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POLSKA AKADEMIA NAUK
ACTA BIOLOGICA CRACOVIENSIA Series Botanica 64/2: 65–74, 2022
DOI: 10.24425/abcsb.2022.143383

THE LONGEST LIVING XYLEM CELLS LOCKED IN LIGNIFIED CELL WALLS – THE CASE OF XYLEM PARENCHYMA IN EUROPEAN ASH (*FRAXINUS EXCELSIOR* L.) STEMS

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Received July 17, 2022; revision accepted January 25, 2023

The investigation described herein discusses the morpho-anatomical characteristics of xylem parenchyma cells of European ash stems undergoing heartwood formation. The research material comprised of cross and radial sections of wood obtained from stems at breast height of 91-year-old ash trees, half of which had visible ash dieback symptoms. The radial section of the wood samples was stained with acetocarmine to detect nuclei and with I_2KI solution to observe starch grains in parenchyma cells, both of radial and axial systems. Additionally, microscopic slides were stained with Alcianblue Safranin-O, and fluorescence microscopy was applied to detect lignified cell walls**.** The color of sapwood and heartwood distinctly differed – heartwood extracts were detected in approx. 47 rings. Most of the parenchyma cells had nuclei present in both wood zones. Also starch grains were detected in the majority of the tree rings of the researched samples. All of the xylem parenchyma cell walls of axial and radial systems were lignified. The research revealed that lignification, parenchyma cells death and the release of heartwood extracts are processes remote in time and space. Furthermore, parenchyma cell walls lignification did not figure as a sign of the upcoming parenchyma cells death. Based on the current research, ash dieback disease might slightly impact the development paths of parenchyma cells. Compared with scores reported for other trees, European ash parenchyma cells longevity is indeed remarkable.

Keywords: heartwood formation, lignification, parenchyma cells longevity, programmed cell death, wood anatomy, xylogenesis

INTRODUCTION

Xylem parenchyma, being the minor part of the wood (present both in radial and axial systems), figures as the only living fraction among dead fibers and tracheary elements (i.e., cells conducting water: tracheids and vessel members). Formation of all these cells undergoes xylogenesis, which is a process enabled by the division of cambial cells and remaining under hormonal control (Wodzicki, 1971; Plomion et al., 2001; Samuels et al., 2006). The main emphasis in xylogenesis studies was put on tracheary elements and their formation, which seems to have been meticulously investigated (Wodzicki, 1971; Fukuda, 1996; Kaneda et al., 2010). It is known that the first stage of their formation is increasing the dimensions of xylem mother cells (Wodzicki, 1971; Samuels et al., 2006). The next stage of differentiation is usually the deposition of secondary walls, consisting of a three-layered structure of cellulose microfibrils, aligned with different angles relative to the cell axis and hemicelluloses, bonding them across (Donaldson, 2001; Samuels et al., 2006; Kaneda et al., 2010).

Apart from the empty cell interior to fulfill conductive and mechanical functions, tracheary elements contain strong, hydrophobic cell walls,

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PL ISSN 1898-0295

which are their only functional structures. To reach this feature, thick cell walls eventually undergo a lignification process. Lignin emergence has significantly contributed to the evolution of land plants by the enablement of long-distance transport (Lei, 2017), due to the rigidity and mechanical support to the axis of the plant and water impermeability provided (Ros Barcelo, 1997). As a durable polyphenolic compound, lignin, that incrusts xylem cell walls, enables their resistance to pathogenic activity (Morris, 2016). Lignification is the last process occurring in the tracheary elements before programmed cell death (PCD); however, some research proves the phenomenon of post-mortem lignification, enabled by the use of monolignols coming from neighboring parenchyma cells (Halpin, 2013).

The longevity of tracheary elements varies from 2-5 days to approx. one month (Wodzicki and Brown, 1973; Bollhöner et al., 2012). By contrast, in some species, xylem parenchyma cells are distinguished by an outstandingly long lifespan, even up to 200 years found in *Rhododendron laponicum* (L.) Wahlenb. (Spicer and Holbrook, 2007; Morris, 2016). Stage sequences of the differentiation processes of xylem parenchyma cells present the same as tracheary elements but expanded in a different space and time dimension. Nakaba et al. (2006, 2012b) proved that positional information and proximity to short-lived tracheary elements are factors involved in the control of xylem parenchyma cells death.

