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# Development of the biomechanical system of the flax stem: mutual game of primary and secondary growth

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## Highlights

- The contributions of phloem fibers and xylem into stem strength change with time
- Younger stem parts mechanically rely more on phloem fibers, older parts – on xylem
- Maximum plant elongation occurs with temporary reduction of secondary growth
- The position of the snap point is independent of the mass of the plant part above it
- The area of stem cavity correlates with the area of bast fiber tertiary cell walls

## Abstract

Flax is one of the oldest cultivated plants and one of the most important fiber crops in the world. Thousands of years of selection have resulted in the unique biomechanics of this plant, the principles of which are not yet fully understood. Detailed anatomical analysis of flax stems at different heights and at different stages of plant development (herringbone, fast growth, flowering) allowed us to follow the dynamics of primary and secondary growth of these plants. The evaluation of the mechanical properties of the stems and their individual parts at the same stages made it possible to identify the tissues that take on the main mechanical load at different periods of the plant's life. In the younger parts of the stems, this tissue is the fiber-enriched phloem, whose fibers rapidly acquire a considerable elastic modulus. Xylem, which takes longer to develop, provides the main mechanical load in more mature parts of the stems and at later stages of plant development. Fast growth of flax stems occurs against the background of delayed xylem development, while the main mechanical support comes from phloem fibers with tertiary cell wall. The onset of cell wall thickening in phloem fibers (the position of the snap point) is not determined by the mass of the plant part above it. The results obtained highlight the contribution of different tissues to the functioning of the plant as a holistic biomechanical system.

**Keywords:** flax, plant fibers, secondary xylem, tertiary cell wall, stiffness, elasticity modulus, stem anatomy, plant biomechanics

## 1. Introduction

According to a perceptive remark by C. Baley and colleagues (2018), plants are an example of a natural biocomposite and high-performance structure and can serve as a source of bioinspiration. Indeed, if we consider the plant as a holistic biomechanical structure designed to optimally capture solar energy,

efficiently propagate offsprings and resist stress, the study of the organizational principles of this system can be extremely enlightening (Speck and Speck, 2021). Even by the standards of the plant kingdom, flax is characterized by an exceptional ratio of stem height to stem diameter (i.e. slenderness) (Goudenhooff et al., 2019a). Many studies have been carried out on the morphology, mechanics and architecture of flax. Most of them were performed at the stage of full maturity, evaluating the changes of the studied parameters along the stem length or their differences between varieties (Goudenhooff et al., 2017; Baley et al., 2018; Réquilé et al., 2018; Nuez et al., 2021). In other researches, measurements were made at different stages of plant development, but the focus of these works was always on phloem fibers as the most valuable renewable raw material obtained from flax (Gorshkova et al., 2003; Brutch et al., 2008; Goudenhooff et al., 2018). However, to fully understand the principles of functioning of any system, it is necessary to consider it in dynamics during its formation, as well as to take into account the possible influence of all its components.

Three main groups of tissues can be distinguished in a cross section of a flax stem (Fig. 1, 2). The peripheral position is occupied by the extraxylary part of the stem, which includes a single-layer epidermis, several layers of parenchyma, conductive elements of the phloem and phloem fibers gathered in bundles; within the paper we designate this part simply as phloem. The xylem, separated from the phloem by a single-layer vascular cambium, occupies an intermediate position. The inner part of the stem consists of pith cells. Over time, a cavity is formed in the center of the stem (Fig. 1, 2). As a dicotyledonous species, flax has both primary and secondary growth. Primary growth is the growth that occurs as a result of cell division in the apical meristems of stems and roots, causing them to elongate and give rise to primary tissues. Secondary growth is the growth that results from cell division in the cambium or lateral meristems, causing the stems and roots to thicken. Tissues produced as a result of such growth are called secondary. The concepts of primary and secondary growth can also be extended to individual plant cells. In this case, primary growth refers to the increase in cell size and the acquisition of a specific shape, and secondary growth refers to the thickening of cell walls and cell specialization (Esau, 1965; Fahn, 1990).

The phloem fibers of flax are exclusively of primary origin, i.e. they are formed only in the stem apical meristem, while the cambium does not produce new cells on the phloem side. After emergence and a brief period of coordinated growth, the fibers begin to grow intrusively, exceeding the elongation rate of adjacent cells and penetrating between them (Gorshkova et al., 2003; Ageeva et al., 2005). Intrusive growth of phloem fibers occurs only in the upper part of the stem, then the fibers progress to the stage of cell wall thickening (Snegireva et al., 2010). A small amount of secondary cell wall is deposited first, followed by a thicker tertiary cell wall (TCW), also called the G-layer (Gorshkova et al., 2010; Gorshkova et al., 2018). The transition to cell wall thickening occurs approximately 10 cm from the stem apex (Ageeva et al., 2005) and is accompanied by a significant increase in longitudinal stem tensile strength (Roach et al., 2011). The point on the stem where this occurs is called the snap point (Gorshkova et al., 2003). At present, it is not clear what determines the position of the snap point on the stem. The stimulus that triggers the phloem fiber transition from primary to secondary growth could be either chemical or physical in nature. For example, it could be the weight of the plant above the snap point, since it has been shown, at least in *Arabidopsis*, that the weight carried by the stem serves as a signal for secondary growth (Ko et al., 2004). It can be assumed that the growing top of the plant becomes too heavy to be supported by turgor and primary cell walls alone, and phloem fibers must begin to thicken their cell walls to support it.

Flax development can be divided into the following stages: 1) herringbone – the period when the plant is characterized by a moderate growth rate and reaches a height of 15-20 cm; 2) rapid growth period–

after reaching a certain height, the plant dramatically accelerates its growth and quickly reaches its final height. At this stage, a snap point can be detected on the stem; 3) flowering – the increase in plant height ends, the inflorescence appears at the top of the plant, the snap point disappears; 4) green ripeness – seed formation occurs; 5) yellow ripeness – phloem fibers complete their maturation; 6) full ripeness – seeds ripen (His et al., 2001, Goudenhoft et al., 2019a).

Mechanical properties of whole flax stems, their xylem and phloem parts, as well as individual phloem fibers and individual layers of their cell walls have been evaluated by various methods (Zeng et al., 2015; Arnould et al., 2017; Goudenhoft et al., 2017, 2018; Réquillé et al., 2018; Petrova et al., 2021; Nuez et al., 2021). However, a comprehensive picture of the formation and functioning of flax as a high-performance structure has not yet been compiled. Our understanding of the relationships between structure, function, and mechanical properties of individual tissues within the plant remains incomplete. In order to establish the possible contribution of different tissues to the functioning of the plant as a holistic biomechanical system, we characterized changes in the morphological and mechanical parameters of the flax stem during plant development.

## **2. Materials and methods**

### *2.1. Plant material*

Flax plants (*Linum usitatissimum* L., cultivar Mogilevsky from the collection of the All-Russian Flax Research Institute, Torzhok) were grown in the open vegetation area (55°47'33.7"N 49°07'15.8"E) in boxes with a 50 cm layer of soil with daily watering and natural photoperiod lighting (16 h of light/8 h of darkness). Plants were watered the night before each study.

### *2.2. Influence of plant mass above the snap point on its position*

Ten flax plants at the period of fast growth, whose height reached 19-20 cm from cotyledons, were used for the experiment. For five plants, the weight of the plant part above the snap point was artificially increased by placing a piece of transparent silicone tube weighing 0.24 g at a level two cm below the apex (Fig. 3). The piece of tube was pre-cut lengthwise so that it could be placed on the plant without damaging the leaves. The transparency of the tube allowed access to sunlight. Five other plants were used as controls. After three days, the weight was removed. The height and the fresh weight of the whole plants were measured, as well as the distance from the apices to the snap point and the fresh weight of this part of the plant for the control and experimental groups.

