicrotome and mounted on nickel grids. The grids were suspended in buffer A (pH 8.2 Tris-buffered saline containing 1% bovine serum albumin and 0.1% NaN_3) for 30 min at room temperature. Subsequently, sections were incubated with LM5 (β-1,4-galactans), LM10 (low substituted xylans) or LM11 (highly substituted xylans), CCRCM1 (fucosylated xyloglucans) antibodies obtained from Plant Probes, Leeds, UK (1:20 dilution in buffer A) for 2 days at 4 °C. Following the washings with buffer A for 15 min each, the grids were incubated with goat anti-rat secondary antibody labelled with 10-nm colloidal gold particles (EM.GAT10/1, BB International, Crumlin, UK) for 2 h at room temperature (1:20 dilution in buffer A). A similar method was followed for labelling xyloglucan except the grids were incubated with goat anti-mouse secondary antibody (EM. GMHL10/1, BB International, Crumlin, UK). The control sections were incubated only with secondary antibody. The labelled grids were washed in six changes of buffer A followed by distilled water for 15 min each. Sections were post-stained with 1% $KMnO_4$ for 30 min, washed with three changes of distilled water for 10 min each and examined under the transmission electron microscope (TEM, Philips, Morgagni M268) at an accelerating voltage of 70 kV.

3. Results

3.1. Light microscopy

The structural characteristics of the cell wall in opposite and tension wood fibres were examined by light microscopy. The tension wood fibres were characterized by a moderately thick non-lignified G-layer which replaces major part of the inner secondary wall in opposite wood (Fig. 1a and b).

3.2. Immunogold labelling of β-1,3-galactans, xyloglucans and xylans

TEM observation of the ultrathin sections of opposite wood fibres labelled with LM5 antibody revealed a weak labelling in the secondary wall. However, β-1,4-galactans labelling was evident in the transition region between compound middle lamellae and S1 wall layer (Fig. 2a). Tension wood fibres showed strong LM5 labelling at the boundary region of S2 wall layer adjacent to the G-layer which was detached during tissue processing (Fig. 2b). Strong β-1,4-galactan labelling was also observed in the G-layer and the compound middle lamellae while the lignified regions of S1 and S2 layers showed a weak labelling (Fig. 2c).

The distribution of fucosylated xyloglucan was detected with CCRCM1 antibody. Opposite wood fibres showed a relatively weak labelling in the compound middle lamellae and cell corners (Fig. 3a and b). A strong labelling for xyloglucans was evident in the CML and boundary between S1 and primary wall of tension wood fibres (Fig. 3c and d). However, we did not detect any labelling in the G-layer (Fig. 3d). Similarly, a weak labelling was evident in the boundary between the S2 and the G-layer suggesting the presence of XyG linkage between secondary and tertiary cell wall layers (Fig. 3e).

Xylan labelling with LM10 antibody showed strong labelling of the

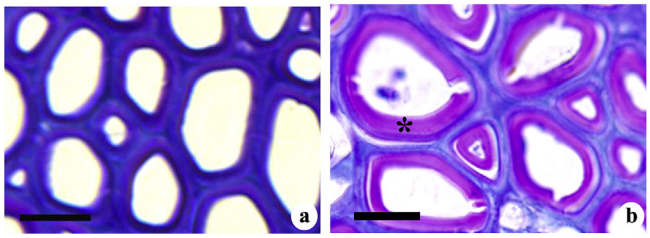

Fig. 1. (a-b): Light (a-b) microscopy of transverse sections from opposite and tension wood fibres in *L. leucocephala* (Toluidine blue O staining).
a. Opposite wood fibres showing thick secondary walls. b
Tension wood fibres showing thick, non-lignified, G-layer (asterisks)