Because of their wide range of functions, living xylem parenchyma cells impressively support the wood tissue in a tree. Their well-known role is to support the conduction and storage of reserved material (Taylor et al., 2002; Słupianek et al., 2021). Due to the ability of tyloses formation into vessels lumen, parenchyma cells may refill embolized vessels (Secchi and Zwieniecki, 2011). The latest investigations indicate that the xylem parenchyma cells may contribute to the aforementioned post-mortem lignification process of tracheary elements on the basis of the good-neighbor hypothesis (Hosokawa et al., 2001; Ros Barcelo, 2005; Blokhina et al., 2019). Furthermore, as the only living fraction of wood may launch active defense mechanisms against pathogens by the compartmentalization of decay in trees (CODIT) (Shigo, 1984), the formation of tyloses and synthesis and accumulation of heartwood extracts, e.g., phytoalexins, phenolic compounds, suberin, demonstrate antimicrobial features and provide the specific color of the heartwood zone (Morris et al., 2016).

Heartwood is the inner part of a tree which does not contain living parenchyma cells and storage material (e.g., starch) or this material was converted into heartwood extracts (IAWA 1964). Heartwood formation is perceived as a secondary process of xylem differentiation (Kampe and Magel, 2013; Ye and Zhong, 2015). The other reported heartwood feature is the difference in moisture content, comparing to sapwood, usually a decrease (Hillis, 1968; Ip et al., 1996; Tomczak et al., 2018). Generally, prior to the heartwood zone, the intermediate wood is distinguished, which, according to IAWA, is described as "the inner layers of sapwood that are transitional between sapwood and heartwood in color and general character" (IAWA 1964). The presence of this zone indicates initiation of the heartwood formation.

European ash drew our attention since it features a remarkably late age of a tree at which it forms heartwood. In European ash, it appears at the age of 70-80 (Bugała, 1995), while e.g., in black locust (*Robinia pseudoacaccia* L.), which has the same morphological type of wood (i.e., ring-porous), at the age of 4-6 (Magel et al., 1994; Dünisch et al., 2010). The heartwood at ash stems is of dark color, but it might be non-dyed as well (Bugała, 1995).

European ash is a precious forest-forming species of humid and fertile sites; however, nowadays its presence in forests is declining due to the common phenomenon of ash dieback that has been observed throughout the whole of Europe (Kowalski, 2006; Szabo, 2009; Woodward and Boa, 2013). The changes of xylem tracheary elements and tree ring increment parameters, which are a response to the disease on the cambium and xylogenesis level, have been reported (Tulik et al., 2010, 2017, 2018).

As a tree of a great cultural and economic importance and supplying valuable timber, European ash is a tree species worth investigating, also due to its share decrease in forests observed nowadays. Knowledge of its wood anatomy remains still incomplete, especially in terms of xylem parenchyma cells. Parenchyma cells, which are the longest-living cells in xylem, express aging of the whole wood tissue. Therefore, tracing their life course enables reaching the aim of the current research, which is a morpho-anatomical description of ash wood senescence. Detailed research purposes are determined as follows: 1) anatomical

characteristics of wood aging with particular attention paid to detection of nuclei and starch grains in order to determine the survivability of parenchyma cells in every tree ring of each studied tree, 2) linked to the first aim, determination of sapwood, intermediate wood, and heartwood zones, based on morphological and anatomical observations, 3) comparison of wood anatomy of healthy ashes to these with visible symptoms of ash dieback.

MATERIALS AND METHODS

In July 2019, six European ash (*Fraxinus excelsior* L.) trees were selected in the Łochów Forest District in Poland (GPS 52°55'N, 21°78'E), where the process of ash decline has been observed for several years. The habitat was described as a wet forest with European ash as a dominant in the species composition of the tree stand. All selected trees presented the same biosocial class (dominant) and were evenly aged; therefore, according to the literature, a heartwood zone was expected to occur. Six trees were selected in total - three of them (classified as dying trees) had lost more than 50% of foliage, which was one of the symptoms of ash dieback, and the other three were healthy, based on the visual method of the defoliation estimation (Tulik et al., 2017). The research material was obtained from the stems of trees as discs (of 50 mm thickness) at the base of the stem and at breast height (DBH = 1.3 m). Then, samples including all annual rings were cut out of each disc along the diameter. After protection from the moisture loss, they were transported to the laboratory.

