

# THE PATTERN OF LIGNIN DEPOSITION IN THE CELL WALLS OF INTERNODES DURING BARLEY (*HORDEUM VULGARE* L.) DEVELOPMENT

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The aim of this paper is to elucidate relationship between anatomical changes and lignin deposition dynamics in the cell wall of barley (*Hordeum vulgare* L.) internodes during four growth stages: heading, flowering, grain filling and ripening. Microscopy was used to analyze anatomical changes related to lignin deposition whereas peroxidase activity was spectrophotometrically determined. In transversal sections lignin was found to be predominant in the sclerenchyma ring in cortex, where particularly lignified cell walls were found. Peroxidase activity was increased in older internodes and their localization in situ was in positive correlation with tissue lignification. Our results showed that, depending on the cultivar, at the flowering and grain filling stages intensive lignin synthesis and deposition occurred. This showed that deposition of lignin in the cell wall at a particular growth stage is in correlation with the lodging resistant phenotype of the investigated cultivars. The results contribute to the understanding of the lignin deposition process during barley development and consequential cell wall thickness.

**Keywords:** barley, lignin, stem, internode, cell wall, growth stage

## INTRODUCTION

Lignification of the cell wall is one of the main processes during stem development. Deposition of lignin in the cell wall takes place after the deposition of cellulose and hemicellulose (Terashima and Fukushima, 1988). Cross-linking of polysaccharide components with monolignols by hydroxycinnamic acids (*p*-coumaric and ferulic acid) signifies the beginning of lignification process and determines its dynamics, which is cell and tissue specific (Bidlack and Buxton, 1992; Morrison and Buxton, 1993). Previous research has shown that the lignification of the cell walls during secondary growth begins in the middle lamella and moves towards the center, therefore reducing the cell lumen (Terashima and Fukushima, 1988). The thickening of the cell wall is

a direct consequence of lignin deposition. Although monocots, particularly grasses (family Poaceae) do not have such intensive secondary growth like dicots and gymnospermae, lignin comprises around 20% of their secondary wall (Barrière et al., 2007; Vogel, 2008).

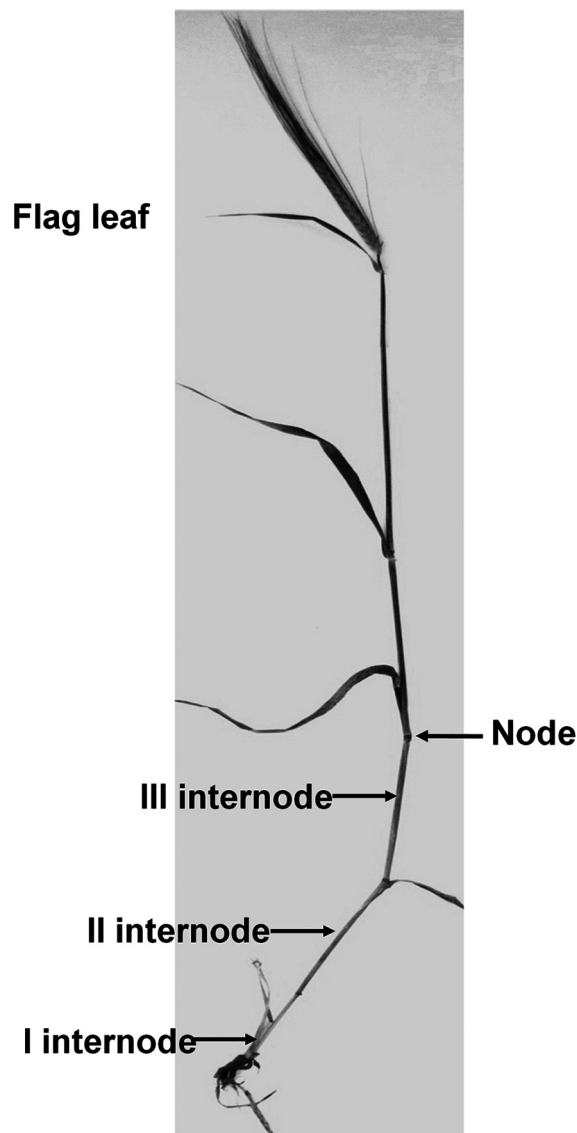
Class III peroxidases in plants are involved in many processes in the cell including lignin biosynthesis. They catalyze the last step in lignin polymerization by oxidizing monolignols using H<sub>2</sub>O<sub>2</sub> as a substrate and they are the most extensively studied enzymes in lignin biosynthesis (Boerjan et al., 2003). Ongoing investigations in gymnosperms and dicots species have aimed to elucidate their specific role in the lignification process (López-Serrano et al., 2004; Marjamaa et al., 2009; Fagerstedt et al., 2010).

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Culm strength is a very important agronomical trait with a direct impact on the crop yield. This trait is a combination of several different traits such as stiffness or bending resistance and elasticity important for a plant to recover from bending (Dunn and Briggs, 1989). Lignin, as a major structural component of the cell wall, is associated with a plant's mechanical strength. Previous research has shown that lignin synthesis and accumulation might be related to stem strength (Jones et al., 2001; Pedersen et al., 2005; Ma, 2009). This creates a major problem in monocotyledonous crop plants in which poor culm strength leads to a lodging phenotype (Hai et al., 2005; Ma, 2009). The selection and breeding processes, which aim to prevent plant lodging, lead to the creation of dwarf and semi-dwarf phenotypes of barley cultivars. The reduction of culm height in such cultivars negatively influenced crop yield (Lalić et al., 2005; Pedersen et al., 2005; Bonawitz and Chapple, 2013), reduced nutritive value of the culm and affected degradability (Travis et al., 1996). Moreover, it is a heritable trait from medium to high with the dominance degree varying from partial to superdominant (Lalić et al., 2005). Based on their phenotype, lodging resistance and agricultural value, three spring barley cultivars were selected for this investigation. Two cultivars used in this study, Astor and Scarlett, had a dwarf phenotype and differed in lodging resistance, being respectively resistant and susceptible. Cultivar Jaran had a longer stem and it was susceptible to lodging.

