Determination of de novo suberin-lignin ferulate deposition in xylem tissue upon vascular pathogen attack

Running head: Xylem ligno-suberin ferulate determination upon vascular pathogen attack

Weiqi Zhang[1,¥], Álvaro Jiménez-Jiménez[1,¥], Montserrat Capellades[1,2], Jorge Rencoret[3], Anurag Kashyap[2,¥], Núria S. Coll[1,2,*]

[1] Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, 08193 Bellaterra, Spain

[2] Consejo Superior de Investigaciones Científicas (CSIC), 08001 Barcelona, Spain

[3] Institute of Natural Resources and Agrobiology of Seville (IRNAS), CSIC, 41012 Seville, Spain

[4] Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam, India, 785013

[¥] These authors contributed equally to the work

[*] Corresponding authors:
Núria S. Coll,
Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, 08195 Bellaterra, Spain
e-mail: nuria.sanchez-coll@cragenomica.es

Anurag Kashyap
Department of Plant Pathology, Assam Agricultural University, 785013 Jorhat, Assam, India
e-mail: anuragkashyap11@gmail.com

1. Introduction

Vascular plant pathogens cause some of the most devastating diseases in plants, ranging from annual herbaceous to big trees (Yadeta & Thomma, 2013). These pathogens adopt different strategies to make their way into xylem vessels. Once the pathogen reaches the vasculature it multiplies profusely inside the xylem tissue and spread vertically and horizontally to the neighboring tissues, resulting in dreadful wilting of the infected plants and eventual death (Bae *et al.*, 2015). However, resistant plants have evolved mechanisms in the xylem vasculature to sense invading pathogens and mount an array of defense responses against these aggressors (Yadeta & Thomma, 2013). One of the major defense mechanisms conferring resistance against vascular pathogens can be attributed to the genesis of physio-chemical blockades (Kashyap *et al.*, 2021). Plants have evolved effective structural defense mechanisms to prevent vessel colonization or movement between vessels, once vascular colonization has occurred (Beckman & Roberts, 1995). Structural barricades such as gels and tyloses prohibit vertical movement of xylem vascular pathogens inside the lumen of vessels. Similarly, vascular pathogen induced reinforcements in secondary cell wall of vascular tissue act as a potent barrier against colonization (Ferreira *et al.*, 2017). Wall reinforcements with phenolic polymers such as lignin and suberin contribute towards preventing horizontal spread of the pathogens to the apoplast and the contiguous active tissues and vessels (Kashyap *et al.*, 2022). Hence, these horizontal and vertical barricades compartmentalize vascular pathogens at the site of infection (Kashyap *et al.*, 2021). Timely formation of these physico-chemical vascular barriers early upon pathogen perception can lead to confinement of the vascular pathogen at the infected vessel, avoiding the spread of wilt diseases (Robb *et al.*, 2008; Zaini *et al.*, 2018; Planas-Marquès *et al.*, 2019). Also, the occurrence of these defense responses are highly intertwined, both spatial and temporally, for avoidance of deleterious repercussions (embolism and cavitation). Hence, these anatomical shifts act as a hallmark of plant defense response against xylem vascular pathogens, which can be vital while exploring resistant germplasms. Such structural defense responses vary based on the host-pathogen interaction but are conserved across the plant kingdom (Kashyap *et al.*, 2021). Hence, an in-depth histopathological characterization gives important insights on the defense responses employed by the host against a pathogen.

We present here protocols based on staining along with bright field and fluorescence microscopy, and on two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy, standardized in our laboratory, to visualize wall reinforcements with phenolic wall polymers namely, lignin, ferulates and suberin that occur in xylem vasculature, in response to pathogen attack. Staining of cross-sections with Phloroglucinol-HCl gives an accurate visualization of changes in lignin accumulation of xylem vasculature in response to infection. Likewise, Sudan IV staining can accurately detect deposition of aliphatic domain of suberin in walls of xylem vasculature in response to pathogen attack. Another important phenolic player in wall reinforcements is ferulate which not only imparts strength to walls by cross-linking but it may also act as lignin-like poly-phenolic domain of suberin. Ferulates constitute a crucial component of the suberin polyphenolic domain and are one of the first compounds deposited in a suberizing tissue, potentially acting as nucleating site for suberin matrix polymerization (Negrel *et al.*, 1996; Graça, 2010, 2015; Boher *et al.*, 2013). Here we also present a simple technique to detect ferulate deposition in walls of vasculature as defense response to pathogens, based on a pH dependent blue to green color conversion of UV autofloresence. On the other hand, 2D-NMR experiments such as the Hetoronuclear Single Quantum Coherence (HSQC) can provide additional valuable information on suberin/lignin structure. 2D-HSQC NMR is considered one of the most powerful tools for plant cell wall structural analysis providing information on the composition and linkages in lignin/suberin polymers (Ralph and Landucci 2010; Correia *et al.* 2020).

