



RESEARCH ARTICLE

Analysis of sinapyl alcohol deposition patterns and lignification regulatory mechanisms in hardwoods using alkynyl-labelled sinapyl alcohol combined with click chemistry technology

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Received: 16 December 2025; Accepted: 05 May 2026; Available online: Version 1.0: 27 August 2026

Cite this article: Xingru W, Dongsheng Z, Haitao Y, Lan Y. Analysis of sinapyl alcohol deposition patterns and lignification regulatory mechanisms in hardwoods using alkynyl-labelled sinapyl alcohol combined with click chemistry technology. Plant Science Today (Early Access). <https://doi.org/10.14719/pst.13230>

Abstract

Lignin is a major cell wall component in vascular plants and the second most abundant biopolymer accounting for 15–30 % of woody biomass. It maintains mechanical strength, supports xylem water transport, and contributes to defense against biotic stresses. Sinapyl alcohol is a major lignin precursor, but its deposition mechanism in hardwoods remains underexplored. In this study, an alkynyl-labelled sinapyl alcohol (S_{ALK}) probe was synthesised. Click chemistry combined with bioorthogonal labelling was applied to develop a visualisation system for tracking lignin monomer deposition in plant cell walls, enabling analysis of lignification mechanisms. Results showed that in *Nerium oleander*, fibre tracheids near the pith were less lignified, while lignin was deposited continuously in ray parenchyma cells. Lignin deposition starts at cell corners and middle lamellae and then moved to the secondary walls. *Broussonetia papyrifera* showed unique lignification process, in which vessel elements lignified non-autonomously, offering an ideal model for studying non-autonomous lignification. For the first time, the alkyne-tagged system developed in this study dynamically visualised sinapyl alcohol deposition in hardwood, revealing lignification differences across developmental stages and tissue types. It provides new technical insights into the autonomous and non-autonomous regulation of plant cell wall lignification.

Keywords: bioorthogonal labelling; click chemistry; hardwood; lignin deposition; non-autonomous lignification; plant cell walls; sinapyl alcohol

Introduction

The 2022 Nobel Prize in Chemistry recognised the development of click chemistry and bioorthogonal chemistry, which enable rapid and selective molecular labelling in complex biological systems. Click chemistry enables rapid assembly of molecular probes, while bioorthogonal reactions allow labelling within living systems without interfering with native biochemical processes (1). The combination of click chemistry and bioorthogonal chemistry has led to significant advances in animal studies and holds great promise for plant science, especially in lignification research.

Early studies introduced phenolic alcohol analogs as chemical reporters to track lignin deposition in plant cell walls (2, 3). Subsequent advances incorporated dual- and triple-labelling strategies, enabling the simultaneous visualisation of multiple lignification events with high spatial resolution (4). Recently, a triple bioorthogonal labelling strategy combined with super-resolution Airyscan microscopy has enabled the parsing of lignin and non-cellulose polysaccharide co-deposition patterns at the subcellular level. The REPRISAL method, integrating fluorescence labelling and AI-based image segmentation, has successfully mapped lignification kinetics in different parts of *Arabidopsis* and *poplar* cells, helping to identify key enzymes involved in the process (5).

Several techniques have been developed to visualise or quantify lignin deposition. Conventional histochemical staining methods, such as phloroglucinol-HCl and Mäule staining, are simple and rapid but lack monomer specificity and cannot distinguish newly deposited lignin from pre-existing lignin (6). Spectroscopic and degradative methods, including pyrolysis-GC/MS and thioacidolysis, provide detailed compositional information but are bulk analytical techniques that destroy spatial context (7). More recently, click-compatible monolignol probes have emerged as powerful tools for tracking lignin deposition. For example, propargyl-labelled coniferyl alcohol has been successfully used to visualise G-type lignin in flax and *Arabidopsis* (2). However, a probe specifically designed for syringyl (S)-type lignin, the predominant lignin in hardwoods and its systematic application to track lignification dynamics across different developmental stages and tissue types in hardwoods has not been reported (8). Our study fills this gap by synthesising an alkynyl-labelled sinapyl alcohol (S_{ALK}) probe and applying it to visualise S-lignin deposition in two representative hardwood species, *Nerium oleander* and *Broussonetia papyrifera*.

Syringyl -type lignin, characterised by its unique dimethoxy aromatic ring, plays a critical role in determining lignocellulose degradability and high-value utilisation potential. Manipulating

key genes such as F5H to raise the S/guaiacyl (G) ratio to 98 % reduces lignin cross-link density, concomitantly enhancing alkaline pretreatment efficiency and enzymatic saccharification yields (9), while reducing non-productive cellulase binding to optimise energy input and processing costs in biorefineries (10). Deciphering the regulatory circuitry that governing S-lignin deposition in *Arabidopsis* (11) provides molecular targets for breeding energy crops that combine high stress tolerance with superior industrial value.

Previous research on lignin deposition mechanisms mainly focused on plants like *flax*, *Arabidopsis*, *tobacco* and *ginkgo* (12), with less attention given to hardwood species. This study selected *N. oleander* seedlings, 10-year-old *N. oleander* and *B. papyrifera* seedlings as research objects. Previous studies have confirmed that *N. oleander* serves as an ideal model plant for investigating lignin crosslinking mechanisms in planta (13). *Broussonetia papyrifera* was selected as an additional model because it exhibited the greatest difference in lignification trend compared with *N. oleander*, thereby expanding the taxonomic coverage of our investigation (14). Based on previous observations, we formulated the following three hypotheses: (i) fibre tracheids and ray parenchyma cells in the xylem of hardwoods exhibit different lignification patterns; (ii) lignification patterns change with plant age; (iii) different hardwood species employ distinct lignification regulatory mechanisms. The study cultivated these plants in a medium with artificially synthesised labelled lignin precursors, using propargyl-labelled coniferyl alcohol as control. Confocal microscopy was used to observe plant sections and investigate lignin deposition patterns in hardwood species.

Materials and Methods

Reagents and chemicals

The reagents employed in this study were all obtained from Aladdin. The cuttings of *N. oleander* and *B. papyrifera* were harvested from Hubei University of Technology (Wuhan, China; coordinates: 30°29' N, 114°18' E; elevation: 25 m; mean annual temperature: 17.2 °C; mean annual precipitation: 1300 mm). Voucher specimens (*N. oleander*: HBUT-MO; *B. papyrifera*: HBUT-BP) have been deposited in the Herbarium of the School of Bioengineering and Food Science, Hubei University of Technology. The *B. papyrifera* samples were collected from the apical tissues of the stems, while the *N. oleander* seedling samples were from 2-year-old plants. Both were cultivated through the sectioning method. In contrast, samples from 10-year-old *N. oleander* branches were obtained using transpiration-driven cultivation method, which involved submerging the branches into the culture medium. A seven-step synthesis of alkyne-labelled S-type lignin monomer S_{ALK} from 2,4-dihydroxybenzaldehyde via bromination, methoxylation, hydroxyl protection, propargylation, Horner-Wadsworth-Emmons olefination, deprotection and DIBAL-H reduction. The overall yield of this route was 21 % (15).

Cultivation protocol for 2-year-old *Nerium oleander* and *Broussonetia papyrifera* stem sections

Healthy 2-year-old *N. oleander* and *B. papyrifera* plants were selected, both of which had differentiated primary xylem but secondary walls that were not yet fully thickened. Stem segments approximately 10 cm below the shoot apex were collected. This region is relatively soft and amenable to manual sectioning.

Sections approximately 200 μ m thick were prepared manually using a surgical blade. One stem segment was collected from each plant and approximately 200 sections were prepared, from which slices of uniform thickness and intact morphology were selected for further use. The selected slices were temporarily placed in cell culture plates containing Murashige and Skoog (MS) solution. A 100 μ M S_{ALK} solution was prepared using MS solution. In a 48-well cell culture plate, 300 μ L of the 100 μ M S_{ALK} solution was added to each well, followed by 8–10 stem sections per well. A total of 10 biological replicates were used. A negative control group (MS solution without S_{ALK}) was also included, with the same number of 10 biological replicates per treatment. Each biological replicate corresponded to one independent culture well. The concentration of S_{ALK} (100 μ M) used in this study was determined in our previous study on flax (15). At this concentration, S_{ALK} provided sufficient fluorescence signal intensity while avoiding obvious inhibitory effects on cell viability or the lignification process.

