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# Development of Hemp Fibers: The Key Components of Hemp Plastic Composites

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<http://dx.doi.org/10.5772/intechopen.70976>

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## Abstract

Plant fibers in general and hemp fibers in particular have great prospects for their use in various innovative applications such as ecological, biodegradable, and renewable resources with unique properties. Such properties together with the increased strength due to high-cellulose content and specific morphological parameters are widely used to produce plant fiber-based plastic composites. The properties of plant fibers that may influence the properties of composites depend on crop processing, but the basis for them is provided during fiber development *in planta*. It is known that two types of bast fibers are developed in the hemp stem: primary fibers formed from procambium cells and secondary fibers that originate as a result of cambium activity. Both types of fibers may significantly vary in their yield and quality depending on the variety and growth conditions. Differences in the anatomical and morphological characteristics of the two types of hemp fibers, together with peculiarities in the composition and architecture of cell wall, influence the technical parameters of the raw material quality. Based on our study of both primary and secondary fiber development in hemp stem that was focused on the two key stages, intrusive elongation and deposition of thick cell wall layers, we suggest the set of parameters that can influence the quality of the mature fibers and trace their biological origin.

**Keywords:** plant fibers, gelatinous fibers, hemp, intrusive growth, plant cell wall, rhamnogalacturonan I

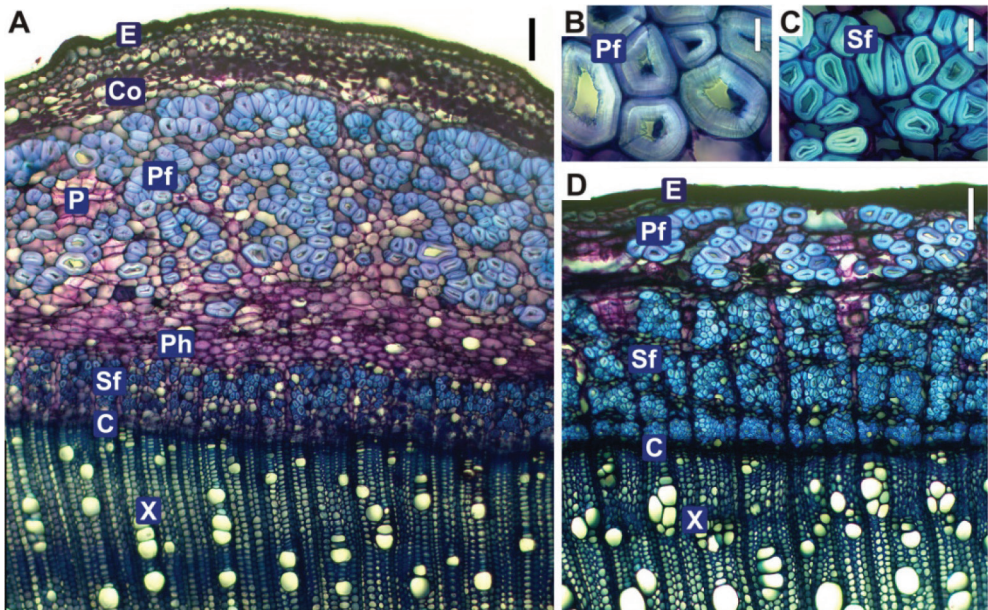
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# 1. Introduction

Hemp plastic is a bio-based composite that may vary in composition and applications. The components of plant material that provide valuable properties and usually constitute 25–65% of the composite [1] are bast fibers. These cells are developed within hemp stem and have specific morphological and mechanical parameters. Hemp fibers, same as fibers of flax, ramie, nettle, and some other fiber crops, are distinguished by high-cellulose content [1–3]. The quality of such fibers varies depending on both the processing and the properties developed *in planta* [4–6]. We will consider the biological determinants of hemp fiber quality, comparing primary and secondary hemp fibers. Being developed within the same plant, primary and secondary hemp fibers differ significantly in morphology, cell wall organization, and as a consequence in quality [7–9], giving the possibility to understand how developmental processes influence quality parameters.