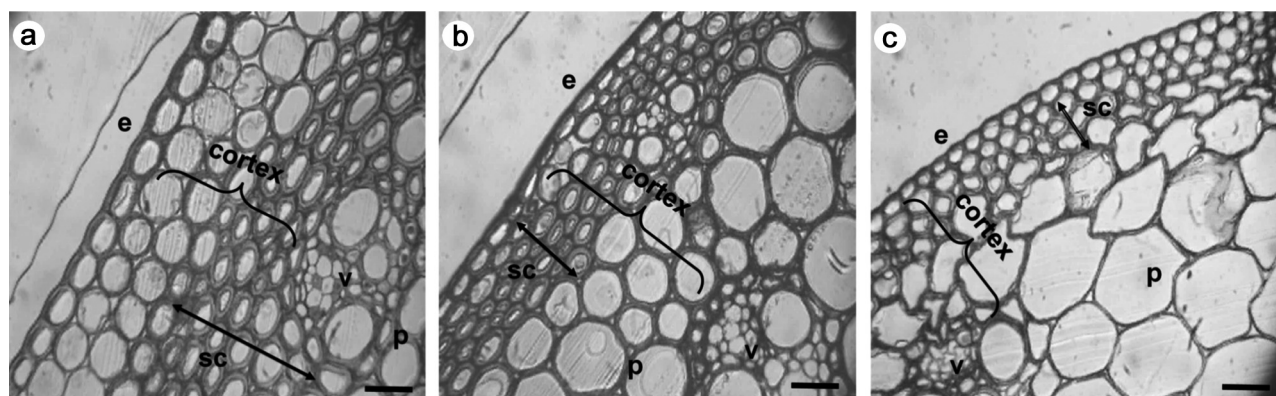
Previous studies investigated different characteristics of the barley stem such as: stem length and diameter, internode length and diameter, overall thickness of the cell wall in internodes and particular tissues as well as the number of vascular bundles during growth. Different internodes at different growth stages were used. Soon after the heading, in the second internode, measurement of the cell wall thickness was performed and four basal internodes were used for the measurement of the total cell wall thickness, total thickness of the sclerenchyma ring and number of the vascular bundles (Dunn and Briggs, 1989). Cenci et al. (1984), measured thickness in the third, fourth and fifth internode only at the ripening stage while Travis et al. (1996) performed measurement in the basal and middle internode at elongation, heading and ripening stages. Jung (2003) showed that in maize deposition of lignin and secondary growth in different parts of the internode had different kinetics and that different developmental profile of the internodes can be observed from top to bottom.

With respect to comprehensive data reported in the literature on cell wall thickness in the stem



**Fig. 1.** The photo shows a barley stem and the position of the internodes used for sampling at the heading, flowering, grain filling and ripening stages.

and differences in lignin deposition at a particular growth stage, which can be related to lodging resistant phenotype, in our study we describe how cell wall thickness of the epidermis, cortex and parenchyma is changing as a consequence of lignin deposition. In addition, we aimed to determine in which direction lignin is deposited in the cell wall, periclinal or anticlinal, in particular tissue of the internode during four growth stages of spring barley and to correlate obtained data with lodging resistant phenotype. The results generate useful information regarding the culm anatomy of barley and will contribute to development of new and improved agronomically important cultivars.



**Fig. 2.** Cross sections (3  $\mu\text{m}$ ) of first (a), second (b) and third (c) internode stained with 0.05% toluidine blue. e= epidermis, sc= sclerenchyma ring, p=parenchyma, v= vascular tissue. Arrows show overall thickness of the sclerenchyma ring and its position in relation to the epidermis. Bar=150  $\mu\text{m}$ .

## MATERIALS AND METHODS

### PLANT MATERIAL

Croatian spring barley cultivars Astor and Jaran and the German cultivar Scarlett, all two-rowed, were grown on an experimental field of the Agricultural Institute in Osijek in a random block design. Plant tissue was collected in 2011 from May until June. Stems of three barley cultivars were harvested at four growth stages according to Zadoks et al. (1974): (1) heading (the spike is emerging from within the flag leaf; Z 51), (2) flowering or anthesis (beginning of flowering; Z 61), (3) grain filling stage (medium milk stage; Z 75) and (4) ripening (kernel hard; Z 91).

At each growth stage three stem internodes, starting from the bottom of the plant, were used in the research. The basal was the first internode (I) also the oldest, the second (II) was the next one proceeding towards the top and the third (III) followed as the youngest internode used (Fig. 1). The middle part of each internode was excised and leaf sheaths were removed prior to histological preparations.

### LIGNIN LOCALIZATION IN THE CELLS

The localization and distribution of lignin were conducted on fresh, hand-cut sections using the Wiesner test (0.5% phloroglucinol (w/v) with addition of HCl) that gives lignin characteristic red staining (Adler et al., 1949). At least ten replicates per each internode were examined.

### PEROXIDASE LOCALIZATION IN THE INTERNODES

In order to determine peroxidase activity in situ fresh tissue sections of ten replicates per each internode were incubated for 2 minutes in a reac-

tion mixture containing 5 mM guaiacol, 5 mM  $\text{H}_2\text{O}_2$  in 0.2 M phosphate buffer (0.2 M  $\text{KH}_2\text{PO}_4$  and 0.2 M  $\text{Na}_2\text{PO}_4 \cdot 12 \text{H}_2\text{O}$ , pH 5.8) (Lepeduš et al., 2004).

