Evidence of the late lignification of the G-layer in Simarouba tension wood, to assist understanding how non-G-layer species produce tensile stress

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Abstract

To recover verticality after disturbance, angiosperm trees produce "tension wood" allowing them to bend actively. The driving force of the tension has been shown to take place in the G-layer, a specific unlignified layer of the cell wall observed in most temperate species. However, in tropical rain forests, the G-layer is often absent and the mechanism generating the forces to reorient trees remains unclear.

A study was carried out on tilted seedlings, saplings and adult *Simarouba amara* trees – a species known to not produce a G-layer. Microscopic observations were done on sections of normal and tension wood after staining or observed under UV light to assess the presence/absence of lignin.

We showed that *S. amara* produces a cell-wall layer with all of the characteristics typical of G-layers, but that this G-layer can be observed only as a temporary stage of the cell-wall development because it is masked by a late lignification. Being thin and lignified, tension wood fibres cannot be distinguished from normal wood fibres in the mature wood of adult trees.

These observations indicate that the mechanism generating the high tensile stress in tension wood is likely to be the same as that in species with a typical G-layer and also in species where the G-layer cannot be observed in mature cells.

Keywords: G-layer, lignification, maturation stress generation, ontogeny, *Simarouba amara* Aubl., tension wood cell wall, tree biomechanics

Introduction

One of the challenges for tree development is to grow vertically towards the light. During this growth, trees are submitted to the double challenge of gravity and external disturbances such as wind (Fournier et al. 2013). Trees must therefore have a structure strong enough to avoid buckling under their own weight (Jaouen et al. 2007), but that can also recover after a disturbance (Alméras and Fournier 2009). So, throughout their life, trees need to correct their posture through imperceptible movements, which enable them to remain upright.

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In angiosperms, this reaction is performed through the differentiation of a special kind of wood, named "tension wood" (Archer 1986), and is often accompanied by an eccentric growth. Tension wood is a peculiar wood tissue, that is under tensile stress, and that is formed in the upper side of leaning trunks and branches. Its tensile stress is a consequence of a strong trend to shrink at the time of its maturation (Archer 1986), and this is therefore named "maturation stress". When tension wood is formed on one side of the trunk or branch, it acts as an internal guy-wire enabling the axis to bend actively.

At the anatomical level, the typical model of tension wood in most temperate species is the development of a "gelatinous layer" or a G-layer in the fibre wall. This layer comes to replace the S_3 layer and part or all of the S_2 layer (Dadswell and Wardrop 1955, Gorshkova et al. 2010). The G-layer has been studied extensively in poplar models (e.g. Jourez et al. 2003, Clair et al. 2006a, Coutand et al. 2007, Fang et al. 2007, Fang et al. 2008a, Fang et al. 2008b, Clair et al. 2011, Yoshinaga et al. 2012, Chang et al. 2014, Coutand et al. 2014, Abedini et al. 2015, Chang et al. 2015). The G-layer is described as being exempt from lignin (Pilate et al. 2004, Yoshinaga et al. 2012) and containing cellulose microfibrils nearly parallel to the fibres' axis. It has the texture of a gel, with mesopores ranging from 2 to 50 nm (Clair et al. 2008), and the matrix is made of numerous specific polysaccharides (Mikshina et al. 2013). It has been shown that the G-layer is the driving force of maturation stress (Yoshida et al. 2002, Yamamoto et al. 2005, Fang et al. 2008a), which involves the swelling of the mesoporous matrix (Chang et al. 2015) and the generation of tensile stress in the cellulosic network (Clair et al. 2011).

However, when observing the diversity of tropical rain forest species, the unlignified G-layer is not commonly observed in tension wood. Indeed, more than half of species do not develop this unlignified layer (Onaka 1949, Fisher and Stevenson 1981, Clair et al. 2006b). Lignified tension wood (known for the absence of a G-layer) was shown to produce lower maturation stress than G-layer tension wood when comparing *Liriodendron tulipifera* with *Prunus spachiana* (Yoshida et al. 2000b). However, no such trend was observable in comparisons between a larger number of species (Clair et al. 2006b).

In these species, the tension wood fibre wall was reported to be lignified, but with a cellulose microfibril angle smaller than that in the opposite wood, *i.e.* as in species producing a G-layer (Yoshida et al. 2000b, Clair et al. 2006b, Ruelle 2006, Ruelle et al. 2006, Ruelle et al. 2007b). Ruelle (2006) showed that *Simarouba amara's* tension wood structure is indistinguishable from normal wood with an optical microscope, and that strong differences between tension wood and normal wood are only visible at the scale of the cellulose microfibrils. He showed that the microfibril angle is nearly parallel to the fibre axis in the inner layer of tension wood, and the crystallite size is higher than in the opposite wood, *i.e.* as in typical G-layer species. The lignin content was found to be almost the same in the tension and normal wood of *S. amara*.

Following Ruelle's works (2006, 2007a, 2007b), we chose *S. amara* as a model for species producing lignified tension wood without a G-layer. Special attention was paid to the fibre maturation stage, which is recognized to be the stage at which tensile stress is generated in G-layer species trees.

This study describes the fibre wall maturation in the tension wood of *S. amara*, and attempts to elucidate the maturation stress generation mechanism in a non G-layer species.

Material and methods

As a preliminary test showed that the anatomy of tension wood presented some strong modifications with ontogeny, experiments were conducted on trees from one year old to 80 cm diameter (estimated to be from 50 to 80 years old). Depending on the tree diameter, three different protocols were needed for the induction of tension wood. Sampling was therefore divided into three groups, corresponding to the growth condition and the type of mechanical disturbance.

Plant material

Seedlings staked in the greenhouse

Seedlings of *Simarouba amara* Aubl. were collected from forests, one and two years prior to the start of the experiment and were cultivated in a greenhouse. The two-year-old seedlings were collected in the forest of the agronomic campus in Kourou (French Guiana); while, the one-year-old seedlings were collected near the Paracou forest research station (50 km from Kourou). At the beginning of the experiment, 50 seedlings (2 ages × 5 angles × 5 replicates) were transplanted in bigger pots. They were then staked and artificially tilted at five angles from vertical: 0° (used as the control), 20°, 40°, 60° and 80° to induce an increasing gravitational stimulus (Fig. 1). The pole fastening enables a controlled gravitational stimulus constant in time and uniform in space to be maintained (Coutand et al. 2014). The trees were tilted on 18/12/2013 and were sampled at regular intervals between 60 and 120 days after tilting. This sampling enables observation of the tension wood development at different stages of maturation. The diameters at 15 cm from the ground ranged from 0.4 to 1.0 cm at the beginning of the experiment and reached 0.5 to 1.3 cm at the sampling dates. Ten of the seedlings had not grown enough at the sampling date (or were dead) to allow the study of the maturation process and thus were not taken into account in the following analysis.