# 2. Origin of primary and secondary fibers in the hemp stem

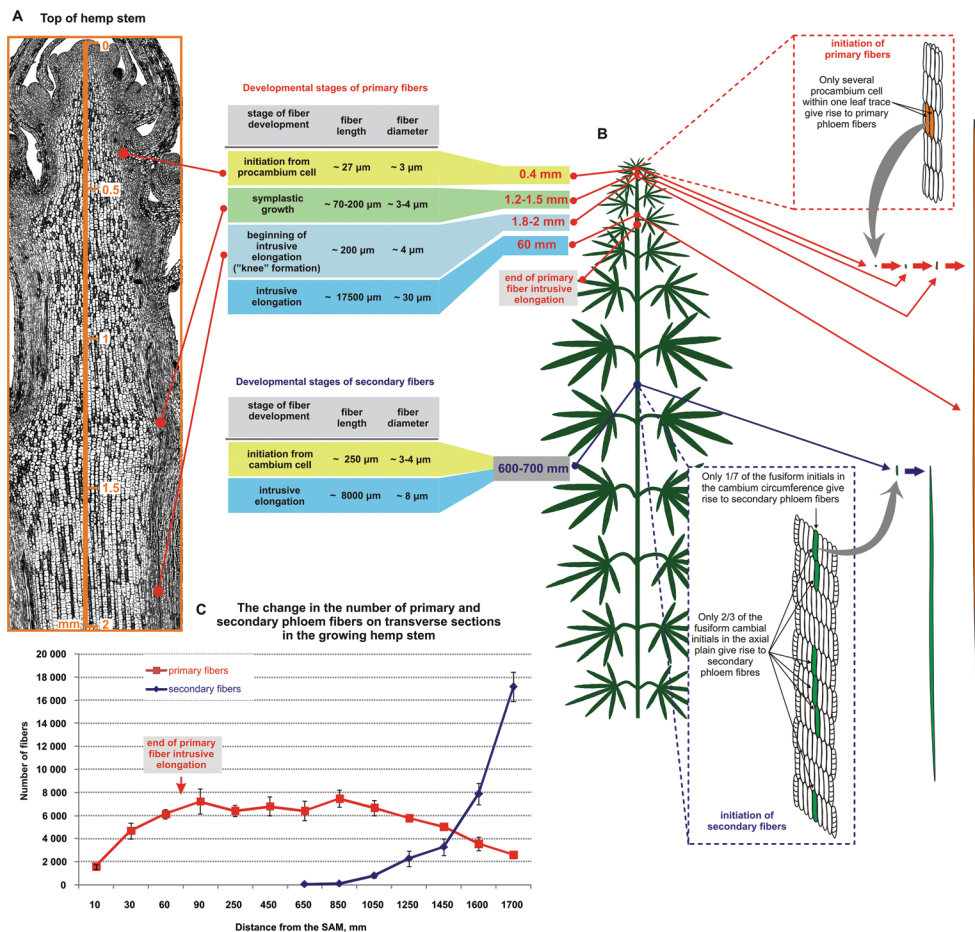
The usually considered primary and secondary hemp fibers are bast fibers, meaning that they belong to phloem and are located closer to stem periphery than cambium (**Figure 1**). Primary phloem fibers located behind the epidermis and collenchyma are larger than the secondary



**Figure 1.** Primary and secondary phloem fibers within hemp stem. Cross-section of hemp stem, stained with toluidine blue. Stem bottom of plant at (A) flower formation and (D) seed maturation stages; (B) primary and (C) secondary phloem fiber bundles. Bar scale = 100  $\mu\text{m}$  (A and D), 20  $\mu\text{m}$  (B and C). C, cambium; Co, collenchyma; E, epidermis; P, parenchyma; Pf, primary phloem fibers; Ph, phloem; Sf, secondary phloem fibers; and X, xylem.

ones (**Figure 1A, B**). Secondary fibers located closer to the cambium and arranged in compact bundles, the number of which grows during plant development (**Figure 1A, C, D**). Hemp stems, same as in many other dicotyledonous plants, have also fibers located within xylem, but they do not form bundles, are mixed with other cell types (vessels, parenchyma) and have different properties and applications [10]. Xylary fibers are not considered further in this chapter.

By definition, primary fibers originate from primary meristem: procambium, in the region close to apical meristem [8]. Their initiation is coupled to leaf trace formation and occurs within the very top millimeters of the developing stem (**Figure 2A, B**). Apical meristem provides the



**Figure 2.** Scheme of initiation and intrusive elongation of primary and secondary phloem fibers in hemp stem. (A) The top of hemp stem (2 mm) [8] with primary fibers at the stages of initiation, symplastic growth, and beginning of intrusive elongation. (B) Primary and secondary phloem fibers at different stages of development, their characteristics, and localization within the stem (on the left); initiation of primary and secondary fibers from procambium and cambium, correspondingly, and morphology of fibers at different developmental stages (on the right); the length of fibers at different stages is scaled to real proportions, while the diameter/length ratio is larger than its actual value. (C) Changes in the number of primary and secondary phloem fibers on transverse section in the developing hemp stem.

increase of stem height [11]. The more downwards the stem, the older the primary fibers. Thus, developmental gradient of primary fiber can be observed along the same stem. However, it is better to characterize it, analyzing fibers at the same height through plant development, same as it was done for flax [12]. This is because primary fibers located at different stem heights originated at different periods of plant development and might be differentially affected by various endogenous and environmental factors. For example, primary fibers from the stem bottom are known to be shorter than the ones from the middle stem part [13]. Fibers traced at the same stem height through plant development have a similar background and can be correctly compared. The total amount of primary fibers in the hemp stem calculated from the total volume of primary fiber bundles and average volume of a fiber cell is around 700–800 thousand [14].

