Fluctuations of cambial activity in relation to precipitation result in annual rings and intra-annual growth zones of xylem and phloem in teak (*Tectona grandis*) in Ivory Coast

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• *Background and Aims* Teak forms xylem rings that potentially carry records of carbon sequestration and climate in the tropics. These records are only useful when the structural variations of tree rings and their periodicity of formation are known.

• *Methods* The seasonality of ring formation in mature teak trees was examined via correlative analysis of cambial activity, xylem and phloem formation, and climate throughout 1-5 years. Xylem and phloem differentiation were visualized by light microscopy and scanning electron microscopy.

• *Key Results* A 3 month dry season resulted in semi-deciduousness, cambial dormancy and formation of annual xylem growth rings (AXGRs). Intra-annual xylem and phloem growth was characterized by variable intensity. Morphometric features of cambium such as cambium thickness and differentiating xylem layers were positively correlated. Cambium thickness was strongly correlated with monthly rainfall ($R^2 = 0.7535$). In all sampled trees, xylem growth zones (XGZs) were formed within the AXGRs during the seasonal development of new foliage. When trees achieved full leaf, the xylem in the new XGZs appeared completely differentiated and functional for water transport. Two phloem growth rings were formed in one growing season.

• *Conclusions* The seasonal formation pattern and microstructure of teak xylem suggest that AXGRs and XGZs can be used as proxies for analyses of the tree history and climate at annual and intra-annual resolution.

Key words: Growth rings, teak, Tectona grandis, vascular cambium, xylem and phloem formation.

INTRODUCTION

Teak, *Tectona grandis*, is a tropical and sub-tropical forest hardwood species, indigenous to regions with monsoon climates in South Asia (Behaghel, 1999; Pardé, 2002). This is a light-demanding and fast-growing species that is prized as one of the most economically important tropical timber trees for its valuable wood (Dupuy *et al.*, 1999; Maldonado and Louppe, 2000; Pandey and Brown, 2000; Cordero and Kanninen, 2003; Rahman *et al.*, 2005). Teak also shows a high potential for carbon storage and biomass production for renewable energy (Kraenzel *et al.*, 2003; Silver *et al.*, 2004).

Teak trees grow best on well-drained and deep alluvium sites with annual rainfall of 900–2500 mm and temperatures between 17 and 43 °C (Behaghel, 1999; Enters, 2000; Pandey and Brown, 2000). Typical growth sites are characterized by a 6 or 7 month rainy season and precipitation >200 mm/month, followed by 5 or 6 months of dry season (Purwanto and Oohata, 2002; Suzuki *et al.*, 2007). In contrast to the Asian distribution area of teak, teak plantations in Ivory Coast experience a shorter main dry season (2–4 months) and a 1 or 2 month secondary period of reduced precipitation (around 70 mm/month) in late summer (August) called the

small dry season. After September follows a period of resumption of rainfall that lasts 2–3 months, with monthly precipitations sometimes equal to those of the major rainy season (Société d'Exploitation et de Développement Aéronautique et de Météorologie – SODEXAM/Côte d'Ivoire: 1972–2000).

In typical growth sites characterized by seasonality of rainfall and temperature, the wood in T. grandis is composed of ring-porous growth rings resulting from seasonal fluctuation of cambial activity (Nobuchi et al., 1996; Borchert, 1999; Priya and Bhat, 1999; Rao and Rajput, 1999). In contrast, irrigated young trees have been found to form diffuse porous rings with less or no distinction between earlywood and latewood (Priva and Bhat, 1999). It can be expected that due to the worldwide distribution of teak plantations in various tropical climates, the pattern of tree ring formation can be diverse. Moreover, while a number of reports addressed the seasonal variation of cambial structure (Rao and Dave, 1983; Nobuchi et al., 1996; Rao et al., 1996; Borchert, 1999; Rao and Rajput, 1999), little is known yet about the environmental factors that affect xylem cell differentiation and growth ring structure. Investigating the time course of xylogenesis in situ and its relationship to climate is a straightforward way to characterize environmental factors controlling the structure

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of wood (Worbes, 1995, 2002; Wimmer, 2002). Knowledge of the seasonal pattern of xylem formation is important for better assessments of forest management and sylvicultural practices, and can provide means to improve the technological features of the wood product (Denne and Dodd, 1981; Bhat et al., 2001; Plomion et al., 2001; Wodzicki, 2001). Moreover, teak is an interesting model for studying the formation of tree rings in the tropics because it typically forms a ring-porous wood structure with distinct annual rings as well as false rings (Priva and Bhat, 1998; Bhattacharvya et al., 2007). Annual growth rings of teak can provide information on productivity and age structure of the forest stands, and enable dendrochronological research (Pumjiumnong et al., 1995; Jacoby and D'Ariggo, 1990; Somaru et al., 2008). Studies on the ecology, growth dynamics and carbon sequestration of tropical forests are often hindered by insufficient information on the structure and periodicity of tree rings. In the case of teak, tree ring analysis can be complicated by variations in xylem structure such as different kinds of false rings (Priva and Bhat, 1998) that can be the result of climatic disturbances or non-climatic factors. such as pathogen attacks (Sudheendrakumar et al., 1993; Managave et al. 2011). With this study, we aimed to reveal the seasonal pattern of xylogenesis in Ivorian teak trees and identify environmental and physiological factors that affect the cambial activity and annual growth ring structure. In particular, we investigated (1) the anatomical features that characterize dormant and active phases of cambial activity; (2) the seasonal time course of growth ring formation and structure of xylem and phloem; and (3) the inter-relationships between cambial activity and phenology in teak, and the climate in Ivory Coast.