### *2.3. Anatomical analysis*

One-centimeter-long stem fragments were taken every 5 cm of stem length for herringbone and every 10 cm of stem length for fast growth and flowering stage (Fig. 1). Level 1 was at a height of 5 cm from the cotyledons. Plant material was fixed in 80% ethanol and stored until use. Transverse sections (50 µm thick) for each stem level were prepared using a VT1000S vibratome (Leica Biosystems, Germany). All samples were examined with a Leica DM1000 microscope (Leica Biosystems, Germany) and photographed with a digital camera. Stem length and weight (with leaves) were recorded at each time point; for time points taken during fast growth, stem length and weight values above the snap point were also measured.

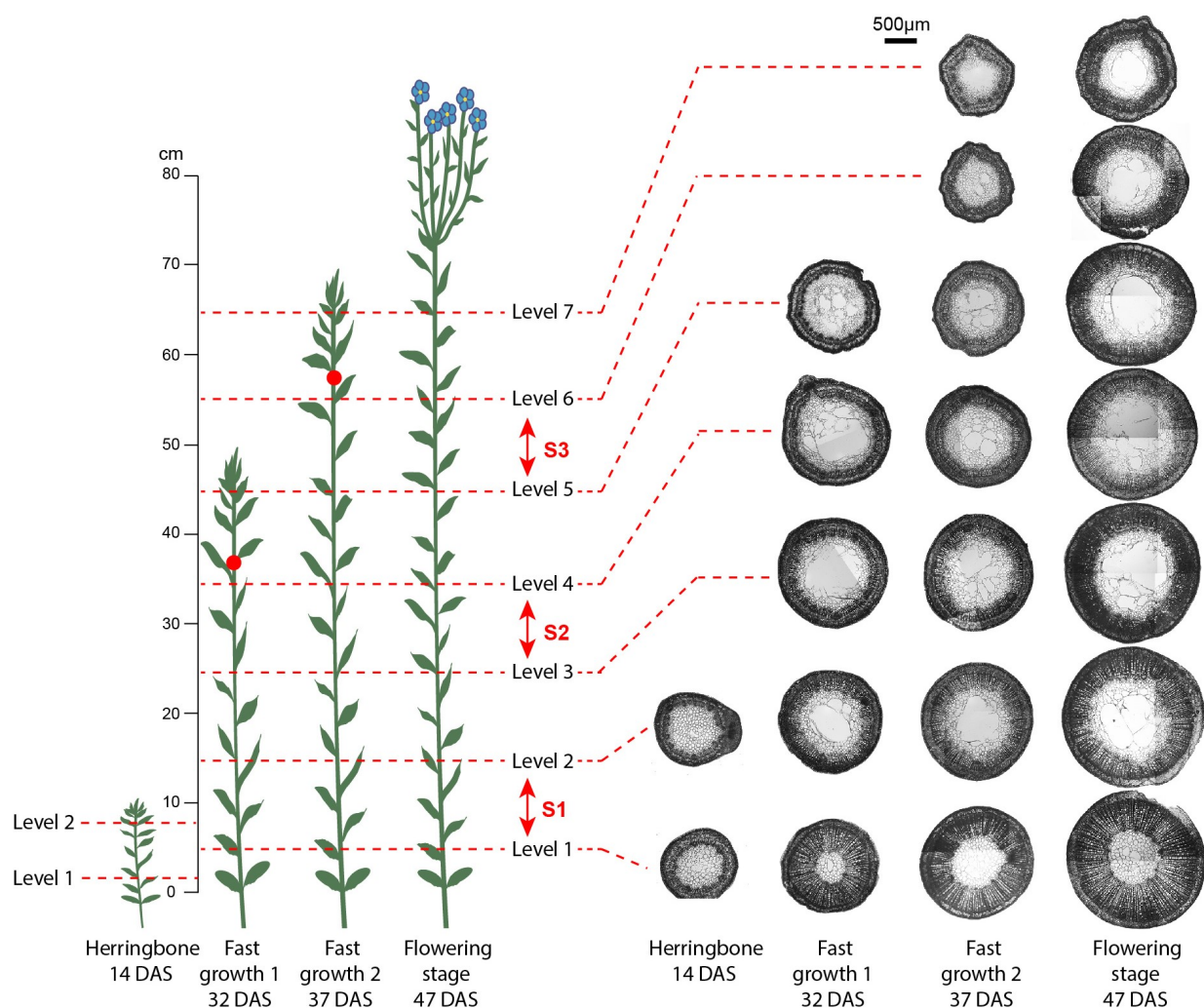


Fig. 1. Scheme of sample collection for stem anatomy analysis (Level 1-7) and measurements of stem tissue mechanical properties (S1-S3) in the course of flax plant development. DAS - days after sowing. Red stars indicate snap point locations. Red double-handed arrows - the parts of the stem taken for measurements of mechanical properties. Dotted lines - the levels of stem used for anatomy analysis.

Various parameters of the stem anatomy were measured on photographs of transverse sections using Image-Pro Plus software (<https://mediacy.com/image-pro/>), as shown in Fig. 2A.