Cultivation and sampling of 10-year-old *Nerium oleander* branches

10-year-old *N. oleander* branches were selected from the plants at the mature xylem stage. The branches were placed in 300 mL of S_{ALK} solution at a concentration of 1 g/L (the molecular weight of S_{ALK} is 264 g/mol, corresponding to approximately 3.79 mM). After the S_{ALK} solution was completely absorbed, fresh distilled water was added daily. Cultivation was carried out under conditions of 25 °C, 50 % relative humidity and a 16 hr light/8 hr dark photoperiod. A total of 10 biological replicates (i.e., 10 independent branches) were used. Control groups were established as follows: cellulase-enzymatic lignin (CEL)_{Blank} was cultured with distilled water without any monomer supplementation; CEL_{Control} was cultured with non-alkynyl-labelled sinapyl alcohol.

After cultivation, a section from the middle of each branch was cut into 100- μ m-thick slices using a freezing microtome (Leica CM1950). The slices were placed in a 6-well cell culture plate containing 4 mL of MS solution and shaken at 180 rpm for 10 min twice to remove impurities.

The samples were designated as follows: CEL₁–CEL₄ for 1- to 4-week cultivation periods, respectively; CEL_{Blank} for the control without any monomer supplementation; and CEL_{Control} for culture supplemented with sinapyl alcohol without alkynyl.

Click chemistry reaction conditions

Freshly prepared CuAAC solution (containing 12.5 mM ascorbic acid, 2.5 mM copper sulfate and 25 μ M Azide-fluor 545 in MS solution) was used for all reactions. All washing and reaction steps were performed in the dark at room temperature (22–25 °C) with shaking at 180 rpm. After click reaction, unbound fluorophores were removed by washing with MS solution, followed by methanol-water washing to reduce non-specific background fluorescence. All slides were observed under a confocal laser scanning microscope (CLSM) and images were analysed using ImageJ software.

For 2-year-old seedling sections (200 μ m thickness)

After incubation, the S_{ALK} solution was aspirated. The slices were washed eight times with 1 mL of MS solution by shaking for 10 min each time. Then, 300 μ L of CuAAC solution (containing 25 mM ascorbic acid, 5 mM copper sulfate and 50 μ M Azide-fluor 545 in MS solution) was added to each well and shaken for 1 hr. The CuAAC treatment at the concentrations used in this study has no obvious

cytotoxicity to plant cells (2). The CuAAC solution was removed and the slices were washed twice with 1 mL of MS solution for 10 min each time. Subsequently, the slices were washed with 500 μ L of 70 % (v/v) methanol-water solution for 1 hr, followed by a second wash for 30 min.

For 10-year-old branch sections (100 μ m thickness)

After cultivation, the MS solution was aspirated and 1.5 mL of CuAAC solution (containing 12.5 mM ascorbic acid, 2.5 mM copper sulfate and 25 μ M Azide-fluor 545 in MS solution) was added. The mixture was shaken for 1 hr. After the click reaction, the CuAAC solution was removed and the slices were washed with 2–3 mL of MS solution three times, each wash lasting 10 min. Then, the slices were washed with 1.5 mL of 70 % methanol-water solution four times, each for 20 min.

After washing, the slices were either stored in an antifluorescence quencher or immediately mounted on slides. Slides were prepared carefully to avoid air bubbles under the coverslip.

Confocal microscopy imaging and image analysis

Confocal microscopy was performed using 20 $\times$ air objective and 63 $\times$ oil-immersion objective to observe plant cell imaging. For the lignin autofluorescence channel, the excitation wavelength was 405 nm and the emission wavelength was 450 nm, with the laser power set to 5–10 % and gain set to 600–800 V. For the Azide-fluor 545 channel, the excitation wavelength was 561 nm and the emission wavelength was 595 nm, with laser power set to 10–15 % and gain set to 650–850 V. For Z-stack acquisition, the Z-step size was set to 1–2 μ m and the number of slices was determined based on sample thickness. After image acquisition, image analysis was performed using ImageJ software.

Extraction of cellulase-enzymatic lignin from vascular cambium of 10-year-old *Nerium oleander* branches

After *N. oleander* branches had completed their growth under artificial lighting and constant-temperature conditions, the bark was rapidly peeled off and dried. The vascular cambium, approximately 5 mm thick, was scraped off. The resulting powder was collected and extracted in a mixture of benzene and ethanol (volume ratio of 1:1). The extracted wood powder was defatted, removing resins, fats and waxes. Subsequently, the wood powder was milled by a planetary ball mill (RETSCH PM 100). The wood powder was then subjected to enzymatic hydrolysis with 70 U/mL cellulase loading (from *Trichoderma reesei*) at 150 rpm and 50 $^{\circ}$ C for 24 hr. After one round of hydrolysis, the products extracted by centrifugation were hydrolysed again with cellulase under the same conditions. The residue was then centrifuged and washed three times with distilled water until the supernatant was clear. Subsequently, phosphate-buffered solution with a pH of 7.4 was prepared and 1 U/mL proteinase K was added to a buffer solution with a concentration of 50 mM. The constant-temperature stirring was set at 150 rpm and 37 $^{\circ}$ C for 24 hr. After incubating with proteinase to remove residual cellulase, the samples were placed in a water bath at 90 $^{\circ}$ C for 2 hr. The precipitate was washed with deionised water until the supernatant was clear and finally freeze-dried to obtain a solid. In the next step, the solid was extracted for 24 hr at room temperature with a mixture of 96 % dioxane and water (volume ratio of 1:10) and the resulting CEL was obtained by rotary evaporation.

Fluorescence spectroscopic analysis

To obtain the fluorescence emission spectrum of CEL, the compound was dissolved in DMSO to a concentration of 5 mg/mL. Measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer (Japan) under the following parameters: excitation wavelength 561 nm, emission scan range 500–700 nm, scan speed 1200 nm/min and slit width 5 nm. Each sample was scanned 3 times and the averaged spectrum was used for analysis. Baseline correction was applied by subtracting the emission spectrum of control (pure DMSO). A total of 3 biological replicates were used, with each biological replicate containing 3 technical replicates.

Processing of confocal fluorescence images

The average fluorescence intensity of 10 parallel samples was taken as the fluorescence intensity of the experimental group for normalising fluorescence intensity against tissue area. For normalising fluorescence intensity against background, background correction and Gaussian Blur were applied. Specifically, CLSM images were opened in LAS X and the required images were selected. The 405 nm, 552 nm channels and merged images were then saved into folders. In ImageJ, the images were opened via File-open (image which need measure). For background correction, Image - Duplicate; Process - Filters - Gaussian Blur; Process - Image Calculator - Operation (subtract) and create new window. Then measure fluorescence intensity: Image - Type - 8 bit; Image - Adjust - Threshold - set - OK; Analyse - Measure - Results (Mean). For analysis of Fluorescence intensity of dot/line in images, click: Analyse - Measure - Results (Mean) to get dots fluorescence intensity. Analyse - Plot Profile click List - File - Saved as to get lines fluorescence intensity change graph. Statistical analysis was performed by IBM SPSS Statistics 27.0 (IBM Corp., Armonk, NY, USA).

Nuclear magnetic resonance (NMR) detection

Two-dimensional NMR detection

A 50 mg of CEL was dissolved in 0.4 mL of DMSO- d_6 . NMR of Bruker Avance 600 MHz NMR spectrometer at 298 K was employed. The experiment employed the Bruker standard pulse sequence, with a ^1H NMR spectral width of 11 ppm and 2048 data points collected. ^{13}C NMR spectral width of 190 ppm and 256 data points were collected, with a total of 56 scans and a delay time of 1 sec. Data processing was performed using MestReNova software.

Semi-quantitative calculation for lignin in NMR spectra

The relative quantitative method involves calculating the relative proportion of each type of linkage unit in the total linkages based on the signal intensity of each linkage unit. This method can determine the relative content of various linkages in lignin samples. The percentage of a specific linkage type k can be calculated using the following formula: $k\% = (I_k / (I_A + I_B + I_C + I_D)) \times 100\%$. Here, I_A , I_B , I_C and I_D represent 2D NMR signals of α -position linkages, including β -O-4, β - β' , β -5 and β -1 linkages, respectively.

