

THE ANATOMY OF »BLUE RING« IN THE WOOD OF *PINUS NIGRA*ANATOMIJA »MODRE BRANIKE« V LESU ČRNEGA BORA *PINUS NIGRA*Alan Crivellaro^{1, 2*}, Marco Reverenna¹, Flavio Ruffinatto¹, Carlo Urbinati³, Alma Piermattei⁴

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Abstract / Izvleček

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Abstract: Tree-ring features are widely used to identify specific climate, environment and stress events affecting plant life. Cold temperatures can mainly affect the last phase of cell differentiation by interfering in the process of cell wall lignin deposition. Recently, it was found out that the effect of cold temperature on lignification is visible in wood microsections double stained with astra blue which stains cellulose, and red safranin which stains lignin. This new tree-ring feature was called »blue ring«. In this study, we investigated the anatomical morphology of blue rings in transverse and longitudinal wood microsections of three European black pine trees (*Pinus nigra* Arnold) growing above the tree-line. We aim to understand if the lack of lignification is consistent along the entire tracheid length and whether or not pits, responsible for water transport from one tracheid to another, are lignified. The results show that the lack of lignification extends along the entire tracheids cell wall, including bordered pits. Based on our observations we speculate that blue ring occurrence decreases the safety of water transport and wood mechanical properties.

Keywords: lignification, tree ring, pointer year, cell wall, tracheids, pits

Izvleček: Lesno-anatomske posebnosti branik pogosto uporabljamo za ugotavljanje specifičnih dogodkov v zvezi s podnebjem, okoljem in stresom, ki jim je izpostavljeno drevo med rastjo. Med pomembnimi dejavniki so nizke temperature, ki lahko vplivajo na diferenciacijo lesa, predvsem na odlaganje lignina. Nedavno so ugotovili, da je učinek nizkih temperatur na potek lignifikacije viden na mikroskopskih preparatih lesa, ki jih dvojno obarvamo s safraninom (ki obarva lignin rdeče) in astra modrim (ki obarva celulozo modro). To novo anatomsko posebnost so poimenovali »modra branika«. V študiji smo raziskali anatomsko zgradbo modrih branik na prečnih in vzdolžnih (radialnih) prerezih lesa črnih borov (*Pinus nigra* Arnold), ki so rasli nad zgornjo gozdno mejo. Cilj raziskave je bil pojasniti, ali je pomanjkljiva lignifikacija vidna po celotni dolžini traheid in ali so obokane piknje, ključne za prevajanje vode, lignificirane. Rezultati kažejo pomanjkljivo lignifikacijo vzdolž traheid in v obokanih piknjah. Na osnovi rezultatov sklepamo, da pojav modre branike zmanjšuje varnost transporta vode in vpliva na slabše mehanske lastnosti lesa.

Ključne besede: lignifikacija, branike, značilno leto, celična stena, traheide, piknje

1 INTRODUCTION

1 UVOD

Tree growth rate and xylem anatomical structure strongly depend on phylogeny, climate and local growing conditions (Schweingruber, 2007). Extreme climatic or environmental events are recorded in tree rings and are detectable in the wood structure (Wimmer, 2002). Bräuning et al. (2016) reviewed all tree-ring features visible in dendrochronological samples (e.g. missing rings, frost rings,

light rings, reaction woods, resin ducts) and suggested for each one an explanation of possible triggering factors. De Micco et al. (2016) focused on intra-annual density fluctuations (IADFs) reviewing how, when, where and why they are formed in tree rings of both hardwood and softwood species. Overall, these studies highlight the possibilities and the need to better understand the effects of climate and environment at intra-annual growth-ring level. Qualitative (Schweingruber, 2007; Schweingruber et al., 2013) and quantitative (von Arx et al., 2016) wood anatomical analyses are ideal tools to infer the factors causing structural changes in wood, because the position of each cell within a tree ring is a time marker of its formation within the growing season.