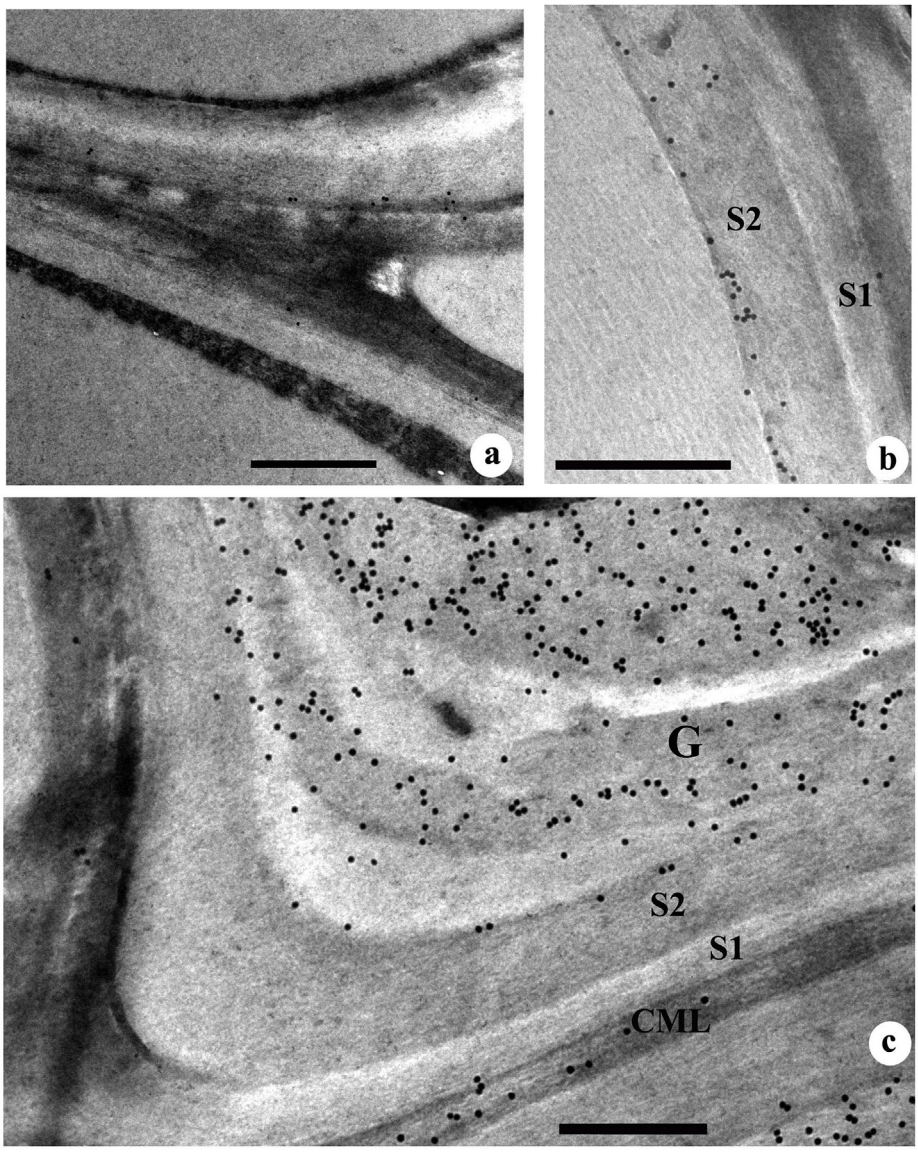

Fig. 2. Immunogold localization of β-(1,4)-D-galactans with LM5 antibody in the opposite (a) and tension wood (b,c) fibres of *L. leucocephala*
a. a. The opposite wood fibres showing β-1,4-galactan labelling at the compound middle lamellae region and inner boundary of S1 layer of secondary wall. Note the weak labelling from S2 and S3 layers of the secondary wall.
b. Tension wood fibre showing strong β-1,4- galactan labelling in the boundary layer between S2 layer of the secondary wall and detached G-layerc
c. The G-layer showing strong β-1,4-galactan labelling. Note the absence of labelling in the lignified secondary wall region and presence of gold particles in the compound middle lamellae region.

secondary wall regions of opposite wood fibres (Fig. 4a). Strong labelling was also noticed in the secondary wall of the tension wood fibres (Fig. 4b). On the other hand, LM10 antibody did not show xylan labelling in the G-layer of tension wood fibres (Fig. 4b). Similar to LM10 labelling results, LM 11 antibody also showed strong labelling of lignified secondary wall regions of the opposite wood fibres (Fig. 4c). Weak LM11 labelling was observed in the compound middle lamellae (Fig. 4c). Tension wood fibres showed strong xylan labelling with LM11 antibody in the lignified S1 and S2 wall layers whereas no labelling was observed in the G-layer (Fig. 4d). The distribution of gold particles was relatively higher in the compound middle lamellae and cell corner region of the G-fibres as compared to the fibres of opposite wood (Fig. 4d).