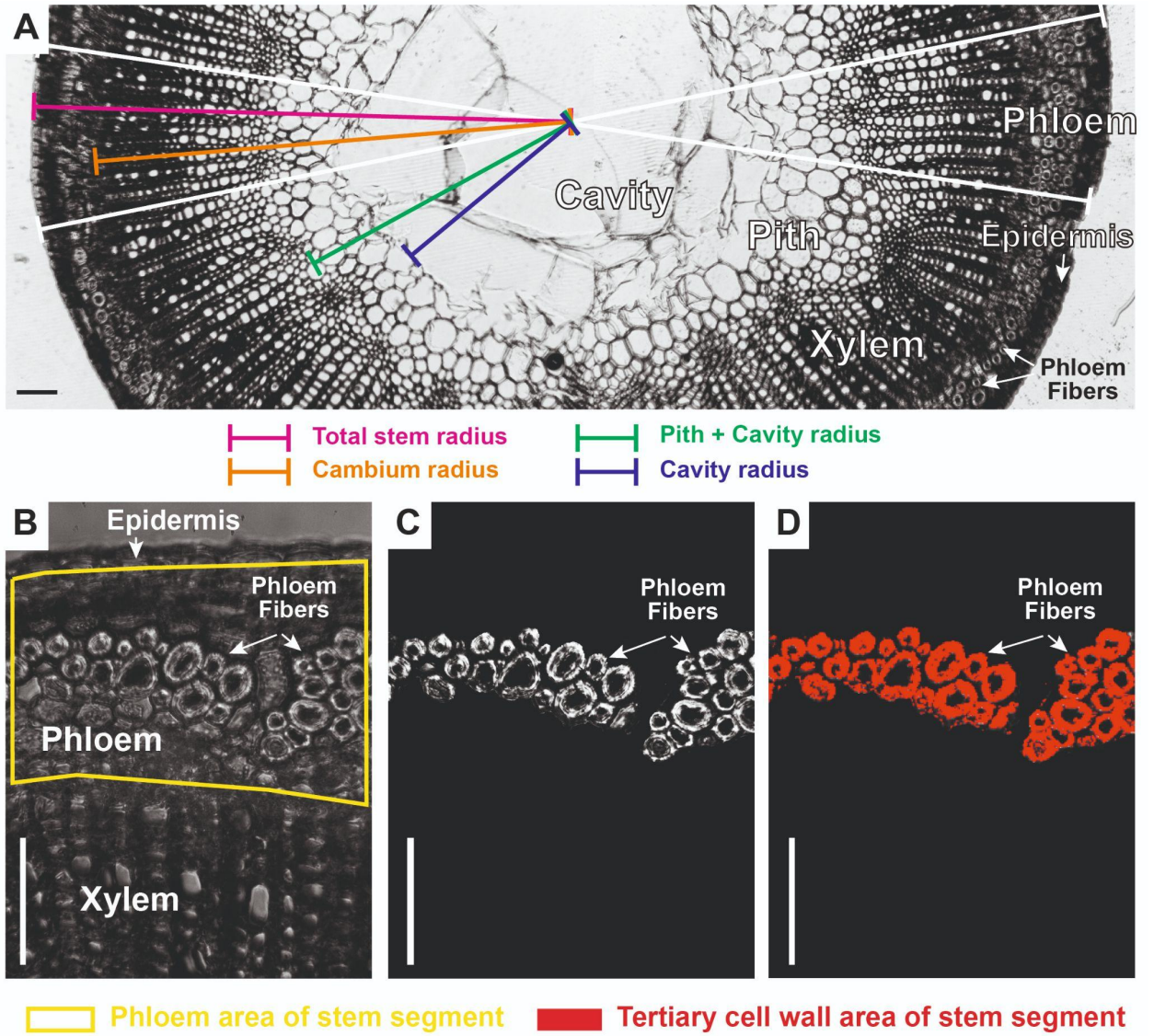


Fig. 2. Scheme for estimating morphological parameters of flax stem tissues. (A) Determination of the radii of different tissues (indicated by colored lines) on a microphotograph of a transverse section of the stem. White lines indicate the two diameters drawn to find the geometric center of the stem. (B-D) Determination of the area occupied by the tertiary cell walls of phloem fibers on a microphotograph of a transverse stem section. (B) Microphotograph of a portion of a flax stem transverse section with the phloem outlined in yellow, (C) the same microphotograph from which all cell types except phloem fibers were removed using Photoshop, (D) a processed microphotograph with the tertiary cell walls of the phloem fibers outlined in red using Image-Pro Plus. The scale bar is 100  $\mu\text{m}$  for all microphotographs.

All measurements were made in three areas of each transverse section as technical replicates. Tissue areas (A) in the flax stem were calculated according to the equations 1-7:

$$A_{stem} = \pi R_{stem}^2 \quad ; \quad (1)$$

$$A_{xylem+pith+cavity} = \pi R_{cambium}^2 \quad ; \quad (2)$$

$$A_{phloem} = A_{stem} - A_{xylem+pith+cavity} \quad ; \quad (3)$$

$$A_{pith+cavity} = \pi R_{pith+cavity}^2 \quad ; \quad (4)$$

$$A_{cavity} = \pi R_{cavity}^2 ; \quad (5)$$

$$A_{pith} = A_{pith+cavity} - A_{cavity} ; \quad (6)$$

$$A_{xylem} = A_{xylem+pith+cavity} - A_{pith+cavity} ; \quad (7)$$

The total area of the tertiary cell wall was determined using Image-Pro Plus and Adobe Photoshop (<https://www.adobe.com/products/photoshop.html>) software. A small fragment of the image of a transverse stem section was used. Using the “polygon selections” tool in Image-Pro Plus, the phloem area was outlined and measured (Fig. 2B). Then all cell types except phloem fibers were removed from that micrograph using Photoshop as shown in Fig. 2C. Then, using the “counting and measuring objects” tool in Image-Pro Plus, the area of the tertiary cell wall of the fragment was determined (Fig. 2D). This operation was performed for four different fragments of the same transverse section as technical repeats. The total area of the tertiary cell wall on the section was determined using the equation 8:

$$A_{TCW} = \frac{A_{TCW}^{stemfragment}}{A_{phloem}^{stemfragment}} A_{phloem} ; \quad (8)$$

#### 2.4. Measurements of mechanical properties

For the analysis of mechanical properties, 10 cm segments of the stems at three time points (fast growth 1, fast growth 2 and flowering) were used (Fig. 1, red double-headed arrows). Segment 1 was located between levels 1 and 2 of the anatomical study, segment 2 was located between levels 3 and 4, and segment 3 was located between levels 5 and 6. Macromechanical properties were investigated using a Python script for vibration evaluation (Nakata et al., 2018, Fig. 3). This approach is based on the fact that the vibration frequency of the stem after twitching is proportional to the stem stiffness. Each plant was cut immediately before the experiment to avoid the effect of water loss on the samples. A drop of red dye was placed on the tip of the sample (Fig. 3A). The bottom centimeter of the sample was carefully clamped. To apply linear bending, the upper part of the stem (3–4 cm from the tip) was grasped with tweezers and pulled sideways at an angle of 20°. The vibration of the stem was recorded for 3 s with the cell phone camera using the high-speed shooting function (240 fps). The experiments were performed first on the defoliated stem segment and then on the same segment with the phloem part removed. Fresh weight (FW) values were measured for each sample (defoliated stem segment and segment with the phloem part removed). The time of each measurement was kept constant and as short as possible (2–3 min) to avoid significant water loss and weight changes. Anatomical analysis was performed on hand cross sections taken right above and below the segment under study.

A script was used to estimate the motion of the red marker at the end of the studied object in each frame (Fig. 3A). The marker coordinates are converted into displacement data (Fig. 3B), which in turn is used to calculate the natural frequency (Fig. 3C).

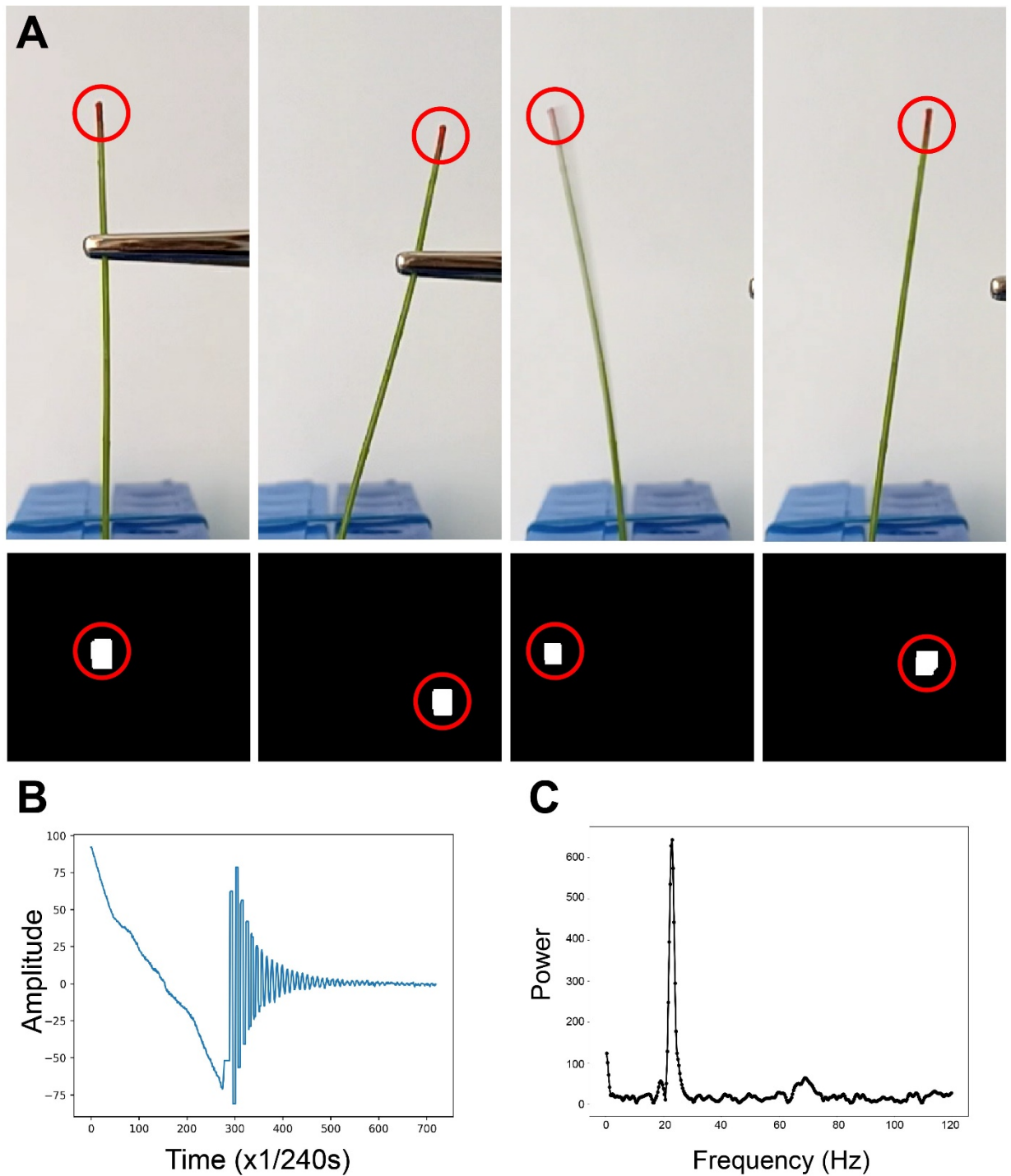


Fig. 3. Scheme of the vibration experiment on flax stem segments. (A) Tracking a red marker at the end of the sample (red circle, top row) and estimating the coordinates of the marker using Python script (red circle, bottom row). (B) Amplitude of oscillations. (C) Natural frequency of the vibration.