Wood samples were inserted in fixative FAA (formaldehyde 10%, acetic acid 5%, etanol 50%) for a few days at room temperature (Broda, 1971). Afterwards, they were rinsed with 70% ethanol twice. With the use of WSL-Core-Microtome, the slides of cross and radial sections at a thickness of approximately 25 μm were prepared.

Sapwood and heartwood zones were initially distinguished by the color of wood of heartwood extracts or their absence. The widths of sapwood and heartwood zones were measured in stitched microscopic images with the use of ImageJ software. The radial sections were stained with acetocarmine to detect the nuclei in the parenchyma cells (Broda, 1971), both of radial and axial systems, as nuclei presence proves the living status of a cell. The shares of living cells in every ring of two healthy and two dying trees were determined, based on the ratio of the number of these cells to the total number of parenchyma cells in a field of view (area equaled 142 890 μm^2) in one tree ring, made on observations of radial sections stained with acetocarmine. All statistical calculations were done with statistical computing environment R in version 4.1.1 (R Core Team, 2021).

In each tree ring the starch presence was checked on the radial sections that were stained with I_2KI solution. The estimation of starch amount served as a supportive way to determine the number of living cells.

Furthermore, radial sections were stained with Alcian Blue with Safranin O [1:1, v/v] to distinguish the lignified and non-lignified cell wall of the parenchyma cells. To obtain reliable results, additional fluorescence microscopy with excitation (λext. = 488 nm) was used to detect lignified walls of parenchyma cells. The sections were observed under an OLYMPUS BX61 light microscope, equipped with a motorized table and a Color View OLYMPUS digital microscope camera, as well as OLYMPUS Cell P software for image acquisition and archiving.

RESULTS

MORPHO-ANATOMICAL DESCRIPTION OF WOOD SAMPLES

Based on the general observation of the cross sections, the wood was characterized as ring porous with well-recognized ring boundaries. In two out of three trees with diminished foliage, which is one of the ash dieback symptoms, up to 20 outermost rings (the closest to cambium) were significantly reduced. These rings featured a well-developed earlywood zone and a diminished latewood zone. Fibers with thick cell walls made up the main part of wood. Axial apotracheal parenchyma was diffuse in aggregates, and paratracheal parenchyma was of a vasicentric character.

The width range of parenchyma rays was from 2 to 30 cells; usually, 8-seriate rays were observed. Most of the rays were composed solely of the procumbent cells (homocellular rays); however, one row of square marginal cells also occurred in some rays (heterocellular rays).

The total number of tree rings at the stem base in every tree equaled 91, at DBH 89. The mean value of tree rings, consisting of the heart-

wood zone, which was detected by the dark color of wood given by heartwood extracts, equaled 47. In turn, the sapwood zone encompassed 42 tree rings on average (Table 1).

HISTOLOGY AND BIOLOGY OF ASH WOOD PARENCHYMA CELLS

Further analysis of the wood proved nuclei presence in a distinct majority of axial and ray parenchyma cells (Fig. 1).

The living parenchyma cells were noted in the majority in every tree increment, both in healthy trees and those with dieback symptoms (Fig. 2). It means that nuclei were detected in most of the cells containing and lacking heartwood extracts, which was of great significance for sapwood and heartwood identification (Fig. 3).

Only in one healthy tree, the non-living parenchyma cells were detected solely in the 53rd and 63rd tree-rings (on the chart – tree ring ranges:

Table 1. Macroscopic characteristics of ash wood samples obtained at stem base. Trees 1-3 were recognized as declining ones, 4-6 as healthy.

Tree number	Sapwood determined macroscopically		Heartwood determined macroscopically	
	Number of rings	Width [mm]	Number of rings	Width [mm]
1	50	23	41	64
2	22	48	69	83
3	50	36	41	75
4	40	72	51	60
5	46	36	45	62
6	44	45	47	59
Mean value	42	43	49	67
Standard deviation	10.5	16.5	10.5	9.7