MATERIALS AND METHODS

Study site

The sampling was carried out from October 2006 to March 2008 (Table 1), in the forest reserve 'Séguié' located in Rubino (Southeast Ivory Coast), between $6^{\circ}10'$ and $6^{\circ}21'N$

and 4°14′ and 4°27′W. The vegetation in the region is classified as an evergreen moist rain forest type and soils are typical acid soils of tropical lowlands (White, 1983). Séguié is the fourth largest of the 48 Ivorian state forest reserves in terms of planted area, covering 19 600 ha, and the second largest teak plantation in the country after Téné. The forest reserve is situated in a predominantly flat terrain and on a hydromorphic soil consisting mainly of sand and clay. The edaphic conditions are classified as the most productive fertility class one (Séguié Forest Management Plan, internal document, 1999).

Rainfall data were recorded daily at the site during the sampling period and compared with two different precipitation data sets (1986-1998, by Séguié Forest Division at the study site; and 1972-2000 by SODEXAM 50 km away from the study site). The mean annual sum of precipitation is 1298 mm, with a mean annual potential evapotranspiration of 1257 mm. About 83 % of the rains that fall infiltrate the soil, which contributes to a sustained water storage (N'Go et al., 2005). Annual mean relative air humidity is between 70 and 80 % (60 % in December and 80 % during the wettest month). Monthly mean temperature is 26.6 °C (minimum in August, 24 °C; and maximum in February-April, 28 °C) over the period 1972-2000 (Société d'Exploitation et de Développement Aéroportuaire, Aéronautique et de Météorologie - SODEXAM/Côte d'Ivoire: 1972–2000). Phenological observations were carried out during the course of the study.

Collection and preservation of plant material

Two or three 36-year-old individuals of *Tectona grandis* were sampled on each sampling date. One block with dimensions approx. $5 \times 5 \times 5$ cm, containing xylem, cambium and inner bark, was removed from the trunk at 1.30 m above the ground using a chisel. Every investigated tree was sampled once following the method of Rao and Rajput (1999). The samples were immediately fixed in FAA [acetic acid 5 %, (37 %) formaldehyde 10 %, distilled water 35 %, and ethanol 50 %]. For analysis of the structure of previously formed growth rings, disks from ten

TABLE 1. Seasonal variation of cambium morphology and width of xylem differentiation zone

Date of sampling	Cambial zone width (µm)	Tangential layers of cambial cells	Fusiform mother cell radial diameter (µm)*	Xylem differentiating zone (µm)
25 November 2006	40.3 ± 7^{de}	$7\pm1^{\rm e}$	$5.5\pm2^{\circ}$	0
27December 2006 and 29	$32.8 \pm 5^{\mathrm{f}}$	$4 \pm 1^{\mathrm{f}}$	$4\cdot 2 \pm 1^d$	0
December 2007				
25 January 2007	34.7 ± 7^{ef}	$7 \pm 1e$	3.8 ± 1^{d}	0
28 February 2007	40.8 ± 7^{de}	9 ± 2^{d}	$5.0 \pm 2^{\circ}$	25.4 ± 11^{e}
29 March 2007 and 27 March	63 ± 6^{b}	12 ± 1^{c}	6.8 ± 2^{b}	176.7 ± 134^{d}
2008				
29 April 2007	$55.4 \pm 6^{\circ}$	11 ± 2^{c}	6.3 ± 2^{b}	$596.1 \pm 117^{\circ}$
31 May 2007	$81.5\pm8^{ m a}$	16 ± 2^{b}	$8.5 \pm 3^{\mathrm{a}}$	607.2 ± 64^{b}
29 June 2007	$86 \pm 7^{\mathrm{a}}$	$19 \pm 2^{\mathrm{a}}$	7.7 1 ^a	1899.1 ± 7^{a}
Coefficient of determination R^2	0.67	0.854	0.429	0,985
F-ratio	133.042	1042.488	44.021	1573,516
Р	0.000	0.000	0.000	0.000

Within each column, values followed by different letters are statistically different (means \pm s.d.; Tukey HSD multiple comparisons, P > 0.05). *Some of the newest division plates may have been overlooked by conventional light microscopy. individuals (Tw59768, Tw59769, Tw59770, Tw59771, Tw59772, Tw59773, Tw60583, Tw60584, Tw60585 and Tw60586; accession numbers of the Tervuren Xylarium) were cut at 1.30 m above the ground. Wood blocks including annual growth rings 5, 10, 15, 20, 25 and 30 as counted from the pith were cut out from each disk and air-dried.

Determination of cambial activity

Light microscopy. Transversal and radial sections with a thickness of 15-30 µm and containing secondary xylem, cambial zone and phloem were cut using a sliding microtome (HM 440E; Microm Laborgeräte GmbH, Germany). The sections were stained with a 0.1 % safranin O (Merck KGaA, Darmstadt, Germany) solution in 50 % ethanol. They were washed in an ethanol series (50 and 75 %, 5 min in each concentration) and then stained with a 1 % fast green solution (Merck KGaA, Darmstadt, Germany) in 96 % ethanol for conventional light microscopy in order to differentiate lignified and cellulosic cell walls in terms of colour. After staining, sections were dehydrated in increasing concentrations of ethanol, mounted on microscope slides in Euparal (Carl Roth Gmbh + Co. KG., Karlsruhe, Germany) and dried in an oven at 50 °C overnight. Observations were made with an Olympus microscope (BX60F-3, Tokyo, Japan), equipped with bright-field and polarized light optics, as well as epi-fluorescence using a mercury arc lamp and an Olympus WU filter cube (excitation 330-385 nm, long-pass emission 420 nm). The polarized light was used to detect birefringence from crystals and developing secondary walls. Digital images were obtained using an Olympus Digital Camera (C-3040 ZOOM; Olympus Co. Ltd, Japan).