The stiffness of the sample was calculated using equation 10:

$$k = (2\pi\omega_n)^2 m_{eq} ; \quad (9)$$

where  $k$  is the stiffness;  $\omega_n$  is the natural frequency;  $m_{eq}$  is the equivalent mass of the oscillator (eq. 11):

$$m_{eq} = \alpha \times 0.9 \times FW ; \quad (10)$$



where  $\alpha$  is the coefficient depending on the taper ratio; 0.9 is the coefficient, which means that only 9 out of 10 cm of the sample participated in the vibration; FW is the fresh weight of the sample.

The stiffness can be converted to Young's modulus using equation 11 (Zebrowski, 1999):

$$k = \frac{nEI}{L^3} ; \quad (11)$$

where  $k$  is the stiffness;  $E$  is the Young's modulus;  $I$  is the moment of inertia;  $L$  is the length of a specimen;  $n$  is the coefficient depending on the taper ratio. Equation 11 was used to calculate  $E_{stem}$  and  $E_{xylem}$ .

The percentage of TCW area from phloem area was used to calculate  $I_{fibers}$  from  $I_{phloem}$ . The moduli of elasticity of the phloem and the fibers were calculated using the rule of mixtures by equations 12 and 13, correspondingly (Réquilé et al., 2018):

$$E_{phloem} = \frac{(EI)_{stem} - (EI)_{xylem}}{I_{phloem}} ; \quad (12)$$

$$E_{fibers} = \frac{(EI)_{stem} - (EI)_{xylem}}{I_{fibers}} ; \quad (13)$$

## 2.5. Statistics

Five plants at each of four time points were used to analyze anatomy. Five plants at each of three time points were used to evaluate mechanical properties. Means with standard deviations among biological replicates are presented. Separation of means was performed with one-way ANOVA followed by t-test or Tukey test at  $\alpha = 0.05$  using SPSS software package (v.21, IBM Corp.). The Pearson correlation coefficient was used to evaluate the correlation between different types of tissues or parts of the flax stem at all stages of development. Correlation coefficients and their significance were calculated using SPSS software package (v.21, IBM Corp.).

## 3. Results

### 3.1. Plant growth and tissue development

The morphological characteristics of the flax stem were measured at the herringbone stage, during fast growth period (1 and 2) and at flowering stage (Fig. 1). The length and weight of the stems for each time point studied are given in Table 1.

Table 1. Length and weight of flax stems during their development. Means  $\pm$  SD are presented. Different letters next to means within the same row correspond to a significant difference according to t-test or one-way ANOVA followed by Tukey test at  $\alpha = 0.05$ . SP - Snap point. DAS - days after sowing.

	Herringbone 14 DAS	Fast growth 1 32 DAS	Fast growth 2 37 DAS	Flowering 47 DAS
Stem length, cm	8.2 $\pm$ 0.3A	48.2 $\pm$ 4.2B	68.5 $\pm$ 1.7C	78.1 $\pm$ 3.5D

Stem length to SP, cm	-	12.3±1.6A	10.3±1.7A	-
Stem length to SP/Stem length	-	0.25±0.02B	0.15±0.02A	-
Fresh stem* weight, g	0.5±0.1A	1.9±0.4B	2.2±0.4B	3.7±0.4C
Fresh stem* weight to SP, g	-	0.47±0.09B	0.29±0.06A	-
Stem weight to SP/Stem weight	-	0.24±0.02B	0.14±0.06A	-

\*together with leaves

As the plants developed, they increased significantly in length and weight. They reached an average of 8.2 cm by the 14th day after sowing (corresponding to a growth rate of 0.6 cm per day). The plants reached 48 cm by day 32 and 68 cm by day 37 (Table 1). Thus, their rate of elongation between the fast growth 1 and fast growth 2 was 4 cm per day. Biomass accumulation was most pronounced between herringbone and fast growth 1, and between fast growth 2 and flowering, while its change between fast growth 1 and 2 was not significant (Table 1). Snap point was well resolved during the fast growth period (both in fast growth 1 and 2). Despite the large increment of stem length, the lengths of stem parts above snap points during this period were similar. At the same time weights of these parts were significantly different between fast growth 1 and 2 (Table 1). This may indicate that the weight of the plant part above the snap point does not serve as a determinant of the snap point position.

To further test this hypothesis, the plant mass above the snap point was artificially increased by placing the silicone tube on top of the plants (Fig. 4A). The weight of the silicone tube was approximately 50% of the plant mass above the snap point during this period. After three days (Fig. 3B), total heights of the control and experimental plants were equal, as were the distances from the top of the plant to the snap point (Fig. 4C). Total plant mass also did not change (Fig. 4D, total). The mass of the plant above the snap point decreased in experimental plants, but only by 10% (Fig. 3D, to snap point). However, because we generally observe a decrease in the mass of plant parts located above the snap point during the fast growth stage (Table 1), this change is unlikely to be due to additional weight.

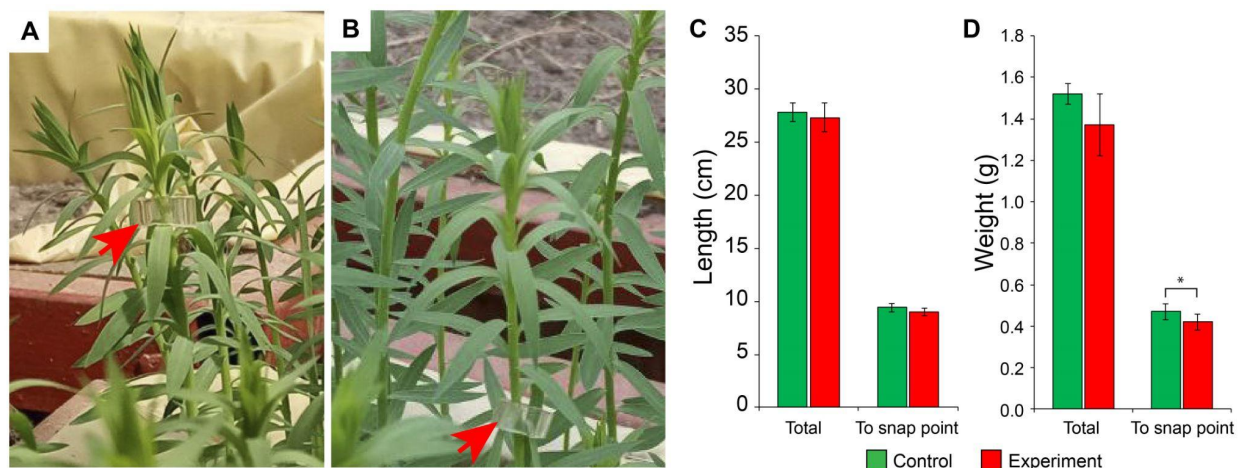


Fig. 4. Artificial increase in the weight of the plant above the snap point. Position of the silicone tube (indicated with red arrows) at the top of the plant on the first (A) and third (B) days of the experiment. Length (C) and weight (D) of control and experimental plants after three days of the experiment. \* - significant difference according to t-test at  $\alpha = 0.05$ .

In order to determine the possible contribution of different tissues to the functioning of the plant as a holistic biomechanical system, changes in the anatomical parameters of the flax stem during plant development were characterized using light microscopy (Fig. 5, 6, Tables S1-S6). In the herringbone stage, there was no significant difference between the stem area at the upper and lower levels (Fig. 5A, Table S1). The flax stem at fast growth 1 had a barrel shape with a maximum stem area at level 3 (Table S1). Such shape largely originated from the formation of a cavity inside the stem (Fig. 1, 6C). At the following stages (fast growth 2 and flowering) the flax stem was conical. Lower levels were significantly larger than the upper levels (Fig. 5A, Table S1).