Within this context, softwood species are more often studied thanks to their homogenous anatomi-

¹ Dept. Territorio e Sistemi AgroForestali, University of Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy

² CNR IVALSIA, Sesto Fiorentino, Italy

* e-pošta: alancrivellaro@gmail.com

³ D3A, Marche Polytechnic University, Italy

⁴ Dept. of Geography, University of Cambridge, UK

cal structure (Evert, 2006). In a softwood transverse section, tracheids, radial and axial parenchyma cells are visible (Richter et al., 2004). Size, shape and distribution of these cells are usually investigated on transversal microsections (Carrer et al., 2016), however tangential and radial longitudinal sections are better suited to understand the three dimensional structure of wood as well as to visually separate wood cell types (Greguss, 1955; Schweingruber, 1978), and to quantify specific anatomical traits (Fonti et al., 2015; Lazzarin et al., 2016). Moreover, specific cell wall staining can inform about its chemical composition (Srebotnik & Messner, 1994): a double staining mix of red Safranin and Astra blue for instance highlights lignin and cellulose presence at once (Gärtner & Schweingruber, 2013). With such staining in a softwood microsection we expect to detect red-stained tracheid cell walls and blue-stained sapwood parenchyma cell walls (Schweingruber & Börner, 2018).

In a study on European black pine (*Pinus nigra* Arnold) growing above the forest line in the central Apennines (Italy), Piermattei et al. (2015) showed that some of the last formed latewood cells stained blue, as a result of an incomplete lignification process. This new anatomical feature was defined as 'blue ring': a continuous layer of unlignified axial tracheids occurring either in the earlywood or in the latewood (Piermattei et al., 2015). Recent technical improvements in the preparation of micro sections of entire increment cores (Gärtner et al., 2015), fostered the detection of blue rings (e.g. Carrer et al., 2016). A recent study conducted on several coniferous species in western North America used frost rings and blue rings to investigate adaptation of trees to cold climate, highlighting the presence of an environmental effect on cell wall lignification (Montwé et al., 2018).

Lignification is the last phase in the xylogenesis process. Xylem formation and tracheid differentiation in conifers are commonly divided in four stages: cambial cell division and post-cambial enlargement of the daughter cells; formation of multi-layered cell walls by polysaccharides macromolecules; deposition of lignin within the cell wall polysaccharides matrix; cell death and autolysis of the protoplasm. Various environmental factors such as temperature (Donaldson, 1992; Gindl et al., 2000, Gričar et al., 2005, 2006), day length (Gindl & Grabner 2000),

drought stress (Donaldson, 2002), and genetics (Donaldson, 1993) strongly influence cell wall lignification. Piermattei et al. (2015) in their study attributed the blue ring occurrence in the lastly formed latewood cells to temperature drop at end of the local growing season (end of October). However, other issues about blue rings still need to be investigated: i) does the lack of lignification involve other cell types besides axial tracheids? ii) is the blue ring consistent along all tracheids' length? iii) are bordered pits unlignified also in blue rings? iiiii) what are the possible implications in tracheids' mechanical properties, conduction efficiency and embolism safety?

Here we provide some preliminary answers by investigating the morphology of blue rings in transverse and longitudinal sections of European black pine in order to assess the blue ring consistency both across different cell types and within the same cell. Our results suggest that the blue ring occurrence can affect the hydraulic and mechanical properties of wood.

2 MATERIALS AND METHODS

2 MATERIAL IN METODE

To compare the anatomy and cell wall lignification in tracheid of lignified and unlignified rings, we investigated nine growth rings of three European black pine trees growing above the forest line on Mount Sirente (2348 m a.s.l., 42°15'N - 13°60'E) in the central Apennines. The age, height and DBH of investigated trees at the sampling time (2012), ranged from 17 to 19 years, 2.25 to 3.2 m and 14 to 19 cm respectively. Increment cores were extracted near the stem base of the trees with a 10 cm long borer. The blue rings occurred in 2006, 2007 and 2009.