Statistical analysis

All data are presented as mean $\pm$ SD. One-way ANOVA followed by the Wallace-Duncan method was used for multiple comparisons ($p < 0.05$) and Student's t -test was used for two-group comparisons. The number of biological replicates ($n = 3$) and technical replicates ($n = 8$ –10) is indicated in the figure legends. Statistical analyses were performed using SPSS 26.0.

Results and Discussion

Fluorescence spectroscopy detection of cellulase-enzymatic lignin

To verify the incorporation of the alkyne group into CEL and its ability to undergo click reaction with AF545, both control and experimental groups were subjected to click labelling. Fluorescence intensity was measured at an excitation wavelength of 561 nm. As shown in Fig. 1, the results indicated that synthesised alkyne-tagged precursor can undergo click reaction with AF545 in plant tissues and be successfully detected. The fluorescence intensity of CEL extracted from *N. oleander* branches after S_{ALK} cultivation was higher than that of blank and control group, while labelled CEL_{blank} (cultivated with water) and $CEL_{control}$ (cultivated with sinapyl alcohol) only exhibited weak fluorescence, which may be due to a small amount of non-specific binding of fluorescent groups. The overall fluorescence intensity of CEL was relatively low compared to synthetic controls. One possible explanation is that the alkyne group may undergo side reactions during enzymatic lignification, such as with quinone methide intermediates (16). However, direct evidence for this mechanism is lacking in the present study and other factors, such as incomplete probe penetration or differential incorporation efficiency, cannot be excluded.

Two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance analysis of cellulase-enzymatic lignin

Two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance (2D-HSQC NMR) technology was employed to systematically analyse molecular structural characteristics of CEL. As shown in Fig. 2, signals can be clearly assigned to two major regions: aliphatic side-chain region ($\delta C/\delta H$ 50–90/2.5–6.0 ppm) and aromatic region ($\delta C/\delta H$ 80–155/6.0–8.0 ppm). The analysis of the aromatic region further verified that although alkyne modification did not have a noticeable effect on the molecular framework of lignin (17).

Semi-quantitative analysis of signals in the aliphatic region showed that lignin linkages were primarily composed of β -O-4 (A), β -5/ α -O-4 (B) and β - β (C) structures, as shown in Fig. 3. The semi-quantitative analysis showed that there were negligible differences in lignin structure between groups (CEL_1 – CEL_4) and the control groups (CEL_{blank} and $CEL_{control}$), indicating that alkyne-tagged precursors had a limited impact on linkages of lignin (Table 1). Specifically, β -O-4 (A) was the main linkage in lignin, with percentages ranging from 78.4–87.1 % in all groups. The percentage ranges of β -5/ α -O-4 (B) and β - β (C) were 6.4–10.0 % and 8.0–12.0 %, respectively. Non-linear changes in β -O-4 bond proportions were observed in alkyne-modified groups (CEL_1 – CEL_4).

Table 1. Quantitative analysis of aliphatic structure based on two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance

CEL	β -O-4(A)	β -5/ α -O-4(B)	β - β (C)
CEL_{blank}	87.1 %	5.1 %	7.8 %
$CEL_{control}$	82.3 %	7.5 %	10.2 %
CEL_1	78.4 %	9.6 %	12.0 %
CEL_2	85.6 %	6.4 %	8.0 %
CEL_3	83.2 %	8.1 %	8.7 %
CEL_4	81.0 %	10.0 %	9.0 %

CEL: cellulase-enzymatic lignin.

over the 4-week cultivation period. The underlying cause remains unclear. While quinone methide intermediates have been implicated in lignin polymerisation dynamics (18), this interpretation is speculative and requires further experimental validation.

Combined with complementary analyses (Fourier transform-infrared spectroscopy (FT-IR) and gel permeation chromatography (GPC); Supplementary Fig. S1 and Table S1), the HSQC data indicate that S_{ALK} incorporation does not significantly disrupt lignin structure, supporting its suitability as a probe for lignification studies. These findings demonstrate the good biocompatibility of the S_{ALK} , which does not interfere with the natural polymerisation process of lignin. Therefore, this alkyne-tagged lignin represents a reliable tool for tracking lignification dynamics in plant systems.

Imaging of *Broussonetia papyrifera* and *Nerium oleander* stem sections

Hardwood lignin is predominantly composed of G and S units. Accordingly, alkynyl-labelled sinapyl alcohol was selected as a precursor to trace S-type lignin deposition. The synthetic pathway of G-type lignin has already been proposed in our previous studies (19).

To investigate the effects of precursor concentration and cultivation time on the lignification of *B. papyrifera* and *N. oleander* cells, concentration of 1, 10, 25, 50, 75 and 100 μ M and time of 0.5, 1, 2, 12, 24 and 48 hr were employed (Supplementary Fig. S2–S5). It was concluded that the total deposition of artificial lignin reached an optimal level under the conditions of 100 μ M concentration and 24 hr of cultivation. Therefore, all subsequent cultivation was conducted under these conditions. As observed under the confocal laser scanning microscope (Fig. 4), artificially synthesised lignin appeared red under an excitation wavelength of 552 nm and its fluorescence intensity varied. In contrast, blue fluorescence under an excitation wavelength of 405 nm represented total natural-deposited lignin in sections before cultivation and remained unchanged. Therefore, in the later image processing, fluorescence information under the two different excitation wavelengths could be obtained by splitting channels of the fluorescence images.

Regarding composition of xylem cell structures, xylem of *B. papyrifera*, unlike that of *flax* and *N. oleander* with radially arranged fibre tracheids and ray parenchyma cells, mainly consisted of neatly arranged large vessels and irregularly arranged parenchyma cells. Lignification in *N. oleander* occurred in both fibre tracheids and ray parenchyma cells, while in *B. papyrifera*, it only occurred in vessels. These differences in xylem tissue cell structures also affected lignin deposition trends.

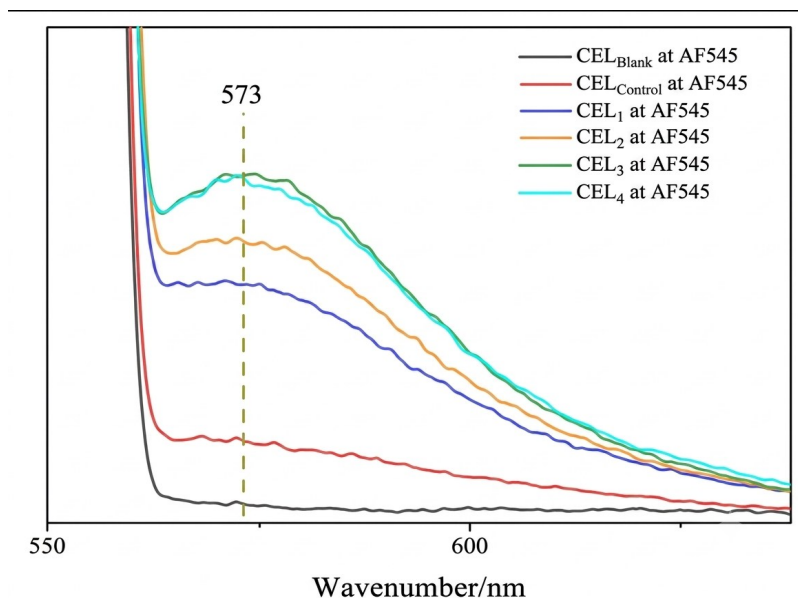


Fig. 1. Fluorescence spectra of cellulase-enzymatic lignin.

