Structural and mechanical design of tissue interfaces in the giant reed *Arundo donax*

Markus Rüggeberg^{1,2,*}, Ingo Burgert² and Thomas Speck¹

¹*Faculty of Biology, Plant Biomechanics Group, Botanic Garden of the University, Freiburg, Germany*

²*Department of Biomaterials, Max-Planck-Institute of Colloids and Interfaces, Potsdam, Germany*

The culms of the giant reed *Arundo donax* represent slender tube-like structures. Several nodes along the culm, a ring of sclerenchymatous fibres in the periphery of the culm wall and numerous isolated vascular bundles enclosed by fibre rings in the culm wall function as stiffening elements. The bundles are embedded in lignified parenchyma. Micromechanical analysis indicated differences in stiffness between the individual tissues of more than one order of magnitude. In case of abrupt transitions in stiffness at the interfaces, stress discontinuities arise under dynamic loads. This eventually leads to critical shear stresses at cell ends, and culm failure may be initiated at these points. Pronounced mechanical differences between individual tissues can be compromised by gradual transitions at their interfaces. Ultrastructural and spectroscopic investigations with high spatial resolution revealed a gradual transition of cell parameters (cell wall area fraction and cell length). However, cell wall parameters (cellulose microfibril angle and lignin content) showed abrupt transitions or remained almost constant across the interfaces between various tissues. The design principles found at the interfaces between tissues in the culm walls of *A. donax* are discussed as an adaptation strategy to mechanical loads at different levels of hierarchy.

Keywords: *Arundo donax*; micromechanics; stiffness; microfibril angle; hierarchy; gradients

1. INTRODUCTION

Plants are able to adjust the mechanical properties of their organs, tissues and cells in response to different environmental conditions and needs. Because of this, adjustments are possible at all levels of hierarchy (Speck *et al.* 1996; Fratzl & Weinkamer 2007). Within plant organs, specialized types of tissues with different mechanical properties have evolved. Monocotyledonous plants contain isolated vascular bundles with fibre caps which are embedded in parenchymatous tissue (Zimmermann & Tomlinson 1972). The vascular bundles function, on the one hand, as water and nutrition-conducting elements. On the other hand, in palms as well as in many other monocotyledonous species, the fibre caps of the vascular bundles are the main stiffening elements. They are stiffer by far than the surrounding parenchymatous tissue (Rich 1987; Tomlinson 1990; Spatz *et al.* 1997). The structure of these plant stems can therefore be regarded as a fibre reinforced composite with a highly inhomogeneous distribution of stresses when loaded. Stress discontinuities arise at the interfaces of fibres and parenchymatous tissue and shear stresses will develop at the ends of

the reinforcing fibres and at free edges in the composites (Hull & Clyne 1996). Therefore, failure might be initiated at these points. In the Mexican fan palm (*Washingtonia robusta*), a gradual transition in stiffness from the central part of fibre caps to the surrounding parenchymatous tissue spanning more than one order of magnitude was found in a bundle type mostly apparent in the stem centre (Rüggeberg *et al.* 2008). This has been interpreted as an adaptive strategy of the plant to lower stress discontinuities and to avoid critical shear stresses at the ends of the fibre cells under the given evolutionary constraints. Furthermore, this gradual transition in stiffness may improve vibration damping due to a (stepwise) reduction in stiffness across the cell layers, leading to micro-friction at their interfaces. These frictional processes may cause an increased energy dissipation which would be mirrored on the macroscopic level of the stem as an improvement in vibration damping of the stem. In other typical peripherally located bundle types, however, there exists an abrupt transition in stiffness (Rüggeberg *et al.* 2009).

The hollow aerial culms of the giant reed (*Arundo donax*), which belongs to the monocotyledonous family of grasses (*Poaceae*), can grow to heights of 4–6 m exhibiting very high growth rates. The culms are very slender with an outer diameter of a few

* Author for correspondence (markus.rueggeberg@mpikg.mpg.de).