### SECTION PREPARATION FOR ANATOMY STUDIES

Segments of the internode's middle part were fixed in a 6% glutaraldehyde (Grade II, Sigma) solution in a 0.05 M phosphate buffer (pH=6.8). The tissue was dehydrated in a series of alcohols and then embedded in metacrylate resin (Leica Histoiresin Embedding Kit). The samples were cut in 3  $\mu\text{m}$  sections with microtome Leica RM2155. The sections were placed in a drop of distilled water on a glass slide, heated until water evaporated which enabled the adhesion of the embedded section. The sections were stained with 0.05% toluidine blue in a benzoate buffer (pH=4.4) (O'Brien and McCully, 1981; Lepeduš et al., 2001). At least 20 sections per each internode were used for measurement.

### MEASUREMENT OF THE CELL WALL THICKNESS

The cell wall thickness was measured in the epidermis, cortex and parenchyma. The number of cell rows in the cortex and parenchyma was counted as well (Fig. 2). The middle part of the internode was used to eliminate the differences in anatomical structure between the upper and lower part of the monocot internode where intercalary meristems are located at the base of the internode to provide growth from the bottom to the top. To correlate lignin deposition with cell wall thickness, at each particular growth stage one hundred cells in the epidermis and two hundred cells in the cortex and parenchyma were measured. The thickness of the cell walls was measured in  $\mu\text{m}$ . The results were expressed as a ratio between the length of the outer

periclinal cell wall (OL) and the length of the inner periclinal cell wall or cell lumen (LL) and the width of the outer anticlinal cell wall (OW) and the width of the inner anticlinal cell wall or cell lumen (Fig. 3). These ratios were used as the lignification coefficient. The ratio between the outer length and lumen length (OL/LL) showed tangential cell wall thickness while the ratio between the outer width and lumen width (OW/LW) showed how thick the cell wall is radially. The sections were observed using a light microscope (Carl Zeiss Jenna, Germany). Measurements were done using a microscopic camera Moticam 350 and analyzed with Motic Images Plus 2.0ML (Motic China Group Ltd.) at 1000x magnification.

#### EXTRACTION AND PEROXIDASE ASSAY

Internodes of each sample type were combined (400 mg) and homogenized using mortar and pestle in liquid nitrogen with an addition of 2.5% polyvinylpyrrolidone (w/v) and 100 mM Tris (pH=8.0) as an extraction buffer. After extraction, homogenates were centrifuged at  $18\,000 \times g$  and  $4^{\circ}\text{C}$ . Supernatants were used for peroxidase activity and protein content measurements. Protein content was determined according to Bradford (1976), using bovine serum albumin as a standard. The reaction mixture for peroxidase activity determination (Siegel and Galston, 1967) contained 5 mM of guaiacol, 5 mM  $\text{H}_2\text{O}_2$  in a 0.2 mM phosphate buffer (pH 5.8). The formation of tetraguaiacol was measured spectrophotometrically by monitoring the increase in absorbance at 470 nm. In each sample type 5 replicates were done.

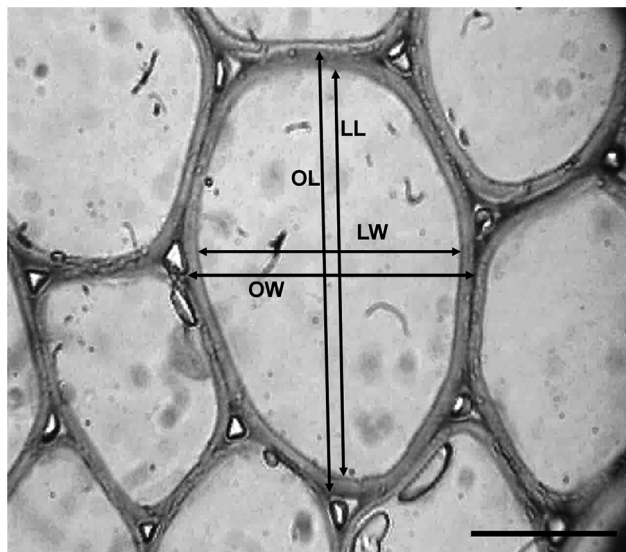
#### STATISTICAL ANALYSIS

ANOVA was used for data analysis: Tukey's HSD test (honest significant difference) for cell wall thickness and Fischer's LSD test (least significant difference) for peroxidase activity. Differences were considered significant at  $P \leq 0.05$ . All statistical analyses were conducted with Statistica 7.1. Software (StatSoft, Inc. 2005).

## RESULTS

#### LOCALIZATION AND DISTRIBUTION OF LIGNIN FROM HEADING TO RIPENING STAGE

Lignin was found to be predominant in the sclerenchyma ring of the cortex at all the studied growth stages and cultivars, but it was also found in the epidermis, parenchyma and vascular tissue (Fig. 4a). The sclerenchyma ring position differs among cultivars but in each it was characterized by thick cell



**Fig. 3.** Measurement of the cell wall thickness in the internode. In epidermis, cortex and parenchyma length of the outer periclinal cell wall (OL), length of the inner periclinal cell wall (LL) as well as width of the outer anticlinal cell wall (OW) and the width of the inner anticlinal cell wall (LW) were measured in  $\mu\text{m}$ . Results were expressed as ratio OL/LL and OW/LW. Bar = 150  $\mu\text{m}$ .