4. Discussion

The primary structural characteristic which distinguishes tension wood fibre from that of opposite wood in *Leucaena* is the presence of a tertiary wall (G-layer) as in several angiosperms (Fig. 1). The LM5 labelling pattern of the tension wood fibres revealed abundant distribution of β-14-galactans in the G-layer which is devoid of lignin. Arend (2008) reported that strong labelling of β-14- galactans was restricted to the interface between the G-layer and adjacent secondary wall in the poplar tension wood fibres and proposed that it may play an important role in cross linking between the G-layer and secondary wall of G-fibres. In willow, immunolabelling analysis revealed LM5 labelling in the G-layer of differentiating tension wood fibres whereas after maturation, β-1,4- galactans were mainly localized in the boundary between the secondary wall and the G-layer (Gritsch et al., 2015). This pattern of differential distribution of high molecular weight β-14-galactans in the newly developing G-layer was proposed to be involved in loose organization of the cellulose microfibrils during the early development of G-fibres (Gritsch et al., 2015; Roach et al., 2011). The main polysaccharide constituent of G-layer matrix is rhamnogalacturanan (RG1) pectin and a smaller fraction of arabinogalactan proteins (AGP) (Gorshkova et al., 2015; Bowling and Vaughn, 2008; Guedes et al., 2017). Gorshkova et al. (2015) reported the co-occurrence of RG-1 backbone and β-1,4-galactan signals in the compound middle lamallae and G-layer using RU2 and LM 5 antibodies and the strongly linked matrix polysaccharides are retained by the cellulose microfibrils in tension wood. The pockets containing these hydrated polymers could be responsible for the characteristic mesoporosity of G-layers (Clair et al., 2011; Chang et al., 2015) which is correlated with development of tensile stress (Clair et al., 2011; Gorshkova et al., 2015). Therefore, the strong labelling of G-layer of *Leucaena* may be confined to galactan residue present in these pectic substances. The galactan and arabinan