Secondary fibers originate from secondary meristem: cambium that largely provides cells for stem thickening [11]. Secondary fibers start to develop more than half a meter away from stem top (**Figure 2B**), as demonstrated for several monoecious varieties [7, 8, 14–16]. Their developmental gradient can be traced within the stem radius: the closer to the cambium, the younger the secondary fibers. Within hemp stem, secondary fibers may form several distinct concentric layers separated by other cell types. The number of such rings may reach 3–4 (**Figure 1D**). The total amount of secondary fibers in the hemp stem is around 2 million, much higher than the number of primary fibers [14]. From that, only small proportion of cambium cells (around 10%) give rise to secondary phloem fibers [8].

### 3. Intrusive elongation of primary and secondary fibers

Plant fibers are distinguished by their extreme length [11, 17–19]. It is mainly attained by the special type of cell elongation: intrusive growth [11, 17, 19]. This process has an enormous, though often overlooked, effect on fiber yield and quality. Intrusive elongation is characterized by the higher rate of a cell growth as compared to its neighbors. It is distinct from symplastic (also called coordinated) growth [20], when all involved cells increase their surface with the same rate, as it happens in the growth zones with most of the tissues [11]. Fibers are the classical example of cells performing intrusive elongation [11, 17, 21, 22]. During such growth, a fiber has to split middle lamellae of the cells on the way and intrude between them. Herewith, new contacts are made along the increased fiber surface, so that the stem tissues do not fall apart.

Primary phloem fibers of hemp stem start intrusive elongation rather soon after being initiated from meristematic cells [8] (**Figure 2A, B**). Only shortly primary fibers grow symplastically with the surrounding cells and then increase the rate of elongation. By symplastic elongation, primary phloem fibers of hemp attain the length of 200  $\mu\text{m}$  [8] (**Figure 2B**). The start of the primary fiber intrusive growth is marked by the formation of the so-called “knee”: the flat tip of the symplastically growing cell is transformed into the tapered one to effectively intrude the surrounding tissues [8, 19, 22]. In hemp stem, such structures are observed at 1.8–2.0 mm from the very top (**Figure 2A, B**). By means of intrusive elongation, primary phloem fibers of hemp stem increase their length roughly hundredfold so that their average length gets



around 18,000  $\mu\text{m}$ . The volume of a fiber is increased even more, since intrusive elongation is accompanied by the increase of fiber width, so that the cell diameter gets 30  $\mu\text{m}$  instead of 3–4  $\mu\text{m}$  at the end of symplastic growth [8] (**Figure 2A**). The final fiber dimensions of primary phloem fibers in hemp may differ depending on the variety and growth conditions. The final length of such fiber is reported between 5000 and 100,000  $\mu\text{m}$ , with modal class being around 20,000  $\mu\text{m}$ , and variations in fiber diameters, which could vary between 15 and 40  $\mu\text{m}$  [2, 15, 23–25]. Thus, the ratio of cell length and cell width in a primary fiber of hemp may constitute several thousands, being among the highest in plant cells.

The duration of intrusive elongation can be traced by the increase in the number of fibers on the stem cross-section. As described above, the formation of new primary phloem fibers occurs only at the region close to apical meristem [8], then during intrusive elongation, the number of primary fibers in the cross-section of hemp stem increases till approximately 60–90 mm from the apex (**Figure 2C**); end of the intrusive elongation occurs within this stem region. Further downward the stem, fibers do not elongate and their number on cross-section does not increase with time.

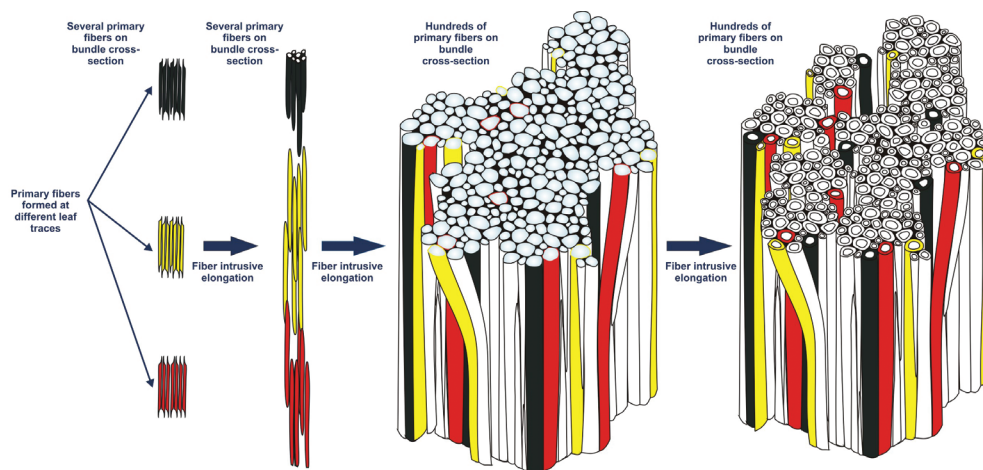
Elongation of fibers goes bidirectionally, as indicated by the presence of “knees” at both cell ends [8, 26]. The intrusive elongation of primary fibers goes for several days and is completely split in time with cell wall thickening, which starts later in the course of fiber development [8, 12, 26]. After the start of cell wall thickening, intrusive elongation cannot be restored any more.