By fluorescence microscopy, yellow or red colour was indicative of lignified cell walls and violet or blue colour indicated cellulosic walls. Whereas safranin is a well-known fluorophore (Donaldson, 1992; Kitin *et al.*, 2003), fast-green staining is used primarily for conventional light bright-field imaging. The effect of violet or blue fluorescence of cambium and phloem was observed after 1 min exposure of the sections to the fluorescent excitation light, possibly due to bleaching of the fast-green stain.

Scanning electron microscopy. For scanning electron microscopy (SEM), 5 mm cubes containing cambium and adjacent xylem and phloem were excised from the samples and rinsed in running tap water overnight. The cubes were planed on the sliding microtome in order to achieve smooth transverse, longitudinal radial and tangential faces of cambium, xylem and phloem. They were then dehydrated using an ethanol series of increasing concentrations (25, 50, 75 and 100 %) for at least 1 h in each concentration. After three changes in 100 % ethanol for 15-20 min each time, cubes were air dried. The samples were coated with gold in a sputter coater and observed with a scanning electron microscope (JSM-6610LV; JEOL, Tokyo, Japan) operated at an accelerating voltage of 10 kV.

Measurements and statistical analysis

Cambial zone thickness (CZ), number of tangential layers of fusiform cambial cells (FC), radial diameter of fusiform cambial cells (Crd) and thickness of the xylem differentiating zone (XD) were measured in bright-field microscopy images of transverse sections (>50 measurements per sample) using AnalySIS Pro 3.2 software (Soft Imaging System Gmbh, Münster, Germany).

To characterize the variation of vessel diameters across annual ring boundaries, earlywood and latewood vessel diameters were measured in annual rings 5, 10, 15, 20, 25 and 30 in five individual trees (>50 vessels per sample). Measurements of Feret diameters (the maximal length straight line that can be fitted into a vessel) were executed automatically in images of transverse sections with ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

An analysis of variance (ANOVA) was carried out on the monthly variations of CZ, FC, Crd and XD. Tukey HSD multiple comparisons were used to determine pairwise similarity probabilities. Pearson's correlation coefficients were calculated for the inter-relationships between CZ, FC, XD and monthly precipitations. All the statistics were performed using SYSTAT 11.5.

Terminology and definitions

The terms cambial zone or cambium indicate all layers of meristematic cells and their intermediate derivatives between the xylem and phloem (Mahmood, 1971; Rossi et al., 2006a). The cells in the cambial zone do not differ from cambial initials in shape. The xylem or phloem differentiating zones comprise the immature xylem cells (newly formed vessel elements, xylem parenchyma, fibres and rays) or phloem cells distinguishable from cambial cells by their shape and the fact that they exhibit various stages of differentiation (growth, primary or secondary wall formation, lignification). The cambial cells and the differentiating cells with primary walls show no birefringence under polarized light and are stained in green or blue after applying our double staining. Cells with developing secondary walls show birefringent walls under polarized light, and lignified cell walls are stained in red after the safranin staining. The lignification of secondary walls was additionally confirmed in controls of unstained sections by the stronger autofluorescence of lignin.

According to a traditional concept, any growth ring or growth zone which is not annual can be designated a false ring. Such a definition, however, is not suitable in climates where species typically form more than one growth ring in a single year. Knowledge on and proper methodologies for how to identify false rings from true rings is fundamental in tree ring studies. The definition of false rings by Kaennel and Schweingruber (1995) is a step forward because it provides a distinction between false rings and growth zones or density fluctuations which are structurally different from true rings. In this study, we use definitions by the Multilingual Glossary of Dendrochronology (Kaennel and Schweingruber, 1995) adapted as the following:

Growth rings: subsequent growth layers of xylem or phloem suggesting periodicity of growth. For classification of xylem growth rings according to structure and distinctive-ness, see IAWA Committee (1989).

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- Annual xylem growth ring (AXGR): a growth layer produced by cambium in 1 year.
- *Growth zone:* a growth layer of xylem or phloem which can be identified by variations in cell shape, size and wall thickness. Unlike growth rings, growth zones are not produced in subsequent layers and often do not go around the entire circumference of the stem. Growth zones with indistinct or diffuse boundaries are defined as density fluctuations by Kaennel and Schweingruber (1995).
- *Intra-annual xylem growth zone (XGZ):* a growth zone within an annual growth ring which is distinct in structure from the annual ring.
- *False ring:* an additional, apparently complete growth zone with well-marked boundaries, formed within one growing season. In contrast to intra-annual growth zones, false rings cannot be morphologically distinguished from true tree rings, and can only be identified by cross-dating or anatomical analysis of cambial activity and annual xylem growth.

RESULTS

Climatic conditions and phenology

The site experiences a 3 month dry season (from mid-November until mid-February) preceding a rainy period (from February until July), then a reduction in precipitation in August, followed by a resumption in rainfall from September until mid-November (Fig. 1). Monthly precipitation data sets collected at the study site over different periods (1986–1998 and 2005–2008) and 50 km away from the site (1972–2000) are in agreement and show no major disturbance

to the typical precipitation pattern during the study period (Fig. 1). The only exception was the month of August (short dry period) in 2006 with only 10 mm monthly precipitation. The rainfall in August of 2007 with precipitation of 66 mm was consistent with the typical pattern.

Our observations, together with phenological data from the Séguié Forest Division (1999), show that Séguié teak gradually sheds leaves during the dry period, but trees do not become completely leafless. Leaf fall starts between mid-October and early November, and lasts until the end of January or mid-February, after which sprouting begins with the start of the major wet season. Development of current-year leaves is continuous from February to April or May. The trees are in full foliage in May before the peak in precipitation. Flowering takes place during full foliage from May to August or September. The fruiting covers 3–4 months from September or October until December.