Saplings guyed in the forest

Twelve saplings of *S. amara*, with diameters at 50 cm from the ground ranging from 1.4 to 6.6 cm, were selected in a natural forest. As these trees are larger than those cultivated in the greenhouse they were therefore presumed older. Unfortunately, as with numerous tropical species, *S. amara* does not produce annual rings and it is therefore not possible to estimate tree age from the growth rings. Ranking between trees is therefore determined by their diameter. These trees were artificially tilted but were not staked (Fig. 1). They were guyed with an angle of approximately 40°. Here, the stimulus was not controlled. The saplings were artificially bent on 9/01/2013, during the rainy season, and were sampled two months after the bending.

Adult trees naturally tilted in the forest

Achieving an artificial lean angle is almost impossible with bigger trees, so eleven naturally tilted *S. amara* were selected to represent adult trees. These trees ranged from 7.5 to 80 cm diameter at 130 cm from the ground (Fig. 1). Special attention was paid to avoid trees that may have been tilted recently (near gaps, broken branches) to ensure a long time of reaction. These trees were sampled without additional delay, assuming that the tension wood formation had already started, at least, several years before.

Both saplings and adult trees were sampled partly in the agronomic campus and partly near the Paracou forest research station.

Methods of measurements

Diameter measurements
Seedlings' diameters were measured with calipers before tilting. This measurement enables the estimation *a posteriori* of the position of the cambium at the tilting date on transverse sections after estimating the bark thickness. This measurement was expected to be necessary in case the tension wood could not be distinguished from normal wood as reported for *S. amara*. The forest trees' diameters were measured with a tape-measure.

For the seedlings and saplings, the trees were cut down and a disc was sampled. For the adult trees, sampling was performed by coring (1 cm diameter) (Fig. 1).

Cross-sections
Semi-thin cross-sections (20 µm thick) were realized with a sliding microtome. For seedlings, the cross-sections were performed on the whole disc at 15 cm from the ground. For the saplings and adult trees, only peripheral wood was examined at 50 cm and 130 cm from the ground, respectively (Fig. 1).

A sub-sample was dehydrated (without prior fixation) with ethanol series and embedded in LR White resin (two exchanges of resin/ethanol mixture for 1 h, followed by two exchanges in pure resin for 1 h and kept overnight at room temperature, then polymerised at 65°C overnight). Thin transverse sections (2 µm thick) were performed with a rotary microtome and diamond knife.

Staining and observations
Semi-thin cross-sections were double-stained with Safranin and Alcian blue 8GX. Safranin stains lignified tissues in red and Alcian blue stains unlignified tissues (*i.e.* the G-layer) in blue. The observations were performed in bright field.

Thin cross-sections were kept unstained. The observations were performed both with bright field and with UV light microscopy. For UV microscopy, light was generated with a Mercury lamp (USH102D USHIO) and the microscope (Olympus BX2) was equipped with Fluorescence Filter Cubes U-MNU2 (Excitation filter: 360–370 nm, Dichromatic mirror: 400 nm, Emission filter: 420 nm). UV light highlights the lignified cell-wall layers as a result of lignin autofluorescence (Lundquist et al. 1978, Albinssona et al. 1999 , Radotic et al. 2006), while the unlignified walls remain dark. In order to highlight the changes in lignin content within a section, a semi-quantitative estimation of the lignin content was performed by recording the mean brightness of several manually contoured G-layer. This comparison is possible within a section but cannot be done from section to section as it would require a perfect control of the thickness of the sections. Cell-wall thickness

Double fibre wall thicknesses (from lumen to lumen) were measured manually from images using ImageJ software (Schneider et al. 2012) on both thick and thin transverse sections. The fibre wall thickness was computed as half of the double wall thickness. Embedding the sample before performing thin sections avoids the swelling of the G-layer, as described by (Clair et al. 2005a). The swelling effect is an artefact occurring on non-embedded transverse section, resulting from the conjugate effect of the high tensile stress level, the absence of the reinforcing S_3 layer and the low transverse cohesion in the G-layer due to the absence of lignin. It results in an overestimation of the thickness of the G-layer. Comparison of the measurements of embedded and non-embedded cross-sections allows one to quantify the swelling effect.

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Raw data of the cell-wall thicknesses were smoothed for a more visual representation with the msir package (Scrucca 2011) for R software (2013). Local trends and their standard deviations were computed using a nonparametric estimation of the mean function.

Results

Identification of tension wood

The wood formed before and after the date of inclination was easily identified in the cross-sections from the seedlings and disks from the saplings. In cross-sections made from seedlings, the wood formed before tilting was recognized from its circular shape centred on the pith. After tilting, the higher growth rate of the upper side (tension wood) compared to the lower side of the tilted stem leads to a well-defined and easily recognizable crescent shape in the newly formed wood. On the tension wood side, fibre walls produced after tilting are nearly twice as thick as those produced before tilting. This tilting position was confirmed with caliper measurements.

The presence of tension wood was also confirmed from a mechanical point of view. When the stake was released at the end of the experiment, the seedlings sprang upward. Yoshida *et al.* (2000b) named this as the "spring-back" phenomenon. It is induced by the release of the growth stress that has been built up during the growing period and confirms the high tensile stress generation in the upper side of the stem.

In saplings, observations were made on the whole disc and clear crescents were observed in the newly formed wood on the upper side of the tilted stem. On the lower side, the limit was not distinguishable as the growth was much slower. These observations confirm that the protocol efficiently stressed the trees and that the trees reacted by forming tension wood, as characterized by the higher growth rate.

The adult trees were not felled and so eccentric growth was not observable; however, the higher activity of the cambium on the tension wood side was confirmed *a posteriori* on anatomical sections.

The identification of tension wood was also confirmed by the lower vessel frequency in the tension wood compared to the opposite wood in each of the ontogenic stages (See Supplementary material, Fig. S1).

Ontogenic variation of the tension wood cell-wall

Observation of the stained sections of the seedlings did not evidenced any trend between tension wood severity (differences in the cell wall thickness or changes in growth eccentricity) and the inclination angles. This would indicate that the reaction of the trees were already maximal for a tilting angle of 20° as already shown by Yoshida et al. (2000b) in *Liriodendron tulipifera* and *Prunus spachiana*. Thereby, all of the seedlings, regardless of the angle of inclination, produced a thick swollen unlignified layer, which looked like a typical G-layer in the vicinity of the cambium. This layer was stained in blue with Safranin/Alcian blue. The cell walls were thick and presented all the characteristic patterns of a G-layer, such as a weak adhesion to the previous layer and an irregular shape on the lumen side (Fig. 2b and 2c1).