Figure 1. (a) Cross section covering the entire culm wall of an internode of *A. donax*. (b) Detail of the outer part of the culm wall with rectangles indicating the regions where cell wall area fraction was determined. (c) Radial-longitudinal section stained with fuchsin, chrysoidin and astrablue. Red staining indicates lignified tissue. The blue rectangles indicate subsequent tissue strips as they were cut out by microlaser dissection for mechanical testing. E, epidermal layer; CP, cortical parenchyma; S, sclerenchymatous ring; PP, pith parenchyma; F, individual fibres within the pith parenchyma; VB, vascular bundle; FR, fibre ring of VB; Ph, phloem; X, xylem.

centimetres and a thickness of the culm walls of a few millimetres (figure 1a). Morphological and anatomical features at different structural levels stabilize and stiffen these tube-like structures. The culms are subdivided into nodes and internodes. The nodes stabilize the entire culms against local buckling in the case of bending (Spatz *et al.* 1993; Spatz & Speck 1994). The ring of sclerenchymatous fibres mainly contributes to the bending stiffness of the culm due to its mechanical properties and its location at the culm wall periphery (high second moment of area; figure 1a,b). Numerous isolated vascular bundles with enclosing fibre rings embedded in lignified parenchyma further stiffen the culm. In tensile tests on macroscopic samples, Spatz *et al.* (1997) measured an elastic modulus of 10 GPa for the periphery of the culm wall, which consisted of the epidermal layer, a narrow layer of cortical parenchyma and the sclerenchymatous ring. An elastic modulus of around 9 GPa was calculated for the inner part of the culm wall consisting of pith parenchyma and vascular bundles. However, the authors suggested that the parenchyma itself should be more flexible, because the embedded vascular bundles with fibre rings act as the main load-bearing elements. They proposed a gradual transition in stiffness from the fibre rings to the lignified parenchymatous tissue to avoid a mismatch of mechanical properties at the interface.

In this paper, we report about micromechanical tests that were performed to resolve the stiffness distribution within the peripheral part of the culm walls of *A. donax* with high spatial resolution. In addition, structural,

ultrastructural and biochemical parameters influencing tissue stiffness at different levels of hierarchy were investigated. With this combined approach, we aimed to unravel the mechanical and structural design of the interface between the different types of tissues in the culm walls of *A. donax*.

2. MATERIAL AND METHODS

2.1. Plant material—conservation and sectioning

A. donax was grown in the Botanical Garden in Freiburg. Two culms were harvested in the full-grown state. The fourth and the fifth internode above soil level were taken for further analysis. In figure 1a,b, a cross section of a segment of the entire culm wall and details of its periphery are shown. From the outer edge inwards different tissue types can be recognized (epidermis, cortical parenchyma, sclerenchymatous ring and pith parenchyma with embedded vascular bundles).

For sample conservation and preparation, tissue blocks of about 1 cm³ were embedded in the water-soluble polymer polyethylene glycol 2000 (PEG 2000). The method has been described in detail in Rüggeberg *et al.* (2008). Thin sections were prepared with a rotating microtome. For further use, the sections were washed in water.

2.2. Mechanical tests

For the mechanical tests, tissue strips were taken from the peripheral zone of the culm walls covering cortical parenchyma, sclerenchyma and pith parenchyma. The fibre rings of the vascular bundles were too small to be subdivided into suitable strips. Therefore, no tests were performed with this tissue. The strips were cut out of consecutive tangential microtome sections using microlaser dissection technology (P.A.L.M. Microlaser Technologies GmbH, Germany, with a pulsed UV-A laser at 355 nm, CryLas GmbH, Germany). The tissue strips were 50 μm wide and approximately 40 μm thick. Figure 1c shows the resulting spatial alignment as it appears in a radial section. The strips were glued onto a plastic foil and fixed in a microtensile testing stage. The test length was 1.6–1.8 mm, and the test speed was 2 μm s^{−1}, resulting in a strain rate of 0.11–0.13% per second. Force and elongation were recorded during the experiment. The detection of the elongation for calculating the applied strain was enabled by video extensometry through black lines on the plastic foil next to the glued ends of the sample (Burgert *et al.* 2003). In order to convert forces into applied stresses which, by dividing through the applied strain, gives the stiffness (elastic modulus) of the sample, the cross-sectional area of the sample had to be determined. Short pieces of the mechanically tested tissue strips were cut apart with the microlaser and flipped by 90°. Images were taken to evaluate cross-sectional areas. The elastic modulus of the tissue was calculated taking into account the entire cross-sectional area of the tissue strip. By using only the cross-sectional area of the cell wall, the elastic modulus of the cell wall itself could

be calculated. The sections were kept wet during preparation and testing.