During intrusive elongation, primary fibers initiated at different leaf traces reach each other, this leads to the formation of fiber bundles (**Figure 3**). The structure of the fiber bundles is almost exclusively determined by fiber intrusive growth [8, 12, 22]. The longer the fibers in the course of elongation, the thicker the fiber bundles.

It is due to intrusive growth that fibers in a bundle are so tightly packed to each other and have no intercellular spaces. The large surface of the contacts between neighboring fibers formed during intrusive elongation permits them to stay together during the retting process [4, 23, 27]. These tight contacts may be further reinforced by the special type of the “glue” between cells—pectic compounds that are constituents of middle lamellae and primary cell walls [28], and may have peculiarities in composition in fibers performing intrusive growth [22]. In the course of intrusive growth that leads to the enormous increase of cell surface, pectins are actively deposited and modified. Low-molecular mass phenolic compounds may also be involved in strengthening of interactions between polymers of fiber primary cell wall [29].

Unfavorable environmental factors, like drought, may influence the extent of fiber intrusive elongation. This would lead to the shorter individual fibers and thinner fiber bundles in the region of the stem that contained elongating fibers [30]. For primary phloem fibers, this region is located at the top of the developing stem, usually constitutes 6–9 cm, and corresponds to the maximum length of individual fibers that are elongating in this region. After the end of unfavorable conditions, this stem portion would contain fewer fibers on the cross-section and would remain the weaker part of the whole bundle until the end of plant development.

Secondary fibers do not have the stage of coordinated growth, since the stem portion ceases elongation before they are initiated from the cambium. The final length of secondary fibers is



**Figure 3.** Formation of primary phloem fiber bundle within the hemp stem. Fibers formed within one leaf trace are given in one color; fibers formed in different tree leaf traces are given in different colors (black, yellow and red); fibers formed within other leaf traces are given in white. The scheme illustrates the formation of primary phloem fiber bundle of hemp stem and participation of fibers formed within tree leaf traces in this process.

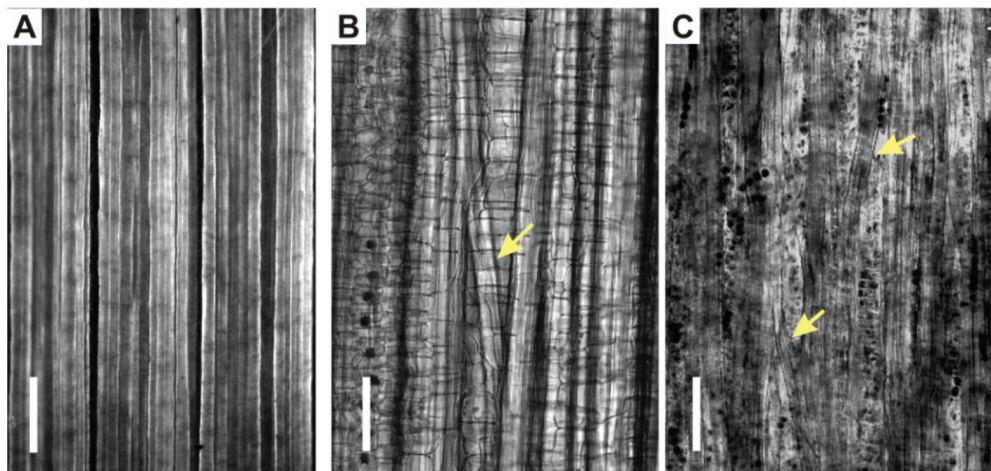
achieved solely by intrusive elongation (**Figure 2B**). The final dimensions of secondary fibers are much lower than that of primary fibers. Their average length accounts for 2000–8000  $\mu\text{m}$ , while the cell diameter is usually about 6–8  $\mu\text{m}$  [8, 10, 15, 24]. However, these smaller dimensions are still considerably larger than those of cambium initials, the length of which in hemp stem was estimated to be around 250  $\mu\text{m}$  [8]. As mentioned above, secondary phloem fibers start to emerge approximately at the middle part of hemp stem (600–700 mm from the apex) and their initiation continues down to stem bottom. The amount of secondary fibers on the stem cross-section increases toward the base of the stem due to both initiation of additional fibers from cambium and intrusive elongation of already existing fibers (**Figure 2C**). Due to smaller size of secondary fibers, their proportion in total yield of bast fibers in the hemp stem does not exceed 45% [10], despite the higher amount of individual cells [14]. This demonstrates the importance of the intrusive elongation stage for the final yield of bast fibers.