2. Materials

2.1 Plant Varieties and Plant Growth Materials

1. Tomato (*Solanum lycopersicum*) plants cultivars Marmande and Moneymaker were used as susceptible control; cultivar Hawaii was used as a resistant control. Transgenic Moneymaker tomatoes overexpressing hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase (THT), a key enzyme in the synthesis of hydroxycinnamic acid amides (Campos *et al.*, 2014) were also used. Plants were grown in controlled growth chambers at 60% humidity, neutral day

photoperiod (12 h day-12 h night) and 27°C (when grown under light-emitting diode (LED) lighting) or 25°C (when grown under fluorescent lighting).

2. Soil mix: 5 parts peat + 3 small parts sand + 3 small part vermiculite.

2.2 Bacterial Strains and Bacterial Culture

1. *Ralstonia solanacearum* strain GMI1000 (Phylotype I, race 1 biovar 3) was used, including luminescent and fluorescent reporter strains previously described in Planas-Marques *et al.*, 2019.

2. Rich B medium: 10 g/L Bacteriological peptone, 1 g/L yeast extract, and 1 g/L casamino acids. For solid media 1.5% agar should be added before autoclaving. Before plating, 0.5% glucose and 0.005% triphenyltetrazolium chloride (TTC) should be added. pH should be adjusted to 7.0. Gentamycin (10 μg/ml) should be supplemented in liquid and solid cultures for selection of reporter strains.

3. Sterile petri dishes.

4. Sterile 50 ml tubes.

5. Spectrophotometer.

2.3 Tissue Sectioning

1. Sterile carbon steel surgical blades.

2. Sterile 2 ml tubes.

3. Ethanol 70%.

2.4 Histological materials

1. Ethanol 70%.

2. Phloroglucinol-HCl: 100 mg of Phloroglucinol dissolved in 8 ml of 95% Ethanol and 8 ml of HCl 37%, stored at room temperature covered in aluminum foil.

3. 1N potassium hydroxide (KOH) (pH above 10).

4. 5% Sudan IV solution: 2,5 g of Sudan IV in 50 ml of Ethanol 70%, filtered and stored at room temperature covered in aluminum foil.

5. Leica DM6B-Z microscope with ultraviolet (UV) illumination (340–380 nm excitation and 410–450 nm barrier filters).

6. Leica MC190-HD-0518131623 digital camera and Leica-DFC9000GT-VSC07341 camera.

3. Methods

3.1 Bacterial inoculation in planta (soil-drenching method)

1. Four- to five-week-old tomato plants are used for inoculation. Two days before inoculation, plants are transferred to a new chamber adapted for infection (27ºC, 60% RH, 12 h/12 h). Do not overwater the plants so the soil is dry enough for the plants to absorb all the inoculum through the roots.

2. One day prior inoculation, set an overnight culture of the *R. solanacearum* strain(s) of interest in Rich B medium (+antibiotics) in an Erlenmeyer flask (see **Note 1**). The amount of inoculum needed depends on the experiment and number of plants you want to inoculate, as well as the final bacterial concentration in the inoculum.

Typically, a concentration of 10^8 colony forming units (CFU)/ml is used for resistant varieties and 10^7 CFU/ml for susceptible ones. Calculations can be made according to the initial concentration in the liquid culture, and the fact that 40 ml of inoculum per plant are used.

3. On the day of inoculation, measure the optical density (OD_{600}) of the culture, and prepare the inoculation solutions by diluting with sterile distilled water to the desired bacterial concentration (see **Note 2**). Poke the soil with a blue pipette tip at each corner of the pot (4 punctures) to inflict root wounding, which facilitates infection.

4. Pour 40 ml of bacterial suspension in each pot, and do not water the plants until they have time to absorb the inoculum (1-2 days, depending on plant size).

4. Afterwards, keep watering the plants regularly (see **Note 3**) and start scoring symptoms at 3 days post infection (dpi), when the susceptible backgrounds begin to show wilting symptoms.

3.2 Histochemical analysis

1. When the desired stage of infection for analysis has come, take the plants, wash the roots with 1% v/v bleach and eliminate the adventitious roots (**Note 4**).

2. Take thin (150 µm) cross-sections with a sterile razor blade or a microtome, at the transition zone between the taproot and the basal hypocotyl, 1.5 cm below the soil line approximately (**Figure 1**).

2. Transfer the sections to tubes containing 300-500 µl of ethanol 70% and incubate at room temperature (RT) 2-5 days (at least) before analysis. This incubation ensures that the components not bound to the cell wall become solubilized with the ethanol and are thus removed from cell wall structures.