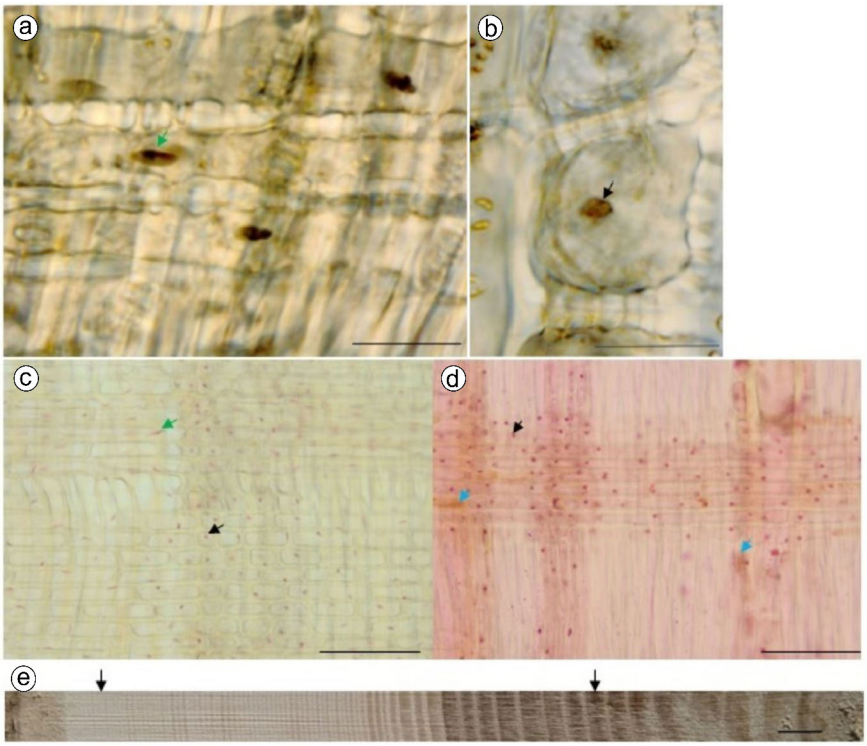

Fig. 1. Micrographs of radial sections of the 15th (**a**), 22nd (**b**), 12th (**c**) and the 81st (**d**) tree ring of ash wood stained with acetocarmine detecting nuclei. Black arrows indicate examples of round nuclei and green arrow – one of the fusiform nuclei. Blue arrows point to heartwood extracts. The left side of the micrograph refers to the side of the stem closer to cambium. Wood samples (**a**,**b**,**d**) derived from healthy trees and (**c**) from declining trees, the height of 1.3 m. Scale bars: 50 µm (**a**,**b**), 100 µm (**c**,**d**). (**e**) Macroscopic sample of ash wood. The first arrow from the left (closer to cambium) indicates the tree ring which the micrograph 1**c**) is derived from, the next arrow – accordingly the micrograph 1**d**). Scale bar: 10 mm