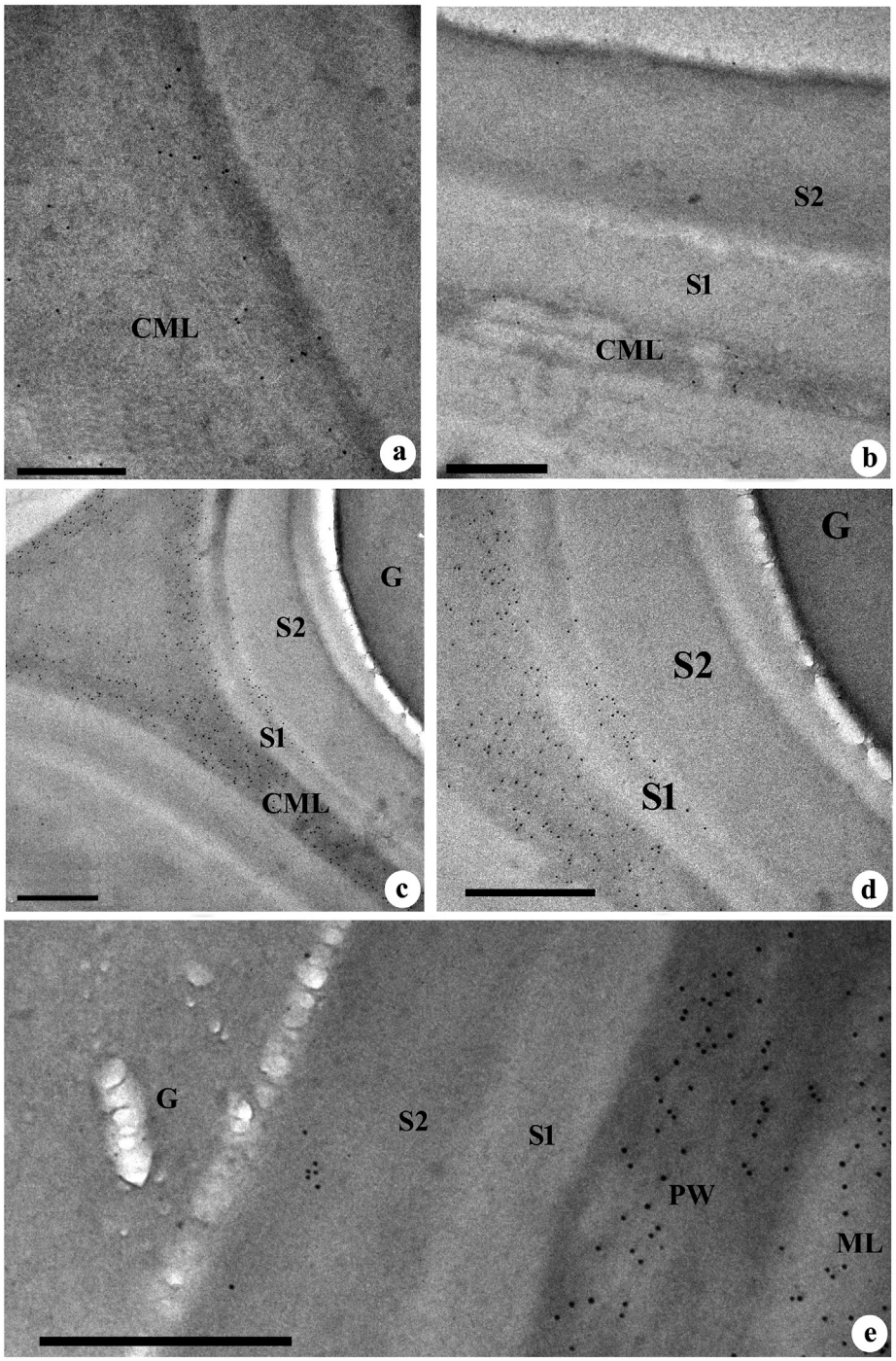

Fig. 3. Immunogold localization of fucosylated xyloglucans with CCRCM1 antibody in the opposite (a,b) and tension wood (c,d) fibres of *L. leucocephala*
a. a. The opposite wood showing xyloglucan labelling in the cell corner middle lamellae (CML).
b. The compound middle lamellae (CML) region of the opposite wood showing relatively weak labelling. Note the weak labelling in the S1 and S2 layers of the secondary wall. c
The CML region of the tension wood showing strong xyloglucan labelling. Note the weak labelling in the secondary wall (S1 and S2 layers) and the G-layer (G).
d. The enlarged view of Fig. 3c showing strong labelling in the CML and the boundary between the primary wall and S1 layer of the secondary wall.
e. The boundary region between S2 layer and the G-layer showing a weak labelling for xyloglucans.

side chains of galactan-rich pectins were proposed to be involved in the cross linking of the cellulose microfibrils (CMFs) (Zykwinska et al., 2007). In *Leucaena*, immunogold labelling showed a higher LM5 labelling in the S2 boundary adjacent to G-layer. This observation was more evident in the S2 boundary from where G-layer has been detached suggesting more β-1,4-galactans are incorporated into the S2 layer prior to G-layer development. On the contrary to previous reports on less relative proportion of β-1,4-galactans distribution in the G-layer of mature tension wood fibre (Gritsch et al., 2015; Roach et al., 2011), we observed high density of gold particles throughout the G-layer suggesting abundance of β-1,4-galactans throughout the G-layer of tension wood fibres of *Leucaena.* The reaction xylem in the *Acacia* root also showed a similar labelling pattern in their gelatinous fibres (Pramod et al., 2014). Some of the identified key elements responsible in force generation in the tension wood fibres are cellulose microfibril angle (Clair et al., 2011) xyloglucan endotransglycosylase (Mellerowicz and Gorshkova, 2012) and fasciclin like arabinogalactan protein (MacMillan et al., 2010). Our results suggest that being a predominant component in the G-layer, β-1,4-galactans could play a major role in mechanical properties of the tension wood possibly by providing the contractile driving force in the G-layer (Gorshkova et al., 2015).