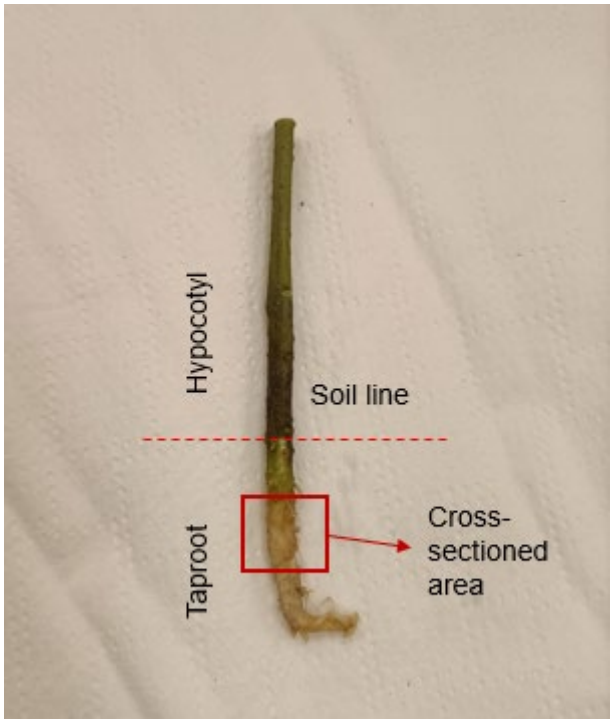

Figure 1. Region of interest for histological analysis. The lower portion of a 4-week-old tomato plant is shown, after throughout washing and eliminating adventitious roots, specifying in red the region of interest for the histochemical analysis.

3.4 Lignin staining

For lignin detection, Phloroglucinol, (**Note 5**), is used for direct visualization of lignin (cinnamaldehyde end-groups of lignin units) (Pomar *et al.*, 2004) as a red-purple coloration in the vasculature (**Figure 2**).

A drop of staining solution is added to the cross-sections and incubated 5 mins at room temperature, until the purple color has appeared. Then, sections are mounted for microscopy using 70% ethanol and subsequently visualized under bright field light in a stereomicroscope (see **Note 6**).

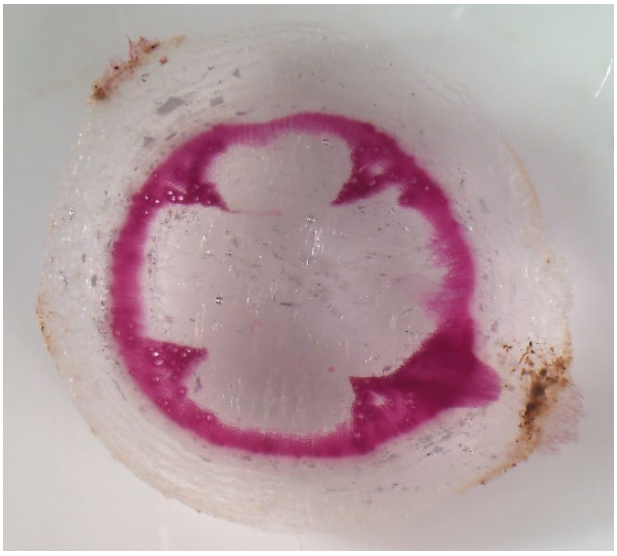

Figure 2. Phloroglucinol-stained samples. Examples cases of cross-sections stained with only Phloroglucinol-HCl observed under brightfield in a stereomicroscope Olympus SX16.

3.5 Detecting ferulate deposition

1. To detect ferulate accumulation (**Note 7**) alkali treatment can be performed, adding one drop of 1N KOH (pH=10) in the cross-sections of interest.

2. After 2 minutes of incubation at room temperature, samples can be mounted in microscopy slides with the KOH and ferulates can be visualized as green regions under

UV light with an epifluorescence microscope. The basic pH is responsible for the blue-to-green shift in fluorescence observed specifically for ferulate deposits (Harris and Trethewey, 2010).

3.6 Detecting suberin aliphatics

1. To detect aliphatic suberin the Sudan IV stain is used. To prepare the samples, put the slices in a tube containing 300 µl of Sudan IV and incubate 15 mins at room temperature.

2. Perform two washes with ethanol 70%, or until the samples do not release more dye.

3. After this, samples can be mounted on slides with ethanol 70% for microscopy. Under UV light in an epifluorescence microscope, suberin deposits can be visualized as brownish regions surrounding the vessels. Alternatively, the same samples can be directly mounted with 1N of KOH instead of ethanol, to combine the techniques in order to localize both parts of suberin barriers (**Figure 3**).