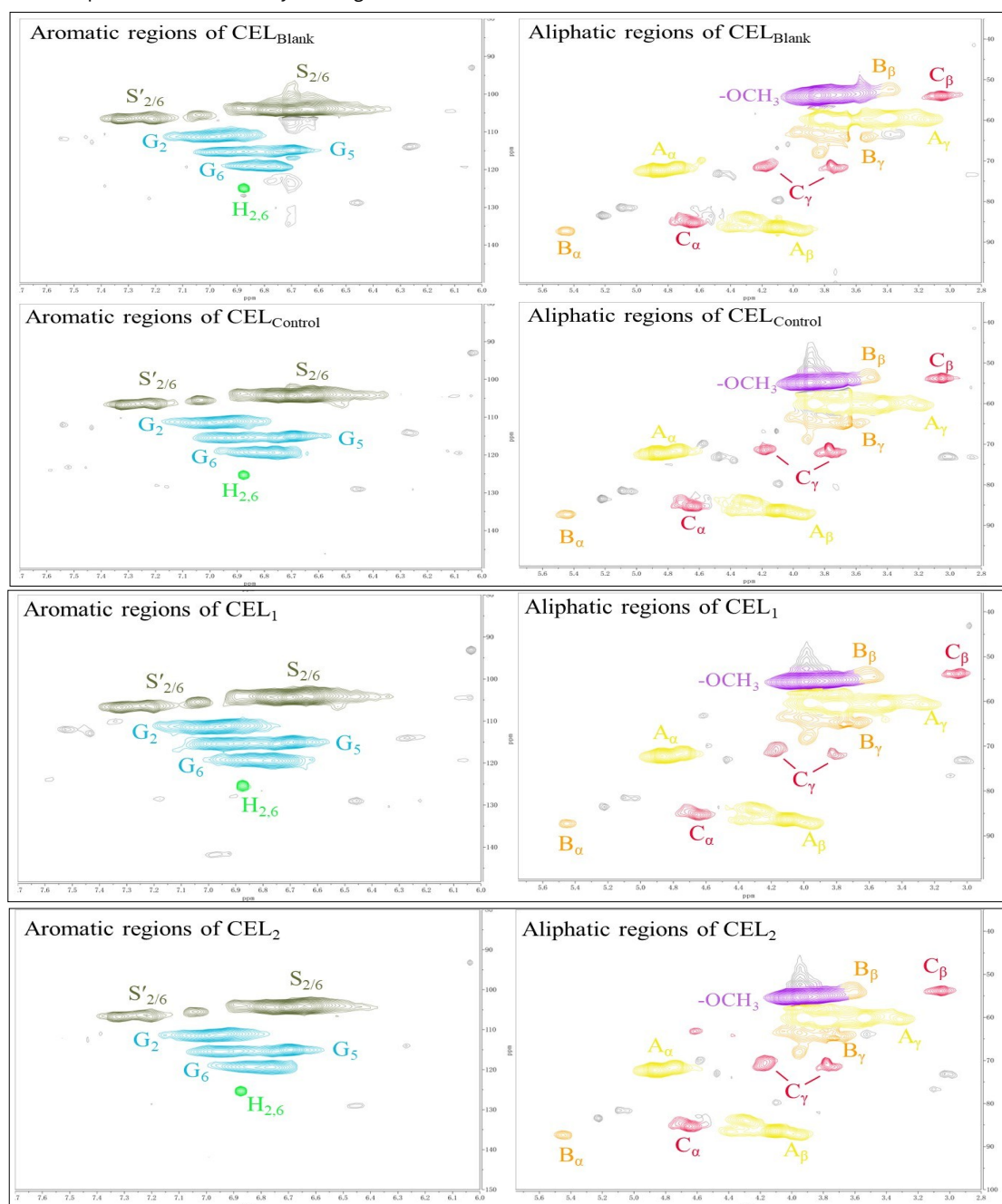


Fig. 2. The two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectra of cellulase-enzymatic lignin.

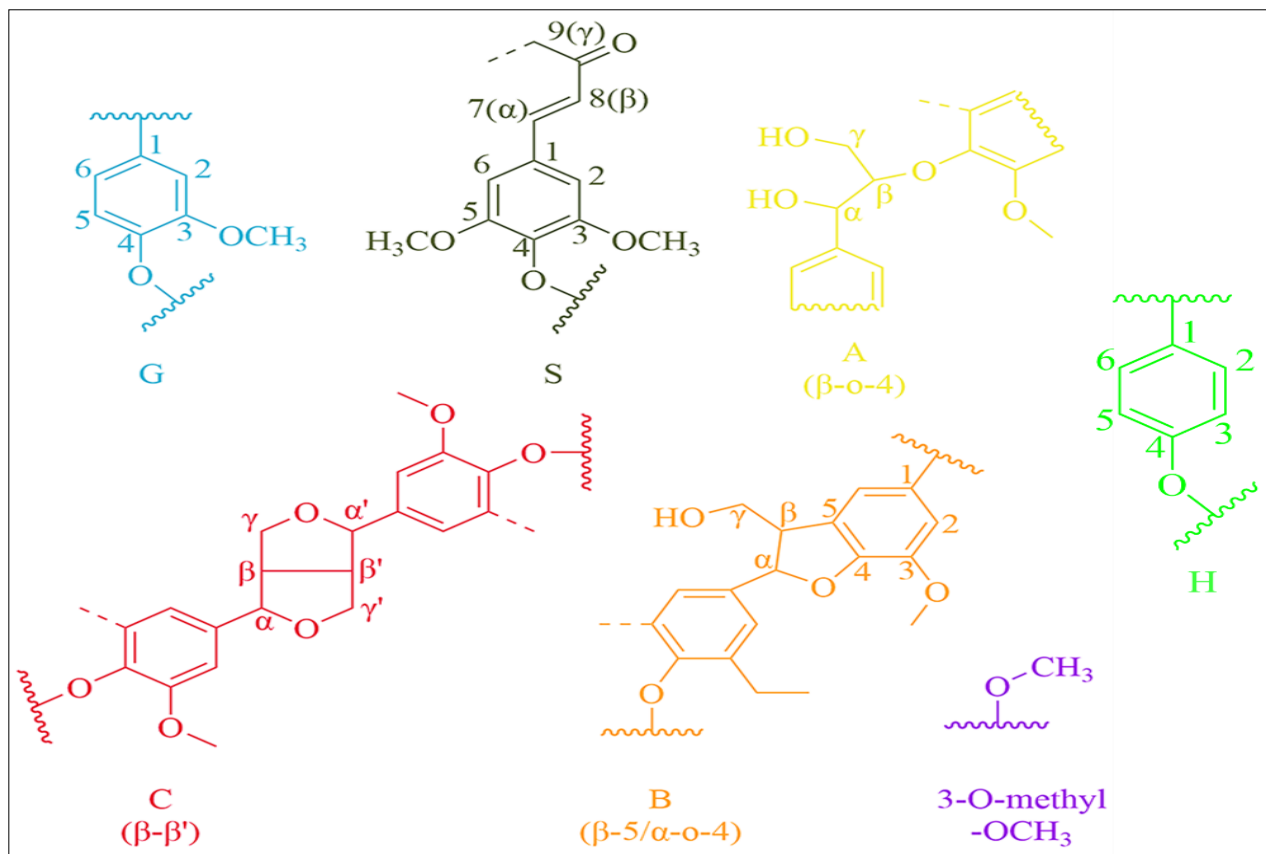


Fig. 3. The structures of cellulase-enzymatic lignin (CEL) in two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectra. Main structures present in the lignins of CEL- A: β -O-4' alkyl-aryl ethers; B: phenylcoumarans; C: resinols; G: guaiacyl units; S: syringyl units; H: 4-hydroxyphenyl unit; X: P-hydroxy cinnamyl end group.