2.3. Cell parameters

Cell wall area fraction across the peripheral zone of the culm wall was determined by image evaluation of cross sections of 20 μm thickness using the software ImageJ, v. 1.38. For the peripheral zone, consecutive regions were defined containing more than 10 cells to calculate average values and standard deviations (figure 1b, red marked rectangles). As a reference line, the outer edge of the culm was taken. Cell lengths were determined in radial–longitudinal sections of 8 μm thickness using the same software.

2.4. X-ray diffraction

Two of the three series of tissue strips tested mechanically were further investigated by wide angle X-ray diffraction to determine the cellulose microfibril orientation. The measurements were performed in dry state using a Nanostar instrument (Bruker AXS), with a beam diameter of 100 μm and a position sensitive detector (HiStar). The wavelength was 0.154 nm, the measuring time 4 h per sample and the sample–detector distance was 4.95 cm. The (002) reflections of crystalline cellulose were used to determine the microfibril angle μ (Lichtenegger *et al.* 1998, 1999).

In addition, radial–longitudinal sections of 40 μm thickness were scanned with synchrotron radiation at the μ -Spot beamline at BESSY II in Berlin, Germany (Paris *et al.* 2007), to resolve the cellulose microfibril orientation across the culm wall with cell diameter resolution. The X-ray beam had a diameter of about 15 μm and the step width was 20 μm . The X-ray wavelength was 0.082 nm and the measuring time was 120 s for each frame. The sample–detector distance was 46 cm, which allowed the wide angle to be captured as well as the small angle scattering signal. As area detector, a MarMosaic 225 (MarUSA, Evanston, USA) was used with 3072×3072 pixels and a pixel size of 73.2×73.2 μm .

2.5. Staining for qualitative lignin detection

Cross sections of 40 μm thickness were stained with phloroglucinol/hydrochloric acid for qualitative lignin detection. Images were taken immediately after staining.

2.6. Ultraviolet microspectrophotometry

The absorbance spectra and the topochemical distribution of lignin and other cell wall phenolics were obtained with a universal microspectrophotometer (UMSP 80, Zeiss). Sample blocks with $1 \times 1 \times 5$ mm size were embedded in Spurr according to Koch & Kleist (2001). Cross sections of 1 μm thickness were prepared for the measurements. Absorbance spectra in the range of 240–400 nm were taken with a spot diameter of 1 μm at different radial positions. The wavelength of the maximum absorbance was then used for two-dimensional scans across the stiffening tissue with a step width of 0.75 μm . For this analysis,

the programme APAMOS (Automatic Photometric Analysis of Microscopic Objects by Scanning, Zeiss) was used (Koch & Kleist 2001).

3. RESULTS

Microtensile tests were performed on three series of consecutive tissue strips from the peripheral zone of three different radial–longitudinal sections. Tissue stiffness and cell wall stiffness were calculated on the basis of sample cross section and cell wall area fraction, respectively (figure 2a–c). The first test series started from the rather thin layer of cortical parenchyma which showed a tissue stiffness of 1 GPa. Within the next two steps, an increase in tissue stiffness to 11 GPa was measured (figure 2a). Further inwards, a pronounced decrease in tissue stiffness to 3.5 GPa was found. This trend is also reflected in cell wall stiffness, which shows values of 2, 12 and 5 GPa at the respective regions. The two other series started further inwards on the culm wall. The second series (figure 2b) started in the cortical parenchyma as well, as indicated by the first value which was much lower than the following ones. The maximum stiffness of both series (figure 2b,c) was similar to the first series for tissue stiffness and cell wall stiffness. Both test series showed a decrease in tissue and cell wall stiffness with increasing distance from the outer edges of the sclerenchymatous ring. Tissue stiffness showed a rather gradual decrease, whereas cell wall stiffness remained high before a pronounced decrease was observed. For the innermost tissue strips, values for tissue stiffness between 400 MPa and 1 GPa were measured.

The tissue strips of the first and second mechanically tested series (figure 2a,b) were further investigated by means of X-ray diffraction under laboratory conditions for determining microfibril orientation. The microfibril angle is shown only for those tissue strips where it could be reliably determined. Microfibril angles and stiffness values did not correlate since the pronounced differences in tissue and cell wall stiffness were not reflected in cellulose fibril orientation.