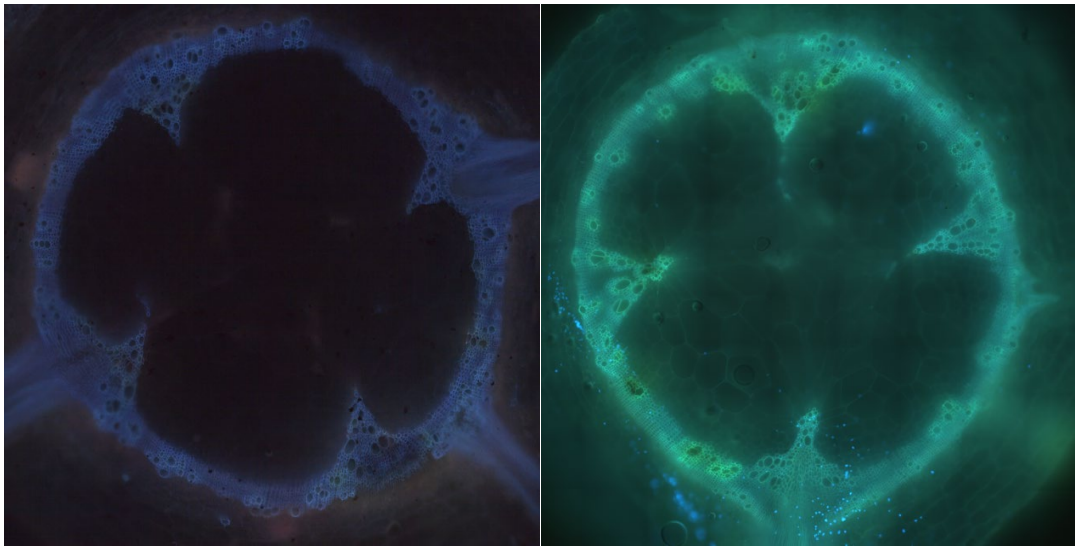

Figure 3. Sudan IV+KOH stained samples. Examples cases of cross-sections stained with only Sudan IV (left) and Sudan IV+KOH (right), observed under UV light in an epifluorescence microscopy Leica DM6.

3.7 Deciphering the composition and structure of the cell wall-deposited compounds

To obtain additional information on suberin and lignin present in tomato plant cell walls, fractions enriched in both polymers were isolated and analyzed by two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR), which currently represents the most powerful tool for the structural analysis of plant cell-wall components (Ralph &

Landucci, 2010; Correia *et al.*, 2020). Prior to 2D-NMR analysis of suberin/lignin polymers, it is necessary to remove the non-structural components of the plant cell wall, as well as the structural polysaccharides that form it.

- *Solvent extraction for removing non-structural plant cell-wall components*. Samples of a pool of tomato plants (taproot-to-hypocotyl region), infected or water-treated, are knife milled and extracted sequentially with distilled water, 80% ethanol, and finally with acetone, by sonicating in an ultrasonic bath, centrifuging and discarding the supernatant.

1. Add ~300 mg of plant material to a 50-ml PTEF Nalgene® centrifuge tubes.
2. Add 40 ml of distilled water and sonicate for 30 min.
3. Centrifuge the samples for 20 min at 8228 *x g* at 4 °C.
4. Remove the supernatant by decanting and discard it.
5. Repeat the water addition, sonication, centrifugation, and supernatant removal two additional times.
6. Add 40 ml of 80% (vol/vol) ethanol and sonicate for 30 min.
7. Centrifuge the samples for 20 min at 8228 *x g* at 4 °C.
8. Remove the supernatant by decanting and discard it.
9. Repeat the addition of 80% (vol/vol) ethanol, sonication, centrifugation, and supernatant removal two additional times.
10. Add 40 ml of 100% acetone and sonicate for 30 min.
11. Centrifuge the samples for 20 min at 8228 *x g* at 4 °C.
12. Remove the supernatant by decanting and then discard.
13. Oven-dry the extract-free tomato plant roots at ~45-50 C (generally, 24 h is sufficient).

- *Ball-milling*. Grind extract-free tomato plant roots (around 200 mg) using a Retsch PM100 planetary mill (Retsch, Haan, Germany) fitted with one 50 ml agate grinding jar and 10 x10 mm ball bearings, set at 600 rpm. A total ball milling time of 3 h, alternating 20 min of grinding with 10 min of rest to avoid heating the sample, is sufficient.

- *Lignin/suberin isolation by enzymatic removal of polysaccharides*. The lignin/suberin fraction can be isolated by enzymatically hydrolyzing the structural polysaccharides that form the plant cell-wall, as described by Chang *et al.* (1975). For this, cellulysin cellulase (Calbiochem), a crude cellulase preparation from *Trichoderma viride* also containing hemicellulase activities, with activity ≥10,000 $FPUg^{-1}$ of dry weight, is used.