The bundle of the secondary fibers within the certain concentric ring is formed by a very similar process as described above for primary fibers (**Figure 3**). Fiber initiated from a cambium cell elongates and joins other intrusively growing fibers that originated from different cambium initials. The structure of fiber bundles is determined not only by the extent of fiber length increase, but also by the direction of elongation. The analysis of fiber-enriched peels demonstrates that the bundles of primary fibers in flax look like “straight columns,” while the bundles in hemp form the ramified net (**Figure 4**). The degree of ramification is especially high for secondary fibers [8, 31]. Such difference is due to the “joint” or “individual” behavior of elongating fibers. In flax, the elongating fibers follow the way made by the “oldest” fiber in the forming bundle. This can be due to the special mechanical properties of middle lamellae that have been already split. For primary fibers of hemp, the situation is rather similar, but

some fibers escape from the bundle, elongating in a different direction and may reach another bundle, leading to formation of a ramified net. Such difference may be related to the large increase of stem circumference in hemp due to secondary growth, which is far less in flax.

The importance of intrusive growth for fiber yield and quality demands the approaches to regulate it. However, the mechanisms of intrusive growth are understood quite poorly. Nothing is known about the mechanisms that trigger and stop it. The peculiarities of fiber physiology at this stage of development are barely characterized. The reason for that is the difficulty to study this process, since it occurs within the depth of tissues and has never been reproduced *in vitro*. Fibers at this stage of development, being quite long cells, have only primary cell wall and can be easily damaged during sample preparation [26, 32]. This makes it quite difficult to obtain intrusively growing fibers for analysis by methods of biochemistry or molecular biology. The important step to identify molecular players involved in various stages of hemp fiber development was performed by the analysis of the transcriptome in hypocotyls of different age [33] and in different parts of young stems [34], both of which may contain intrusively growing fibers. However, the analyzed samples contained complex mixture of tissues and the early stages of fiber development were not fully identified. The indication of the stage-specific participants of fiber intrusive elongation in hemp may come from the analysis of whole transcriptome of intrusively growing fibers of flax. Such fibers were obtained by cryosectioning of stem and further, laser microdissection of fibers specifically at the stage of intrusive elongation [35]. However, elucidation of the mechanisms that perform and regulate the intrusive growth of fibers still has a long way to go.

To summarize the importance of fiber intrusive elongation, it (1) determines the final size of each individual fiber, (2) leads to the formation of fiber bundles and dictates their structure,



**Figure 4.** Comparison of phloem fiber bundles in flax and hemp. Structure of (A) primary phloem fiber bundles of flax, (B) primary and (C) secondary phloem fiber bundles of hemp [8] on the strips peeled off from the stems. Fiber bundles of hemp frequently split and merge along the stem forming numerous anastomoses (marked by arrows) unlike fiber bundles of flax. Bar = 100  $\mu\text{m}$ .

- (3) provides the tight contacts between the fibers that help to withstand retting process, and
- (4) provides the large surface for the further deposition of thick cell wall that is the major component of mature fibers.