Xylem area increased both from top to bottom within one stage of plant development (except for herringbone) and at the same level over time (Fig. 5B, Table S2), with one notable exception. An increase in xylem size between fast growth 1 and 2 was not observed at any of the levels studied (Fig. 5B, Table S2).

In comparison, there was a clear increase in phloem area between herringbone and fast growth 1, and the increase was also evident between the fast growth 2 and flowering on the upper levels of the plants (Fig. 5C, Table S3). However, no increase in phloem area was observed between fast growth 1 and fast growth 2 and between fast growth 2 and flowering at levels 1-5 (Fig. 5C, Table S3). In general, both xylem and phloem increased significantly in size between the herringbone and fast growth 1; fast growth was not accompanied by significant changes in the dimensions of these tissues; and the transition between fast growth and flowering was characterized by an increase in xylem area, but not in phloem area except two upper levels.

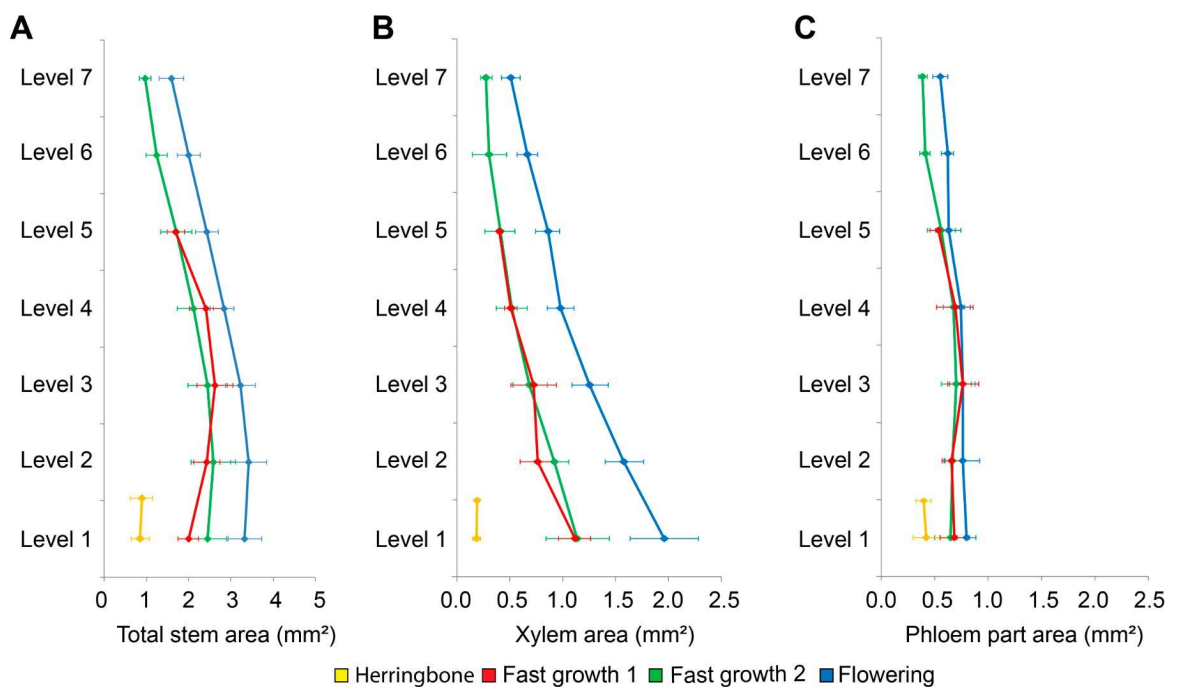


Fig. 5. Areas of stem (A), xylem (B) and phloem (C) during the development of flax. Means  $\pm$  SD are presented.

The distinguishable tertiary cell wall appeared in phloem fibers at the lowest level of flax plants already at the herringbone stage (Fig. 6A, Table S4). The deposition of tertiary cell walls was quite prominent between herringbone and fast growth 1 (Fig. 6A). Level 5, which roughly corresponded to the snap point

location at fast growth 1, was characterized by a negligible area of tertiary cell walls. The largest areas of tertiary cell walls were observed at levels 1, 2 and 3 at this stage (Fig. 6A, Table S4). At the same levels, the area of tertiary cell walls did not increase significantly between fast growth 1 and 2 (Table S4), whereas upper levels (4-7) displayed tertiary cell wall deposition (Table S4). Between fast growth 2 and flowering, a significant increase in tertiary cell wall area was observed in all levels of the plant except level 1 (Table S4). At the flowering stage, the area of tertiary cell walls became rather uniform along the entire length of the stem. The ratio of phloem to xylem area seemed to be important for the development of the tertiary cell walls. Tertiary cell wall area and the ratio of phloem to xylem areas were inversely correlated, with a correlation coefficient of -0.80 (Table 2).

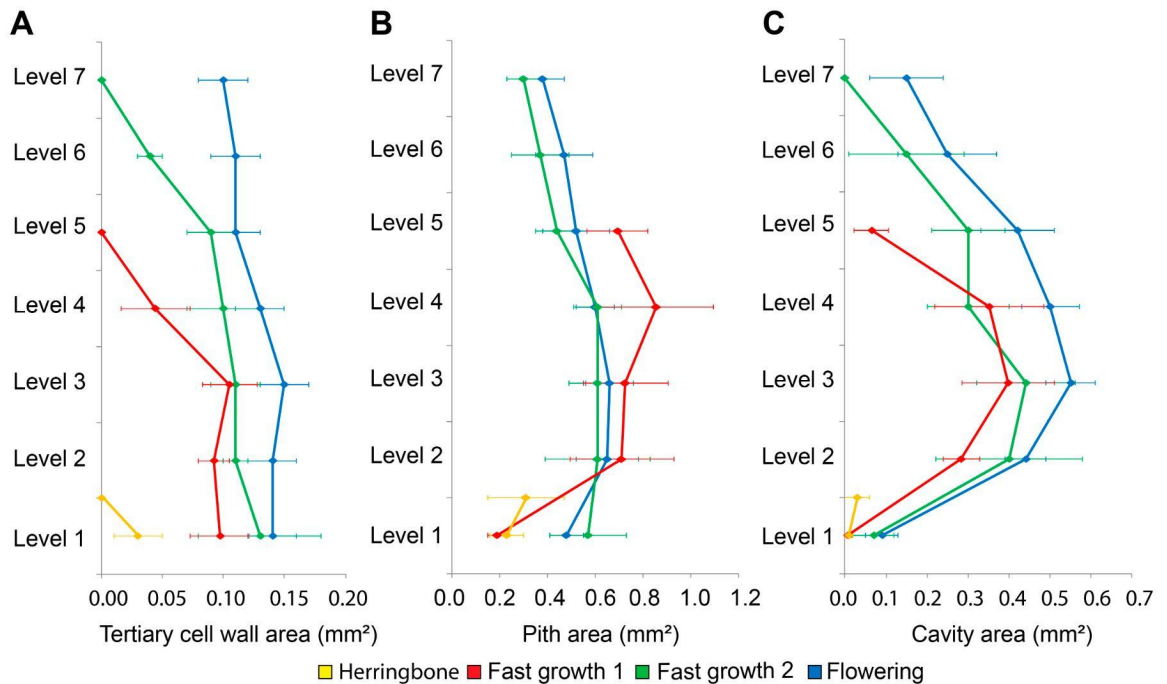


Fig. 6. Areas of tertiary cell walls of phloem fibers (A), pith (B) and cavity (C) during the development of flax. Means  $\pm$  SD are presented.

The central part of the flax stem is occupied by the pith and the cavity (Fig. 1, 2, 6, Table S5, S6). Unlike the phloem or xylem, the cavity and pith had the largest areas in the middle of the stem (levels 2 – 4, Fig. 6B, C) and were smaller at the top and bottom.