1. Add ~200 mg of extractives-free ball-milled tomato roots to a 50-ml PTEF Nalgene® centrifuge tube.
2. Add 30 ml of 20 mM sodium acetate (pH 5.0) buffer and 7.5 mg of Cellulysin cellulase.
3. Incubate the reaction slurry at 30ºC for 48 h, with constant agitation.
4. Centrifuge the samples (8228 *x g*, 4ºC, 20 min) and discard the solvent by decantation.
5. Repeat the process with fresh buffer (30 ml) and enzyme (7.5 mg) two additional times.
6. Finally, wash the residue (enriched lignin/suberin fraction) with distillated water, recover it by centrifugation and freeze dry it.

- *2D-NMR analysis*. Transfer approximately 20 mg of enzymatically isolated lignin/suberin preparation to a 5 mm NMR tube and add 0.6 ml of DMSO-d_6. Sonicate the NMR tube in an ultrasonic bath for 30-60 min until complete sample dissolution. Acquire 2D ^{1}H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectra on a cryoprobe-equipped Bruker Avance III 500 MHz instrument, using a standard Bruker adiabatic-pulse program ('hsqcetgpsisp.2') that enabled a semiquantitative analysis of the different ^{1}H-^{13}C- correlation signals. 2D-HSQC spectra are acquired from 10 to 0 ppm in F2 (1H) using 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 1 s, and from 200 to 10 ppm in F1 (^{13}C) using 256 increments of 32 scan, for a total experiment time of 2 h 34 min. The $^{1}J_{CH}$ used is 145 Hz. Processing uses typical matched Gaussian apodization in ^{1}H (LB=-0.1 and GB=0.001) and a squared cosine bell in ^{13}C (LB=0.3 and GB=0.1). The central residual DMSO peak (δ_C/δ_H 39.5/2.49) is used as an internal reference.

- *Assignation and quantitation of 2D-HSQC correlation signals*. HSQC cross-signals are assigned by literature comparison (Rencoret *et al.*, 2018; del Río *et al.*, 2018; Mahmoud *et al.* 2020; Youngsung *et al.*, 2021). A semiquantitative analysis of the volume integrals of the HSQC correlation peaks can be performed using Bruker's Topspin or other equivalent NMR-processing software such as MNova. The ^{1}H-^{13}C correlation signals from the aromatic/unsaturated region (δ_H/δ_C 5-8/90-150 ppm) of the spectrum are used to estimate the lignin composition in terms of guaiacyl (G) and syringil (S) units (**Figure 4**). The correlation signals of G_2 and $S_{2,6}$ are used to estimate the content of the respective G- and S-lignin units (as the signal $S_{2,6}$ involves two proton-carbon pairs, its volume integral is halved). The Cα/Hα correlation signals of the β–*O*–4′ alkyl aryl ethers (Aα), phenylcoumarans (Bα), and resinols (Cα) in the aliphatic-oxygenated region of the

spectra (**Figure 4**) are used to estimate their relative abundances (as per 100 aromatic units), whereas the Cγ/Hγ correlation signal of the cinnamyl alcohol end-units (Iγ) is used to estimate its relative abundance (as per 100 aromatic units); as signal Iγ involves two proton-carbon pairs, its volume integrals is also halved. Suberin/lignin (Sub/L) ratio can be roughly estimated by integration of all the HSQC signals in the aliphatic region (in which most of suberin signals appear) and referring this value to the total aromatic lignin units (G+S) integration.