For studying microfibril orientation with single cell resolution, radial–longitudinal sections were analysed with synchrotron X-rays. In figure 3a, one radial–longitudinal section which was used for a line scan is shown with the corresponding graph of the line scan below, showing the microfibril orientation (figure 3b). Two further line scans are shown in figure 4.

The line scans revealed abrupt changes in microfibril angles at the interfaces between cortical parenchyma and sclerenchymatous fibres (figure 4a) and sclerenchymatous fibres and pith parenchyma (figure 3b, second vertical line, 430 μm ; figure 4). The changes from small to large microfibril angles (and vice versa) occurred within the step width of the scan, which is in the range of the diameter of a single cell. Owing to very low signal intensities of the measurements in the parenchymatous tissue, only part of the diffraction images could be evaluated.

Figure 2. (a–c) Cell wall stiffness (closed circles) and tissue stiffness (open circles) of three series of tissue strips. For (a) and (b), the microfibril orientation was investigated. Values of microfibril angle (MFA, grey columns) are given for strips whose orientation could be clearly determined.

The sclerenchymatous fibres showed a rather constant microfibril angle below 10° . In terms of the parenchymatous tissues the results were less consistent. For most measurements values between 15° and 30° were calculated, whereas a few diffraction patterns resembled those typical of the sclerenchymatous fibres. These values might be accounted for by some single fibre cells embedded in the pith parenchyma (figure 1c). The small microfibril angles beyond the third vertical line in figure 3 (620 μm) and in figure 4a at the right end of the scan belong to the fibrous ring of a vascular bundle which is embedded in the parenchyma.

Next to the microfibril orientation, cell parameters such as fibre length and cell wall area fraction were studied with high spatial resolution as well. The determination of cell length (figure 3c) was performed on a consecutive section to the one used for the line scan of figure 3a,b. Epidermal fibres were approximately 500 μm long. The cortical parenchyma cells were significantly shorter with cell lengths between 60 and 150 μm . Close to the interface between cortical parenchyma and sclerenchymatous ring an increase in cell length to 680–890 μm was measured. A further gradual increase in cell length within the sclerenchymatous ring to a maximum of 3.5 ± 0.9 mm was detected, before fibre length decreased towards pith parenchyma to a value of 1.4 ± 0.5 mm close to the interface. The fibres in the sclerenchymatous ring showed considerable variation in cell length. The length of pith parenchyma cells decreased from 600 μm close to the interface to 320 μm further inwards.

Cell wall area fractions were determined as a relative measure of tissue density. Although they were performed on (two) different cross sections (figure 1b), the results were integrated in figure 3 because, as in the radial–longitudinal sections, individual measurements could be addressed to distinct distances from the outer edge of the culm (figure 3d). Considerable differences in absolute values existed between the two culms, but the overall trend was very similar. A pronounced increase in cell wall area fraction was found from cortical parenchyma to the sclerenchyma followed by a gradual decrease within the sclerenchyma towards the pith parenchyma. Cell wall area fraction for sclerenchymatous tissue reached values of up to 95 per cent. Values in the range of 30–65% for the cortical parenchyma were slightly higher than those of the

pith parenchyma, which varied between 20 per cent and 45 per cent.

Lignification of the culm wall was revealed qualitatively in cross sections of two different culms by means of staining with phloroglucinol/hydrochloric acid. In figure 5a, only the cross section of one culm is shown, since both sections consistently indicated a lignification of the whole culm wall.

The lignification seemed to be higher in the sclerenchymatous ring than in the cortical parenchyma and in the pith parenchyma. The fibre rings enclosing the vascular bundles showed the highest level of lignification gradually decreasing towards the pith parenchyma. In addition, two-dimensional UV scans with a wavelength of 282 nm were performed across the peripheral part of the stem (figure 5b). The absorbance decreased gradually across the sclerenchymatous ring and the adjacent parenchymatous tissue from approximately 0.6 to 0.4. The epidermal layer showed the highest absorbance values of up to 0.8, whereas for the cortical parenchyma values around 0.3 were obtained. The staining intensity (figure 5a) correlated reasonably well with the absorbance pattern of the UV scans.