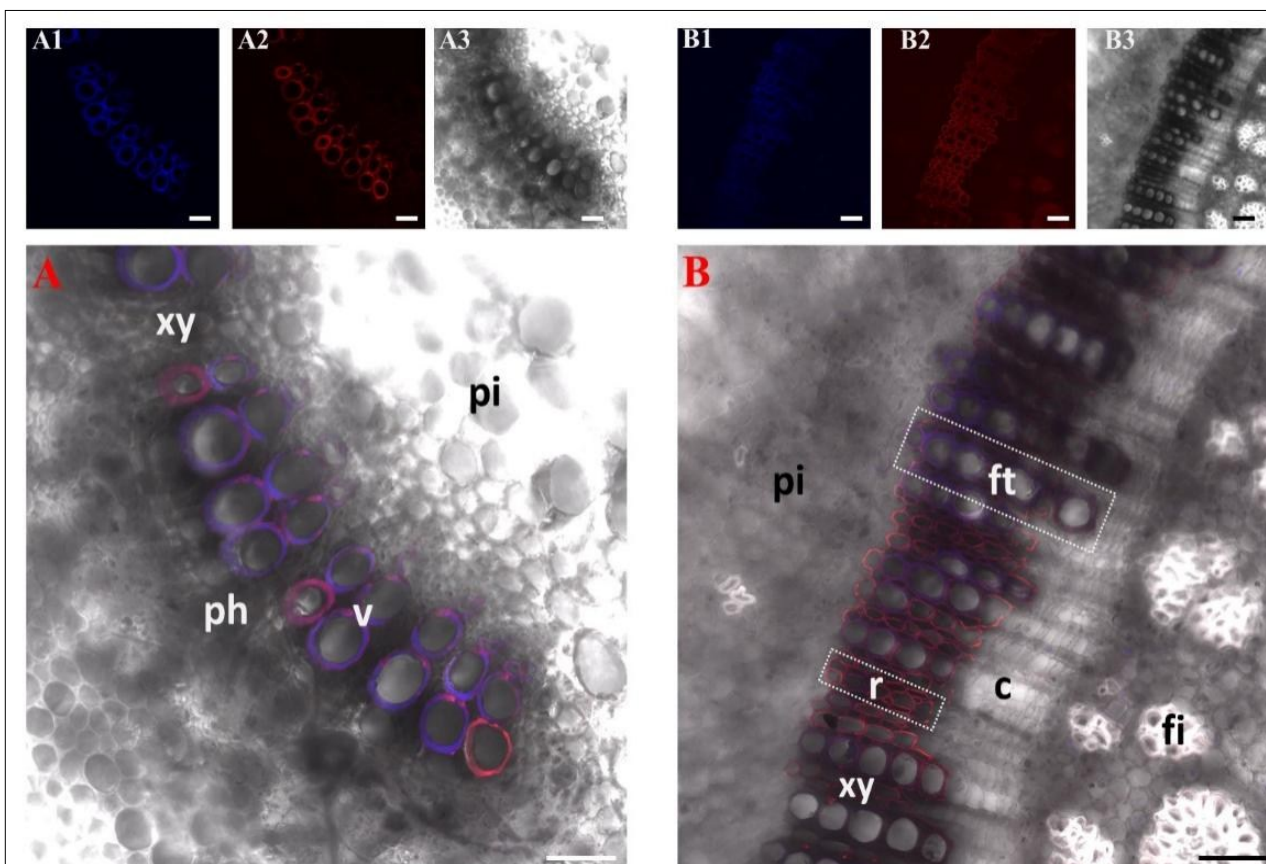


Fig. 4. Fluorescence images of *Broussonetia papyrifera* and *Nerium oleander* stem sections under different excitation wavelengths. A1: Autofluorescence image of *B. papyrifera* under 405 nm excitation; A2: fluorescence image of synthetic lignin in *B. papyrifera* under 552 nm excitation; A3: bright-field image of *B. papyrifera* section; A: composite image of A1, A2 and A3; B1: autofluorescence image of *N. oleander* under 405 nm excitation; B2: fluorescence image of synthetic lignin in *N. oleander* under 552 nm excitation; B3: bright-field image of *N. oleander* section; B: composite image of B1, B2 and B3. xy: xylem; pi: pith; ph: phloem; v: vessel; ft: fibre tracheid; r: ray parenchyma; c: cambium; fi: fibre; Scale bar: 50 μ m.

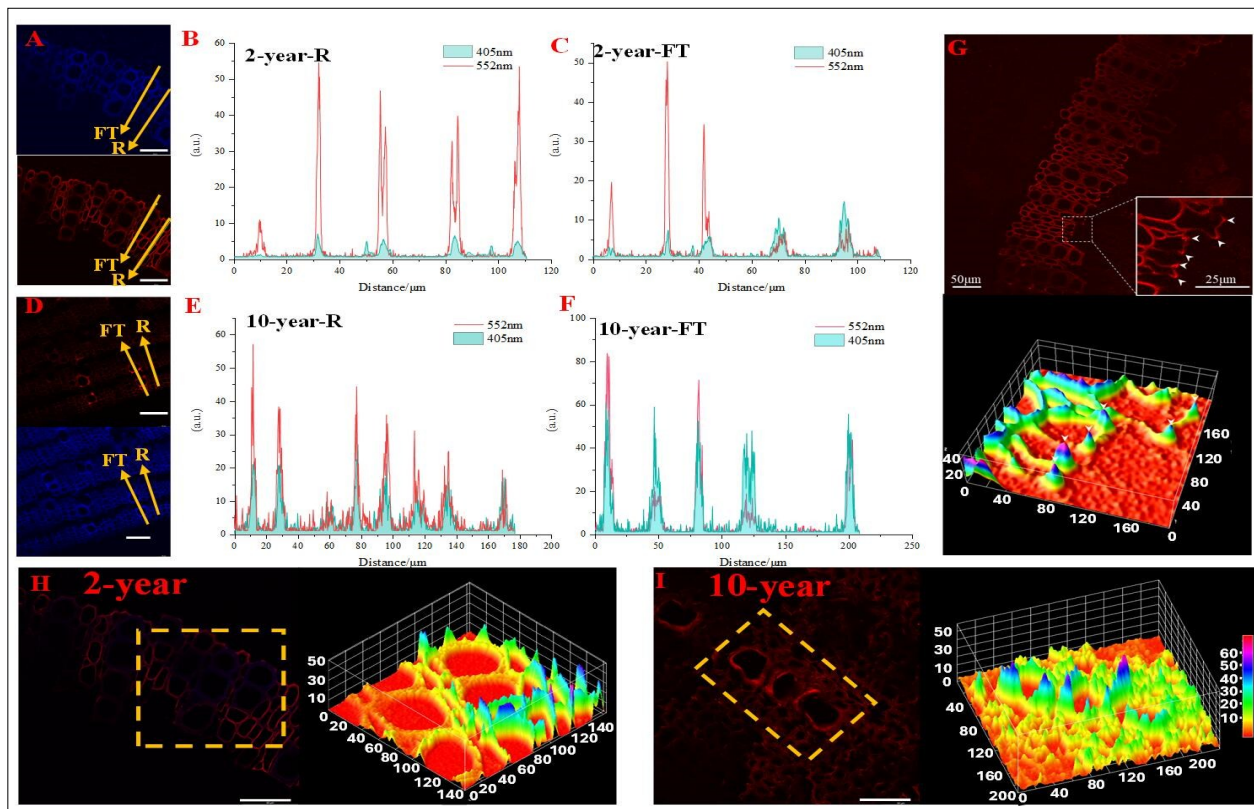


Fig. 5. Lignification patterns of fibre tracheids and ray parenchyma cells in *Nerium oleander* seedlings and 10-year-old *N. oleander*. A–C: Fluorescence quantification images of fibre tracheids and parenchyma cells in 2-year-old *N. oleander* seedlings; D–F: Fluorescence quantification images of fibre tracheids and parenchyma cells in 10-year-old *N. oleander* branches; G: 3D fluorescence quantification image of cambium marginal cells in 2-year-old seedlings; H: 3D fluorescence quantification image of cambium in 2-year-old seedlings; I: 3D fluorescence quantification image of cambium in 10-year-old branches. FT: fibre tracheids; R: ray parenchyma cells. Yellow arrow indicates regions traversing a column of longitudinally arranged cells. Yellow dashed boxes outline fluorescence-quantified areas. Scale bar: 50 µm. Imaging: Natural lignin autofluorescence was excited at 405 nm (emission: 420–480 nm), appearing blue; alkynyl-labelled sinapyl alcohol-lignin was excited at 552 nm (emission: 570–620 nm), appearing red.

The lignification differences between 2-year-old and 10-year-old *Nerium oleander*

As shown in Fig. 5A–F, in *N. oleander*, both fibre tracheids and ray parenchyma cells exhibited varying degrees of lignin deposition. During cultivation, the total amount of precursors absorbed by ray parenchyma cells was significantly higher than that absorbed by fibre tracheids. This difference was reflected in the overall fluorescence intensity, which was much lower in fibre tracheids than in ray parenchyma cells. Such cell-type-specific differences in lignification activity are consistent with genetic evidence showing that down-regulation of specific peroxidases, such as NtPrx60 in tobacco, can reduce total lignin content by up to 50 %, underscoring the essential role of peroxidases in lignin polymerisation (20).