Reaction xylem has been an attractive model system to study the interaction between the cell wall polymers. Previous immuno-localization studies suggest that increased lignification in softwood tracheids may have a close relationship with distribution of galactan (Schmitt et al., 2006; Mast et al., 2009; Altaner et al., 2010; Donaldson and Knox, 2012). The influence of the matrix polysaccharides in lignification process was also demonstrated in the recent studies on mannans and

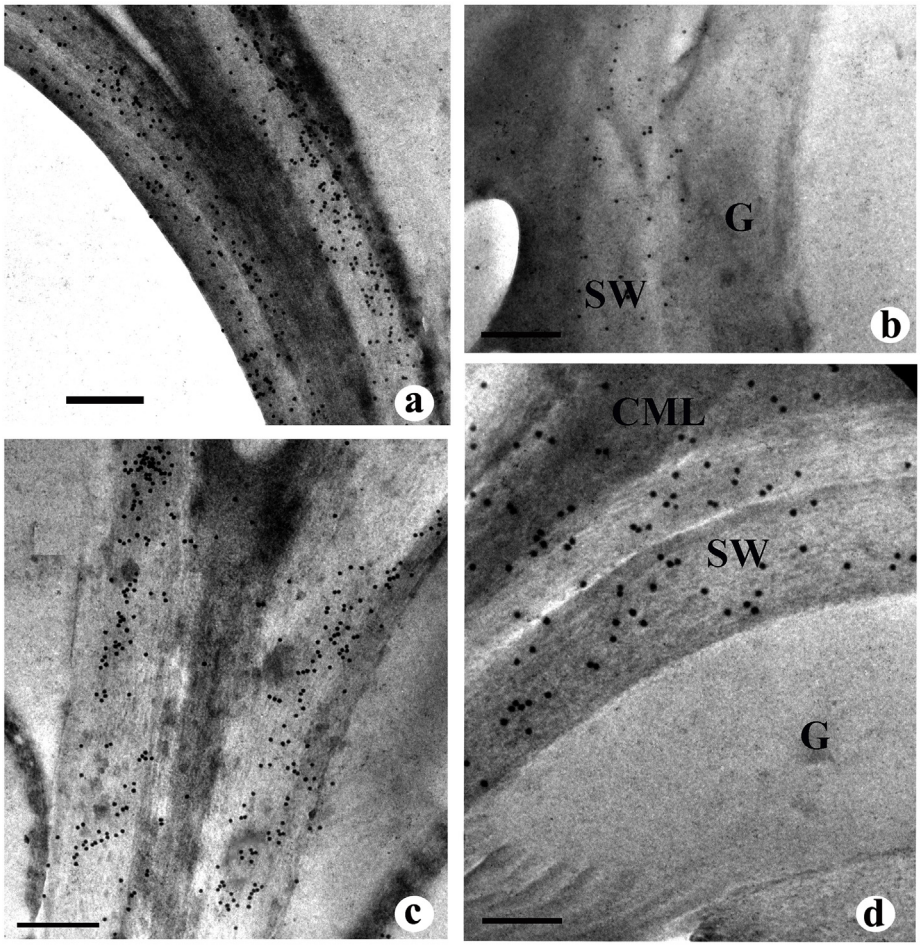

Fig. 4. Immunogold localization of xylans with LM10 (a-b) and LM11 (c-d) in the opposite and tension wood fibres of *L. leucocephala*
a. The opposite wood fibre showing strong labelling for ls ACG Xs in the lignified secondary wall regions. Note the weak labelling in the compound middle lamellae (CML) and the cell corner regions.
b. The tension wood fibre showing strong labelling for ls ACG Xs in the lignified secondary wall. Note the absence of gold
particles in the G-layer, CML and the cell corner regions.
c. The secondary wall of the opposite wood fibre showing strong labelling for hs ACG Xs with LM11 antibody. Note the weak labelling from CML region.
d. The tension wood fibre showing strong labelling for hs ACG Xs in the lignified secondary wall (SW) regions and CML. Note the absence of labelling from the G-layer (G).