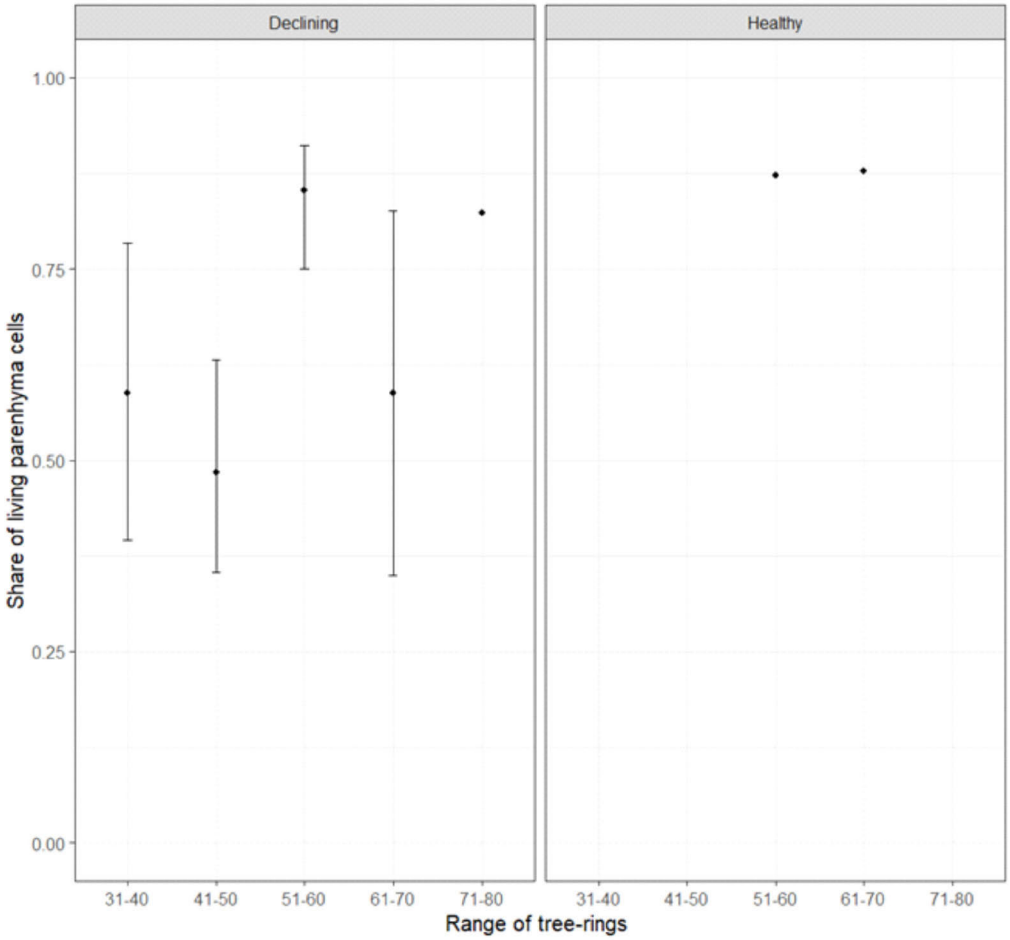

Fig. 2. Share of living parenchyma cells in selected tree-rings in declining and healthy ash stems. The points are marked only in these ranges of tree rings, at which any of the xylem parenchyma cells lacked nuclei. In the declining trees such cells were observed from the age of 31, whereas in the healthy trees – 51. The range 81-90 is not included, since the nuclei in these tree rings were in most cases hard to be detected, due to abundance of heartwood extracts.

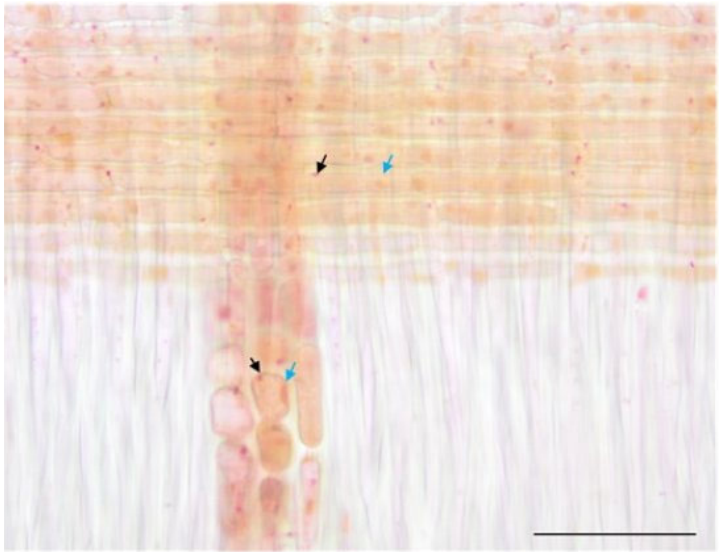

Fig. 3. Micrograph of radial sections of the 76th tree ring of healthy ash wood stained with acetocarmine detecting nuclei. Black arrows indicate examples of nuclei and blue ones – heartwood extracts. The left side of the micrograph refers to the side of the stem closer to cambium. Scale bar: 100 µm

51-60 and 61-70) and the share of living parenchyma cells in them amounted to 87% and 88%, respectively. In all other tree rings of both healthy trees, nuclei were present in the xylem parenchyma cells.

The youngest xylem parenchyma cells lacking nuclei were observed in dying ashes, already in the 37th tree ring (on the chart visible within the range: 31-40). The share of living cells did not consequently decrease in the subsequent tree rings, but unordered values occurred.

The least share of living parenchyma cells (i.e., 48%) was noted also in one of the dying ashes within 41-50 tree ring range.

Within a distance from cambium, nuclei tended to change their shape from fusiform to rounded: this referred mainly to procumbent cells elongated in radial direction (Fig. 1a). Square and procumbent cells, shorter in radial direction, contained rounded-shaped nuclei in every increment.