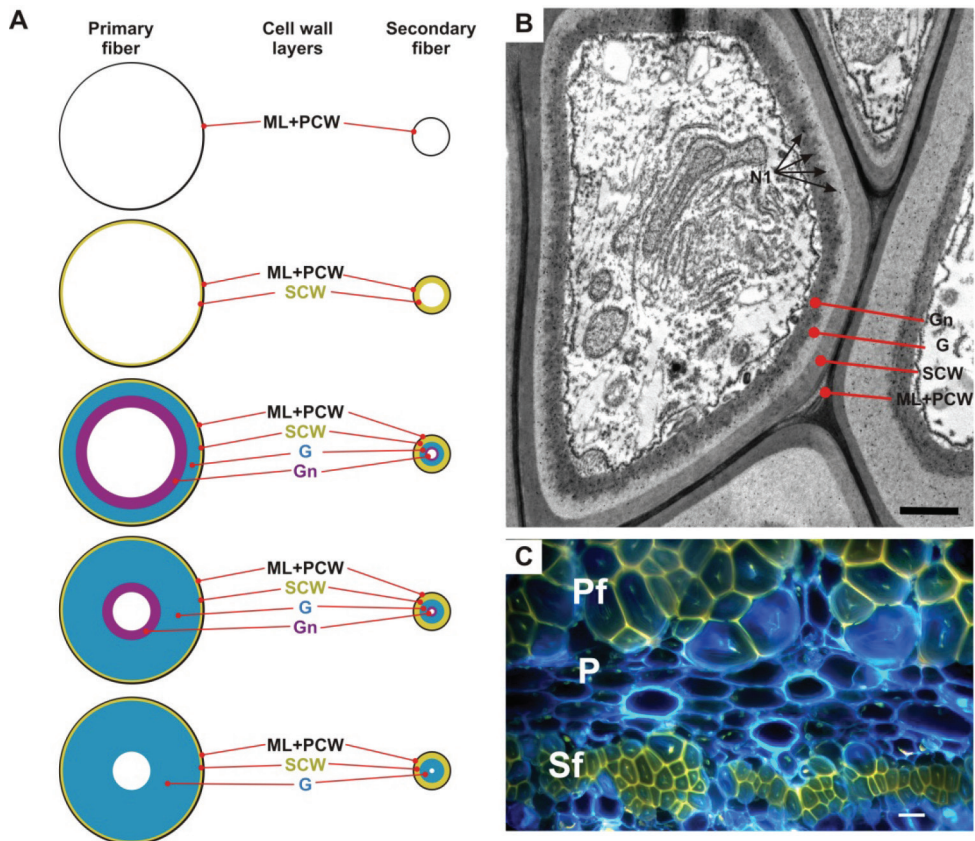
## 4. Cell wall thickening

The mechanical properties of mature individual fiber largely come from thickened cell wall. In hemp fibers, cell wall may get 15  $\mu\text{m}$  thick and occupy over 90% of cell cross-section [15]. The thick cell wall is not uniform and contains several layers with distinct properties. After the primary cell wall that is formed during fiber elongation, the secondary cell wall layer (S1) is deposited (**Figure 5**). The rest of the two layers of the thickened cell wall in hemp fibers are often also considered as secondary cell wall layers and named correspondingly, S2 and S3 [15, 31]. However, they differ significantly in composition and structure from S1. As revealed by fluorescent microscopy, LM10 and LM11—antibodies specific for xylan [36]—label only the outer layer of fiber cell wall [37]. Electron microscopy coupled with immunocytochemistry demonstrates that only S1 of hemp phloem fibers, both primary and secondary, is labeled by anti-xylan antibody [38]. The rest of the thickened cell wall does not contain epitopes for anti-xylan antibodies (**Figure 6**). This is very distinct with xylem cells, which are heavy labeled by these antibodies throughout all secondary cell wall layers [37].

The difference between S1 and the other layers of thickened cell wall is also obvious after labeling with antibody against fiber-specific galactosidase: S1 is not labeled, while the epitope is quite abundant in the inner two layers (**Figure 5B**). The antibody was raised against the enzyme isolated from flax, but it also binds cell wall in hemp phloem fibers, same as G-layers of tension wood fibers in poplar [39]. This tissue- and stage-specific  $\beta$ -1,4-galactosidase is necessary for maturation of cell wall structure in flax fibers, and is involved in partial trimming off the  $\beta$ -1,4-galactan side-chains from the backbone of rhamnogalacturonan I [40]. Similar polymer—rhamnogalacturonan I with  $\beta$ -1,4-galactan side-chains is also present in hemp fibers [38, 41]. Same as in flax [42, 43], fraction of this polymer is so tightly retained by cellulose that can be obtained only after complete cellulose degradation [38]. The antibody RU2 against rhamnogalacturonan I backbone [44] does not recognize any epitopes within S1 layer, but binds to the thick inner cell wall layers of hemp fibers, both primary and secondary, same as to the primary cell wall/middle lamellae region [38] (**Figure 6**). Cytochemical staining for pectin is also positive in the inner layers of hemp fibers [6]. Presence of acidic component indicates that inner layer of hemp fibers is similar to G-layers of tension wood and flax fibers [45, 46]. Notably, hemp fiber cell wall is not labeled by LM5 antibody specific for  $\beta$ -1,4-galactan [37, 38], despite the fact that the presence of corresponding polymer is biochemically proven [38, 41].

Difference between S1 and the rest of the thickened cell wall layers is further evidenced by the character of hemp fiber lignification. As usual for secondary cell wall layers, S1 gets lignified, especially at an advanced stage of hemp fiber development (**Figures 5C and 6**). Lignification occurs only in the outer cell wall layers of hemp fibers (middle lamellae, primary cell wall and S1): autofluorescence that is characteristic of lignin under UV light (**Figure 5C**), and staining for