Among the saplings, the six saplings with the smallest diameters also produced a thick G-layer like that observed in the seedlings.

The other five larger saplings, together with the naturally tilted adult trees, also presented an unlignified cell-wall layer in the tension wood up to around 1 mm from the cambium; whereas in the opposite wood, this distance was around 0.1 mm. However, the unlignified layer was not thick but was characterized by a torn-apart aspect of the inner layer (Fig. 3c1 and 4c1) like in the non-mature cell-wall layer of seedlings. The torn-apart aspect was not visible on sections prepared on embedded wood blocks. Observations with bright field microscopy of embedded wood sections (Fig. 3e1 and 4e1) showed a distinct and well-defined wall layer. The torn-apart aspect is therefore an artefact, consequence of damages during the preparation of the non-embedded samples, similar to what is observed in mature G-layer (Clair et al. 2005a, Clair et al. 2005b).

Following these observations, we distinguished two main types of tension wood cell wall near the cambium depending on the ontogenic stage: (i) a thick G-layer in seedlings and young saplings and (ii) a thin G-layer in older saplings and adult trees.

Effect of the duration of development on the lignification of the cell wall

Normal wood and opposite wood
Normal wood was observed in seedlings maintained in an upright position (0°) and in the opposite side of saplings and adult trees. Observations of semi-thin sections after the double-stain provided evidence of the lower growth rate in normal/opposite wood compared to tension wood.

Observations on the thin cross-sections in bright field and under UV light indicate that lignification occurs all along the cell wall thickening (see Supplementary material, Fig. S2).

Tension wood in tilted seedlings
In the seedlings sampled 60 days after tilting, a G-layer was observed in double-staining in the whole portion of wood produced after tilting, up to the normal wood formed before tilting (see Supplementary material, Fig. S3). In the seedlings sampled later than 60 days after tilting, a transition zone occurred between the G-layers and normal wood with reddish thick fibres (Fig. 2c2). In this zone, fibres look neither like normal wood nor like the G-layer (Fig. 2b, 2c2 and 2c3). From cambium to normal wood, four stages can be distinguished: (i) fibre wall thickening; (ii) wood with a thick G-layer; (iii) a reddish area of unknown cell type; and (iv) normal wood (Fig. 2).

Observations with UV light (Fig. 2d) confirmed the previous results. The comparison of the same fibres in bright field (Fig. 2e) and under UV (Fig. 2d) clearly revealed that the thick G-layer did not emit any fluorescence signal, confirming the absence of lignin in this layer. In these fibres, middle lamella and cell corners already showed a strong lignin signal. In the transition zone, the fluorescence signal increased with the increasing lignin content of the G-layer. It can be noticed that the lignin content in the transition zone did not reach the level observed in normal wood cell-walls.

Tension wood fibres are also characterized by the absence of a distinct S_2 layer in the outside of the G-layer. The observation methods used in this study do not allow us to draw conclusions about the presence of the S_1 layer.

Figure 2a shows the double fibre cell wall thickness as a function of the distance from the cambium measured in a seedling. The measurements were performed both on semi-thin cross-sections (20 µm) and on thin cross-sections embedded in resin (2 µm) sampled from

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the same wood block. The distance between the two different cross-sections did not exceed 1 or 2 mm along the fibre direction.

Measurements performed on the embedded sample afforded the distinction of three stages from cambium to mature wood: (i) cell wall thickening up to ca 500 μm from the cambium; (ii) tension wood fibres with nearly constant thickness (2.5 ± 0.5 μm) up to ca 1500 μm from the cambium (this area includes fibres with a thick G-layer and the transition zone with reddish fibres); and (iii) normal wood with a constant cell-wall thickness (1.5 ± 0.4 μm) but approximately 40% thinner than in tension wood .

Measurements performed on a non-embedded wood block showed similar cell-wall thickening near the cambium, but this increase continued up to 1000 μm, reaching nearly twice the thickness measured on the embedded samples in the same area (4.8 ± 0.8 μm). This increasing thickness stopped when cell wall lignification starts, and then decreased up to the transition with normal wood. In normal wood, the thickness remained constant and equivalent to the thickness measured on the embedded samples (1.7 ± 0.3 μm).

Saplings with a thick G-layer
Saplings with a G-layer presented the same pattern in tension wood as seedlings. The late lignification of the G-layer was even better characterized in double-staining as no trace of Alcian Blue remained at the end of the lignification, contrary to the seedlings, where the reddish area keeps a trace of Alcian blue (Fig. 2c3).

Saplings without a thick G-layer
For the bigger saplings, a similar pattern as trees with a thick G-layer was observed in double-staining, but the classical thick G-layer pattern was not visible. The inner unlignified layer was neither thick nor swollen. Hence, we could also distinguish four stages: (i) fibre wall thickening; (ii) unlignified fibres; (iii) reddish fibres of lignified fibres; and (iv) normal wood (Fig. 3b).

Thickness measurements performed on the embedded samples show three stages (Fig. 3a) from cambium to mature wood: (i) cell wall thickening up to ca 900 μm from cambium; (ii) tension wood fibres with nearly constant thickness (2.2 ± 0.3 μm) up to ca 2 mm from the cambium (this area includes fibres with a thick G-layer and a transition zone with reddish fibres); and (iii) normal wood with a constant cell wall thickness (1.6 ± 0.3 μm), but approximately 25% thinner than in tension wood. The difference between normal wood and tension wood is smaller than for the seedlings.

The thin inner layer (noticed as torn on the semi-thin cross-section) did not emit a UV fluorescence signal all along its thickening. The lignification occurred only after the cell walls reached their final thickness (Fig. 3a, 3c and 3d). This phenomenon was also observed in seedlings and saplings with the late lignification of the thick G-layer, contrary to normal wood, where the lignification of the secondary wall occurs all along the wall thickening.

Adult trees
In adult trees, normal wood is not expected to be observable on the sections made on the upper side of the tree. Indeed, the trees chosen for the tests had been tilted for a long time and normal wood was not expected to be present in the first centimetre. The number of cells remaining blue after the cambium appeared much higher than in the opposite wood (40 vs ca 10 cells), indicating a higher growth rate or a longer maturation process. The torn-

apart aspect of the inner layer disappeared when the cell wall was lignified (Fig 4b3 and 4b4).