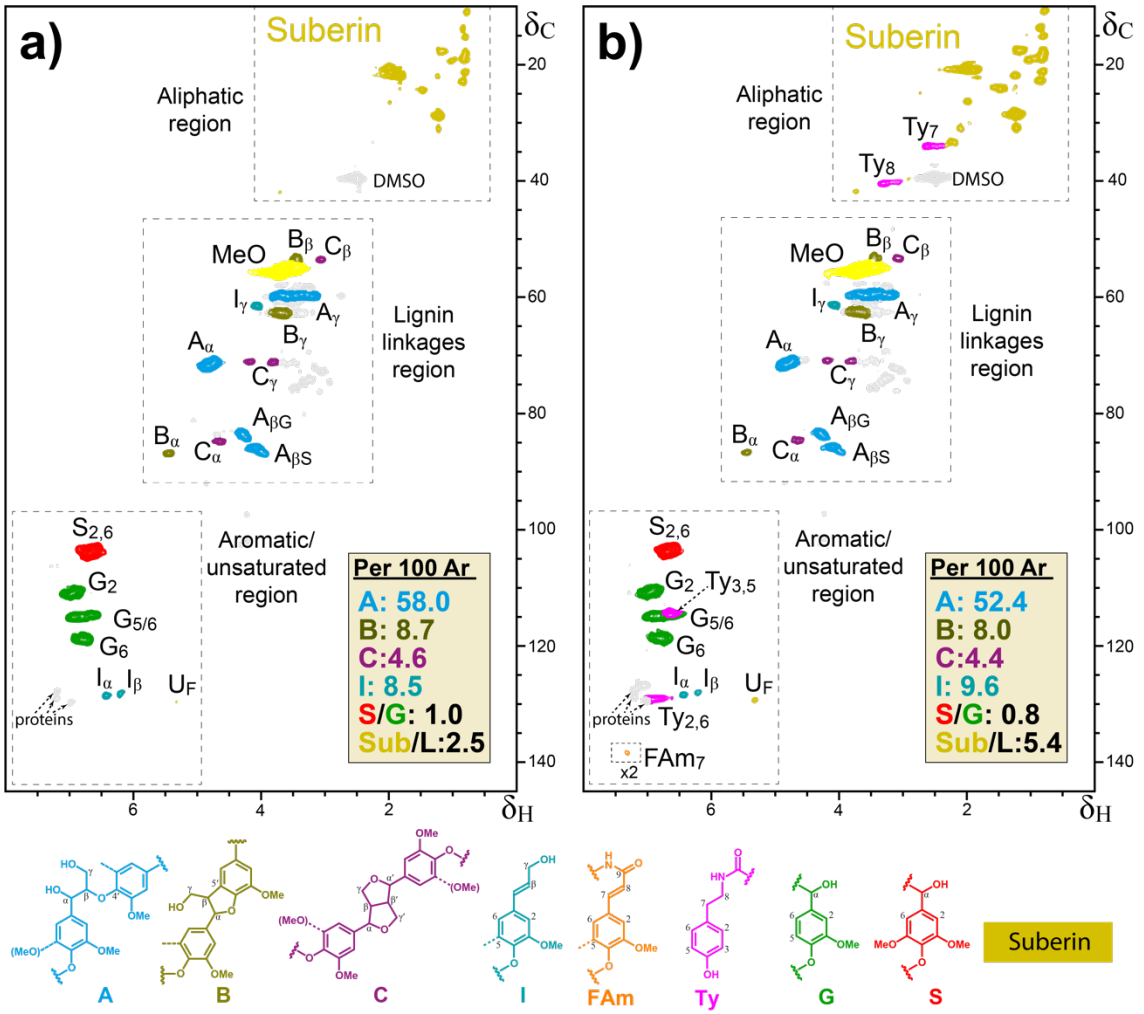

Figure 4. 2D-HSQC spectra of enzymatically isolated lignin/suberin fractions from mock-treated and *R. solanacearum*-infected taproots of resistant H7996 tomato. (**b**) Main lignin/suberin structures identified: β–*O*–4′ alkyl aryl ethers (A), β–5′ fenylcoumarans (B), β–β′ resinols (C), cinnamyl alcohols end-groups (I), feruloyl amides (FAm), amides of tyramine (Ty), guaiacyl lignin units (G), syringyl lignin units (S), as well as unassigned aliphatic signals from suberin. The structures and contours of the HSQC signals are color coded to aid interpretation. To detect the FAm7 signal, the spectrum scaled-up to 2-fold (×2) intensity. The abundances of the main lignin linkages (A, B and C) and cinnamyl alcohol end-groups (I) are referred to as a percentage of the total lignin units (S + G = 100%). Image reproduced from Kashyap *et al.*, 2022 with permission.

4. Notes

1. Always use a bigger Erlenmeyer than the desired volume (2.5 times-5 times) to ensure a good aeration of bacterial culture.

2. Do not use tap water, that can contain hypochlorite and other compounds toxic to bacteria.

3. Drought stress interferes with the response to bacterial wilt disease, so it is essential to ensure proper watering of the plants during infection.

4. In terms of analyzing the contribution of barrier formation on resistance, always take samples where both susceptible and resistant lines carry the same bacterial load.

5. Phloroglucinol is also known as the Weisner stain.

6. Phloroglucinol is easily oxidized (green coloration starts to appear) after a couple of weeks, so always use freshly stained samples.

7. At the molecular level, suberin is generally divided into an aromatic fraction composed mainly of ferulates, metabolic derivatives from the phenylpropanoid pathway, and an aliphatic part composed of long carbon chains (Nomberg *et al.*, 2022).