Seasonal changes of the cambial zone morphology

Number of tangential layers of fusiform cells (FC). The cambial zone contained the least FC in the middle of the dry period in December (Table 1). In contrast, the largest FC occurred during the peak of cambial activity in June. There was the same FC at the end of cambial activity in October and at the onset of reactivation in February. The FC during new leaf development in March and April were also statistically different from other months of the year (Table 1, Fig. 2; Supplementary Data Fig. S1).

Cambial zone width (CZ). The CZ in October and November (end of the growing season) was significantly thinner compared with the CZ at the peak of the rainy season in May





and June. The CZ was further significantly decreased in December and January (a >2-fold decrease relative to the cambium at the peak of the rainy season). At cambial reactivation in February, the CZ increased and became statistically

similar to that at the end of cambial activity in October and November. (Table 1, Fig. 2; Supplementary Data Fig. S1). A strong linear correlation was found between the FC and CZ (Fig. 3). There were considerable variations in CZ between



FIG. 2. Seasonal development of cambium in teak in Séguié Forest. (A) Dormant in November. (B) Cambial reactivation in February. The arrow shows an expanding xylem cell. (C) Image of active cambium in April. The black arrow shows developing phloem fibres, and white arrows show developing vessels. (D) Matching image of the sample in (B) viewed with polarized light. Methods: TS (A–D), FL (A) and (C); BF (B) and PL (D). Scale bars = $25 \,\mu$ m (A); 50 μ m (B–D). Abbreviations in all figures. Xylem and phloem development: ca, cambium; df, developing fibres; dp, developing phloem; dxy, differentiating xylem; dxy1, xylem differentiation zone of primary cell wall development and expanding cells; dxy2, xylem differentiation zone of secondary cell wall development and lignification; dxy3, lignified xylem of the current year; f, fibres; phl, phloem; xy, xylem of the previous year. Growth ring structure: XGR, xylem growth ring XGZ, xylem growth zone. Methods: RS, radial longitudinal section; TLS, tangential longitudinal section; TS, transverse section; BF, bright-field light microscopy; FL, epi-fluorescence; PL, polarized light.



FIG. 3. Correlations between morphometric features of cambium and precipitation. (A) Number of tangential layers of cambial cells and cambial zone width (P < 0.01). (B) Cambial zone width and width of xylem differentiation zone (P < 0.05). (C) Cambial zone width and monthly precipitation (P < 0.01). Trendline equations are shown in the graphs.

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different trees (Table 1). The seasonal change in cambium morphology (CZ and FC) showed a significant correlation with the precipitations of the corresponding sampling months (Fig. 3).

Fusiform mother cell radial diameter (Crd). The Crd in January (before reactivation) was the smallest, and at the onset of reactivation in February was significantly increased (Table 1, Figs. 2; Supplementary Data Fig. S1). Newly derived (daughter) cells may have been overlooked in the measurements because new division plates were not easily visualized by light microscopy. Uneven thickness of the radial walls of cambial cells (primary pit fields) was evident in the period November–February (see dark and bright spots in the radial walls of cambial cells in the radial section in Supplementary Data Fig. S1f).

Relationships between the cambial morphology and the growth ring development in the stem

The seasonal changes in the morphology of the cambial zone were associated with corresponding changes of the XD (Fig. 3).

Dormant cambium. There were no enlarging or differentiating cambial derivatives during the main dry period of the year from October until February (Fig. 2A, B; Supplementary Data Fig. S1a, b). However, the CZ in October and November was still significantly thicker than the CZ in December and January (Table 1). Cell walls of cambial cells, phloem rays and phloem parenchyma showed little birefringence, in contrast to developed xylem and sieve tube elements which allowed for a clear differentiation between these different types of elements under polarized light (Figs 2D and 4F).

Cambial reactivation and initiation of xylem and phloem formation. The first cell divisions were seen in February at the phloem side of the cambium. The reactivation of cambium was preceded by an apparent swelling of the cambial zone (about 17 % increased thickness relative to the January samples) which was associated with increased FC and Crd (Table 1, Fig. 2B; Supplementary Data Fig. S1b, c). The first expanding cambial derivatives were seen in February at the xylem side (Fig. 2B; Supplementary Data Fig. S1c). There was no secondary wall formation in the differentiating cells, indicated by the lack of birefringence in their walls (Fig. 2D).

Active cambium. The swelling of the cambial zone in February was followed by an increased mitotic activity evidenced by a statistically significant increase of the FC. In March, the radial enlargement of the CZ was an 83 % increase compared with the dormant stage in January (Table 1, Fig. 2; Supplementary Data Fig. S1). In March, the xylem differentiating zone included a single layer of earlywood vessels (Fig. 4A). Some vessels were still growing while others were fully expanded with developing secondary walls. Lignification was evident in the fully expanded vessels but not in the surrounding developing cells (Fig. 4A; Supplementary Data Fig. S2a, c).

The zone of differentiating xylem in April was enlarged >200% compared with that in March, and included 2-3

layers of earlywood vessels (Table 1, Fig. 4A, B). However, the amount of newly produced xylem cells varied at different positions in the samples. Several layers of xylem cells had developing secondary walls, and lignification was evident in the cell walls of fully expanded vessel elements and adjacent-to-vessels fibres (Fig. 4B). Similarly, Ridoutt and Sands (1994) in eucalypts noted that fibres close to vessels started secondary wall development sooner than fibres further away.