Fibre wall thickness measurements performed on embedded samples allowed us to distinguish only two stages (Fig. 4a) from the cambium to mature wood: (i) cell wall thickening up to ca 800 μm from cambium; (ii) tension wood fibres with nearly constant thickness (1.7 ± 0.3 μm).

In adult trees, as in the largest saplings, the thin inner G-layer (also noticed as torn on the semi-thin cross-sections) did not emit any fluorescence signal along the length of its thickening. The lignification occurred only after the cell walls had reached their final thickness (Fig. 4a and 4c).

In adult trees, in mature wood far from the cambium, fibres with a G-layer pattern were not observed and no difference could be found at the anatomical level between tension wood and normal wood, except for a slight difference in wall thickness, as reported by Ruelle (2006).

Figure 5 summarises the patterns of the fibre wall thickness observed in tension wood at the three ontogenic stages and in the normal wood of an upright seedling.

Discussion

Simarouba produces a G-layer that is lignified at a late stage during the maturation process

Previous reports revealed *S. amara* to be a model for species forming tension wood without a G-layer. The secondary wall layer described in the results has all the common characteristics of a G-layer. It is unlignified, thick and is prone to swell during sectioning. Therefore, these results show the ability of this species to produce a G-layer. No distinct S_2 layer was observed using bright field microscopy, suggesting that the cell-wall structure corresponds to the model S_1 + G reported by Dadswell and Wardrop (1955). The presence of a G-layer and its thickness appears not to depend on the stimulus intensity in the range of the tested angles.

In the seedling and small saplings of the sample, the G-layer is thick and presents all of the common anatomical features of G-layers from species such as poplar. When trees were maintained tilted for a long enough time, observations showed that maturation of the G-layer continues up to its lignification. This result is attested by the observations of lignin autofluorescence under UV light. Fibre wall thickness measurements on the resin-embedded cross-sections show that the G-layers had already reached their final thickness before the lignification starts. Measurements on the semi-thin cross-sections without embedding showed a much thicker cell wall of the G-layer before lignification. This overestimation on the non-embedded samples was already described for the G-layer in poplar tension wood and named the "swelling effect" or "border effect" (Clair et al. 2005a). It is attributed to: (i) the high longitudinal tensile stress in the G-layer generating large transverse strains when the stress is released by cutting and (ii) the absence of reinforcement of the cellulose network by lignin in the unlignified G-layer. When fibres begin to be lignified, the wall is reinforced transversally and the swelling effect is less marked, and so disappears when the fibre walls are fully lignified. In normal wood, no significant difference in the cell wall thickness could be measured between the embedded and non-embedded wood sections.

Both with embedded and non-embedded samples and with both seedlings and small saplings, a mature lignified tension wood was observed with a thicker fibre wall than in normal wood.

In older saplings, the wall thickness is much thinner and the observation is consequently less obvious. However, we showed that a G-layer can also be observed with a similar pattern as observed in seedlings based on three arguments: (i) in the early stage of development, tension wood is highly sensitive to cutting artefacts that produce its torn-apart aspect on non-embedded wood blocks, exactly as in seedlings and saplings, (ii) at this stage, the tension wood secondary wall layer is unlignified, as attested by UV microscopy, and (iii) the lignification of the G-layer starts after the end of its thickening, as in seedlings and saplings. On the contrary, in normal wood, the lignification occurs all along the wall thickening, as classically reported in the literature (Takabe et al. 1981, in Yoshida et al. 2000a, Grünwald et al. 2002, Zhong and Ye 2015). After lignification of the G-layer, the wall is reinforced and never appears torn apart.

In adult trees, there is no significant difference in thickness between tension wood and normal wood cell wall thickness, as reported by Ruelle (2006). However, we still found three other aspects of a G-layer that are also found in seedlings: (i) tension wood highly sensitive to cutting artefacts (ii) an unlignified secondary wall layer, and (iii) lignification, which starts after the end of the wall thickening.

The G-layer in *S. amara* is therefore a temporary stage of the cell wall, which occurs during the maturation and is later masked by lignification. Non-G-layer species differ from G-layer species by the absence of mesoporosity (Chang et al. 2009). Our observations are consistent with the assumption that lignin fills the mesopores of the G-layer.

This late lignification would indicate that a functional process occurs during the cell maturation in its G-layer form and needs to be completed before lignification. This process may presumably be linked to the maturation stress generation.

Tension wood cell-wall thickness change during ontogeny
The tension wood fibre wall is around 1.5-times thicker than normal wood cell-wall in the seedling stage. This decreases to 1.3-times in the sapling stage and ends with a thickness similar to normal wood in old trees. It could be suspected that the change in cell wall thickness is the result of a change of mechanical stimulus which is not controlled in our experiment. Mechanical stimulus has been shown to be trigged by both the local inclination and the local stress (Coutand et al. 2014). In our experiment, saplings were tilted with an angle larger than some of the seedlings and the stress applied during the guying of the sapling is much higher than in tilted stacked seedling, therefore stimulus is in both cases higher in saplings than seedling. Even so, the seedlings produced a thicker cell wall than the saplings in tension wood. We therefore conclude that change in thickness is related to ontogenetic stage.

After completing the maturation of tension wood fibres, no differences can be observed at the anatomical level between tension wood and normal wood in adult trees. Only at a finer scale do some traces of its past as a G-layer remain visible: Ruelle et al. (2007b) reported that the orientation of cellulose microfibrils is nearly parallel to the fibre axis in *S. amara* tension wood, as commonly observed in the G-layer, and that the crystallites sizes are also significantly higher in tension wood, as in *Eperua falcata*, a species producing a G-layer.

At the anatomical level in adult trees, the only parameter allowing one to distinguish tension wood is the kinetics of the maturation process, which is clearly different than in normal wood. In normal wood fibres, lignification occurs simultaneously with the fibre wall thickening, whereas in tension wood, maturation appears to take much longer and it can be clearly observed that lignification of the secondary wall (G-layer) starts after the end of the fibre wall thickening.

The effect of ontogeny on the wall thicknesses was also evidenced by the observation of both patterns in a single naturally tilted sapling tree. In this individual, a crescent of tension wood was recognizable near the pith, characterized by thick lignified cell walls, whereas far from the pith, the presence of tension wood was confirmed by the eccentric growth, but no significant difference in thickness was recorded with the opposite wood.

Generalization to other "non-G-layer" species

The generalisation of these observations to all species known to produce tension wood without a G-layer may be questionable. In previous studies reporting on the diversity of tension wood (Onaka 1949, Fisher and Stevenson 1981, Clair et al. 2006b), more than 50% of species were described as being without a G-layer. In these studies, only mature wood was studied, and probably only on adult trees. It is therefore probable that the unlignified wall occurring before the end of the maturation process was not evidenced.