5. Acknowledgements

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6. Figure legends

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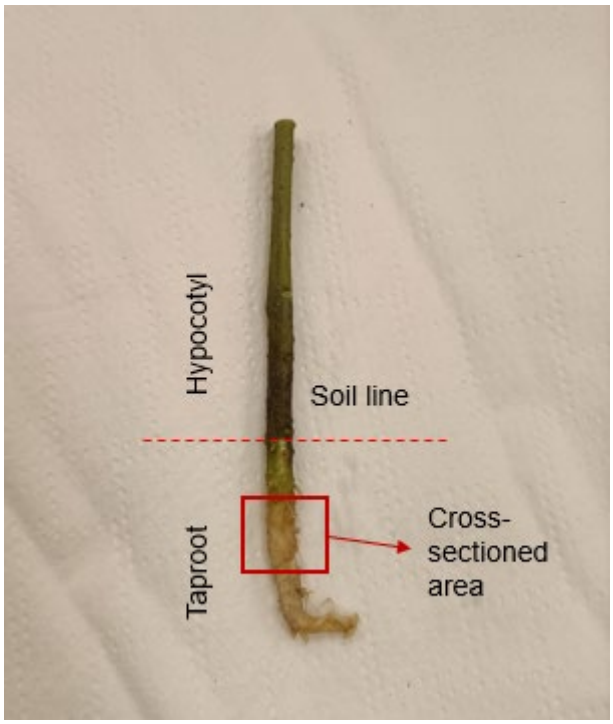

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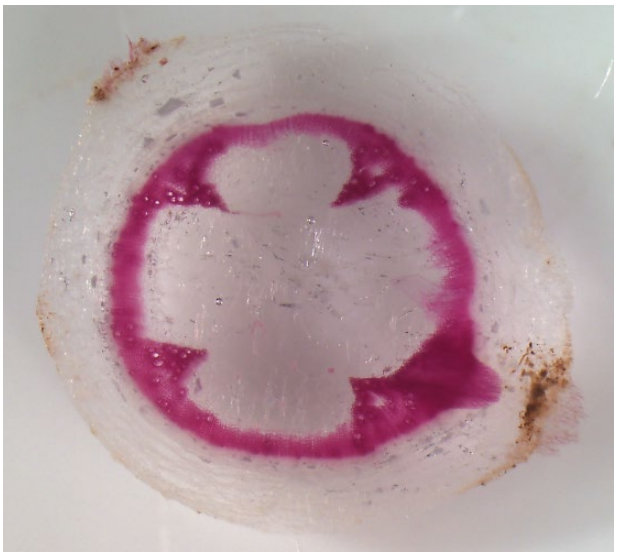

Figure 2. Phloroglucinol-stained samples. Examples cases of cross-sections stained with only Phloroglucinol-HCl observed under brightfield in a stereomicroscope Olympus SX16.

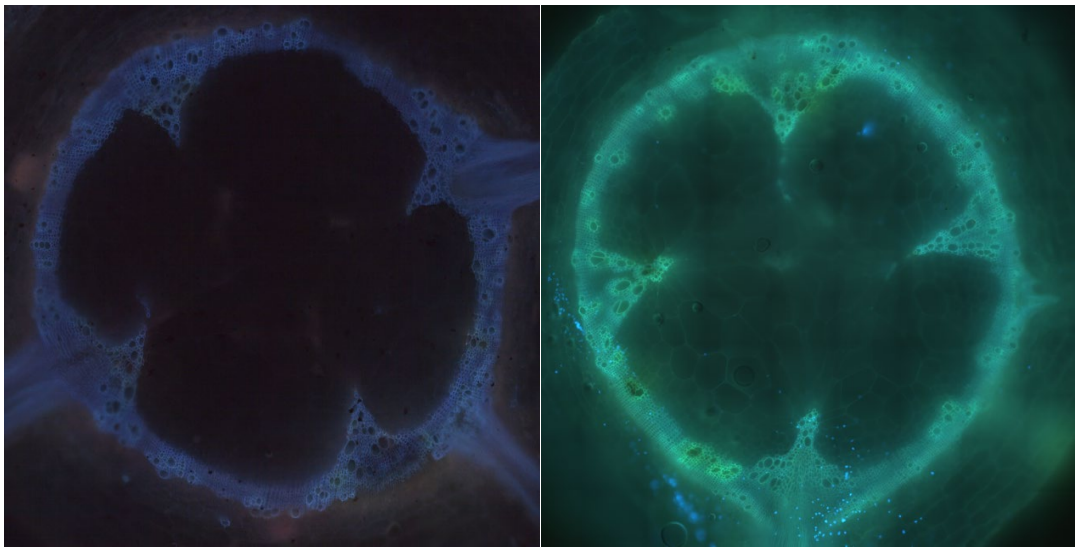

Figure 3. Sudan IV+KOH stained samples. Examples cases of cross-sections stained with only Sudan IV (left) and Sudan IV+KOH (right), observed under UV light in an epifluorescence microscopy Leica DM6.