A Reichert sliding microtome has been used to cut thin sections from the three cores. Cross sections were cut upon application of a 10:8:7 mixture of corn starch, water and glycerol to avoid mechanical damage to cellular structure (Schneider & Gärtner, 2013). Longitudinal sections were cut at 15-25 micrometres in thickness to ensure the best visibility of minute anatomical features.

All sections were bleached with sodium hypochlorite (60%), stained in a mixture of Safranin and Astra blue (1 g of Safranin powder in 100 ml of distilled water; 0.5 g of Astra blue powder and 2 ml of acetic acid in 100 ml of distilled water; 1:1 ratio),

dehydrated with an ethanol series (50%, 75%, 96%), treated with the clearing agent Bioclear and permanently mounted with Eukitt (Bio-Optica). Light microscopy observations were carried out on a Nikon microscope (Eclipse 80i, Nikon, Tokio, Japan), and images were recorded with a high-resolution camera (2.07 pixels/ μm) mounted on the same microscope.

Wood anatomical descriptions followed the IAWA list of microscopic features for softwood identification (Richter et al., 2004). Anatomical features of the blue rings were compared to the ones of adjacent growth rings. Results are presented in a descriptive form, while Table 1 highlights the observed differences.

3 RESULTS

3 REZULTATI

On sanded transverse sections, growth rings are macroscopically visible thanks to an abrupt change in colour intensity from the lighter earlywood to the darker latewood (Ruffinatto et al., 2015). The blue rings did not show any macroscopic difference compared to previous and following rings

(Piermattei et al. 2015), the color difference between earlywood and latewood is not affected by the presence of a blue ring. To this respect, blue rings differ from the so-called light rings, featuring a latewood zone with thin-walled cells, macroscopically detected in trees at northern and subalpine treelines (Filion et al., 1986; Montwé et al., 2018).

A microscopic transverse view of blue ring tracheids (Fig. 1; Tab. 1) showed blue-stained inner cell wall layers, as opposed to the red-stained fully lignified tracheid cell walls in regular rings (Fig. 1A). Unlignified cell wall areas usually include the layers towards the cell lumen only (Fig. 1C), rarely extending towards the middle lamella (Fig. 1C). In the most extreme cases where lignification lacks, the red-stained areas are located near the tracheid corners (Fig. 1D). Although the single cell wall layers (M, P, S1, S2, S3, Tab. 1) normally cannot be differentiated by using light microscopy (Prislan et al., 2009) we interpreted the outermost cell wall layers M, P and S1 as one cell-wall complex, while S2 and S3 were easier to distinguish due to the higher thickening of S2 compared to S3.

Blue ring axial tracheids appear also in longitudinal radial sections with different degrees of ligni-