In the literature, the presence of traces of lignin in G-layer has been several times reported (see review by Fagerstedt et al. 2014) however these studies did not evidenced that non-G-layer species may be the result of the lignification of the G-layer as these studies did not followed the maturation process of the tension wood cell wall.

It is interesting to note that in a study on seedlings of *Liriodendron tulipifera* Linn.*,* a species that does not produce a G-layer in tension wood, Yoshida et al. (2000b) did not observe an unlignified G-layer. If the pattern is common to *S. amara* seedlings, a thick unlignified G-layer may have been visible near the cambial zone. Unfortunately, for the need in their experimental set-up, the authors removed 'the smooth outer surface of the secondary xylem', including the 'cambial zone, and differentiating xylem', thus they were not able to observe these stages.

In order to support the generalization of our results with respect to more species, additional sampling was performed in a tropical rain forest (French Guyana). Naturally tilted seedlings were sampled and observed after double-staining with Safranin/Alcian blue. In several species, tension wood exhibited fibres with a G-layer at the vicinity of the cambium, which was later lignified during the maturation process (see Supplementary material, Fig. S4). Therefore, preliminary observations could lead to the conclusion that the lignification of the G-layer is a mechanism shared by other species. However, the question of the generalisation to all species still classified as non-G-layer species remains open and will need specific large-scale studies in the future.

A common mechanism in tension wood with an unlignified and lignified G-layer

Tensile maturation stress is generated by the trees to counter the gravitational stress (naturally occurring or experimentally induced). Up to now, all research about the generation of maturation stresses in tension wood has focused on G-layer species, with poplar as a model plant. Numerous observations have proven the involvement of the G-layer to produce stress in these species. It was also shown that maturation stress generation takes place during the development of this G-layer, as observed in poplar wood (Clair et al. 2011). Several mechanistic models have therefore been proposed (Mellerowicz et al.

2008, Alméras et al. 2012, Mikshina et al. 2013, Fournier et al. 2014, Chang et al. 2015), and all of them consider the G-layer as the driving force of maturation stress generation. The question of the absence of a G-layer in some species and therefore the mechanism of the generation of stress in these species has been totally ignored. This present study has proved that in *S. amara,* a G-layer is also formed specifically in tension wood. It can therefore be hypothesized that a common mechanism would be involved in other non-G-layer species. However, most studies insist on the role of specific polysaccarids of the G-layer in stress generation. It will therefore be necessary to identify if the polysaccarids from *S. amara*'s G-layer are the same as those in an unlignified G-layer, such as in poplar. If so, it will be very interesting to understand how trees are able to reprogram the cellular machinery of cell wall synthesis, in order to delay the lignification process in *S. amara* or even entirely suppress it in G-layer species.

Considering that the stress generation is achieved within the unlignified stage, a second process involving lignification would then start. No evidence can be given that the lignification does not contributes to the tensile stress generation. However, the few studies where maturation stresses were measured on species with and without G-layer did not evidenced a higher level of maturation stresses in non-G-layer species (Yoshida et al. 2000b, Alméras et al. 2005, Clair et al. 2006b, Ruelle et al. 2007a). To give a clear answer to this question, a measurement of the strain of cellulose microfibrils during the lignification of the cell wall in an experiment similar to what was done on poplar (Clair et al. 2011) would be required. If lignification do not contribute to the stress generation; it would rather be dedicated to another function, like in normal wood, such as the reinforcement of the wall in the transverse direction or protection of the wall against pathogens. We will need to clarify the function of the late lignification for the tree, examining which trade-offs become possible thanks to this late lignification to justify its higher construction costs to the species.

This study allowed a new step in understanding the mechanism of maturation stress generation of non-G-layer species. However, there still remain some questions, especially concerning the steep change in the G-layer thickness from the seedling to the adult tree. Does this change express the mark of an ancestral character as noticed in other organs such as leaves that can be deeply modified from seedling to adult plant? Or is it an adaptation to the new needs of the plant, for mechanical support or conduction that changes during ontogeny? These questions remain.

Acknowledgements

Funding

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Supplementary material figure legends

Figure S1. Vessel frequency measured in tension wood (blue square) and in the opposite wood (red circles) of 19 *Simarouba amara* trees from seedlings to adult trees. Vessel frequency is plotted as a function of the distance to the pith to clarify the link with ontogeny and to evidence the lower frequency in the tension wood compared to normal wood in a given class of diameter.

Figure S2. Normal wood of *Simarouba amara* observed in saplings maintained in an upright position (0°). Fibre wall thickening, microscopic observation of the semi-thin section after double-staining with Safranin/Alcian blue and the thin cross-sections observed under UV light indicate that lignification occurs all along the cell wall thickening.

Figure S3. Transition from normal wood formed before tilting (right) to tension wood formed after tilting (left) observed in saplings of *Simarouba amara* tilted at 60° for 60 days. Double-staining with Safranin/Alcian blue. Images width: 1100 µm.

Figure S4. Observation with bright field microscopy after double-staining with Safranin/Alcian blue of the formation of tension wood cell-wall in six species sampled on naturally tilted seedlings in a tropical rain forest (Piste de St Elie research station, French Guiana). In these species, a classical G-layer is formed and later lignified during the maturation process. In some species, some cells remain partly unlignified. The number of cells in the G-layer stage depends on the growth rate of the tree at the sampling date. Images width: 1100 µm.

Figure Legends

Fig. 1. Schematic representation of the biological material of *Simarouba amara*: staked saplings, guyed young trees and naturally tilted old trees. Samples have been taken at different positions in trees with different methods depending on tree height and diameter.