4. DISCUSSION

To analyse structure–function relationships in the giant reed, mechanical tests have so far been conducted only with the entire culm and culm wall or with macroscopic parts of the culm wall (Spatz *et al.* 1997). With the present set-up, we gained considerably higher resolution following the course of stiffness within the different tissues and across the interfaces of tissues. A maximum tissue stiffness of 11 GPa was measured for tissue strips of the sclerenchymatous ring. This is well in the range of the 10 GPa obtained by Spatz *et al.* (1997) for the entire outer part of the culm wall. The tissue stiffness of the parenchyma was in the range of 0.4–1 GPa. This confirmed the assumption of Spatz and co-workers that the value of 9 GPa which they calculated for the inner part of the culm wall was not representative for the stiffness of the pith parenchyma, because of the additional stiffening effect by the vascular bundles scattered within the pith parenchyma. Thus, differences in stiffness of more than one order of magnitude exist between the sclerenchymatous ring and the parenchyma.

Figure 3. (a) Image of the radial section used for the line scan with synchrotron radiation. (b) Microfibril angles as calculated from the diffraction diagrams of the line scan. (c) Mean cell length measured in a consecutive section taken in a matching position to the section above. (d) Cell wall area fraction versus distance from epidermal layer measured in two different culms. The interfaces of sclerenchyma and parenchyma are indicated with vertical lines. For convenient comparison (coincidence of the tissue interfaces, vertical lines), the x -axis of (d) has a different scale from (a–c). E, epidermis; CP, cortical parenchyma; S, sclerenchyma; PP, pith parenchyma; FR fibre ring; X, xylem.

For severe changes in stiffness at tissue interfaces, a gradual transition should be beneficial, as this limits stress discontinuities and reduces the probability of failure under mechanical loading (Neubrand & Rodel 1997; Waite *et al.* 2004). In terms of the culm walls of *A. donax*, the interfacial characteristics were studied by examining the distribution of micromechanical, ultrastructural and biochemical parameters. The course of stiffness across the culm wall (figure 2) indicated gradual increases and decreases within both parenchymatous and sclerenchymatous tissues. The decrease in elastic modulus across the sclerenchymatous ring could be less pronounced in the intact culm than measured on the consecutive tissue strips in the

mechanical tests. The cross-sectional area of the individual cells increased across the sclerenchymatous ring towards the pith parenchyma (figure 1b). This decreased the number of fully intact cells per individual tested tissue strip across the sclerenchymatous ring, whereas the amount of partly cut cell walls can be considered as constant in good approximation. According to Mark (1967), a lower elastic modulus is measured for partly cut cells than for fully intact cells. As we considered a high spatial resolution to be more important for obtaining more detailed mechano-structural information, we took a possible (slight) overestimation of the decrease into account, which should not have any major influence on our conclusions. The gradual

Figure 4. The graphs show the results of two additional line scans with synchrotron X-rays similar to the one presented in figure 3b.

Figure 5. (a) Cross section taken close to the one used for the UV measurements stained with phloroglucinol/hydrochloric acid that stains lignified tissues in red; (b) bottom: composite image of a toluidine blue-stained cross section and two-dimensional UV-absorbance scans of an unstained cross section taken at 282 nm. Top: absorbance values along the red line marked in the two-dimensional scans. Violet, absorbance 0; indigo, absorbance 0.1; dark blue, absorbance 0.2; light blue, absorbance 0.3; dark green, absorbance 0.4; light green, absorbance 0.5; yellow, absorbance 0.6; orange, absorbance 0.7; red, absorbance 0.8.

changes in elastic modulus within the individual tissues pointed towards smooth transitions at the interfaces. However, since consecutive tangential strips were mechanically tested, it cannot be excluded that tissue

strips taken directly in the region of the interface contained both sclerenchymatous and parenchymatous tissues, resulting in (artificial) intermediate values of tissue and cell wall stiffnesses. Therefore, the resolution

achieved with the mechanical test set-up was high enough to follow the alterations of stiffness within the individual tissues of the culm wall towards the interface, but it might have been too low for exactly revealing the tissue stiffness at the interfaces.