In ray parenchyma cells, a weaker fluorescence intensity was observed on the first cell wall, followed by a high-intensity single peak on the second cell wall. It should be noted that the numbering of “first”, “second” and subsequent cell walls refers to the sequential order along the fluorescence intensity scanning path (from the tail to the tip of the arrow). Subsequent cell walls also maintained a high intensity level, showing a bimodal pattern. Cells at the edge of the vascular cambium showed fewer lignin precursors polymerising in the middle lamella during the differentiation stage. In the next stage, the focus shifted to the middle lamella and then to the S1/S2 layers.

Fibre tracheids showed a different pattern. The fluorescence intensity changes in the first and second cell walls were similar to those of ray parenchyma cells, but the following three cell walls exhibited a stepwise significant decrease.

As shown in Fig. 5G, 3D fluorescence analysis of differentiating cells at the edge of the vascular cambium revealed that fluorescence intensity in cell-corner regions (marked by white arrows) reached the highest level of 30–40. Additionally, scattered weak fluorescence was observed in the middle lamella regions.

In Fig. 5H, I, when comparing 10-year-old *N. oleander* branches with 2-year-old seedlings, the fluorescence signal intensity of natural lignin at 405 nm and that of S_{ALK} at 552 nm were highly similar. In the ten-year-old sample, S_{ALK} -labelled fibre tracheids and parenchyma cells both exhibited a distinct fluorescence attenuation pattern: as distance from the vascular cambium towards the pith increased, the red fluorescence intensity decreased stepwise. However, at 405 nm, the fluorescence intensity of both cell types remained high and was evenly distributed in space. For fibre tracheids in the ten-year-old sample, even when sampled close to the vascular cambium, two peaks were observed where natural lignin fluorescence intensity was higher than that of S_{ALK} labelling. Furthermore, 3D fluorescence analysis revealed a distinct “gap” in lignin deposition in areas where fibre tracheids contacted adjacent parenchyma cells.

The changes in fluorescence intensity in the area marked by the yellow arrow likely reflect the lignin deposition pattern of ray parenchyma cells, suggesting a dynamic process from the middle lamella to the S1/S2 layers. The stepwise significant decrease in the later cell walls of fibre tracheids indicates that lignification hardly occurs in these walls during cultivation, with no detectable precursor polymerisation and deposition. Similar phenomena have been reported after fluorescence analysis of flax sections (12).

A possible factor for the difference in lignin deposition trends between the two cell types is their distinct physiological functions. Fibre tracheids primarily transport water and inorganic salts and their programmed cell death occurs significantly faster than that of ray parenchyma cells, which continue to play key roles in growth, development and metabolism (21).

The intensity patterns observed in Fig. 5G suggest that the starting point of S-type lignin deposition is located at cell corners. The initiation of polymerisation at specific sites such as cell corners may be directed by wall-bound phenolic compounds like ferulate, which are proposed to act as nucleation sites, with peroxidases catalysing their cross-linking to growing lignin polymers (22). Combined with fluorescence intensity changes in longitudinal cell walls, this deposition pattern is consistent with that reported for flax, where lignification of S-type substructures progresses from cell corners to the middle lamella and then to the secondary cell wall (23).

The similarity in fluorescence signals between natural and S_{ALK} -labelled lignin in mature branches indicates that the total amount and spatial distribution of natural lignin deposition tend to align with those of artificially synthesised lignin. The stepwise decrease in S_{ALK} -labelled red fluorescence intensity from cambium to pith suggests that lignification activity weakens with increasing cell maturity. In contrast, the consistently high and even distribution of natural lignin fluorescence at 405 nm reflects that natural lignin had already been deposited in a stable and homogeneous manner in mature tissues before S_{ALK} cultivation.

This age-dependent difference likely relates to cell development stage. In contrast to the dynamic lignification process observed in seedlings (sequential deposition from cell corners to middle lamella to secondary walls), cells in 10-year-old branches appear to have completed programmed development, with

secondary wall thickening tending to stop (24). Especially, the two peaks where natural lignin intensity exceeded S_{ALK} labelling in fibre tracheids, as well as the distinct “gap” in lignin deposition at contacts with adjacent parenchyma cells, may relate to the physiological need to improve lateral nutrient transport efficiency (25). These observations further suggest that mature cells gradually lose the ability to continue lignification during programmed cell death (26).

Incorporation of alkyne-tagged lignin precursors in 10-year-old *Nerium oleander* branches

Due to the large xylem diameter in 10-year-old branches, the entire radial transect from cambium to pith could not be captured in a single high-resolution image. We therefore sampled multiple contiguous regions along this axis and quantified the overall fluorescence intensity of each region to assess the lignification gradient. As shown in Fig. 6A, B, fluorescence imaging revealed that red fluorescence was restricted to the cambium and declined sharply toward the pith. In the xylem itself, almost no signal was detected.

In Fig. 6C, when epidermal cells of *N. oleander* were analysed under fluorescence microscope, these cells exhibited strong autofluorescence, while almost no fluorescence signal was detected under an excitation wavelength of 552 nm. In addition, distortion of epidermal cell morphology was observed, likely resulting from outward expansion growth of the plant stem.

The observation that red fluorescence was restricted to the cambium and declined sharply toward the pith (Fig. 6A, B) suggests that cells in the cambium are actively engaged in lignin biosynthesis and deposition, whereas xylem cells contribute much less to this process. Quantitative analysis further supports a differential lignification gradient from pith to cambium: the closer to the cambium, the stronger the trend of lignin deposition (12).

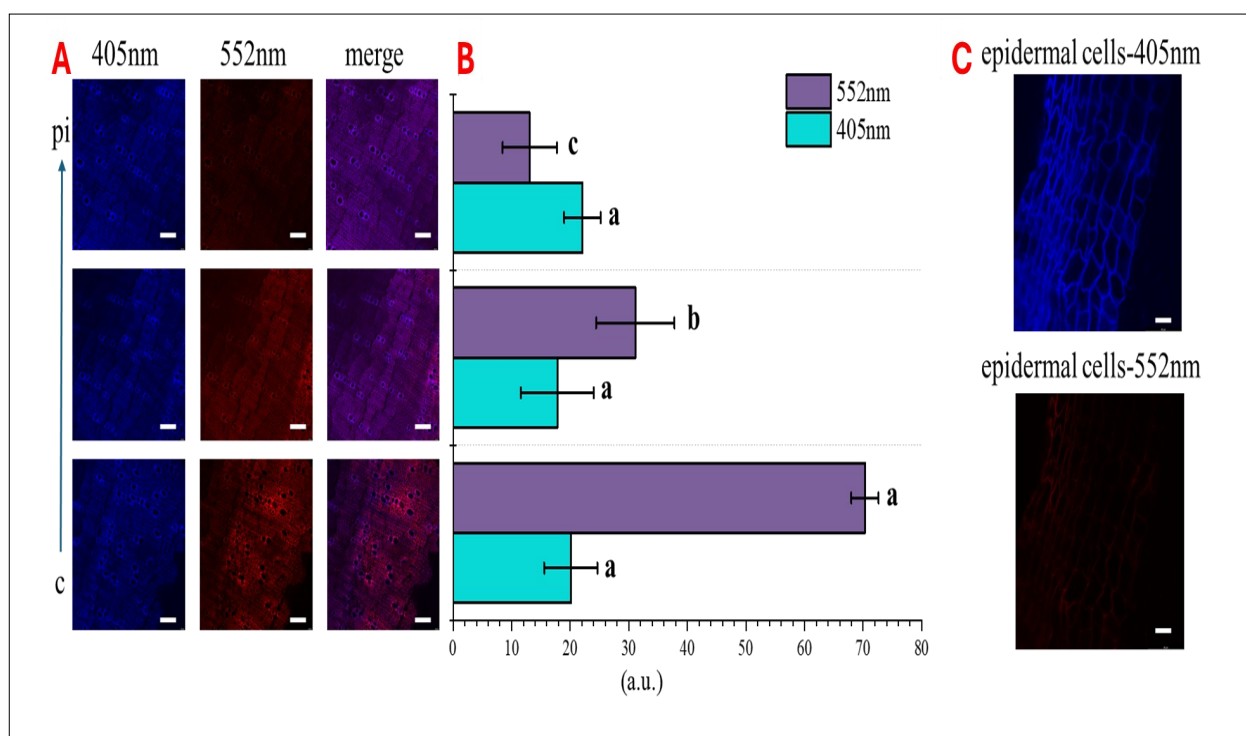


Fig. 6. Changes in lignin deposition in 10-year-old *Nerium oleander* stem cultivated in S_{ALK} . (A) Fluorescence imaging from the cambium to the pith; (B) quantitative fluorescence intensity analysis corresponding to the regions shown in A; (C) fluorescence image of epidermal cells. Scale bar: 100 μ m. Pi: pith; c: cambium. Scale bar: 200 μ m. Letters indicate significant difference ($p < 0.05$). The data were analysed using one-way ANOVA followed by the Wallace–Duncan method for multiple comparisons ($n = 8–10$). Imaging: Natural lignin autofluorescence was excited at 405 nm (emission: 420–480 nm), appearing blue; S_{ALK} -labelled lignin was excited at 552 nm (emission: 570–620 nm), appearing red.