The new xylem layer in May had a relatively small increase compared with that in April, showing that xylem formation in May was relatively slow, although statistically significant (Table 1, Fig. 4B, C). In June, the cambial activity had become very intensive, resulting in a 3-fold enlargement of the XD compared with the previous month (Table 1, Fig. 4D). Moreover, three distinct zones of cell differentiation could be defined in the current xylem ring in June (Fig. 4D-G): (1) a zone of expanding cells (dxy1) where only some fully expanded vessel elements had birefringent walls (Fig. 4D-F; Supplementary Data Fig. S2c); (2) a zone of secondary wall development (dxy2), where all cells were birefringent and only the vessel elements and a few layers of vessel-adjacent cells had lignifying walls (Fig. 4D-G; Supplementary Data Fig. S3); and (3) a zone of lignified xylem (dxy3) where all cells were lignified and appeared fully developed (Fig. 4D, G; Supplementary Data Fig. S3). In dxy1, most of the expanding vessels were large, indicating that earlywood was still formed in June. Vessels with small diameters and developing secondary walls occurred near the cambium (arrows in Fig. 4D, F), which indicated that latewood formation was also initiated in June. Xylem deposition appeared completed in October in both 2006 and 2007.

Phloem differentiation

The inner bark of teak consists of alternating layers of sieve tube–companion cell complexes, ray and axial parenchyma cells, and fibre bands (Figs 2 and 5). These alternating sclerenchymatic and parenchymatic layers form regularly spaced phloem rings (Fig. 5C). The developed sieve tubes and fibres were easily distinguishable with their birefringent cell walls from the parenchymatic tissue and cambium (Figs 2D and 4E, F). Two or three layers of axial parenchyma occurred at the inner and outer sides of each fibre band (note the dark nonbirefringent layer of cells between the phloem fibres and sieve elements in Fig. 2B, D, and the less-stained layers of parenchyma cells in Fig. 5A). The axial parenchyma cells adjacent to fibres always contained prismatic crystals (arrows in Fig. 5A, B).

In the November–February samples, the sieve elements in all phloem rings contained callose at their sieve plates, which was detected by blue fluorescence (Fig. 5D). Callose disappeared from the inner rings during the growing season. Development of new phloem started at the end of February simultaneously with the initiation of the growth of new xylem cells (Fig. 2B; Supplementary Data Fig. S1b). Developing bands of phloem fibres, which mark a growth ring boundary, were firstly seen in the April samples (Figs 2C and 4B). The formation of new phloem was more intensive in May and June when trees were in full leaf at the peak



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FIG. 4. Intra-annual pattern of xylem differentiation. (A) Development of earlywood in March. Vessel elements at different stages of differentiation (black arrows). Fibre bands of phloem from previous years (white arrows). (B, C) Development of earlywood in April (B) and May (C). (D) Development of earlywood and initiation of latewood in June. The differences in colour indicate various stages of differentiation: ca, dxy1, dxy2, dxy3 and xy (see abbreviations in the legend to Fig. 2). (E) Polarized light image of June cambium and differentiating xylem and phloem. Note the unstained developing fibres (df) and stained red lignified fibres (f). Birefringence is seen in differentiating xylem (dxy2), phloem fibres (df, f) and sieve elements (phl) but not in the cambial zone (ca), and not in differentiating xylem with primary walls (dxy1) or in rays. Arrows point to vessels in different stages of development (red colour indicates lignifying vessel walls). (F) A magnified view of June cambium and differentiating xylem and phloem by polarized light. The arrow points to a latewood vessel with developing secondary wall indicated by birefringence and red staining. (G) A polarized light image of the sample in (D) showing the border between dxy2 and dxy3 (lignifying cell walls stained red). Methods: TS (A–G), PL (A, E–G), BF (B–D). Scale bars: (A–E, G) = 220 μ m, (F) = 100 μ m. Abbreviations are as in the legend to Fig. 2.

of the rainy season. In the June samples, a second phloem layer was developing despite the fact that not all fibres from the previous phloem ring had completed differentiation (Figs 4E and 5A, B; Supplementary Data Fig. S2c). In Fig. 4E, the two developing phloem fibre layers of the current year are indicated with the letters 'df'; note also the occurrence of lignified

(safranin-stained) and non-lignified (unstained) fibres in the first-formed ring of phloem. The size of developing crystals in the parenchyma cells adjacent to fibres was indicative of the stage of fibre development. For example, the small crystals and thin fibre walls in Fig. 5A show an early development and undifferentiated fibres of the second phloem ring in June.



FIG. 5. Development of phloem fibres of teak. (A, B) Current-year fibres in June. Red colour indicates cell wall lignification and arrows point to developing crystals. The cambium side is towards the bottom in (A), and to the right in (B). (C) Sample in January. Arrows point to growth rings in phloem marked by phloem fibres. (D) Sample in February. Arrows point to sieve plates with callose. Methods: TS (A, C), RS (B, D), BF (A, B), PL (C), FL (D). Scale bars: (A) = $20 \,\mu$ m, (B) = $50 \,\mu$ m, (C) = $200 \,\mu$ m, (D) = $40 \,\mu$ m. Abbreviations are as in the legend to Fig. 2.