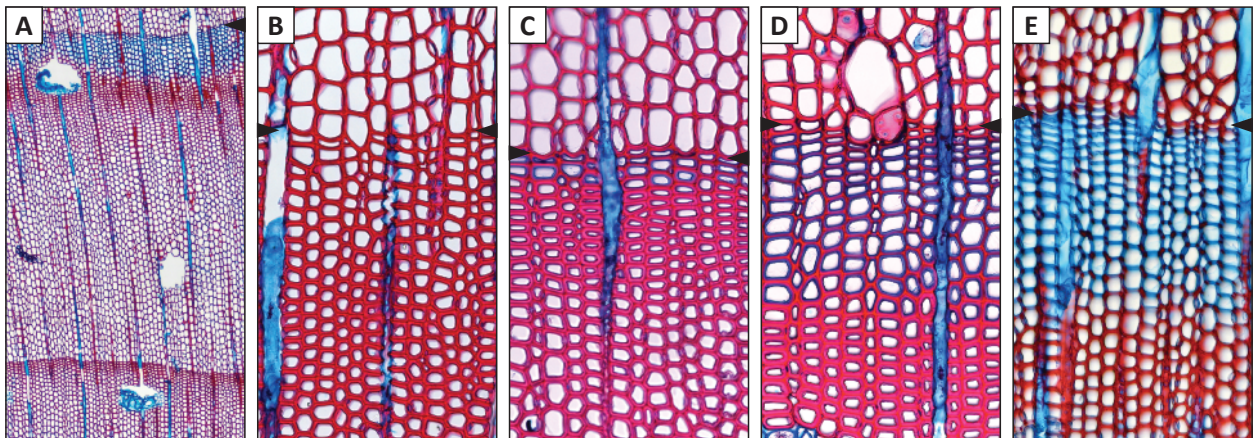


Figure 1. Cross sections of different European black pine (*Pinus nigra* Arnold) trees. (A) complete tree ring with a blue ring; (B) standard latewood and (C-E) blue rings with (C) few axial tracheids lacking lignin only in the innermost wall layer; (D) with a larger number of tracheids partly lacking lignification and (E) last formed unlignified tracheids, with lignin present only at cell corners. Black arrows indicate tree-ring boundaries.

Slika 1. Prečni prerezi lesa različnih dreves črnega bora (*Pinus nigra* Arnold). (A) izgotovljena branika z modrim pasom; (B) običajen kasni les ter (C-E) modre branike. (C) v nekaj zadnjih traheidah branike je lignifikacija delno izostala v notranjih slojih celične stene, (D) lignifikacija je delno izostala v širšem pasu traheid ter (E) zadnje nastale traheide so v splošnem nelignificirane, nekaj lignina je le v celičnih vogalih. Črne puščice kažejo letnice.

fication (Fig. 2 A-D) from fully lignified (Fig. 2A), to partially lignified where only innermost cell wall layer is lacking lignin (Fig. 2B), to larger number of cells included in the blue ring (Fig. 2C). Bordered pits of axial tracheids in blue ring show unligified pit borders (Fig. 2C; 2D), as opposed to fully lignified bordered pits in standard wood. In the observed samples the torus is always non-lignified.

As opposed to axial tracheids, blue ring ray tracheids do not show any difference compared to standard ones. The same holds true for ray parenchyma cell walls that are normally not lignified and stain blue.

4 DISCUSSION

4 RAZPRAVA

Our results show that the reduced lignification occurring in blue rings concerned axial tracheids and their bordered pits, but not parenchyma ray cells,

nor radial tracheids. This xylogenetic phenomenon can be related to decreasing temperature during the cell maturation phase (Piermattei et al., 2015). The consequences of unligified axial tracheids in blue rings deserve further analysis because they can affect their hydraulic and mechanical properties.

The hydraulic function of conifer tracheids provides water transport under strong negative pressure and limit cavitation spread over the sapwood (Choat et al., 2008; Pittermann et al., 2006). A quantification of the strength of axial tracheids cell walls is provided by the thickness-to-span ratio (Hacke et al., 2001). However, this index implicitly assumes that cell wall mechanical properties are constant, which is not the case for unligified cell walls in which the lack of lignin decreases their rigidity and strength (Voelker et al., 2011). Therefore, unligified cell walls in the blue rings may increase the probability of mechanical failure under the negative pressure needed for water transport from the soil to the leaves.