Fig. 2. Synthesis of results for a two-year-old *Simarouba amara* seedling tilted at 40° and sampled after 110 days. (a) Fibre wall thickness and UV brightness of G-layers (1, 2, 3) and S_2 layer (4) (arbitrary unit). (b) Double-staining with Safranin/Alcian blue. (c) Zoom on the semi-thin section observed in the bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

Fig. 3. Synthesis of the results for a 4 cm diameter *Simarouba amara* sapling. (a) Fibre wall thickness and UV brightness of G-layers (1, 2, 3) and S_2 layer (4) (arbitrary unit). (b) Double-staining with Safranin/Alcian blue. (c) Zoom on the semi-thin section observed in bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

Fig. 4 - Synthesis of the results for an adult *Simarouba amara* tree 25 cm diameter. (a) Fibre wall thickness and UV brightness of G-layers (arbitrary unit). (b) Double-staining with Safranin and Alcian blue. (c) Zoom on the semi-thin section observed in bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

Fig. 5. Schematic evolution of the process of fibre thickening and maturation during the ontogeny of *Simarouba amara*. The seedlings produce a classical thick G-layer, which is lignified later. The older the tree, the thinner the G-layer is. At a given stage during ontogeny, the G-layer becomes unrecognizable as the tension wood cell wall thickness is