Starch grains were detected in parenchyma cells, both of axial and radial systems, in most of the tree increments of the studied sections. The most abundant in starch grains were cells in the youngest tree rings (Fig. 4). The largest drop was noted in approximately 10 oldest tree rings of declining tree ashes.

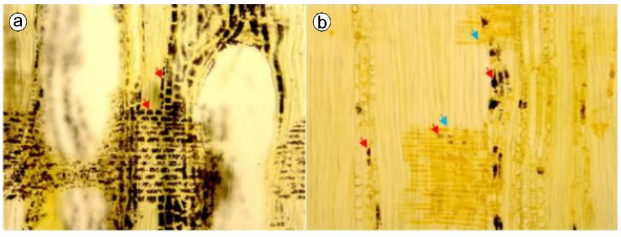

Fig. 4. Micrographs of a radial section of the 2nd (**a**) and 70th (**b**) tree ring of the ash wood stained with I_2KI solution to detect starch (example starch grains in axial and radial parenchyma cells indicated with red arrows). Blue arrows point at heartwood extracts. Wood samples obtained from the dying ash tree at the stem base (**a**) and 1.3 m (**b**). The left side of the micrograph refers to the side of the stem closer to cambium. Scale bars: 200 µm

The starch appeared mostly in the form of clusters, but also single grains were observed. Like nuclei observation, starch grains were detected, accompanied by heartwood extracts in the same cells.

A conspicuous feature of the studied sections were thick parenchyma cell walls at the very beginning of the xylem tissue, both axial and radial systems, in every studied tree. The Alcian Blue and Safranin O staining (Fig. 5a), as well as lignin autofluorescence observation (Fig. 5b), showed that this compound was present in the walls of all parenchyma cells.

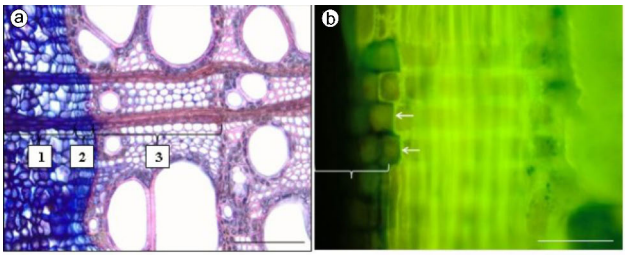

Fig. 5. Micrograph of a cross section of conductive tissues (**a**) stained with Alcian blue showing cellulose cell walls of phloem (indicated by the first bracket) and cambium (the second bracket) cells and safranin detecting lignified cell walls of the xylem cells (the annual xylem increment indicated by the third bracket). Autofluorescence of lignin (**b**) visible in radial section of the wood on the boundary with cambium (indicated with the white bracket). Lack of autofluorescence encompasses the zone of cambial cells (in particular differentiation zone). White arrows show lignified walls of ray parenchyma cells. Wood samples (**a**,**b**) derived from the healthy tree, the height of 1.3 m. The left side of the micrograph refers to the side of the stem closer to cambium. Scale bars: 200 µm (**a**), 50 µm (**b**).

Starting from cambium, it can be seen that parenchyma cell walls lignification takes place within the current increment, aproximately at the same time as in vessels and fibers, and irrespective of the neighborhood or lack thereof with the vessels of the wood (Fig. 5). It is noteworthy that every single parenchyma cell of axial and radial systems had lignified cell walls, while most of them remained alive. Lignin incrustation begins from the inner wall (situated further from cambium) of the parenchyma cells in each studied row (Fig. 5b).

DISCUSSION

The current research enabled us to follow a part of the xylem parenchyma cells life course in the ash tree stem, which, on the background of the literature data, presents itself remarkably. An interesting issue to consider at first is parenchyma cell walls lignification. According to the previous re-

search made on *Abies sachalinensis* and *Populus sieboldii* × *P. grandidentata*, xylem parenchyma cell walls lignification depends on positional information and lasts within two years, and they die no later than after ten years (Nakaba et al., 2006, 2012b) (Fig. 6). Lignification and PCD of parenchyma cells in Scotch pine xylem are nearly subsequently occurring events during heartwood formation (Bergström et al., 2003), whereas in sapwood, a few lignified parenchyma cell walls were also observed (Bamber and Fukazawa, 1985; Zimmer and Treu, 2021).