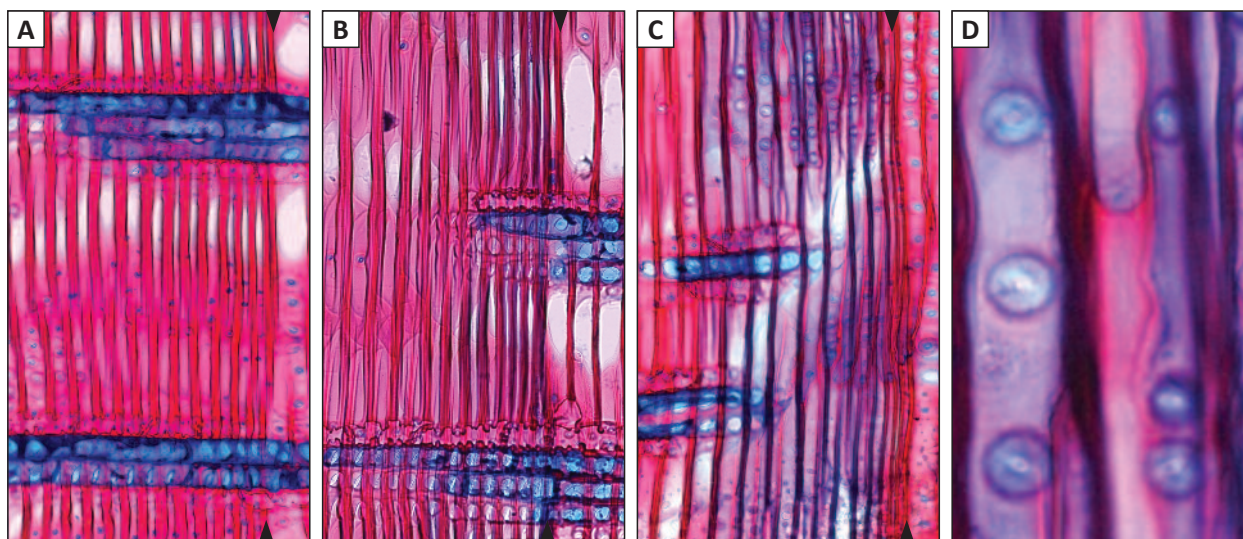


Figure 2. Longitudinal radial wood sections of different European black pine (*Pinus nigra* Arnold) trees (A, B, and C) showing different degrees of lignification; (A) completely lignified tracheids with latewood tracheids stained red, torus of axial tracheids bordered pits unligified (blue stained), parenchyma cells of the rays unligified (blue stained); (B) decreasing lignification towards the formed last axial tracheids; (C) larger number of unligified tracheids; (D) bordered pits of unligified axial tracheids (at larger magnification). Black arrows indicate tree-ring boundaries.

Slika 2. Vzdolžni radialni prerezi lesa različnih dreves črnega bora (*Pinus nigra* Arnold) (A, B in C) z različno stopnjo lignifikacije celic. (A) popolnoma lignificirano tkivo, kjer so traheide kasnega lesa obarvane rdeče, torusi obokanih pikelj aksialnih traheid so nelignificirani (modri), parenhimske celice trakov pa so nelignificirane in obarvane modro; (B) stopnja lignifikacije upada v smeri proti zadnjim aksialnim traheidam v braniki, (C) večje število nelignificiranih traheid; (D) obokane piknje nelignificiranih aksialnih traheid pri večji povečavi. Črne puščice kažejo letnice.

Table 1. Observed anatomical features on double stained transverse and longitudinal sections of Pinus nigra wood in regular and blue rings.

Preglednica 1. Anatovski znaki na dvojno obarvanih prečnih in vzdolžnih prerezih lesa normalnih in modrih branik črnega bora (Pinus nigra).

Anatomical feature		Regular tree ring	Blue ring	Figures
Axial tracheid cell wall layers	Middle lamella - M	Lignified	From lignified to partially unlignified	Fig. 1D
	Primary wall - P	Lignified	From lignified to unlignified	Fig. 1D
	Secondary wall - S1	Lignified	From lignified to unlignified	Fig. 1D
	Secondary wall - S2	Lignified	From lignified to unlignified	Fig. 1C, 1D
	Secondary wall - S3	Lignified	Unlignified	Fig. 1B, 1C, 1D
Bordered pits in axial tracheids	Pit borders	Lignified	From lignified to unlignified	Fig. 2C, 2D
	Torus	Present, disc-shaped, unlignified	Present, disc-shaped, unlignified	Fig. 2D
	Tracheid pitting in radial wall in earlywood and in latewood	Uniseriate	Uniseriate	Fig. 2A
Ray tracheids	Ray tracheids	Present, lignified	Present, lignified	Fig. 2B, 2C
	Pit borders	Lignified	Lignified	Fig. 2B, 2C
	Torus in ray tracheids	Present, unlignified	Present, unlignified	Fig. 2C
	Indentures on cell walls of ray tracheids	Present, lignified	Present, lignified	Fig. 2B, 2C
Ray parenchyma cells	Cell walls of ray parenchyma cells	Unlignified	Unlignified	Fig. 2A, 2B, 2C