Formation of annual xylem growth rings (AXGRs) and xylem growth zones (XGZs)

The transition between earlywood and latewood within AXGRs is characterized by a gradual change of vessel diameter, whereas an abrupt shift in vessel diameter marks annual ring boundaries (Fig. 6A, B; Supplementary Data Fig. S5a). Measurements of vessel diameter defined latewood zones with a mean vessel diameter of $110.2 + 8 \,\mu\text{m}$ and earlywood zones with a mean vessel diameter of $212.5 \pm 13 \,\mu\text{m}$. These vessel characteristics of the growth increment determine the ring-porous/semi-ring-porous feature of teak wood and the distinct pattern of the annual growth rings. In addition, AXGR boundaries were marked by an abrupt change from thickwalled and flattened terminal fibres at the latewood side of the growth ring boundary to initial axial parenchyma at the earlywood side (Fig. 6B; Supplementary Data Fig. S4a, e, f). In contrast, thin-walled axial parenchyma at the XGZ boundaries was usually not found, or, if present, it was confined to not more than 2-3 layers of cells (Fig. 6E; Supplementary Data Fig. S4b-d). The AXGR consisted of mostly earlywood and smaller proportions of latewood (Fig. 6A). The samples of developing xylem in June showed that most of the AXGR, the entire earlywood plus several cell layers of latewood, was already deposited by the end of June (Fig. 4D). In fully differentiated AXGRs, vessels with small diameters, such as those shown by arrows in Fig. 4D, F, occur near the growth ring boundary within <15 % of the entire width of the annual increment (Fig. 6A). This observation indicates that the outer 15 % of the AXGR, which is composed of latewood, is formed in the period July-October. A careful observation of dxy3 in all three sampled trees in June (Fig. 4D, G; Supplementary Data Fig. S3) revealed a tendency for a decrease of the vessel size from the beginning towards the end of the dx3 zone. The widths of the dxy3

zones as well as the small shift in vessel size were similar to those in the developing xylem in the April and May samples (Fig. 4B, C), indicating that dxy3 zones were formed prior to May during the development of new leaves. Moreover, dxy3 appeared as separate growth rings because of the different developmental stages of the fibres and different vessel sizes on both sides of the dxy3/dxy2 boundary (see Fig. 4D; Supplementary Data Fig. S3). These growth increments (dxy3) are XGZs and not false annual rings because they were distinct in structure from the true annual rings (for definitions, see the Materials and Methods). The most apparent difference between AXGRs and XGZs was that the vessels at the end of the dxy3 zone were considerably larger than the typical latewood vessels (see xy zone, Figs 4D and 6A, B; Supplementary Data Fig. S4a).

Similar XGZs in the initial part of the earlywood in 2007 were found in each of the nine sampled trees after June 2007 (100 % incidence). Distinct XGZs were also seen adjacent to dormant cambium in two trees in December 2006 (Supplementary Data Fig. S4c, d). The occurrence of XGZs was investigated from 2006 back to 1971 in polished stem disks from ten trees of the same plantation. This observation revealed a high frequency of XGZs which were found in each year until the age of 14, and in 64 % of the years after the age 14 (Fig. 7). The results also showed that the incidence of XGZs among individuals decreased with age. Up to 14 years of cambial age, 45 % of the total number of annual xylem rings that we investigated had XGZs, and at between 15 and 35 years of cambial age the occurrence of XGZs was 12.4 % (Fig. 7).

Unlike the true annual rings, the XGZs were variable in structure and often indistinct. Some XGZs were marked by differences in the wood fibre density (Fig. 6A, C; Supplementary Data Fig. S4b), or by several layers of flattened fibre cells (Fig. 6B; Supplementary Data Fig. S4c, d), also by slight



FIG. 6. Variations in the morphology of xylem growth zones (XGZs) in teak. (A) The current annual xylem growth ring (AXGR) of a sample in December 2007. The lower arrow points to the previous AXGR boundary. The upper arrow points to an indistinct XGZ marked by a variation in the fibre cell wall thickness. (B) Distinct XGZ boundary (upper arrow) in the earlywood of a sample in October 2006. The distinct XGZ boundaries are marked by variations in vessel diameter, flattened fibre cells and tangential widening of rays. The lower arrow points to the previous AXGR boundary. Note the difference in structure between the XGZ boundary and the AXGR boundary (for discussion, see text). (C) An enlarged view of the sample in (A) showing the indistinct XGZ boundary (arrow). (D) Distinct XGZ boundary (arrow) in the latewood of the previous AXGR of a sample in March 2008. (E) An enlarged view of the XGZ boundary (arrow) of the sample in (D). Methods: TS and BF. Scale bars: (A–D) = 500 μm, (E) = 100 μm. Abbreviations are as in the legend to Fig. 2.



FIG. 7. Occurrence of xylem growth zones in ten 35-year-old teak trees in the Séguié forest.

widening of the rays and differences in vessel diameters in both sides of the growth zone borders (Fig. 6B, D, E). The distinctiveness of the XGZs varied between trees and even within the same sample block, therefore, the XGZs sometimes appeared discontinuous. The XGZs were apparent when marked by variation in vessel diameters (Fig. 6B, D), but could be easily overlooked when marked by variation of the fibre cell wall thickness (Fig. 6A, C; Supplementary Data Fig. S4b). However, the XGZs were easily identified in the samples of developing xylem increments because of the distinct developmental stages of the xylem cells on both sides of the growth zone borders (Fig. 4D; Supplementary Data Fig. S3).

DISCUSSION

Seasonal changes of the cambial zone morphology

The occurrence of seasonal fluctuations of cambial activity and morphology has been well studied in temperate trees, but is comparatively little understood in tropical species (Barnett, 1992; Catesson, 1994; Larson, 1994; Worbes, 1995; Frankenstein et al., 2005; Rossi et al., 2006a, b). Seasonal changes in cambial activity associated with seasonality of the climate have been reported for a number of tropical species in Asia and America (Venugopal and Krishnamurthy, 1987a, b; Fujii et al., 1999; Rao and Rajput, 2001; Worbes, 2002; Yáñez-Espinosa et al., 2006; Venugopal and Liangkuwang 2007; Marcati et al., 2006, 2008) as well as in Africa (Amobi, 1974; Rogers, 1981; Worbes et al., 2003; Verheyden et al., 2004; Schmitz et al., 2008). In deciduous teak in India, Rao and Rajput (1999) found six cell layers in dormant cambium and ten or 12 cell layers in the reactivation phase and during the full active phase, respectively. In another study highlighting an effect of moisture conditions, Priya and Bhat (1999) counted 6-10 cells in the dormant cambial zone of irrigated teak, 11-14cells in the reactivation phase and 15-20 cells in the full active phase. Variations in cambial cell number could be the result of differences in the age of the investigated trees. The reports so far often include young trees, while more research would be needed to reveal the effects of cambial age on the cambial structure. Moreover, our study showed considerable variations in the width of the cambial zone between individual trees.