Stem, phloem, xylem and tertiary cell wall areas had strong direct correlations with each other (Table 2), as phloem and xylem developed synchronously and both made a significant contribution to total stem area. Stem, phloem, and xylem expanded from stage to stage and from top to bottom along the length of the stem with a slowdown in their development between the fast growth of 1 and 2 (Fig. 5). A strong direct correlation was also observed between the pith and the cavity areas (Table 2). Direct significant though moderate correlation was also observed between the tertiary cell wall and cavity areas. This may indicate a link between the formation of the cavity and the development of the tertiary cell wall of the fibers.

Table 2. Correlations between areas of different types of tissues or part of flax stem at all stages of development. TCW is a tertiary cell wall. \* – the correlation is significant at  $\alpha = 0.05$ . \*\* – the correlation is significant at  $\alpha = 0.01$ .

	Stem	Phloem	Xylem	TCW	Phloem/Xylem	Pith	Cavity
Stem		0.92**	0.85**	0.79**	-0.77**	0.53*	0.60**
Phloem	0.92**		0.74**	0.77**	-	0.50*	0.55*
Xylem	0.85**	0.74**		0.74**	-	0.07	0.16
TCW	0.79**	0.77**	0.74**		-0.80**	0.12	0.57**
Phloem/Xylem	-0.77**	-	-	-0.80**		0.01	-0.19
Pith	0.53*	0.50*	0.07	0.12	0.01		0.60*
Cavity	0.60*	0.55*	0.16	0.57**	-0.19	0.60**	

### 3.2. Mechanical properties of different stem parts during flax development

Stiffness of the stem fragment increased with maturation and from top to bottom along the length of the plant (Fig. 7A). Xylem stiffness also increased with time and basipetally (Fig. 7B). The values of stem and xylem stiffness had a strong direct correlation with a correlation coefficient of 0.97. The stiffness of both whole segments and the xylem part separately increased most dramatically between the fast growth 2 and flowering stages (Fig. 7).

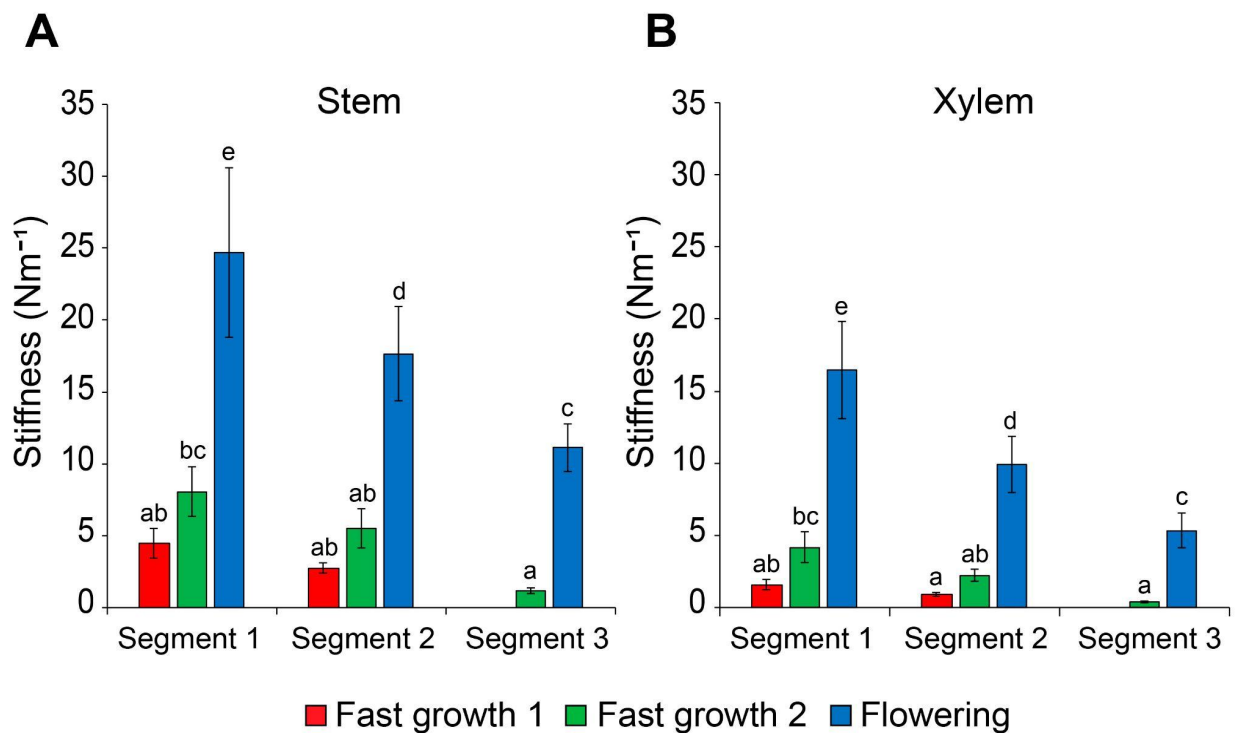


Fig. 7. Stiffness of stem (A) and xylem (B) during the development of flax. Means  $\pm$  SD are presented. Different letters above the bars within the same graph correspond to a significant difference according to one-way ANOVA followed by Tukey test at  $\alpha = 0.05$ .

Removal of the phloem part always resulted in a significant reduction in stiffness (t-test at  $p = 0.01$ ), since the phloem in general, and the phloem fibers in particular, contribute to the overall stiffness of the system. However, the magnitude of the phloem removal effect differed for stem segments of different ages and taken at different heights. For example, segment 2 at fast growth 1 had a stiffness of  $2.7 \text{ Nm}^{-1}$ , the xylem part of this segment had a stiffness of  $0.9 \text{ Nm}^{-1}$  (Fig. 6). Thus, the relative stiffness reduction due to phloem removal was over 68%. A similarly significant reduction in segment stiffness, after phloem removal was observed for segment 1 at fast growth 1 - 65%. For more mature segments (e.g. segments 1 and 2 at flowering, whose stiffness was  $24.7$  and  $17.7 \text{ Nm}^{-1}$ , respectively), phloem removal resulted in a much smaller decrease in relative stiffness than in the younger parts of the stem. The xylem parts of these segments had a stiffness of  $16.5$  and  $9.9 \text{ Nm}^{-1}$ , which corresponds to a 33% and 44% reduction in stiffness, respectively. Thus, as plants develop, their stems and the individual parts of these stems (xylem and phloem) become increasingly stiff. However, while in young plants and in the upper segments of the older plants, most of the stiffness is provided by the phloem, in more mature plants and in the lower segments, most of the stiffness of the stem is provided by the xylem.

Elastic moduli of the stem, its xylem and phloem parts, as well as of phloem fibers, were calculated from stiffness and sample geometry (Fig. 8). In most cases, the values increased with time and from the top to the bottom of the plant (Fig. 8). Stem moduli always had an intermediate elasticity compared to xylem and phloem parts, in accordance with the rule of mixtures. A pairwise comparison of phloem and xylem moduli in the same stem segments revealed several significant differences. At fast growth 1, in segments 1 and 2, and at fast growth 2, in segment 1, the phloem modulus was higher than that of the xylem ( $p = 0.05$ ). With maturation, the difference between phloem and xylem moduli became insignificant. At flowering, these values were almost identical in all segments. The moduli of elasticity of the fibers were calculated using the rule of mixtures, assuming that the tertiary cell wall of the phloem fibers is the stiffest component of the flax phloem. Phloem fibers in the youngest parts of the plants tested (namely, segment 2 of fast growth 1 and segment 3 of fast growth 2) were characterized by the elasticity moduli around 30 GPa. Upper levels of these segments (namely level 4 in fast growth 1 and level 6 in fast growth 2) contained a lower amount of tertiary cell walls compared to lower levels (Table S4). However, even having tertiary cell walls which were still developing these fibers displayed a prominent elasticity. Older segments selected for analysis contained stiffer fibers whose moduli were about 50 GPa and reached up to 75 GPa in one segment (segment 3 at flowering).