Bordered pits are canals in the secondary cell walls of xylem tracheids with a pit border, composed of secondary wall that overhangs and partially encloses the pit cavity (Beck, 2010). The primary walls and intervening middle lamellae of two opposing cells make up the pit membrane, which lies in the centre of the pit pair (Choat et al., 2008; Li et al., 2016). In most conifers, the pit membrane has two distinct regions. The central region consists of a lenticular thickening, called the torus, which is considered impermeable, while the outer region of the membrane, the margo, is porous and permeable to xylem sap. Pits' features greatly affect cavitation resistance of connected tracheids (Jansen et al., 2012). In fact, unlignified pit border may weaken the bordered pits to the point that their ability to limit spread of cavitation to adjacent cells might be reduced or even compromised. In fact, to be fully functional, the pit border and the torus should seal together, enclosing the air bubble within a limited number of tracheids (Domec et al., 2006; Delzon et

al., 2010). In the analysed samples blue rings occurred in the latewood portion, where the hydraulic function is less important (Domec & Gärtner, 2002). However, Piermattei et al. (2015) found blue rings located also in earlywood where the effects on sap uplift efficiency and safety can be more relevant (Carrer et al., 2016).

Softwoods' mechanical properties are known to be very high in relation to their density (FPL, 2010) due to their unique anatomical structure and to their cell wall composition. The reduced lignification observed in blue rings could negatively affect wood mechanical strength. The occurrence of blue rings could be envisaged as a spot of structural weakness in tree stems as well as in timber. Unfortunately, blue rings cannot be easily detected in timber, but their effect might be visible as ring-shakes, which are known to occur in some softwood species and have been attributed to cold events (Laacke, 1990).

It is not so evident why wood formation ends so early in the growing season, especially in cold envi-

ronments such as at high elevation. Previous studies showed that maturation and lignification of last formed axial tracheids in the current year tree ring is completed much later than the end of cambial production of wood are recorded (Gričar et al., 2014; Schmitt et al., 2003). Our observations show that early ending of wood production leaves enough time to complete cell wall differentiation up to full lignification, before air temperatures decrease significantly, withdrawing lignin deposition. In this way, there is a sufficient amount of time for cell walls to build a hydraulically and mechanically efficient wood.

5 CONCLUSION

5 SKLEP

The effects of climate change on woody plants include a wide variety of consequences and interactions. As climate warming and changing phenology interact with increasing temperature variability, frost damage is expected to keep being an issue in the future (Montwé et al., 2018). Our study on blue ring anatomy pointed out the important consequences of a lack of lignification caused by an abrupt cold event (Piermattei et al., 2015) with lower temperatures affecting new-formed xylem cells in the final stage of lignification. Such consequences involve both hydraulic functioning of the plant and mechanical properties of its wood, and therefore timber. Further analyses are needed to quantify the loss of hydraulic efficiency in trees with blue rings.

6 SUMMARY

6 POVZETEK

Rast dreves in anatomija lesa sta odvisna od fitogenije, podnebja in rastiščnih razmer. Ekstremni klimatski ali okoljski dogodki se zabeležijo v anatomskih posebnostih lesa in jih je mogoče prepoznati pod mikroskopom. Kvalitativne in kvantitativne lesno-anatomske raziskave omogočajo prepoznavanje dejavnikov, ki povzročajo strukturne spremembe v lesu, z določitvijo njihovega položaja v branikah pa je mogoče ugotoviti, kdaj (v katerem letu in sezoni) so nastale. Položaj posamezne celice znotraj branike zato predstavlja časovni mejnik nastanka lesa oz. posameznega znaka v rastni sezoni. Les iglavcev je zaradi homogene anatomske zgradbe v tem pogledu boljše raziskan kot les listavcev. Poleg beleženja po-

sebnih struktur na nivoju celic in tkiv lahko spremljamo tudi posebnosti celične stene. Dvojno barvanje z barviloma safranin, ki obarva lignin rdeče, in astra modro, ki obarva celulozo modro, v lesno-anatomski praksi uporabljamo za oceno kemijske zgradbe celične stene. Dve barvili uporabimo zaporedno ali hkrati in tako na istem preparatu poudarimo prisotnost lignina in celuloze.