The investigated cell and cell wall parameters, which all influence tissue stiffness, could be measured with higher resolution. Thus, their profiles across the culm wall can provide further information about the structural basis of alterations in stiffness both within the individual tissues and directly at the interfaces, namely abrupt or gradual. The measurements revealed different kinds of profiles for the respective parameters at tissue and at cell wall level. Parameters at the tissue level, namely cell wall area fraction (which can be taken as a relative measure of tissue density) and cell length, showed gradual alterations within the different tissues and at the interfaces of the tissues. But such a trend was not observed for the cell wall parameters investigated. The orientation of the cellulose microfibrils showed no trends within the tissues and an abrupt change from small microfibril angles in sclerenchymatous tissues and fibre rings of vascular bundles to high values in parenchymatous tissues. In terms of lignification, a slight gradual decrease within the sclerenchyma ring and towards pith parenchyma was found by staining and UV microspectrophotometry. However, the effect of lignification on the axial tensile stiffness does not only depend on its degree but also on the cellulose fibril orientation in the cell wall. At high microfibril angles, cellulose microfibrils are sheared under tensile loads (Keckes *et al.* 2003; Fratzl *et al.* 2004). In consequence, a higher shear modulus of the matrix leads to a higher axial stiffness, whereas this effect is much less pronounced at low cellulose fibril angles. Lignification effectively leads to an increase in axial cell wall stiffness in particular for high microfibril angles, because it leads to an increase in the shear modulus and the shear strength of the matrix (Rüggeberg *et al.* 2008). For *A. donax*, this may explain the high stiffness of the parenchyma which was up to two orders of magnitude higher than values reported for non-lignified parenchyma of potato tubers (Niklas 1988). But, as an abrupt change in cellulose fibril orientation was observed between sclerenchymatous fibres and parenchyma, lignification probably contributes to only a minor extent to a more gradual stiffness transition. It may contribute to the observed stiffness variations only within parenchymatous tissues.

The mechanical and structural results obtained allow conclusions to be drawn about the profile of stiffness within the individual tissues of *A. donax*, whereas the interfacial characteristics could not be exactly resolved in the micromechanical tests. The resolution of these tests was too low to address the latter question. Whereas cell parameters indicated a gradual transition in tissue stiffness, cell wall parameters did not. Moreover, it is difficult to weigh the influence of the individual parameters on the local material properties. Both cellulose microfibril angle and cell wall area fraction are well known to play a dominant role in adjusting the axial tissue stiffness (Ashby 1983; Reiterer *et al.* 1999). It is striking that one of these parameters (cell

wall area fraction) seems to contribute to a gradual transition in axial stiffness within and between the different tissues, whereas the other (cellulose microfibril angle) does not. It can be summarized that, by gradual changes of cell parameters within the individual tissues, the differences in tissue stiffness at the interfaces should be much smaller than the maximum differences measured between parenchyma and sclerenchyma. This can be regarded as a strategy to achieve rather low stress discontinuities at the interfaces to avoid critical shear stresses at the ends of the fibre cells and sufficient flexural stiffness of the thin culm with its high aspect ratio. The latter is mainly maintained by the thin sclerenchymatous ring (only a few fibre rows wide) at the periphery of the culm wall, i.e. with a high relative contribution to axial second moment area. Therefore, one can hypothesize that higher cellulose microfibril angles at the transitions to the parenchyma might cause deterioration of its main function in providing bending stiffness to the culm.

5. CONCLUSIONS

The hollow aerial culms of *A. donax* are optimized towards high flexural stiffness by the morphology of the culm and the anatomy of the culm wall. By means of microtensile tests, large differences in stiffness were detected between sclerenchymatous and parenchymatous tissues. This puts constraints on the design of transition zones between tissues and especially of the interfaces, as a mismatch in mechanical properties evokes stress discontinuities resulting in high shear stresses at the ends of the fibre cells. These stresses endanger the integrity of the culm under dynamic wind and gust loads. The profiles of stiffness in the individual tissue types indicate that gradual changes in stiffness exist within parenchymatous and sclerenchymatous tissues. Analyses of structural cell and cell wall parameters across the periphery of the culm wall showed that these gradual changes in stiffness are mainly caused by alterations at the tissue level.

The characteristics of the interface between the different tissues could not be clearly resolved by the mechanical tests. However, it can be concluded that, owing to adaptations at the tissue level, an abrupt transition between the sclerenchymatous fibres and the parenchyma is unlikely. On the other hand, the cellulose microfibril orientation which represents a (mechanically) very important cell wall parameter does not show any gradual transition, i.e. it has obviously not evolved in a way that contributes to a gradual stiffness transition within the tissues and at the tissue interfaces. This might be explained by an 'evolutionary strategy' to compromise between avoiding stress discontinuities, brought about at least to a given extent by variations at the tissue level, and 'optimizing' bending stiffness of the entire culm.

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