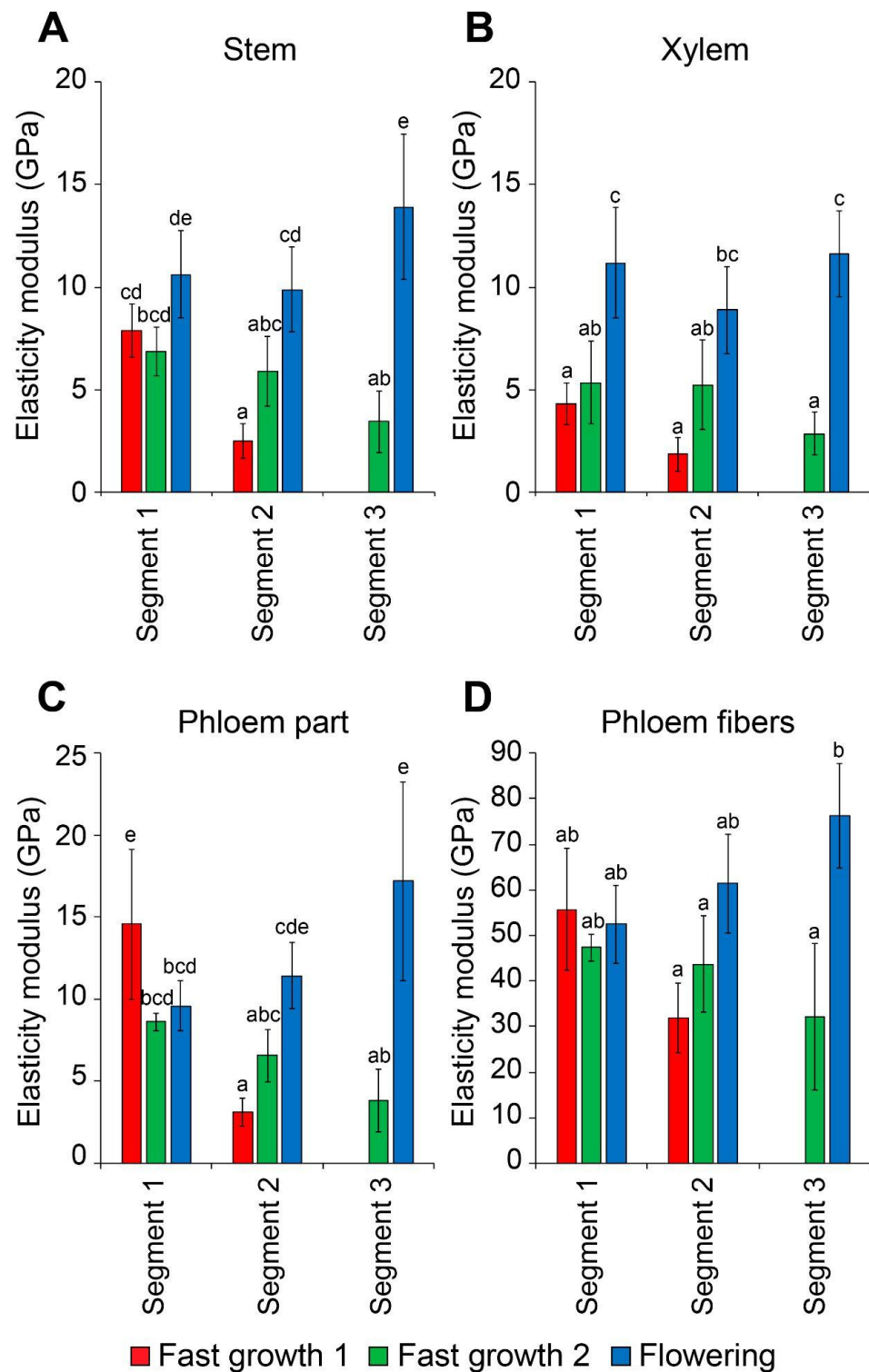


Fig. 8. Elastic moduli of the stem (A), xylem (B), phloem part (C) and phloem fibers (D) during the development of flax. Means  $\pm$  SD are presented. Different letters above the bars within the same graph correspond to a significant difference according to one-way ANOVA followed by Tukey test at  $\alpha = 0.05$ .

#### 4. Discussion

The biomechanical system of the flax stem is largely based on the strength of phloem fibers and xylem. However, the development of these two tissues has different dynamics.

##### 4.1. Xylem

We observed that the xylem increases in area between stages (excluding transition between fast growth 1 and 2) and from the top to the bottom of the plant within a single stage (Fig. 5B). This increase in area is due to the activity of the cambium, which produces secondary xylem (Esau, 1965), and has been reported in flax before either basipetally or between stages (Baley et al., 2018; Goudenhooff et al., 2019b; Nuez et al., 2021). Xylem stiffness also increased from stage to stage and from top to bottom in our studies (Fig. 7B). In contrast to the stiffness, the longitudinal elastic moduli did not differ significantly in different segments taken at the same stage (Fig. 8B). They were lower or close to 5 GPa during the fast growth and reached approximately 10 GPa at the flowering stage for all segments examined (Fig. 8B). These latter values are in agreement with those obtained by Réquillé et al. (2018) (7.5-11 GPa) in three-point bending tests, although the colleagues used a later stage of plant development (full maturity). It can be assumed that in flax, the xylem reaches its final and maximum possible longitudinal elastic modulus already at the flowering stage.

Theoretically, xylem stiffening can occur as a result of the following processes: i) production of new cell layers by the cambium, leading to an overall increase in tissue area (probably affecting xylem stiffness but not elasticity); ii) thickening of the secondary cell walls (probably affecting both); iii) lignification of the secondary cell walls and the middle lamella (probably affecting both stiffness and elasticity). As shown in poplar, the thickening of the secondary cell walls ends before they reach their final stiffness, which they apparently acquire during lignification (Arnould et al., 2022). In a study of hemp xylem, it was shown that the lower internodes are characterized by higher Klason lignin content, higher density and require more energy to break, i.e. they are tougher than the upper ones (Beaugrand et al., 2014). According to Day and colleagues (2005), the lignin content in the alcohol insoluble residue of the flax xylem core continues to increase between the flowering and full maturity stages of flax development, but only in the basal segments of the plants. (iv) Increase in secondary cell wall thickness achieved by each new generation of xylem cells. Nuez et al. (2021) observed that the thickness of secondary cell walls in the xylem of a mature flax stem increases from the pith to the cambium, i.e. over time, newly formed flax xylem cells are able to form progressively thicker secondary cell walls (this process can probably lead to an increase in both stiffness and elasticity).

Thus, we observe that in flax, xylem stiffness (note that stiffness is a property of a body with a certain geometry) increases from stage to stage and from top to bottom. Apparently, it continues to increase in later stages of plant development as long as the cambium is active. At the same time, the longitudinal modulus of elasticity, which is a bulk property of the xylem material, increases slowly but reaches its final possible values already in the flowering stage.

#### 4.2. Phloem

In the lower parts of the stems, an increase in phloem area was observed between the herringbone and fast growth stages, but in contrast to xylem, there was no further significant increase in its area between the fast growth and flowering stages (Fig. 5C). In the upper levels of the plants, there was a moderate increase in phloem area between fast growth and flowering (Fig. 5C, levels 6 and 7). Since secondary growth does not occur in the phloem of flax (Esau, 1965; Fahn, 1990), this increase is probably due to radial growth of parenchyma cells as well as intrusive growth of primary phloem fibers (the latter occurs only above the snap point). The Young's modulus of the phloem part of flax stems was about the same in the lower portions of stems at all three stages (Fig. 8C, segment 1) and was about 10 GPa, i.e., comparable to that of mature xylem (Fig. 8B). In the upper segments of the stem, an increase in phloem modulus was observed between stages of fast growth and flowering (Fig. 8C, segments 2 and 3). This



increase appeared to be due to both an increase in the area of the tertiary cell walls and their maturation.