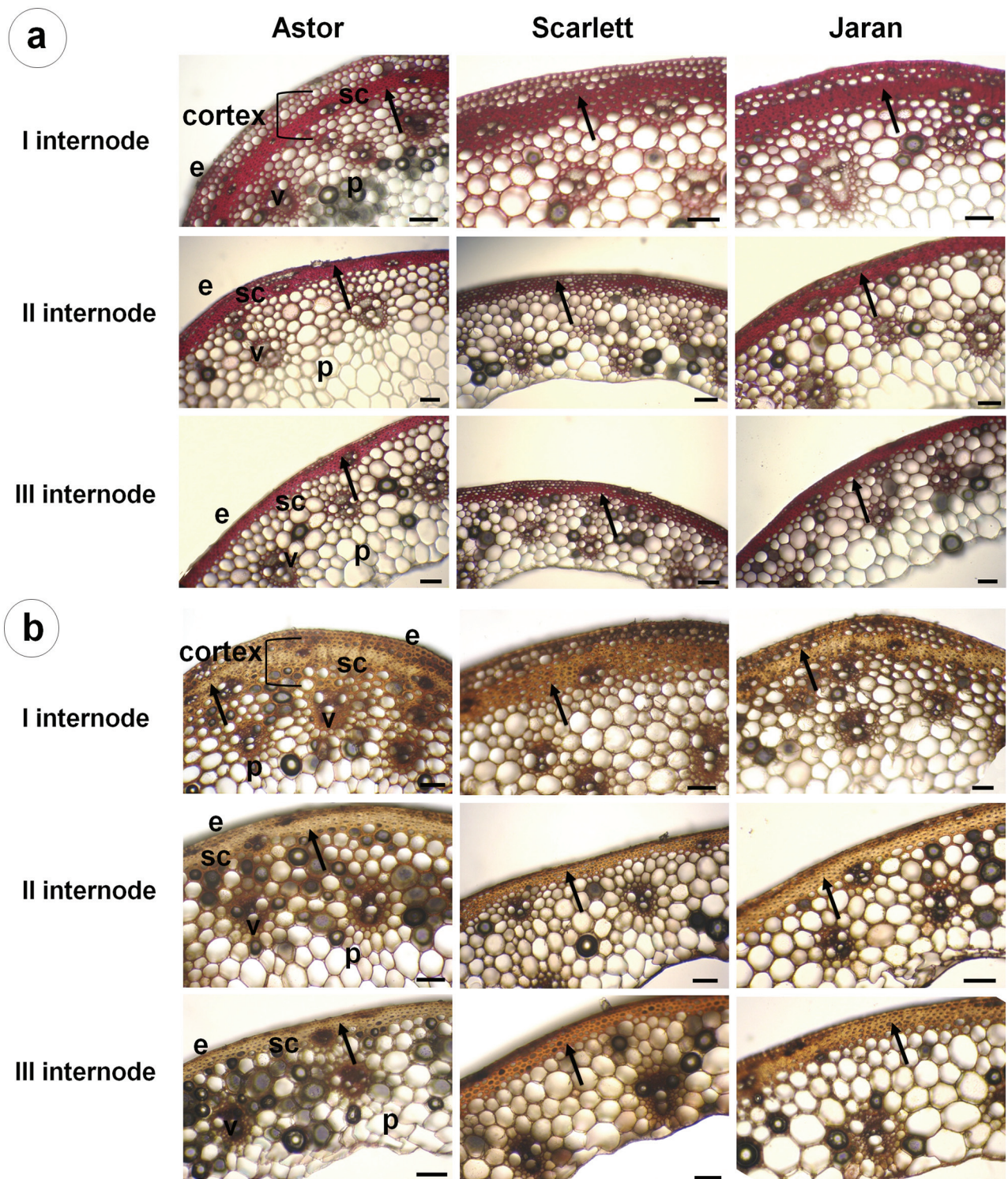
walls with particularly narrow cell lumen. In lower internodes the ring was positioned far from the epidermis while in the upper internodes it was closer to it. In the lowest (first) internode of cultivars Astor and Scarlett the sclerenchyma ring was built-up of 2–3 cell rows and in cultivar Jaran of 3–4. The second internode had an identical number of rows (4–5) in all the investigated cultivars. Similar results were obtained for the third internode which had 3–4 cell rows. Cells of the sclerenchyma ring had particularly lignified cell walls with incorporated smaller vascular tissue. In this layer cells were very densely packed and smaller – compared to the epidermis or parenchyma.

The number of cell rows in the parenchyma also differed between cultivars. The largest number of cell rows (5–7) was counted in the first internode of cultivar Jaran, while in the other cultivars and internodes parenchyma tissue contained 2–3 cell rows. The number of cell rows in the sclerenchyma ring and parenchyma was not changed between the studied growth stages.