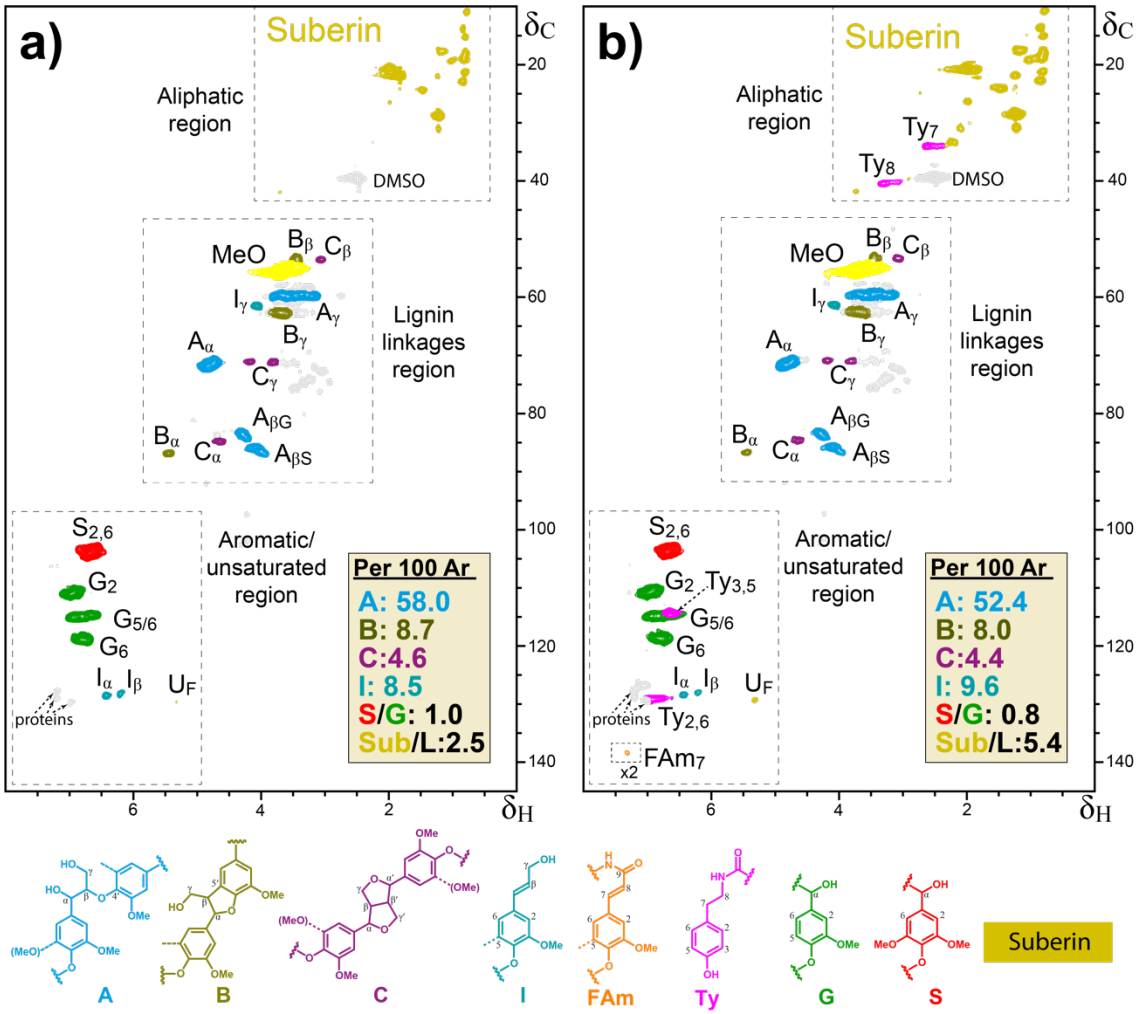

Figure 4. 2D-HSQC spectra of enzymatically isolated lignin/suberin fractions from mock-treated and *R. solanacearum*-infected taproots of resistant H7996 tomato. (**b**) Main lignin/suberin structures identified: β–*O*–4′ alkyl aryl ethers (A), β–5′ fenylcoumarans (B), β–β′ resinols (C), cinnamyl alcohols end-groups (I), feruloyl amides (FAm), amides of tyramine (Ty), guaiacyl lignin units (G), syringyl lignin units (S), as well as unassigned aliphatic signals from suberin. The structures and contours of the HSQC signals are color coded to aid interpretation. To detect the FAm7 signal, the spectrum scaled-up to 2-fold (×2) intensity. The abundances of the main lignin linkages (A, B and C) and cinnamyl alcohol end-groups (I) are referred to as a percentage of the total lignin units (S + G = 100%). Image reproduced from Kashyap *et al.*, 2022 with permission.