**Figure 5.** (A) Scheme of the sequential deposition of cell wall layers during thickening of primary and secondary phloem fibers of hemp stem. Primary and secondary fibers differ significantly in diameter as well as in the ratio of cell wall layers. The thickness of the primary cell wall and middle lamellae (ML + PCW) is comparable in primary and secondary fibers. Later deposited layer of secondary cell wall (SCW) is more developed in secondary fibers and exceeds that of primary fibers in width roughly twofold. Then, newly deposited layer of the tertiary (gelatinous) cell wall (Gn) is formed, which is later transformed into mature layer of tertiary (gelatinous) cell wall (G). In mature fibers, the layer Gn can be absent. (B) Cross-section of hemp secondary phloem fibers with different cell wall layers labeled with antibodies N1 raised against flax fiber-specific  $\beta$ -galactosidase [39]; label is absent in SCW, but present in G and especially Gn layers. (C) Primary and secondary phloem fibers on the cross-section of hemp stem stained with Calcofluor White, under UV light (lignified layers of cell walls look yellow due to lignin autofluorescence, nonlignified cell wall layers look blue); only outer layers of fiber cell wall are lignified. Bar = 1  $\mu$ m (B) and 20  $\mu$ m (C).

lignin by specific dyes like phloroglucinol are not observed in the thick inner layers, though they are obvious for S1 [6, 15]. The rest of cell wall remains nonlignified even in the fully formed fibers, which is especially obvious for primary fibers. Since the proportion of S1 layer in total cell wall is higher in secondary fibers than in primary ones, the degree of their lignification is higher [6, 15, 38]. This makes secondary fibers more coarse and rigid, and is often considered as the major reason for their lower quality [9].

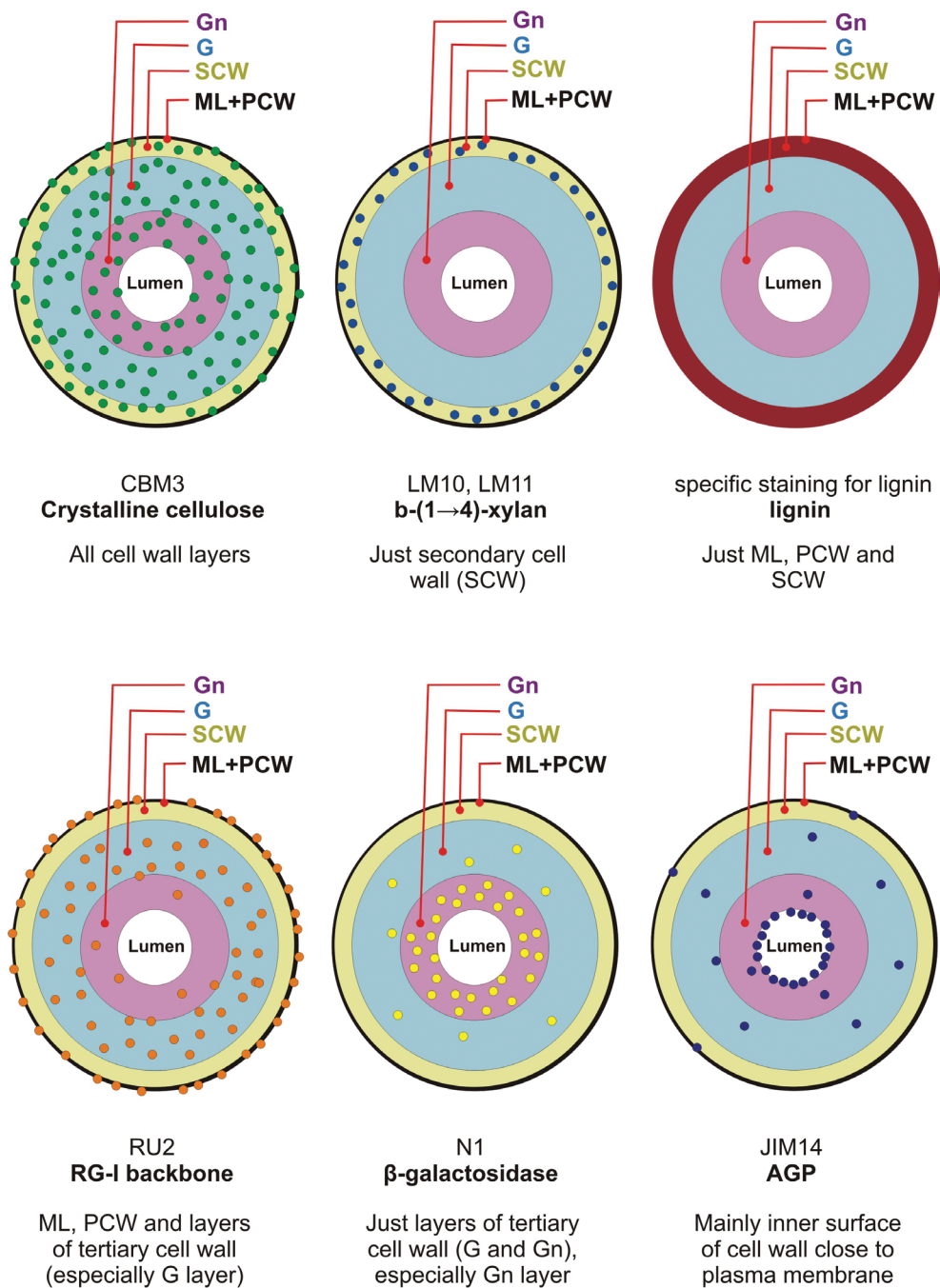
The main component of the inner layers of cell wall is cellulose. This is evidenced by binding of CBM3—carbohydrate-binding module specific for crystalline cellulose [37] and also by high content of cellulose in hemp fibers [2, 3]. The content of hemicelluloses in hemp fibers is reduced at advanced stages of hemp fiber development [15], which may be due to the increasing proportion of cellulose-enriched inner cell wall layers. The average microfibril angle (MFA) toward the longitudinal fiber axis in hemp fibers is low; in the major cell wall layer (G/S2), it constitutes an average of  $2.65^\circ$  [47], meaning that the orientation of all microfibrils is close to axial. In S1, MFA is over  $80^\circ$  [47].