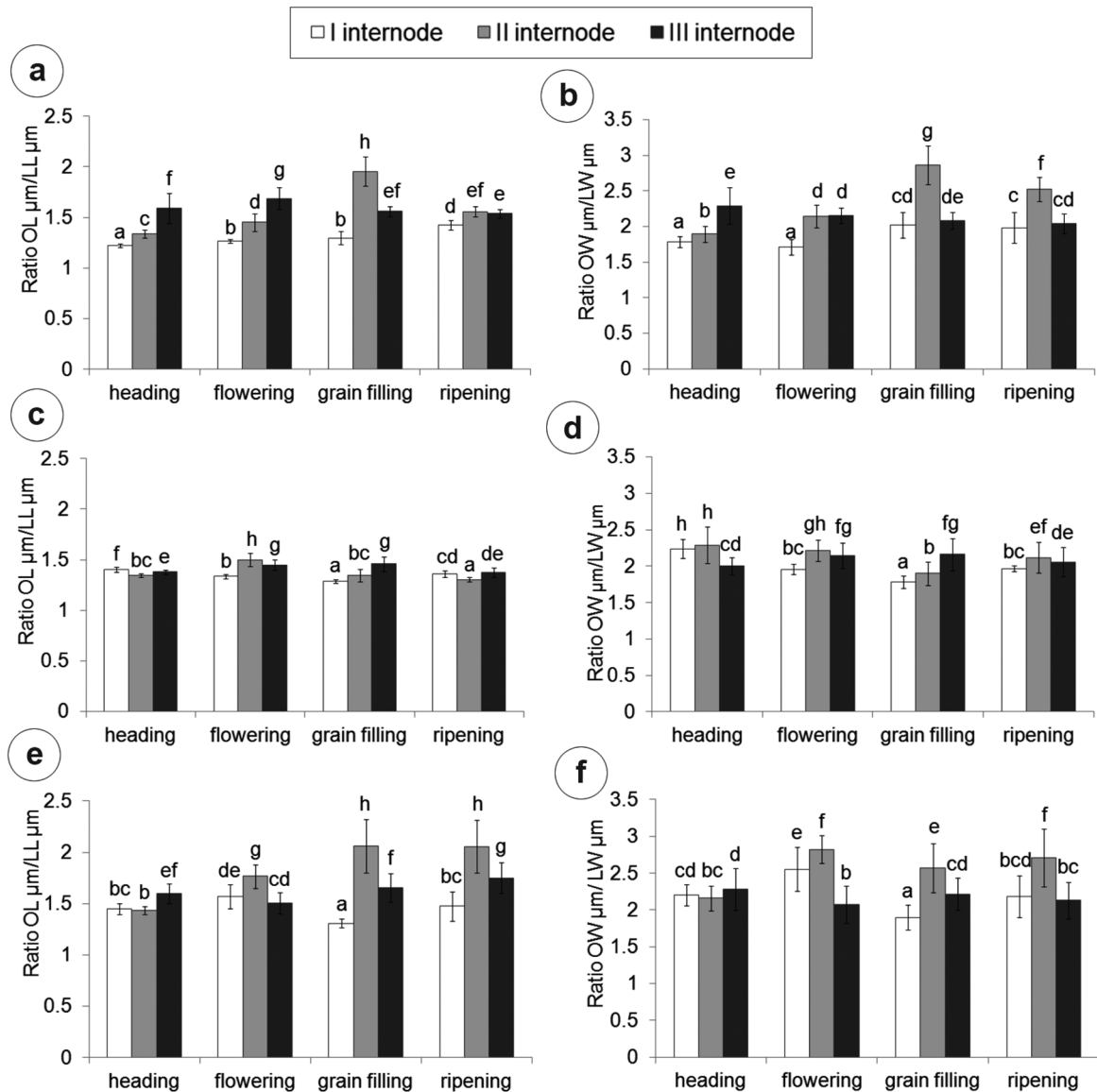
#### LIGNIN DEPOSITION AND CELL WALL THICKNESS

##### Epidermis

In the barley stem the epidermis was composed of a single cell row in which cell wall thickness increased in both directions (periclinal and anticlinal) during



**Fig. 4.** (a) Histochemical localization and distribution of lignin in the internodes of three spring barley cultivars. Lignin (red) was localized in the epidermis (e), sclerenchyma ring (sc) and vascular tissue (v). Most of the lignin was deposited in the sclerenchyma ring (sc) in cortex in all internodes (arrows). (b) Peroxidase activity in situ (brown color) co-localized with more lignified tissue in the internodes especially sclerenchyma ring (arrows). The image shows only the ripening stage because there were no changes in localization and distribution of lignin during all four growth stages. Bar=500  $\mu$ m.