xylans (Kim et al., 2010, 2011) suggesting their possible chemical bonding with lignin that results in the formation of lignin-carbohydrate complexes (Tenkanen et al., 1999; Barakat et al., 2007). The initiation and extension of the lignification could be regulated by the spatial distribution of β-1,4-galactans (Donaldson and Knox, 2012). In the present study, we detected the presence of gold particles in the compound middle lamellae region. However, we noticed a weak labelling in compound middle lamellae region of fibres where lignin distribution is maximum. Similarly, the lignified secondary wall also shows a weak labelling of β-1,4-galactans suggesting the possibility of either a weak interaction between β-1,4-galactans and lignin in SW or due to masking effect of lignin on labelling.

One of the most important matrix polymers associated with the G-layer is xyloglucan (Mellerowicz et al., 2008). Xyloglucan (XyG) has been proposed to be involved in tightening the G-layer in a fibre cell by facilitating the attachment of CMFs into adjacent wall layers bearing the tensile stress (Mellerowicz et al., 2008; Nishikubo et al., 2007). Present study revealed that the xyloglucan distribution is more in the tension wood fibres as compared to that of opposite wood fibres of *Leucaena*. The strong labelling was evident in the compound middle lamellae and boundary between the primary wall and S1 layer. The G-layer did not show XyG labelling. The recognition site for CCRC-M1 monoclonal antibody is the terminal fucosyl residue of xyloglucan (Puhlmann et al., 1994). The fucosylation occurs at β-D-Gal in the side chain attached to the third glucose in the XyG subunit (Zabotina, 2012). The absence of XyG labelling in the β-1,4-Dgalactan-rich G-layer of *Leucaena* also confirms the specificity of CCRCM1 to the fucosylated residue in the xyloglucans. Guedes et al. (2017) also reported absence of XyG detection in the G-layer of poplar tension wood fibres. However, we noticed few gold particles in the boundary of S2 and the G-layer detached from the secondary wall (Fig. 3e) suggesting the presence of XyG linkage between the G-layer and the secondary wall of the tension wood fibres in *Leucaena*. Baba et al. (2009) noticed the incorporation of XyG started at the inner surface of the S1 and S2 layers and labelling was apparent until the development of complete G-layer. However, labelling disappeared from these regions apart from faint traces in the inner surface after maturation of the fibre cells (Lafarguette et al., 2004). Therefore, the weak labelling observed for XyG in the G-layer found in the mature tension wood fibres of *Leucaena* could be due to some masking of CCRCM1 epitope associated with the cell wall maturation process. Based on the observed pattern of distribution of XyG and XET during G-fibre development, Baba et al. (2009) proposed that the inner surface of the wall is coated with XyG where XET could be the internal regions of secreted XyG during wall layering. Subsequently cleaving leads to the transfer of the newly generated reducing end to the non-reducing end of wall bound XyG. This wall bound XyG form a large network during endotransglycosylation occuring at the inner surface. On the other hand, the XyG terminals remain more exposed at the outer surface of the cell wall. The abundant AGPs in the G-layer create an appropriate moisture environment in the cell wall which promotes XET activity for several years even after cell death (Mellerowicz et al., 2008). AGP also play a role in maintaining the integrity of XyG inter-linkage which is essential for efficient tensile stress transfer between cell wall layers (Mellerowicz et al., 2008; Takeda and Fry, 2004). In poplar, the reinforcement of the cross linking between primary wall and S1 layer could be facilitated by xyloglucan endotransglycosylation (Mellerowicz et al., 2008). We could detect relatively more XyG distribution in CML and the boundary between the primary wall and the S1 layer in the tension wood fibres supporting the concept that XyG linkage could be a key element for force generation

during the growth stress (Groover, 2016; Mellerowicz and Gorshkova, 2012).