Our results showed statistically significant annual fluctuations in the thickness of the cambium (CZ and FC) in teak following the transitions from wet to dry periods. These fluctuations of cambium size could be explained by changes in the mitotic activity and the number of cambial cell layers, and they showed the active and dormant periods of cambial activity. Furthermore, the widest cambial zone occurred simultaneously with the widest cambial zone in June morphologically represented the most active period for stem diameter growth when most of the current growth ring was deposited (Fig. 4D, E). Independent measurements with dendrometers in this teak plantation also showed the occurrence of a steep increase in the intensity of radial growth in June (Séguié Forest Management Plan, internal document).

Teak grows naturally in monsoonal climates characterized by high precipitation during half of the year and long dry periods during the other half. In contrast to the Asian natural growth sites of teak, the Séguié site experiences a shorter dry season, less cumulative rainfall during the rainy period, and a 1 or 2 month second small dry period. Regardless of the occurrence of the short dry season in August, the growth of the vegetation lasts about 8-9 months from the end of February until October or November. In addition to the rains, the availability of ground moisture is probably substantial (N'Go et al., 2005). It is noteworthy that cambial reactivation started at the end of the dry season in February when precipitation was still low, and xylem and phloem formation had ceased in October when precipitation was still relatively high. These facts are suggestive of a complex physiological and genetic regulation of the duration of radial growth in Séguié teak. Despite the fact that secondary growth had

stopped, the cambium in October and November was statistically thicker than the cambium in December and January, which might be an indication that due to favourable moisture conditions the cambium in November was not yet in the resting stage.

As discussed earlier, dendrometer measurements showed that growth activities reach their peak during the precipitation peak, which is in June (for the entire Southern part of the country and our study site location) and in July (for the deciduous forest zone: Central-West of the country). Growth activities stop in September–October. These observations have allowed the establishment of management strategies in teak plantations. Therefore, forest inventories (diameter and height measurements for wood biomass estimate) take place at the onset of the dry season (December) when there seems to be no growth of trees. In this way, the annual growth increment that has started with the onset of rains (March in the case of the Southern part of the country) can be measured (Dr Anatole Kanga, Centre National de Recherches Agronomiques, previously CTFT: Centre Technique Forestier Tropical, pers. comm.).

Xylem growth zone (XGZ) formation and structure

Teak is an interesting model species for studying how growth rings develop in tropical trees because it forms distinct ringporous rings as well as XGZs and variations in xylem structure. For example, it was found that irrigated trees form diffuse porous rings in the juvenile phase (Priya and Bhat, 1999). The same authors reported that old trees show a shorter duration of annual cambial activity than juvenile trees and suggested that this could explain the absence of pronounced ring porosity in very young trees. The annual nature of the ringporous xylem rings in teak is well documented in Asia (cf. Somaru *et al.*, 2008; Managave *et al.*, 2011, and references cited therein). However, surprisingly little information is available on the intra-annual pattern of xylem formation.

Priya and Bhat (1998) identified four types of false rings in young teak trees (from 1 to 12 years old) based on anatomy and location within annual rings. The XGZ at the initial part of the AXGR in our study corresponded to variations of the 'false ring types 1, 2, or 3' described by Priya and Bhat (1998). Some of the XGZs that we observed in the latewood were similar to the 'false ring types 3 and 4'. Because the XGZs in our samples were very distinct in structure from the annual rings, we refer to them not as 'false rings' but a 'growth zones' for consistency with the definitions proposed earlier in the Materials and Methods.

Our observations in teak confirmed the results of previous studies of many different species that have shown the vessel elements to be the first cells to expand and form lignified secondary walls, attributed to their importance in water conduction (Suzuki *et al.*, 1996; Kitin *et al.*, 2003; Marcati *et al.*, 2006) or the pattern of auxin transport (Ridoutt and Sands, 1994). When development of new leaves initiated in March, a partial wall lignification was evident in fully expanded new vessels but not in the surrounding developing fibres (Fig. 4A; Supplementary Data Fig. S2). Therefore, the new xylem in March was not completely functional for water conduction because lignification is necessary for the proper functionality of woody cells (Hacke *et al.*, 2001; Voelker *et al.*,

2011). SEM observation of vessel elements with developing secondary walls revealed perforation-partition membranes (Supplementary Data Fig. S2b), which were not present in fully developed vessels.

Formation of XGZs at the beginning of the growing season clearly coincided with the period of leaf growth, and these XGZs were fully developed at full leaf before the most intensive radial growth. It was previously proposed that insufficient rainfall soon after the onset of cambial activity might be the reason for intra-annual rings within earlywood (Priva and Bhat, 1998). However, the monthly rainfalls in the Séguié teak plantation during the course of our study were typical for the region and sufficient for growth. Therefore, we propose that the XGZs at the start of the annual rings in our sampled trees might be a physiological response to the new foliage growth and to an increased demand for xvlem water transport. In the same samples, tyloses were observed in the previous-year xylem, suggesting that older xylem rings were not fully functional. The early maturation of the XGZs in June (dxy3 in Fig. 4) may indicate that new functional xylem is needed to provide the water transport at an accelerated transpiration rate and diameter growth in full leaf. Similarly, in temperate ring-porous woods, early development of new functional xylem takes place before the full-leaf phenological phase of the seasonal development (Funada et al., 2001; Kitin et al., 2003). Xylem with ring-porous growth rings is compartmentalized structurally and physiologically, which is regarded as an advanced safety feature for water transport in climates with distinct seasonality of moisture availability (Tyree and Zimmermann, 2002; Kitin et al., 2004; Ellmore et al., 2006).