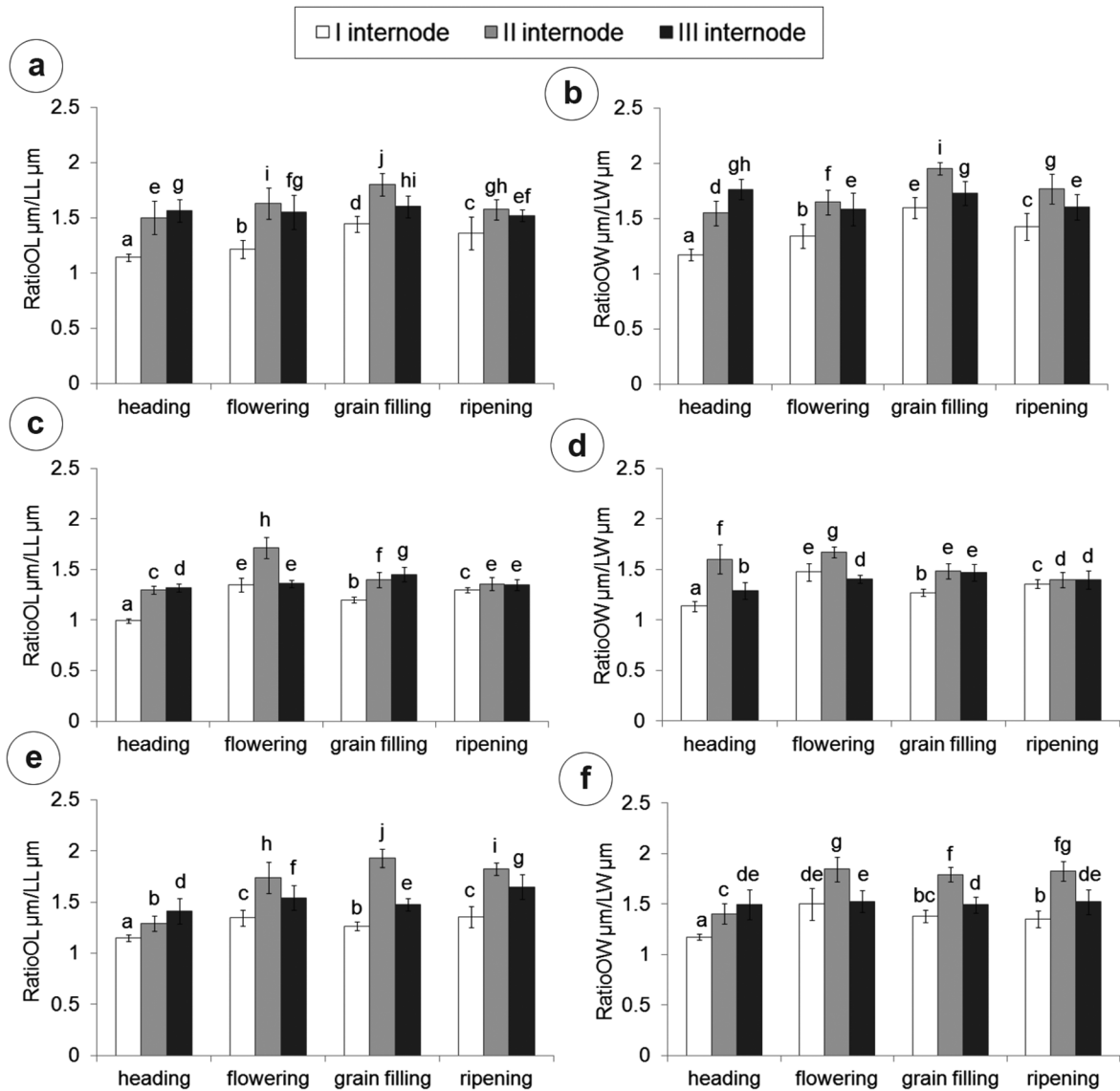


**Fig. 5.** Changes in the cell wall thickness of the epidermis in the internodes in four growth stages expressed as a ratio between outer length (OL) and lumen (LL) length (a–c), and ratio between outer (OW) and lumen (LW) width (d–f). Cultivar Astor (a, b), Scarlett (c, d) and Jaran (e, f). Different letters represent significance at  $P \leq 0.05$ . Error bars represent mean values  $\pm$  standard deviation ( $n=100$ ).

stem development in all internodes as a consequence of lignin deposition and it was cultivar dependent. In the epidermal cells of cultivar Astor (Figs. 5a, b), from heading to grain filling, the cell wall was becoming thicker as a result of lignin deposition in the tangential and radial direction of the cell. Epidermal cells in the first internode, which is the oldest, were less lignified than the cells in the second and third internode in the tangential and radial direction of all growth stages (Fig. 5a). The cell wall of epidermal cells in cultivar Astor was the thinnest in the first internodes although stage-

dependant increase was significant (Figs. 5a, b). The most significant increase of cell wall thickness in cultivar Astor was in the second internodes at the grain filling stage. In contrast to the first and second internodes, the cell walls in the third internode decreased at later growth stages (Figs. 5a, b).

In cultivar Scarlett the thickest cell walls were found in the first internode at heading, in the second at flowering and the third internode had the thickest cell walls at flowering and grain filling stage, but all these differences were not as intense as in cultivar Astor (Figs. 5c, d).



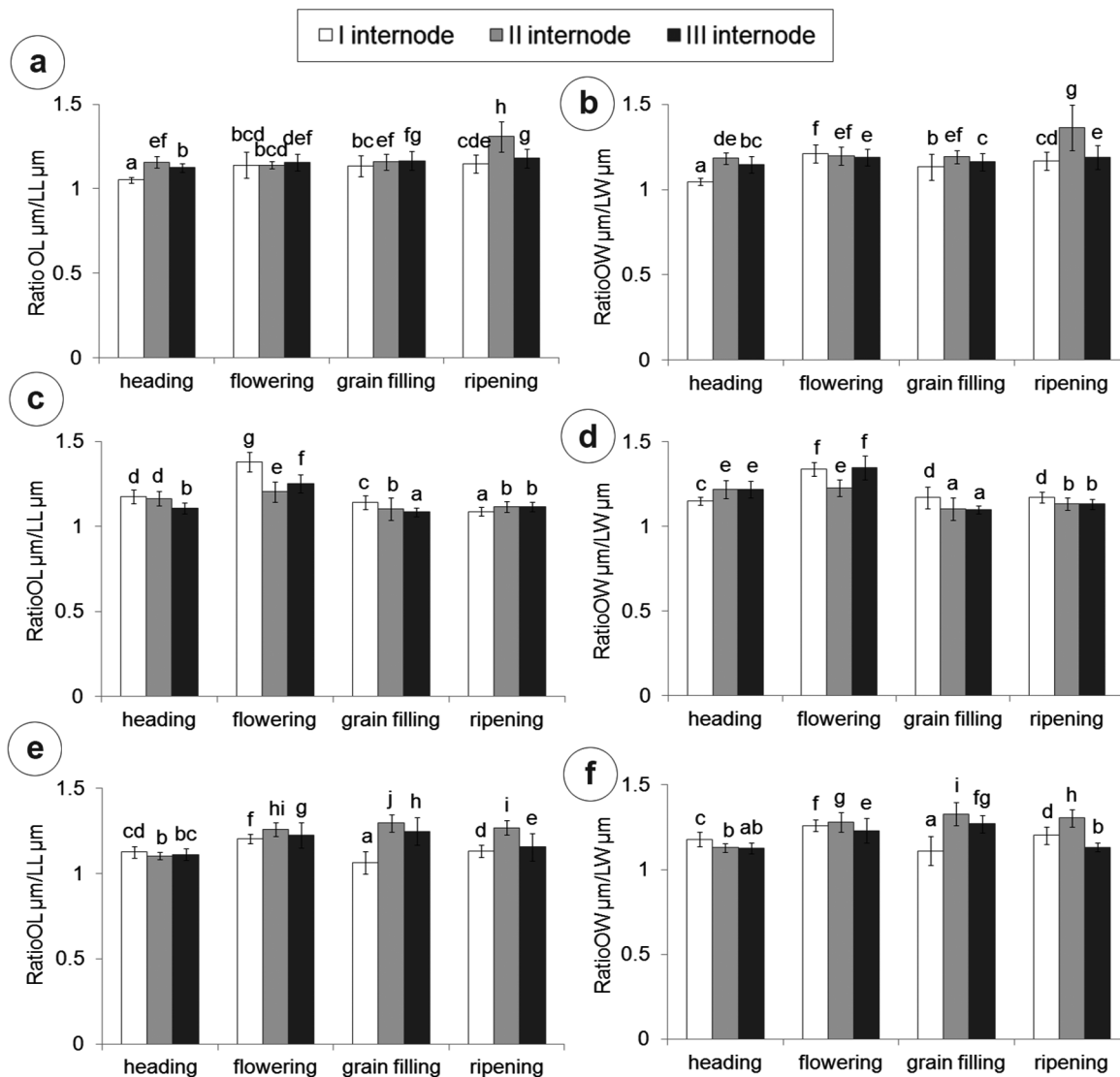
**Fig. 6.** Changes in the cell wall thickness of the cortical sclerenchyma in the internodes at four growth stages expressed as a ratio between outer length (OL) and lumen (LL) length (a–c), and ratio between outer (OW) and lumen (LW) width (d–f). Cultivar Astor (a, b), Scarlett (c, d) and Jaran (e, f). Different letters represent significance at  $P \leq 0.05$ . Error bars represent mean values  $\pm$  standard deviation ( $n=200$ ).