Tertiary cell wall area increased basipetally within one stage and at the same level of plant height between stages (Fig. 6A, Table S4). This corresponds to a well known dynamics of tertiary cell wall development in flax phloem fibers (Gorshkova et al., 2003; Goudenhooff et al., 2017; Gorshkova et al., 2022). At lower levels of the plant, tertiary cell wall deposition was paused between stages of fast growth 1 and 2, but resumed between fast growth 2 and flowering (Fig. 6A, Table S4). Two minimum values of tertiary cell wall elastic moduli were observed for segment 2 in fast growth 1 and segment 3 in fast growth 2 (Fig. 8D). These two segments were the youngest included into the study. However, even at this age, the elastic modulus of their phloem fibers had already reached significant values (about 30 GPa). In all other segments analyzed, the elastic moduli of the fibers were not significantly different from each other and ranged from 40 to 75 GPa. These values are in agreement with the data of Réquillé et al. (2018), who found that the elastic modulus of flax fibers was around 55 GPa. Similar values were also obtained for individual flax fibers in tensile tests (31-69 GPa for different flax varieties Aslan et al., 2011; Goudenhooff et al., 2017; Chabi et al., 2023).

It is known from studies of tension wood and gravitropic reaction of flax that the rate of accumulation and stiffening of tertiary cell walls is higher than that of secondary cell walls in the same plant (Abedini et al., 2015; Petrova et al., 2021; Arnould et al., 2022). Thus, unlike xylem, flax phloem is able to rapidly deposit tertiary cell walls and acquire significant mechanical strength.

#### *4.3. Phloem and xylem work together to create a holistic biomechanical system for the flax stem*

The early stages of flax plant development as well as the upper (younger) parts of plants at more advanced stages of development are characterized by a higher Young's moduli of the phloem part, compared to xylem (Fig. 8) and by a greater contribution of phloem to stem stiffness (Fig. 7) than in the later stages of plant development. The tertiary cell walls in the phloem fibers are deposited and mature faster than the secondary cell walls in the xylem, which allows the phloem to take the main mechanical load in the early stages of plant development while the xylem has not yet acquired sufficient stiffness and elasticity. Between the fast growth and flowering stages the xylem dramatically increases its volume (Fig. 5B) and stiffness (Fig. 7B), xylem modulus reaches the highest values (Fig. 8). At this point, the xylem probably takes the main mechanical load and supports the plant in an upright position.

#### *4.4. Some specific features of flax*

##### *4.4.1. The fast growth stage*

In our study, we sampled plants twice at the fast growth period (fast growth 1 and fast growth 2). Only 5 days elapsed between these two time points, but during this time the height of the plants increased by 20 cm, which corresponds to a primary growth of 4 cm per day (Table 1). At the same time, the plant mass was almost identical at these two time points (Table 1). Moreover, the observed changes in total stem area and xylem area were negligible at this time (Fig. 5A, B). It should be noted that the accumulation of xylem and tertiary cell walls between the herringbone and fast growth 1 were significant, while primary growth (plant elongation) was moderate. Then, during the fast growth, flax plants exhibit primary growth of exceptional magnitude and almost no secondary growth. The xylem, which takes considerable time to mature, is unable to support the rapidly growing plants during this period. However, the phloem fibers, which rapidly deposit a tertiary cell wall that also matures rapidly, can provide sufficient strength. Nevertheless, flax is particularly susceptible to lodging during the fast

growth and flowering stages (Menoux et al., 1982). Shorter plants with shorter internodes and fibers are less susceptible to lodging (Menoux et al., 1982). However, since increasing plant height and fiber length is desirable for breeding, these traits are in conflict (Goudenhooff et al., 2019a).

#### 4.4.2. *The snap point*

The snap point is a natural marker for the transition of phloem fibers from intrusive growth to cell wall thickening (Gorshkova et al., 2003), i.e. switching from primary to secondary growth within a particular cell type. The existence of the snap point provides a suitable background for the search for molecular, physiological and physical factors that determine the switching of cellular developmental programs (Gorshkova et al., 2003; Ageeva et al., 2005; Gorshkova et al., 2018). We hypothesized that the position of the snap point on the stem might be determined by the mass of the plant above it. However, we found that during fast growth, the mass of the part of the plant above the snap point changed, while the distance from the snap point to the apex did not (Table 1). Artificially increasing the mass of the plant above the snap point using a fragment of silicone tube also did not change the position of the snap point on the stem (Fig. 4C). It should be noted that the additional mass placed on the stem in this experiment was approximately 50% of the plant mass above the snap point, i.e., the increase was substantial. Thus, the position of the snap point appears to remain relatively constant during fast growth, and the transition from phloem fibers to cell wall thickening is rather triggered when they reach a certain distance from the apex, but not because the weight of the plant above the snap point becomes too high to be maintained without the involvement of tertiary cell walls.

#### 4.4.3. *The cavity*

Hollow structures can be beneficial to plants for several reasons: i) material economy, ii) mass reduction and increase and buoyancy, iii) efficient gas exchange. Hollow stems, petioles and peduncles are observed in a wide range of plants belonging to different taxonomic groups (Newcombe, 1894). Cavities can be thought of as very large intercellular spaces and they typically originate in the parenchyma. There are three main mechanisms for their formation: lysigenous, schizogenous and rhexigenous (Esau, 1965). The lysigenous way of formation assumes that a part of cells self-destructs, freeing the occupied space, schizogenous - that as a result of disruption of contacts between cells under the action of enzymes they can move away from each other in the presence of external forces. Rhexigenous cavities are created by tearing or breaking of cells, for example, in cases when these cells have already finished their expansion, while the neighboring cells have not. Often different pathways are combined and it is difficult to assess the contribution of each pathway.

Phloem fibers in flax are known to generate tension in the longitudinal direction (Alméras et al., 2020). We hypothesized that fiber formation and maturation may correlate with cavity development, as partial release of accumulated tension may occur through transversal stem expansion. Thus, we hypothesized that the cavity in flax plants is at least partially rhexigenous. Indeed, a significant positive correlation was observed between tertiary cell wall area and cavity area (Table 2). However, the magnitude of the correlation coefficient was rather moderate compared to other parameters that are correlated with each other (Table 2). It is also known that phloem fibers are longer in the middle of the flax stem than at the bottom and top (Goudenhooff, 2018). Longer fibers can generate more tension for the same area and modulus of elasticity. This may be an additional reason why the cavity is the largest at the middle of the stem while being smaller at the top and bottom. However, the idea of tertiary cell wall deposition and maturation in phloem fibers as the driving force for cavity enlargement is not consistent with the fact that at the lower levels of the plant, where the tertiary cell walls mature earliest, there is no cavity at all. Furthermore, in flax plants, cavities begin to be detected above the snap point, where the amount of

tertiary cell walls is minimal (Fig. 1, 6C). All of this may suggest that other mechanisms contribute to cavity formation in flax, but more research is needed.

## 5. Conclusion

The formation of the biomechanical system of the flax stem goes through several stages, each of which is characterized by a specific, strictly defined intensity of primary and secondary growth. For example, maximum plant elongation occurs with temporary reduction of secondary growth. Upon completion of the fast growth period, secondary xylem accumulation and cell wall thickening resume with new vigor. Both xylem and phloem fibers increase in stiffness and Young's modulus with maturation. However, from a mechanical point of view, younger plants and upper (younger) parts of the stem rely primarily on the stiffness of the phloem fibers, while older plants and lower parts of the stem rely more on the xylem. At the same time, despite the need to support the young part of the stem, its mass is not the stimulus that determines the transition of phloem fibers from cell elongation to thickening of cell walls (the position of the snap point). The complex dynamics of primary and secondary growth during the development of flax plants opens up new perspectives for possible manipulation of this process to improve fiber and by-product quality.

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## Author contributions

Conceptualization AP, LK, TG; Data curation AP; Formal analysis AP; Funding acquisition TG; Investigation AP, LK, TC; Methodology AP, LK; Visualization AP; Writing – original draft AP, LK; and Writing – review & editing AP, LK, TC, TG.

## Conflict of interest

Authors declare that there is no conflict of interests

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