The absence of labelled fluorescence in epidermal cells at 552 nm (Fig. 6C) indicates that these cells did not undergo new lignin deposition. The observed distortion of epidermal cell morphology is consistent with outward expansion growth of the plant stem, as cells must constantly adjust their morphology to adapt to environmental changes; restriction or disturbance of this adjustment can lead to abnormal cell morphology, such as twisting or deformation (27).

Incorporation of alkyne-tagged lignin precursors in *Broussonetia papyrifera*

After analysing fluorescence intensity of radial vessel elements in the xylem of *B. papyrifera*, no fixed pattern of lignin deposition was observed, even in adjacent radially arranged vessel elements within the same section (Fig. 7). V1 showed a trend of first increasing and then decreasing, while V2 showed a trend of continuous increase. This pattern differed from that observed in flax and *N. oleander*, in which both fibre tracheids and parenchyma cells exhibited a fixed trend of lignin deposition.

Unexpectedly, no fixed lignin deposition pattern was found in adjacent vessel elements of *B. papyrifera*, a phenomenon that prompted us to further investigate its underlying causes. This absence of a fixed pattern may be explained by the species' special xylem structure and organisation. Specifically, a large number of parenchyma cells surround the vessel elements and may provide enzymes and substrates needed for the lignification process of vessel elements. Consequently, the lignification of vessel elements may be regulated by adjacent parenchyma cells, while the vessel elements themselves provide relatively limited enzymes and substrates. Moreover, there appears to be no unified regulatory mechanism controlling the amount or specific location of lignin deposition in parenchyma cells, which ultimately leads to heterogeneous and irregular lignin deposition in vessel elements. In botanical terms, this phenomenon is consistent with the characteristics of non-autonomous lignification (28).

Non-autonomous lignification mechanism in *Broussonetia papyrifera*

As shown in Fig. 8, cell types adjacent to the 1-side and 3-side were vessel elements. The cell walls of these vessel elements all showed red fluorescence, indicating S_{ALK} deposition, while no fluorescence was observed in the surrounding parenchyma cells. Neighbouring cells on the 2-side and 3-side were parenchyma cells that did not undergo lignification. Lignin deposition on the 3-side was mainly located in cell corners and the middle lamella, while on the 2-side, it

was mainly located in the secondary cell walls of both cells. The average fluorescence intensity on the 3-side was significantly higher than that on the 2-side. The secondary cell wall of vessel elements showed relatively uniform fluorescence intensity, with lignin deposition mainly in the secondary cell wall.

When vessel elements were adjacent to parenchyma cells that did not undergo lignification, the location of lignin deposition and fluorescence intensity varied. For example, in vessel elements adjacent to such parenchyma cells, lignin deposition was concentrated in cell corners and the middle lamella or mainly located in the secondary cell walls of both cells.

The lignification process of plant cells involves both autonomous and non-autonomous mechanisms. Autonomous lignification refers to a cell's ability to synthesise its own monolignols and oxidative enzymes (laccases/oxidases) and deposit lignin in its own cell wall; this is typically observed in living cells such as differentiating fibre tracheids and ray parenchyma cells. Non-cell-autonomous lignification (also termed the "good neighbour" mechanism) occurs when a cell, often a dead or dying cell, such as a vessel element or tracheid, relies on neighbouring living cells (e.g., parenchyma cells) to supply monolignols and/or oxidative machinery for its lignification. The oxidative machinery supplied by neighbouring cells may include laccases, which in *Arabidopsis* have been shown to exhibit substrate specificity; for instance, LAC17 preferentially affects guaiacyl lignin deposition and alters the S/G ratio (29). Additionally, laccases are thought to polymerise monolignols into oligolignols during early lignification stages, whereas peroxidases may function later when H_2O_2 is generated (30). In the xylem of *B. papyrifera*, vessels are adjacent to non-lignified parenchyma cells, providing a useful model for studying this mechanism. In this structure, the lignification of vessel elements depends not only on their own enzymes and substrates (18, 31–34) but also on the influence of adjacent parenchyma cells.

In positions where vessel elements were adjacent to other vessel elements (1-side and 3-side), lignification appeared to be primarily regulated by the vessel elements' own enzymes and substrates, characterised by lignin deposition mainly in the secondary cell wall and uniform fluorescence intensity, features consistent with autonomous lignification.

When vessel elements were adjacent to non-lignifying parenchyma cells (2-side and 3-side), the influence of non-autonomous lignification became significant. The observed

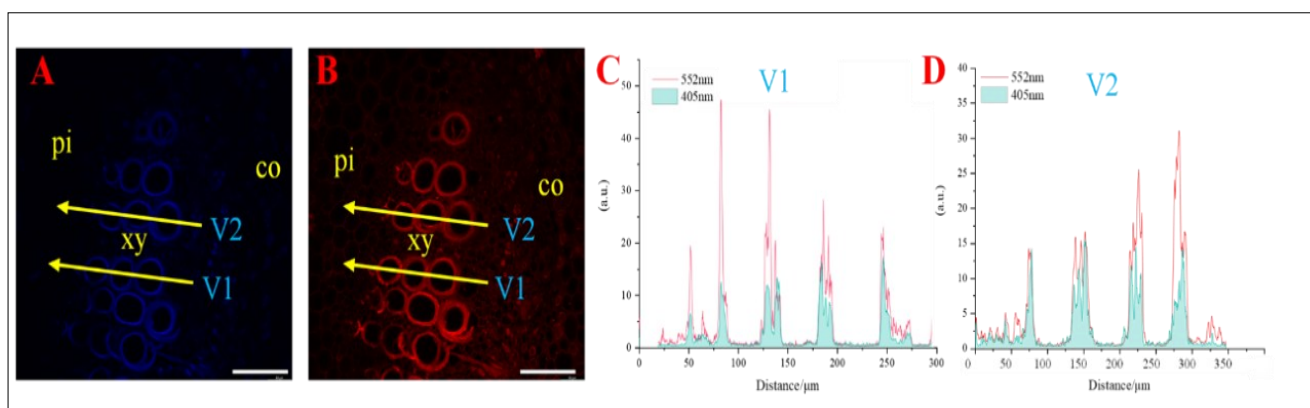


Fig. 7. Fluorescence intensity quantification of vessel elements in *Broussonetia papyrifera*. A, B: Fluorescence images of *B. papyrifera* sections under 405 nm and 552 nm excitation wavelengths; C, D: fluorescence quantification line charts of S_{ALK} and natural lignin. V1 and V2 represent two groups of radially arranged vessel elements. Scale bar: 50 μm. Imaging: natural lignin autofluorescence was excited at 405 nm (emission: 420–480 nm), appearing blue; S_{ALK} -labelled lignin was excited at 552 nm (emission: 570–620 nm), appearing red.