As described earlier, the wood formation in May was slow, which was not related to a decrease in the moisture availability because monthly precipitation was in fact increasing from February to May in each of the study years. New leaves were growing in May, which may have diverted photosynthetic assimilates toward foliage development at the expense of radial growth. In contrast, a sharp increase in the rate of wood deposition was observed from the end of May to June, after the new foliage was fully developed. Our microscopic observations were in accordance with results from several years of dendrometer measurements in the same plantation which have shown significant increases in the rates of diameter growth after the trees develop full leaf (Séguié Forest Management Plan, internal document).

Formation of distinct XGZs was frequently observed in young trees. During the first 3 years of cambial age, XGZs were present in almost all growth rings of the investigated trees (Fig. 7). Similarly, Priya and Bhat (1998) observed that 73 % of 8-year-old teak trees displayed intra-annual growth rings in their second annual increment. In another sample of nine 12-year-old trees, the maximum numbers of intra-annual rings were found in the second and third annual rings (Priya and Bhat, 1998). It is generally agreed that physical factors, such as drought during the growing season, which may disturb the normal activity of cambium, may also trigger formation of false rings. Priya and Bhat (1998) showed that induced drought during the growing period, as well as transplanting, causes formation of false rings in 1-year-old seedlings. Younger trees are more likely to be influenced by physical disturbances which could explain why the frequency of XGZs was negatively correlated with cambial age (Fig. 7). In addition to direct physical factors, the developing xylem in young tree stems is strongly influenced by hormonal signals from the leaves (Aloni, 2010).

The XGZs in the earlywood were easily observed in the June samples of developing wood because of the distinct differentiation stages of the wood increments before and after May (dxy2 and dxy3 in Fig. 4 and Supplementary Data Fig. S3). The XGZ boundaries can become indistinct in fully developed annual rings, but through careful observations we were able to detect them in the earlywood zone in all other trees during the study years 2006 and 2007 (Fig. 6A-C; Supplementary Data Figs S3 and S4b). In our study, as well as in previous reports (Priva and Bhat, 1998), XGZs were observed in the latewood of teak. Compared with the XGZs in the earlywood, those in the latewood were always distinct and easier to detect in microscope slides or disks (Fig. 6D, E; Supplementary Data Fig. S4c, d). In two of our samples from December 2006, the occurrence of XGZs at the end of the annual rings might be related to a temporal interruption of the cambial activity due to the exceptional drought in August 2006 (10 mm monthly precipitations). However, the response to drought is either varied among individuals or the XGZs form incomplete rings, because the other sampled trees in 2007 and 2008 did not display XGZs in the latewood of 2006.

Differentiating phloem

The phloem structure in teak is characterized by distinct phloem growth increments that have relatively equal thickness. Our observations, together with investigations in different locations in India, indicate that secondary phloem and xylem differentiation initiate simultaneously or within a month after the emergence of new leaves (Venugopal and Krishnamurthy, 1987b; Priya and Bhat, 1999; Rao and Rajput, 1999). Similar phloem differentiation patterns in relation to the phenology were reported for other tropical species in seasonal climates (Rao and Rajput, 2001; Marcati et al., 2008). In addition, our study showed that teak trees form at least two growth increments of phloem during one growing season. Therefore, the last two or three phloem growth increments always contain relatively new phloem with non-collapsed sieve elements. An interesting observation which would need additional investigation was the occurrence of callose in all phloem rings during the dormant period but not in the inner phloem rings during the growing period. A characteristic of teak phloem is the consistent occurrence of crystals in the adjacent-to-fibres parenchyma cells. The dimensions of those crystals together with the thickness of the fibre cell walls vary during the growing season and indicate the stage of development of the corresponding phloem increment.

Conclusions

This study confirmed the annual nature of growth rings in teak, showing their potential for analysis of tree age, carbon sequestration and reconstructions of forest growth dynamics and climate. The most active cambium and radial growth occurred during the peak of the rainy season in June. Phloem Page 12 of 13

formation took place simultaneously with the formation of xylem. At least two growth rings of phloem, marked by alternating layers of sieve tubes and sclerenchyma bands, were formed during one growing season. Morphometric features of cambial development, such as cambial zone width, number of tangential cell layers and width of the xylem differentiation zone, were strongly correlated with each other and showed a linear relationship with monthly precipitation.

Xylem growth zones were formed within earlywood or latewood of true annual rings. The annual xvlem increments started with XGZs that were formed while new leaves of the semi-deciduous trees were developing and moisture conditions were favourable. When the trees achieved full leaf and radial growth rate accelerated, these XGZs were fully differentiated. The cause of the XGZ formation in earlywood remains to be experimentally identified. We propose that XGZs functional for water transport must be developed early in the growing season in order to supply sufficient water for increased transpiration, photosynthesis and radial growth when teak trees achieve full foliage. These XGZs can be easily detected in differentiating xylem, but their boundaries are structurally less distinct and may appear discontinuous in mature xylem. The XGZs are morphologically different from the true annual rings and represent a potential feature for intra-annual tree ring analysis.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: morphology of dormant and active cambium. Figure S2: cell wall development of differentiating xylem and phloem cells. Figure S3: illustrations of growth zone formation in the earlywood of different individuals of teak. Figure S4: illustrations of the structure of xylem growth zones and annual growth rings in teak.

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