Same as in flax phloem fibers, cell wall of hemp fibers is a dynamic structure with intensive post-synthetic modifications of the deposited RG-I (**Figure 5**). The newly deposited portions, designated as Gn look rather loosened and contain larger amount of electron-dense material than the mature G-layer. Gn is a transient layer and is transformed into G in the course of fiber development, while the new portions of Gn are deposited (**Figure 5A**) [38]. The transition of Gn- into G-layer is coupled to the action of fiber-specific galactosidase [40] (**Figure 5**).

Together with the cell wall polymers discussed above (xylan, lignin, rhamnogalacturonan I with  $\beta$ -1,4-galactan side chains), immunodot analysis of isolated cell wall constituents reveals other polysaccharides, namely glucomannans, polygalacturonic acid, arabinogalactan proteins, and some xyloglucans [38]. These polymers are unevenly distributed between cell wall layers. For instance, arabinogalactan proteins are mainly detected at the inner surface of fiber cell wall, close to plasma membrane (**Figure 6**) [37]. The structural peculiarities of these polymers in hemp fibers, same as the possible differences in their structure in fibers of contrast quality are still to be characterized.

Absence or low content of xylan and lignin, axial orientation of cellulose microfibrils, presence of pectic components, processes of cell wall maturation with the involvement of tissue- and stage-specific galactosidase make to consider that the overall structure of hemp fibers resembles that of tension wood fibers and of flax fibers [48, 49], and that the inner layers of thickened cell wall may be viewed as tertiary cell wall. Tertiary cell wall, also named G-layer, is a fiber-specific cell wall type [19]. Its structure is based on the entrapment of RG-I by laterally interacting cellulose microfibrils. Tertiary cell walls are formed in fibers of various plant species and in many ecophysiological situations [50]. Secondary and tertiary cell walls have different mechanical properties, such as the lignified secondary cell wall provides rigidity, while tertiary cell wall adds flexibility due to the tension of cellulose microfibrils. The selection of fiber crops, like hemp, flax, ramie, has led to the extreme development of tertiary cell walls in fibers of their stems.

Summarizing, the major revealed differences between thickened cell wall of primary and secondary hemp fibers lay in the total cell wall width and in the proportion of S1 layer relative to the rest of cell wall. In secondary fibers, the cell wall width is considerably lower than in primary ones, while the proportion of S1 layer is higher. Since it is mainly S1 layer that gets lignified, secondary fibers have higher lignin content and due to that they are coarser than primary fibers. Importantly, the nanomechanical properties of tertiary cell wall as such are similar in primary and secondary fibers, as revealed by peak-force quantitative nanomechanical property mapping (PF-QNM) and micro tomography [31]. The major parameters of cell wall thickening that influence the yield and quality of hemp fibers are (1) the amount of the



**Figure 6.** Scheme illustrating the differences in distribution of polymers between cell wall layers in hemp fiber. Occurrence of cell wall epitopes for carbohydrate-binding module CBM3, antibodies LM10, LM11, RU2, N1, and JIM14, and localization of lignin deposition in various layers of phloem fiber cell wall in hemp stem.

deposited cell wall (cell wall thickness), (2) the ratio between the cell wall and cell lumen on the fiber cross-section, (3) the proportion of S1 layer in total cell wall thickness, and (4) the set and peculiarities of structure of cell wall polymers.

## Acknowledgements

We thank the colleagues from the Chuvash Research Institute of Agriculture (Tsivilsk, Russia): Dr. G.S. Stepanov, Dr. I.V. Romanova, and N.A. Trofimova—for growing the hemp plants. We also thank Dr. Paul Knox (University of Leeds) for the provided LM5 and LM11 antibodies and Dr. Fabienne Guillon (INRA, France) for the provided RU2 antibody. The research on the topic of the chapter was supported by the Russian Science Foundation, project 16-14-10256 (TG, NI, TC), and by the Russian Foundation for Basic Research, projects 15-04-02560 (PM) and 15-04-05721 (TC, MA).

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