Similar to dwarf Astor, Jaran had the thickest cell walls in the epidermis of the second internode, when compared to the first and third ones. Thickness increased at later growth stages including the ripening stage (Figs. 5e, f).

#### Cortex

The cortex was the most variable layer between the three cultivars and included the area from the epidermis to the parenchyma, sometimes with incorporated vascular tissue (Fig. 2). Cells in the sclerenchyma ring in the first internode of cultivar Astor

were less lignified in comparison to the second and third internodes at all four growth stages (Figs. 6a, b). Examination of the cell wall thickness in cultivar Astor showed that the cell wall thickness increased during development until the grain filling stage in all internodes. Therefore, most of the lignin was deposited at the grain filling stage in both the radial and tangential directions in all internodes. Decrease in lignin deposition was observed at ripening (Figs. 6a, b). In cultivar Scarlett significant lignin deposition was visible already at the flowering stage (Figs. 6c, d) as well as in cultivars Astor (Figs. 6a, b) and Jaran (Figs. 6e, f). In the second internode of



**Fig. 7.** Changes in the cell wall thickness of the parenchyma cells in the internodes at four growth stages expressed as a ratio between outer (OL) and lumen (LL) length (**a–c**), and ratio between outer (OW) and lumen (LW) width (**d–f**). Cultivar Astor (**a, b**), Scarlett (**c, d**) and Jaran (**e, f**). Different letters represent significance at  $P < 0.05$ . Error bars represent mean values  $\pm$  standard deviation ( $n=200$ ).

cultivar Jaran lignin deposition also significantly increased at the grain filling and it was 40% higher, compared to the heading stage.

#### Parenchyma

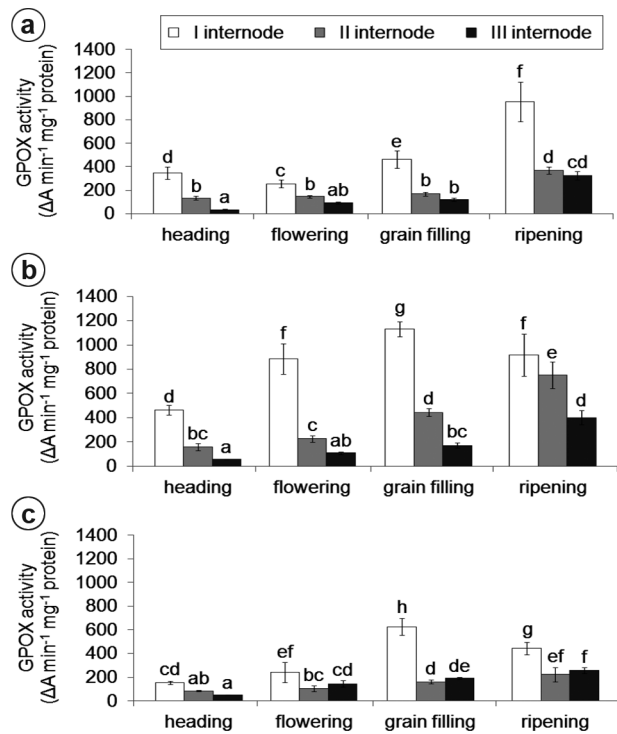
Parenchyma cells had thinner cell walls compared to the cells in the epidermis and the cells in the sclerenchyma ring. The cells varied in size and dimensions. Close to the pith the cells were larger with very thin walls, while those closer to the surface were smaller and more lignified (Fig. 4). In all internodes, the lignification and cell wall thickness of the parenchyma cell walls in the three cultivars

(Fig. 7) started to increase at the flowering stage. In general, the maximal lignification of the first and third internodes was lower compared to the second one in cultivars Astor and Jaran, in which the cell walls were thickest in the second internode at the ripening and grain filling stages respectively. In cultivar Scarlett maximum cell wall thickness was reached at the flowering stage with the thickest first internode.

#### PEROXIDASE ACTIVITY IN THE INTERNODES

The activity of guaiacol peroxidase was compared in three internodes of spring barley cultivars at four





**Fig. 8.** Changes in guaiacol peroxidase activity in the internodes of three spring barley cultivars Astor (a), Scarlett (b) and Jaran (c) at four growth stages: heading, flowering, grain filling and ripening. Different letters represent significance at  $P \leq 0.05$ . Error bars represent mean values  $\pm$  standard deviation ( $n=5$ ).

growth stages. When comparing specific activity between internodes, the highest activity was measured in the first internode in all cultivars at each growth stage (Fig. 8) with the maximum during either the grain filling or ripening stage. In cultivar Astor (Fig. 8a) peroxidase activity in the first internodes increased significantly at the grain filling stage reaching its maximum at the ripening stage in which a significant increase was visible in all internodes (Fig. 8a). In cultivar Scarlett (Fig. 8b) and cultivar Jaran (Fig. 8c) the highest peroxidase activity was measured at the grain filling stage in the first internode, while in the second and third internode the maximum was measured at the ripening stage. Comparing all three spring barley cultivars, Jaran had the lowest peroxidase activity (Fig. 8).

H<sub>2</sub>O<sub>2</sub>-dependant oxidation of guaiacol was used in order to visualize peroxidase activity in situ. The results showed that peroxidase activity in situ co-localizes with lignified tissue in the internodes (Fig. 4b). Increased activity was correlated to older and more lignified tissue in the internodes, especially in the first internode and in the scler-

enchyma ring of all three internodes as well as in vascular bundles.