Strong labelling for LM10 and LM11 in the opposite wood fibres provided evidence for xylan as the major matrix polysaccharide. The previous reports of analysis with several antibodies specific for xylan present in the secondary wall (LM10, LM11 and AX1) suggest that xylan is restricted to the secondary wall layers in gelatinous tension wood fibres of several species (Berlyn and Mikshe, 1976; Decou et al., 2009). According to Nishikamo et al. (2007), xylan is not detected in the linkage analysis of isolated G-layers. LM10 and LM 11 labelling tension wood fibres of *Leucaena* shows distribution of both low and highly substituted xylans mainly confined to the secondary walls while the absence of labelling in the G-layer suggesting difference in chemical composition between G-layer and the secondary wall. The tension wood fibres of poplar also showed a similar pattern of xylan distribution (Kim and Daniel, 2012). Kim and Daniel (2012) suggested the possibility of less impact of tensile stress on the secondary wall of the tension wood fibres even though xylan is the major hemicellulos in hard woods. A weak LM11 labelling has been reported in the G-layer of poplar tension wood indicating the presence of hs ACG Xs in the G-layer and suggested that xylans may not be completely absent in G-layer (Kim et al., 2012). In the tension wood fibres of *Leucaena*, we did not detect LM10 and LM11 labelling in the G-layer. The lignified secondary wall regions of the opposite fibres also showed strong labelling with both LM10 and LM11. Interestingly, the middle lamellae regions and the cell corner regions of secondary wall in tension wood fibres showed relatively more hs ACG Xs labelling than that of opposite wood. The lignified region of the tension wood fibre is characterized by more quantity of condensed guaiacyl lignin monomeric units (Pramod et al., 2013). Hence, distribution pattern of xylan epitopes in the lignified and non-lignified (G-layer) regions of the cell wall suggests that xylans could be the major wall polysaccharide component which determines the lignin distribution in the secondary wall of fibres in *Leucaena leucocephala*. Kim et al. (2012) also reported the possibility of heterogeneity in composition of xylan distribution within the secondary wall layers of xylem fibres of poplar which was evident from a strong labelling for ls ACG Xs in the outer SW than the inner layer whereas uniform distribution of hs ACG Xs throughout the SW. In *Leucaena*, the distribution pattern of immunogold particles suggests abundance of hs ACG Xs in the outer corner region of SW while ls ACG Xs distribution was uniform throughout the SW of mature fibres. This variation pattern in the xylan heterogeneity within the same cell type reflects the plausibility of interspecific variation between trees growing in the temperate and tropical climates (Pramod et al., 2014) or due to different modes of deposition of ls ACG Xs and hs ACG Xs (Awano et al., 2002) during the cell wall assembly.

5. Conclusion

The interaction of matrix polysaccharides with cellulose microfibrils in the tension wood fibres believed to play the key role in tensile stress generation which is crucial in regulating the growth pattern of trunk and organs in order to maintain the posture of perennial trees. The relative abundance of β-1,4-galactans and xyloglucan in the fibre wall of tension wood compared to that of opposite wood of *Leucaena* suggests the possible role of these matrix polymers in the tensile stress generation within the G-layer of tension wood fibres. Although, xylan did not show any significant variation between their labelling pattern in the secondary wall of TW and NW, the weak labelling in the non-lignified G-layer suggests that xylans may have a major role in lignification of the secondary wall in the xylem fibres of *L. leucocephala*. Along with variation in CMFs, the regulation of spatial distribution of β-1,4-galactans, xyloglucan, xylans and lignin appears to be the most important factor determining the cell wall architecture and its viscoelastic properties, which in turn make the possibility of adaptive growth of trees under various physical, physiological and environmental cues.

Contribution of Co-authors

S. Pramod: Designing and conducting the experiments, interpretation of results and manuscript preparation.

K. S. Rao and Kishore S. Rajput: Designing the experiments, supervising the work and critical evaluation of the manuscript.

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