V predhodnih raziskavah lesa črnega bora (*Pinus nigra* Arnold), iz dreves, ki rastejo nad gozdno mejo v osrednjih Apeninih v Italiji, so zabeležili, da se je nekaj nazadnje oblikovanih traheid v braniki obarvalo modro, kar nakazuje nepopolno lignifikacijo celičnih sten. To novo anatomsko posebnost, kjer se pas lesa v braniki obarva modro, so poimenovali »modra branika« (ang. blue ring). Modra branika torej vsebuje strnjen sloj pomanjkljivo lignificiranih aksialnih traheid, ki se lahko pojavijo v ranem ali kasnem lesu (Piermattei et al., 2015). Pojav, ki ga ne vidimo s prostim očesom ali lupo (makroskopsko), je bil doslej raziskan predvsem na mikroskopskih preparatih prečnih prereзов.

V tem prispevku prvič predstavljamo morfološko modrih branik na prečnih in vzdolžnih (radialnih) prereзов lesa črnega bora, da bi ocenili prostorsko porazdelitev modrih nepopolno lignificiranih območij v tkivih in posameznih celicah. V ta namen smo raziskali devet modrih branik iz debel treh dreves črnega bora, ki so rasla nad zgornjo gozdno mejo na gori Monte Sirente v osrednjih Apeninih. Izdelali smo preparate vzdolžnih (radialnih in tangencialnih) prereзов modrih in normalnih branik. Preparate smo obarvali v mešanici barvil safranin in astra modro, da bi ločili lignificirane in nelignificirane celice. Celice in tkiva v modrih branikah so bila anatomsko podobna tistim v sosednjih branikah, razlike so bile samo v stopnji lignifikacije. Naši rezultati kažejo, da je pomanjkljiva lignifikacija v modrih branikah značilna za aksialne traheide in njihove obokane piknje, ne pa za parenhimske celice trakov (ki so po naravi manj lignificirane) in za trakovne traheide.

Naši rezultati nakazujejo tudi najverjetnejše posledice pojava modrih branik (oz. pomanjkljive lignifikacije) za hidravlične in mehanske lastnosti lesa. Nelignificirane aksialne traheide so manj trdne, zato obstaja nevarnost porušitve traheid zaradi tenzije vodnih stolpcev, povezane s prevajanjem vode. Slednje bi lahko oviralo prevajanje vode v drevesu. Pomanjkljiva lignifikacija pikenjskih obokov bi lahko

zmanjšala možnost omejevanja kavitacij (širjenja zračnih mehurčkov), ki se lahko razširijo v sosednje celice in jih onesposobijo za prevajanje vode. Pomanjkljiva lignifikacija traheid tudi zmanjšuje trdnost lesa. Glede na navedeno menimo, da modre branike najverjetneje predstavljajo šibka mesta v lesu s hidravličnega in mehanskega vidika.

Modre branike žal niso vidne s prostim očesom ali lupo, zato jih v lesu ne prepoznamo kot napako ali možen vir napak. Vidimo lahko samo njihov učinek, saj vplivajo na nastanek napak kot so kolesivost (krožne razpoke), ki jih pri iglavcih pogosto štejejo za posledico nizkih temperatur.

Ker segrevanje podnebja in spreminjanje fenologije vplivata na vse večjo variabilnost temperature, pričakujemo, da bo v prihodnosti škoda zaradi zmrzali predstavljala vedno večji problem. Naša študija anatomije modrih branik nakazuje kakšne bi lahko bile posledice pomanjkljive lignifikacije, ki je posledica prekinitve procesa lignifikacije zaradi kratkotrajnih obdobij z izredno nizkimi temperaturami.

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