similar to that of normal wood and lignification prevents the swelling effect. Simarouba was therefore classified as a non G-layer species. The distance to the cambium is given in arbitrary units and the length of the stages cannot be compared between schematics.
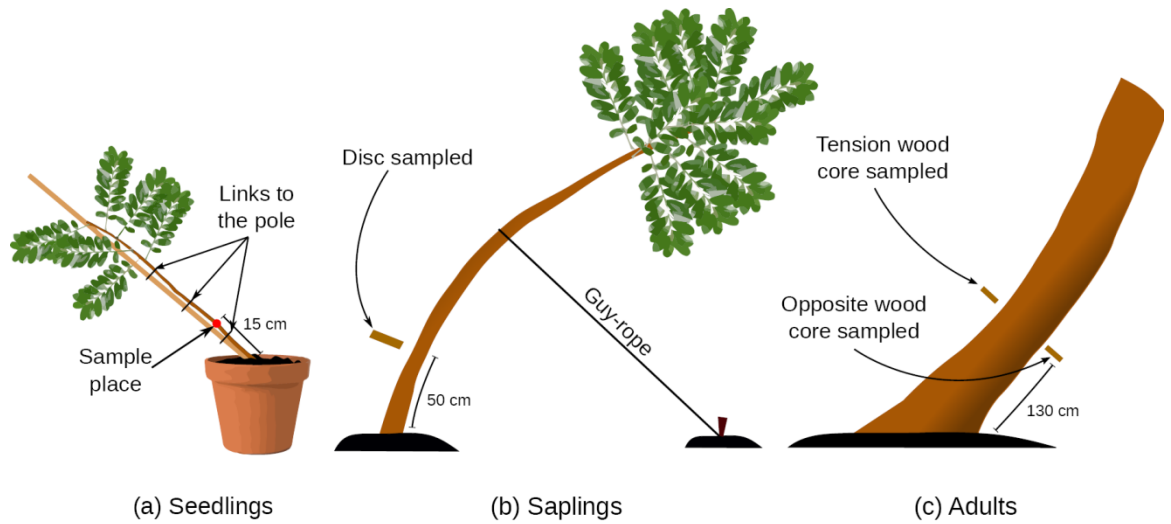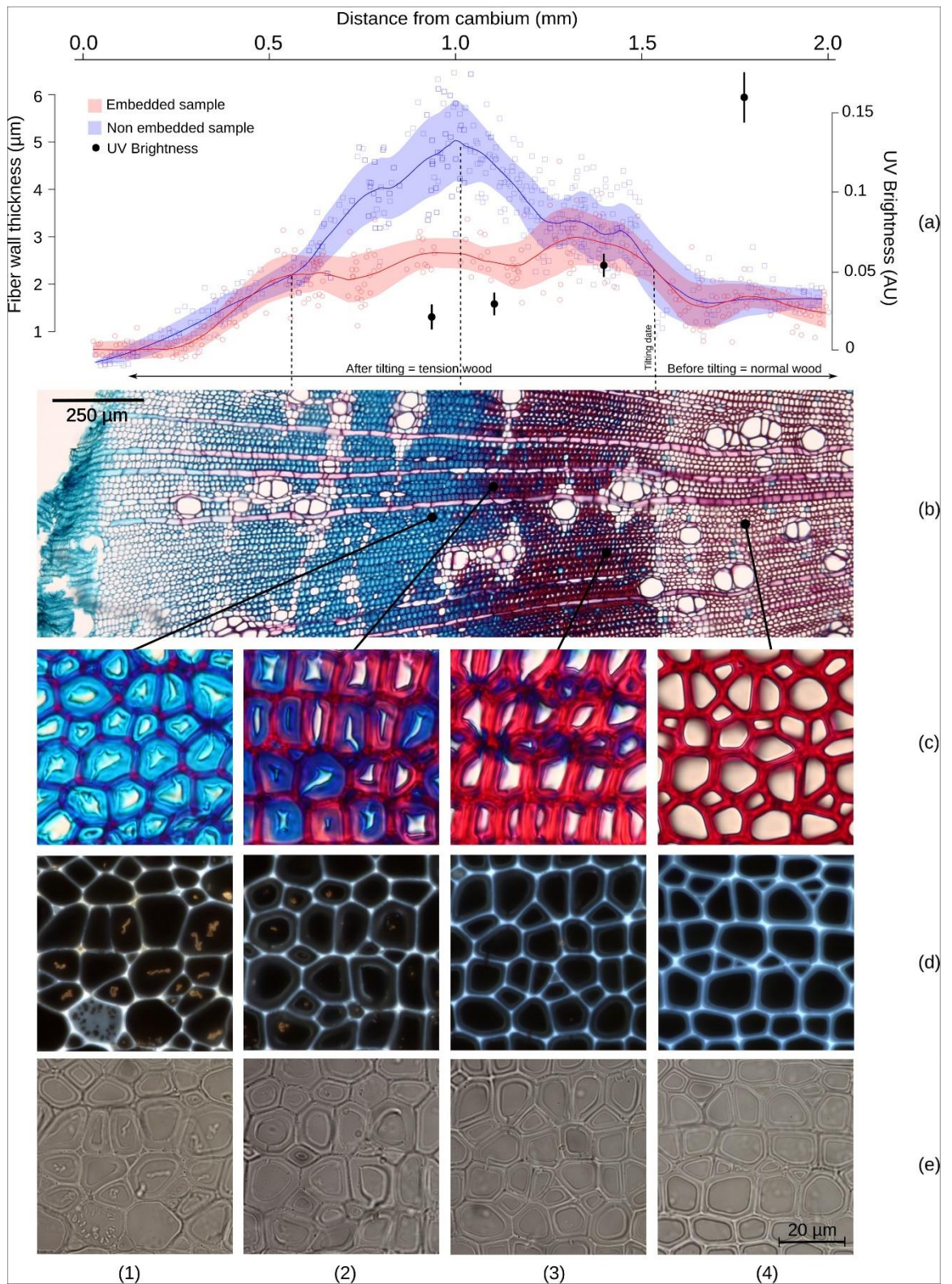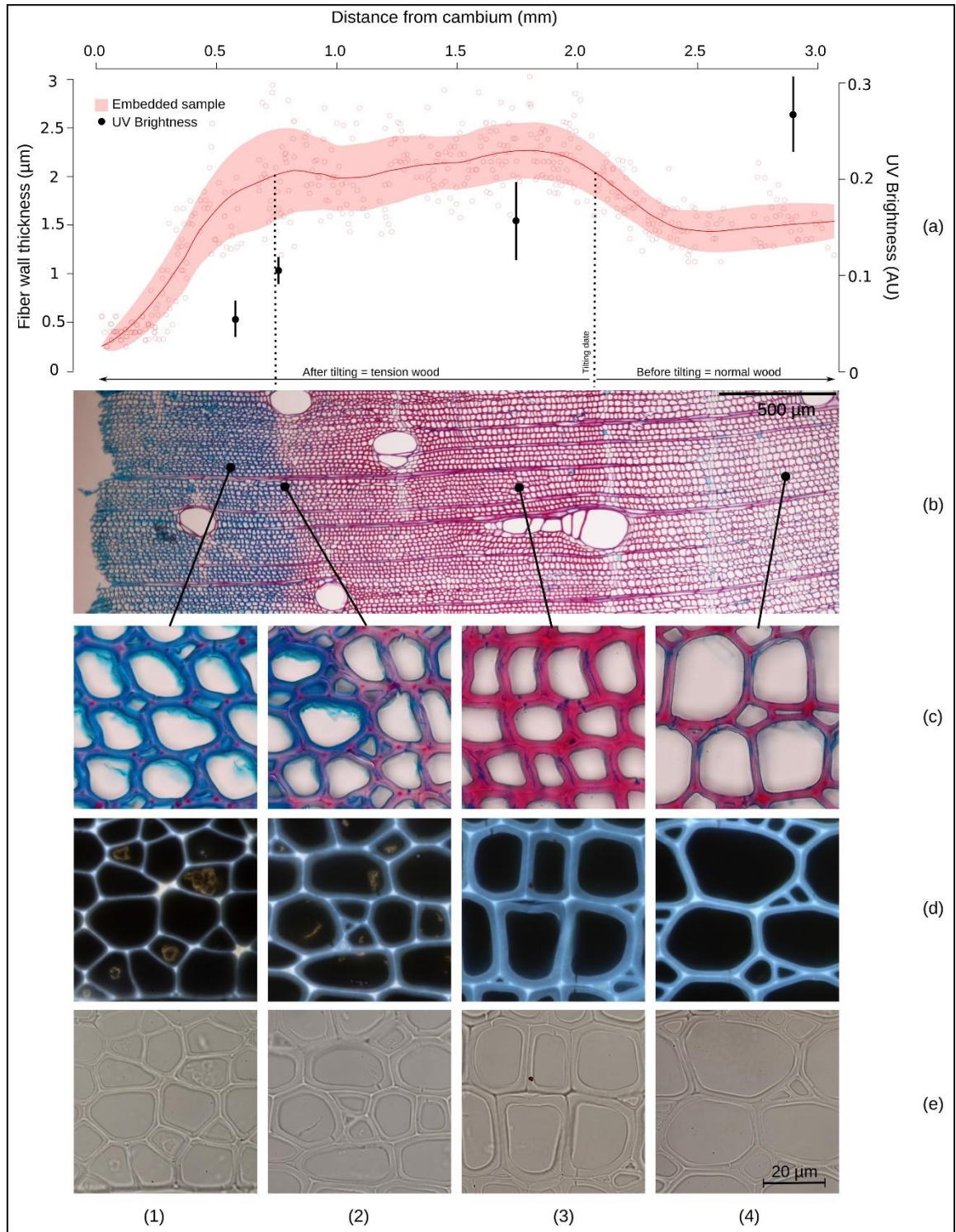
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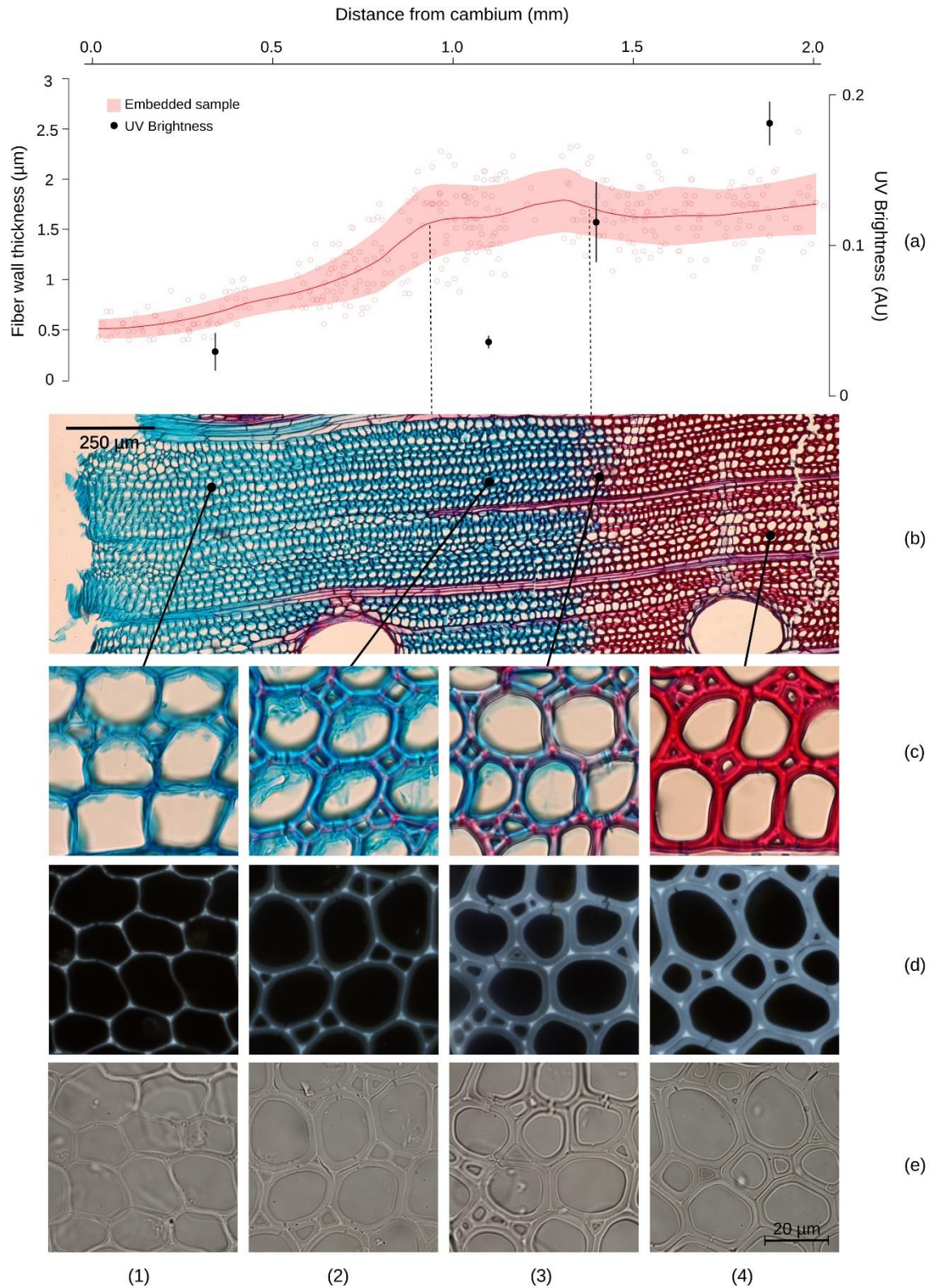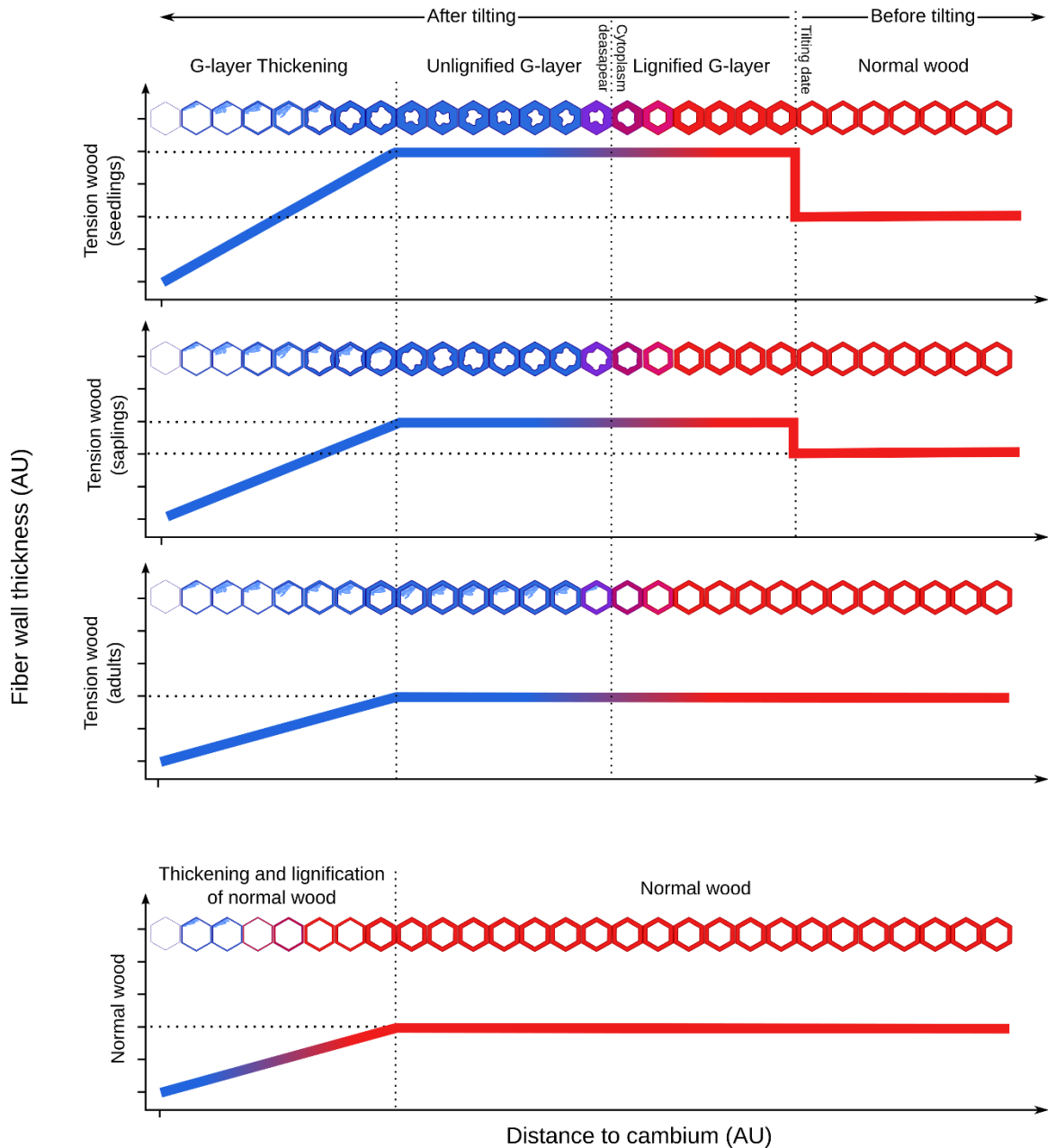
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Fig. 2. Synthesis of results for a two-year-old *Simarouba amara* seedling tilted at 40° and sampled after 110 days. (a) Fibre wall thickness and UV brightness of G-layers (1, 2, 3) and S2 layer (4) (arbitrary unit). (b) Double-staining with Safranin/Alcian blue. (c) Zoom on the semi-thin section observed in the bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

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Fig. 3. Synthesis of the results for a 4 cm diameter *Simarouba amara* sapling. (a) Fibre wall thickness and UV brightness of G-layers (1, 2, 3) and S2 layer (4) (arbitrary unit). (b) Double-staining with Safranin/Alcian blue. (c) Zoom on the semi-thin section observed in bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

Fig. 4 - Synthesis of the results for an adult *Simarouba amara* tree 25 cm diameter. (a) Fibre wall thickness and UV brightness of G-layers (arbitrary unit). (b) Double-staining with Safranin and Alcian blue. (c) Zoom on the semi-thin section observed in bright field after double-staining with Safranin/Alcian blue. (d-e) Details on the thin section of the same cells observed under UV light (d) and in bright field (e).

Fig. 5 Schematic evolution of the process of fibre thickening and maturation during the ontogeny of *Simarouba amara*. The seedlings produce a classical thick G-layer, which is lignified later. The older the tree, the thinner the G-layer is. At a given stage during ontogeny, the G-layer becomes unrecognizable as the tension wood cell wall thickness is similar to that of normal wood and lignification prevents the swelling effect. Simarouba was therefore classified as a non G-layer species. The distance to the cambium is given in arbitrary units and the length of the stages cannot be compared between schematics.