Lignin, as the second (after cellulose) most abundant terrestrial biopolymer (Boerjan et al., 2003; Barros et al., 2015), was considered to occur in xylem mainly in tracheary elements and fiber walls (Weng and Chapple, 2010). However, the European ash case proves that parenchyma cells contribute to the overall share of lignin in the wood. The current studies show that in European ash stems, all parenchyma cells had lignified cell walls immediately after leaving the cambium. Contrary to all previous research, parenchyma cell walls lignification, heartwood extracts synthesis, and parenchyma cells PCD are processes astoundingly distant from each other in time and space. Surprisingly, these cells, that started the cell walls lignification in the first increment, reach the age of 91. This phenomenon remains inconsistent with the common statement that lignification is a sign of the cell death (Bergström et al., 2003). Due to the fact, that in the studied species cell wall lignification occurs along with the outstanding longevity of nuclei, we assume that lignified cell walls seem to function as a shield protecting outstandingly long-lived protoplasts.

Considering recent research on zinnia (*Zinnia elegans*) and Norway spruce (*Picea abies* (L.) H. Karst) (Iakimova and Woltering, 2017; Blokhinaet al., 2019), ash parenchyma cells providing lignin for their own cell wall lignification do not seem to act as a good neighbor, while in the afore mentioned plants, lignin coming from parenchyma cells is supplied just to adjacent tracheary elements. This leads to the question, whether the good neighbor theory can be justified in the case of ash xylem parenchyma cells.

It is crucial to emphasize that nuclei are also detected in the parenchyma cells, in which storage material has been converted into heartwood extracts. This diverges with heartwood definition (IAWA 1964). A similar observation of living parenchyma cells containing heartwood extracts has already been made (Nobuchi et al., 1984). From the anatomical point of view, the wood zone, in which heartwood extracts appear, parenchyma cells remain alive, and the storage material is present, is the intermediate wood. Heartwood formation had already started, and this is indicated by the occurrence of the heartwood extracts themselves.

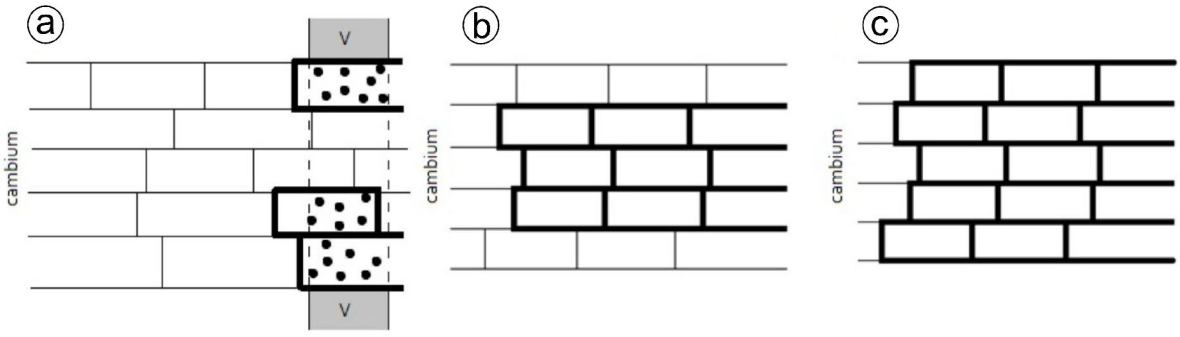

Fig. 6. Diagrams of ray parenchyma cell walls thickening and lignification in the current tree increment of different tree species. Squared boxes signify ray parenchyma cells and bold lines refer to lignified cell walls. Black dots in the subfigure (**a**) show half-simple pits, that enable connection of contact cells with a vessel element, and the grey tube in the background signed with the letter "V" presents a vessel element. (**a**) in *Populus sieboldii* × *P. grandidentata* parenchyma cells live 5 years at maximum and contact cell walls thicken and undergo lignification process at first (based on Nakaba et al., 2012b); (**b**) in *Abies sachalinensis*, cell walls' thickening and lignification is delayed at upper and lower lines of the ray; parenchyma cells live no longer than 10 years (based on Nakaba et al., 2006); (**c**) in *Fraxinus excelsior*, the case described herein, all parenchyma cells gained lignified cell walls simultaneously in the current tree increment and live for decades.