## DISCUSSION

Lignin synthesis and deposition in the cell wall depends on multiple factors including genotype and growth stage (Frei, 2013). In order to determine the presence of lignin deposition we measured, at four growth stages, the cell wall thickness and peroxidase activity in the internodes of spring barley cultivars differing in their stem height and resistance to lodging. The comparison between cultivars showed that cultivar Jaran, the highest cultivar, had the thickest cell wall at the flowering, grain filling and ripening stages in epidermis in all internodes and in sclerenchyma ring in the second and third internode at the ripening stage, while the shorter cultivars Astor and Scarlett had thinner cell walls (Figs. 5, 6). Similar results were obtained by Cenci et al. (1984) in their investigations of changes in the anatomical structure of the stem of different barley cultivars. They measured plant height, internode length and thickness of the epidermal and sclerenchyma cell walls in four cultivars with different stem length at the ripening stage and showed that cultivars with longer stems had thicker cell walls unlike cultivars with shorter stems where the cell walls were thinner.

Histochemical detection of lignin in the cell walls of internodes (Fig. 4a) showed that the cortex, particularly the sclerenchyma ring, was a dominant place of lignin deposition in all internodes. The thickness of the sclerenchyma ring along with cell wall thickness significantly influence stem strength (Cenci et al., 1984) and regarding the thickness it is the most variable area in the internodes of different wheat and barley cultivars (Travis et al., 1996).

Previous reports have shown that peroxidase activity plays an important role in lignin biosynthesis (Christensen et al., 1998; Marjamaa et al., 2009; Martínez-Cortés et al., 2011). Our results show that peroxidase activity increased with the development from the heading to ripening stage (Fig. 8). The thickening of the cell wall and increased peroxidase activity were visible at grain filling and ripening in cultivar Astor, and already at the flowering stage in cultivars Scarlett and Jaran. A comparison of the different internodes showed that developmentally older internodes had increased peroxidase activity. Similar results in peroxidase activity during development of sugarcane internodes were *ENREF\_26* observed by Cesarino et al. (2012). They showed that developing and mature internodes, as opposed to younger, had increased peroxidase activity. Our results showed that enhanced peroxidase activity in the internodes is positively correlated with lignin deposition in the cell wall during development.

The results show that lignin deposition in the cell wall depends on the growth stage and cultivar. By comparing the dynamics of lignin deposition between barley cultivars analyzed in this study, through four growth stages, we showed that in the epidermis the deposition of lignin increased from the heading to the grain filling stage in cultivar Astor and Jaran, especially in the second internode (Fig. 5), while in cultivar Scarlett a significant decrease is visible at the grain filling stage in the first and second internodes in both the radial and tangential directions (Figs. 5c, d). In the cortex, lignin deposition increased at the flowering stage as opposed to the heading stage in all cultivars. At this growth stage in cultivar Scarlett the thickest cell walls were measured in the second internode, while in cultivar Jaran and Astor maximum cell wall thickness was reached at the grain filling stage. Moreover, lignin deposition in cultivar Astor was most evident at the grain filling stage in all three internodes in both directions, tangential and radial. Shen et al. (2009) and Sarath et al. (2007) noticed that in switchgrass lignification increased at the stem elongation and reproductive stages in the cell walls of older internodes. Similar lignification pattern results were also observed in maize (Jung, 2003; Jung and Casler, 2006) and in tall fescue (Chen et al., 2002).

Kong et al. (2013) highlighted the significance of mechanical tissue in the resistance to lodging in different wheat cultivars. Peng et al. (2014) in their study also suggested that lignin accumulation is related to culm strength of wheat and thus a lodging resistant phenotype.

It is also important to emphasize the growth stage in which lodging occurs since it was shown that lodging at the heading or growth stages after heading have a strong negative impact on the yield (Kelbert et al., 2004; Acreche and Slafer, 2011) and that the stage was correlated with the synthesis and deposition of lignin in the cell walls. After completion of the internode elongation in maize, secondary cell wall of sclerenchyma and parenchyma is becoming more lignified (Jung and Casler, 2006). Comparing different cultivars at the ripening stage showed that the thickest cell walls in parenchyma were found in cultivar Astor (Figs. 7a, b) and the thinnest in cultivar Scarlett (Figs. 7c, d). Parenchyma cell wall thickness, in other growth stages, did not show any specific pattern but varied between cultivars and growth stages.

## CONCLUSION

The study found significant differences in the anatomical characteristics and peroxidase activity as well as dynamics of lignin deposition in the cell

wall between the three spring barley cultivars. Developmentally, the flowering and grain filling stages were phases when the whole plant was very active with numerous biosynthesis in process. Our results demonstrated that these two growth stages were the stages of intensive lignin synthesis and its deposition in the cell walls. Lignin deposition significantly increased at the flowering stage in all cultivars, but in cultivar Astor an additional significant increase was visible at the grain filling stage. This cultivar proved to be more resistant to lodging than the others and therefore we assume that this additional step in lignin deposition in the cell walls at the grain filling stage could be the cause for enhanced culm strength which contributes to the lodging resistant phenotype of cultivar Astor.

Changes that took place at the anatomical and physiological levels in the barley stem concerning lignin deposition in the cell wall are essential for the future selection and breeding of new spring barley cultivars with better culm strength. They are also important for understanding the development of cell walls as a function of lignin deposition through four growth stages in spring barley cultivars differing in their stem height and lodging phenotype and could be used as criteria for their selection.

## AUTHOR'S CONTRIBUTION

All authors declare that there are no conflicts of interest. LB performed most of the experiments and wrote the manuscript. JR helped in preparation of sections for microscopy. HL, DLL took part in interpretation of the results and manuscript preparation. VC designed and directed the study and revised the manuscript.

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