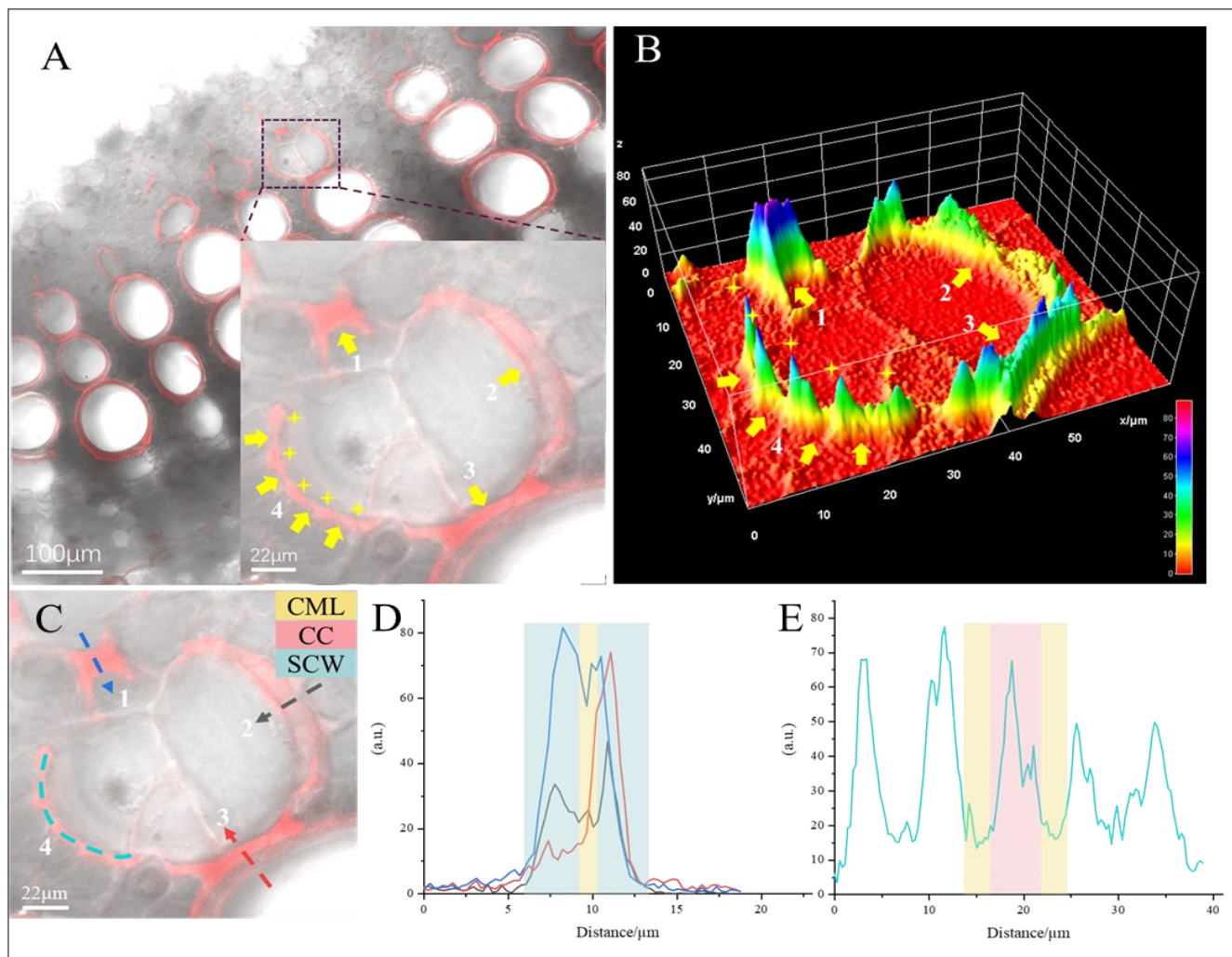


Fig. 8. Analysis of lignification trends in *Broussonetia papyrifera* cells at the single-cell level. A: Overlay of bright-field and click chemistry fluorescence images. Asterisks indicate cell corners; arrows indicate middle lamella; 1, 2, 3 and 4 represent four sides of the same cell wall. Scale bars: 100 and 22 μm; B: 3D quantification of fluorescence intensity in (A); C: Four differently colored arrows passing through the four sides of the cell wall; D, E: Quantification of fluorescence intensity in cell walls 1, 2, 3 and 4. Scale bar: 22 μm. CML: middle lamella; CC: cell corner; SCW: secondary cell wall. Imaging: Natural lignin autofluorescence was excited at 405 nm (emission: 420–480 nm), appearing blue; S_{ALK}-labelled lignin was excited at 552 nm (emission: 570–620 nm), appearing red.

differences in deposition location and fluorescence intensity between the two sides suggest that the adjacent parenchyma cells modulate the lignification process (35–39). The higher average fluorescence intensity on the 3-side compared to the 2-side may indicate stronger or more active lignification on that side. Similar phenomena have been observed in loquat fruit flesh sections after click fluorescence during cold storage (28).

The combination of large, lignified vessels and non-lignified parenchyma cells in *B. papyrifera* offers a useful system for observing and testing the “good neighbour” mechanism. This structure provides an opportunity to further investigate the role of neighbouring cells in the lignification process and the importance of non-autonomous lignification in plant growth and development.

Conclusion

In this study, alkyne-tagged syringyl lignin monolignols (S_{ALK}) were used to investigate lignification in *Broussonetia papyrifera* and *Nerium oleander* of different ages. Species-specific lignification patterns were observed. In 2-year-old *N. oleander*, the lignification level of fibre tracheids decreased from the cambium toward the pith, whereas ray parenchyma cells

maintained a high lignification level throughout. In contrast, 10-year-old *N. oleander* exhibited a uniform lignification distribution, indicating that lignification pattern varies with growth stage.

In *B. papyrifera*, an unexpected finding emerged: lignification was mainly concentrated in vessel elements but followed no fixed pattern, even between adjacent radially arranged vessel elements. This serendipitous observation led us to explore its underlying mechanism. We found that this disordered lignification was influenced by adjacent non-lignified parenchyma cells, suggesting the presence of non-cell-autonomous lignification (the “good neighbour” mechanism). In comparison, lignification in *N. oleander* fibre tracheids and ray parenchyma cells followed predictable, cell-intrinsic patterns consistent with autonomous lignification.

The S_{ALK} probe enabled simultaneous visualisation of newly deposited S-lignin alongside pre-existing natural lignin, allowing direct comparison of lignification dynamics across cell types and developmental stages. Comparison with existing lignin imaging techniques underscores the advantages of this probe. Traditional staining methods are rapid but cannot resolve monomer types or deposition timing. Spectroscopic methods

provide quantitative data but sacrifice spatial resolution. Among click-compatible probes, the propargyl-labelled coniferyl alcohol probe has been successfully applied to G-type lignin imaging in model plants. In contrast, the S_{ALK} probe offers three specific advantages: (i) it targets S-type lignin, which is quantitatively dominant in hardwood and critically influences biorefinery efficiency; (ii) it enables direct comparison between newly deposited and pre-existing lignin; and (iii) it is applicable across different tissue types and developmental stages.

While this approach is not the first click-compatible monolignol probe, it is, to our knowledge, the first systematic application of an S-type-specific alkyne-tagged probe for visualising lignification dynamics in hardwoods. These findings lay a foundation for future studies on lignin dynamics, including further exploration of the molecular mechanisms underlying non-cell-autonomous lignification, a mechanism first brought to our attention by the unexpected observations in *B. papyrifera* and the broader application of S_{ALK} in other plant systems.

To further elucidate the deposition mechanisms of lignin, the following issues need to be addressed in the future work. The efficiency of the CuAAC click reaction was not quantitatively assessed and variations in labelling efficiency across different cell types or tissue regions cannot be completely excluded. Our experiments were performed at specific time points after S_{ALK} cultivation, providing snapshots rather than continuous real-time tracking of the lignification process. Additionally, the molecular mechanisms underlying the observed age-dependent and species-specific lignification patterns were not investigated in this study. Despite these limitations, to our knowledge, our study is the first systematic application of an S-type-specific alkyne-tagged probe for visualising lignification dynamics in hardwoods. These findings lay a foundation for future studies on lignin dynamics, including further exploration of the molecular mechanisms underlying non-cell-autonomous lignification, a mechanism first brought to our attention by the unexpected observations in *B. papyrifera* and the broader application of S_{ALK} in other plant systems.

Acknowledgements

The authors appreciate the financial support from the National Natural Science Foundation of China (No. 21978074) and Joint Funding Program of Hubei Provincial Department of Science and Technology (2024AFD121).

Authors' contributions

XW conducted research on the synthesis of alkyne-labelled isopentanol, participated in the construction of the visualisation system and the study of the lignification mechanism, and wrote the first draft of the paper. DZ conducted an analysis and determination of the material properties. HY participated in the design of the study and performed the statistical analysis. LY conceived of the study and participated in its design and coordination. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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