Dead parenchyma cells occurred earlier in declining ashes. On the basis of this observation, it can be concluded that the ash dieback disease, to some extent, can trigger modifications in development paths of these cells.

Nuclei changing their shape within a distance from cambium prove the senescence of the cells, and this observation is in line with other investigations (Nakaba et al., 2012b).

In the studied case, the detected life stages of xylem parenchyma include lignification as the first process after cambium leaving and, as the next step, heartwood extracts synthesis. Determination of most of the cells' lifespan was not possible. Some authors reported that longevity of axial parenchyma cells in ash stems amounted to more than 45 years (Spicer and Holbrook, 2007; Morris, 2016). In the case of black locust (*Robinia pseudoacaccia* L.), the xylem parenchyma cells were found to live for four years (Nakaba et al., 2012a) and in white ash (*Fraxinus americana* L.), they reach the age of 48 (Spicer and Holbrook, 2007). With regard to conifers, Sakhalin fir (*Abies sachalinensis* F. Schmidt) xylem parenchyma cells were reported to live for 10 years (Nakaba et al., 2006), in white pine (*Pinus strobus* L.) – 20 (Spicer and Holbrook, 2007) and in Scotch pine (*Pinus sylvestris* L.) – 42 years (Tulik et al., 2019). The obtained results for different tree species prove the remarkable longevity of European ash xylem parenchyma cells, all of which had lignified cell walls. In angiosperms trees, xylem parenchyma cells may live for 100 years (Spicer, 2005), and ash trees seem to reach this score.

The life course of xylem parenchyma cells, the only living cells, reflects the processes occurring in the wood, including the senescence of the whole tissue. Surprisingly, in ash stems, xylem parenchyma PCD is not related to the release of polyphenols and other compounds, significant for heartwood formation (Pinto et al., 2004). As has been noted, in trees affected by ash dieback disease, a tendency toward less survivability of xylem parenchyma cells is observed. The lowest survivability rate amounted to nearly 0.5, hence the living parenchyma cells still occurred abundantly.

This research provides a novel insight into heartwood formation, as most of xylem parenchyma cells remained alive while releasing of heartwood extracts. Furthermore, the main source of energy for the cell, which is starch, was present in the xylem parenchyma cells of the oldest tree increments. This provides further evidence for the longevity of the studied cells, which is the occurrence of metabolic reactions, for which energy stored in starch grains is used.

A considerable issue is the biological sense of such remarkable longevity of the parenchyma cells. Taking into account lignin features and the role fulfilled in tracheary elements, in the studied case we consider its main function as mechanical support. Lignin incrusting all cell walls of parenchyma cells, which constitute approx. 20% of the wood tissue (Morris, 2016), must strongly contribute to the stability of a tree.

CONCLUSIONS

Considering the mentioned research aims, only sapwood and intermediate wood were distinguished within the microscopic samples of the studied material. The presence of intermediate wood zone indicates initiation of the heartwood formation; however, heartwood in which all parenchyma cells were dead, was not observed. In terms of ash dieback disease, the first non-living xylem parenchyma cells appeared earlier in the dying trees, than in the healthy ones.

Moreover, the current research presents the phenomenon of the radial and axial parenchyma cells with lignified walls and extremely long-lived protoplasts. We conclude that it reveals a new case of the cells undergoing several decades of development, acting as an energy reservoir, as well as actively participating in strengthening the mechanical and protective functions of the complex wood tissue of European ash.

AUTHORS' CONTRIBUTION

MT designed the study conception. AB acquired funding to conduct the research. AB and MT performed data collection. AB prepared wood sections for microscopic investigation. AB conducted the research on nuclei and starch detection, analyzed obtained results and prepared photographs included in Fig. 1 and 3. MT investigated lignin presence, and took photographs included in Fig. 4. AB prepared illustrations for the manuscript. AB drafted and wrote the manuscript. MT provided the comments on the manuscript. Both authors read and approved the final version of the manuscript. The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

Special thanks to Prof. Vladimir Gryc and Ing. Radim Rousek from the Faculty of Forestry and Wood Technology at Mendel University in Brno for their invaluable help in preparing wood samples and to Dr. Rafał Wojtan for great assistance in statistical analysis. We also owe acknowledgements to the workers of the Forest District Łochów for obtaining the research material.This work was partially supported by Own Scholarship Fund of Warsaw University of Life Sciences SGGW.

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