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Host cell wall composition and localized microenvironment implicated in resistance to basal stem degradation by lettuce drop (*Sclerotinia minor*)

Ivan Simko^{1*†}, Bullo Erena Mamo^{2,4†}, Clifton E. Foster^{3,5}, Neil D. Adhikari^{1,2,6} and Krishna V. Subbarao^{2*}

Abstract

Background *Sclerotinia* spp. are generalist fungal pathogens, infecting over 700 plant hosts worldwide, including major crops. While host resistance is the most sustainable and cost-effective method for disease management, complete resistance to *Sclerotinia* diseases is rare. We recently identified soft basal stem as a potential susceptibility factor to *Sclerotinia minor* infection in lettuce (*Lactuca sativa*) under greenhouse conditions.

Results Analysis of stem and root cell wall composition in five *L. sativa* and one *L. serriola* accessions with varying growth habits and *S. minor* resistance levels revealed strong association between hemicellulose constituents, lignin polymers, disease phenotypes, and basal stem mechanical strength. Accessions resistant to basal stem degradation consistently exhibited higher levels of syringyl, guaiacyl, and xylose, but lower levels of fucose in stems. These findings suggest that stem cell wall polymers recalcitrant to breakdown by lignocellulolytic enzymes may contribute to stem strength-mediated resistance against *S. minor*.

Conclusions The lignin content, particularly guaiacyl and syringyl, along with xylose could potentially serve as biomarkers for identifying more resistant lettuce accessions and breeding lines. Basal stem degradation by *S. minor* was influenced by localized microenvironment conditions around the stem base of the plants.

Keywords Lignin, Hemicellulose, Monosaccharides, Stem strength, Guaiacyl, Syringyl, Xylose

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Introduction

Lettuce (*Lactuca sativa*) is the world's leading leafy vegetable but is vulnerable to diseases such as downy mildew, Fusarium and Verticillium wilts, and lettuce drop [1] caused by *Sclerotinia minor* and *S. sclerotiorum*. *Sclerotinia* spp. infect over 700 hosts worldwide [2, 3] and pose serious threats to production of lettuce and other crops [4]. Once infected, lettuce plants collapse within a few days [5, 6]. Despite efforts to control it using cultural, antifungal, and biological methods [7], lettuce drop remains challenging due to the pathogens' ability to overwinter in soil and windborne nature [8, 9]. Host resistance is the most effective strategy of managing *Sclerotinia* diseases, but it is limited in economically important crops [10]. Although, a complete resistance of lettuce to *S. minor* has not been identified, some cultivars exhibit partial resistance linked to certain developmental traits [11]. These traits, including plant foliar density, rosette architecture, small leaf area, altered growth habit, plant height, rapid bolting, flowering and maturity [11–15], are often considered undesirable in modern lettuce cultivars [16–19].

Sclerotinia spp. have evolved mechanisms to overcome host immunity and procure nutritional sources [20, 21], including the production of phytotoxins and host cell wall-degrading enzymes (CWDEs). These enzymes facilitate rapid infection and nutrient acquisition [20, 22] by degrading host tissues [23], including the stem. In other crop species, stem mechanical strength is strongly correlated with resistance to lodging and soil-borne fungal diseases [24–27]. Soft basal stem have also been recently identified as a plausible susceptibility factor to *S. minor* in lettuce, leading to what we term 'plant architecture- or stem strength-mediated resistance' (PAMR) [14]. However, the causal relationship between stem mechanical strength and resistance to lettuce drop remain largely elusive. Given the absence of basal stem and root degradation in lettuce accessions with strong stems [14], we hypothesize that this may be due to differences in host tissue composition or cell wall architecture [28].

The plant cell wall acts as the first line of defense against pathogens [29–32]. Cellulose, hemicellulose, and lignin, the primary components of the cell wall, contribute to its structural integrity [24, 33, 34], playing an essential role in disease resistance [24, 30, 35, 36]. Several studies have demonstrated a positive correlation between lignin content and resistance to *Sclerotinia* spp. in *Brassicaceae* (*Brassica napus*, *B. oleracea*, and *Camelina sativa*) [37–43]. For instance, significant differences in the constitutive expression and regulation patterns of genes involved in the synthesis of lignin monomers (syringyl and guaiacyl) were observed in response to *S. sclerotiorum* infection in false flax (*C. sativa*) [40]. In oilseed rape (*B. napus*), the transcript abundance of a gene catalyzing

a step in the lignin biosynthesis pathway decreased in a susceptible cultivar inoculated with *S. sclerotiorum* [41]. Furthermore, in oilseed rape cultivars transformed with cDNA expressing recombinant proteins involved in lignin biosynthesis, there was an increase in the lignin content which correlated with enhanced resistance to *Sclerotinia* [39]. In cabbage (*B. oleracea*), cell wall degradation was inhibited in F₂ population resistant to *S. sclerotiorum* [43].

However, contradictory results have also been reported regarding the role of lignin in host resistance to *Sclerotinia* spp. Some studies show a negative correlation or unreliable prediction of resistance based on stem lignin content, as observed in soybean (*Glycine max*) [44], peanuts (*Arachis hypogaea*) [45], and oilseed rape [46]. In soybean infected with *S. sclerotiorum*, resistance appears to be regulated by reprogramming of the phenylpropanoid pathway, diverting its flux from lignin to lignin-intermediates, anthocyanins and phytoalexins [47]. This suggests that lignin intermediates, such as caffeic and ferulic acid, along with other compounds with antifungal activity (e.g., phytoalexins), are crucial components of the resistance response. The results were also confirmed at the transcript level, where several genes, including those encoding phenylalanine ammonia-lyase (PAL), lignin biosynthetic enzymes, chalcone synthase (CHS), and flavonol synthase (FLS), were down-regulated in the resistant line [47].

To investigate the biological basis of PAMR to *S. minor* in lettuce, we analyzed the carbohydrate polymers, monosaccharides, lignin, and monolignol composition in plant's basal stems and roots. Additionally, we compared the cell wall composition of lettuce drop-tolerant and susceptible accessions to understand the progression of basal stem degradation after *S. minor* infection. Furthermore, we quantified fungal DNA in the basal stems of lettuce accessions to determine the influence of canopy architecture on pathogen growth and development.

Materials and methods

Plant material

Six accessions representing different horticultural types of lettuce were used for the study: Eruption (Latin type), Reine des Glaces (Batavia subtype of crisphead lettuce), Salinas (iceberg subtype of crisphead lettuce), Da Ye Wo Sun (stem type), PI 251246 (primitive oil-seed type), and 11-G99 (*L. serriola*, the wild progenitor of cultivated lettuce). These accessions exhibit varying rates of stem elongation and field reactions to lettuce drop. Eruption, Reine des Glaces, Salinas, and 11-G99 (in fall) are slow-bolting. Da Ye Wo Sun and 11-G99 (in spring/summer) have intermediate rates of stem elongation; while PI 251246 bolts rapidly. Eruption, PI 251246, and 11-G99 (in spring/summer) are partially resistant to lettuce drop, although

Eruption shows high disease severity once infected. Reine des Glaces, Salinas, Da Ye Wo Sun, and 11-G99 (in fall) are susceptible to lettuce drop. Seeds of all accessions were obtained from the USDA-ARS lettuce germplasm collection in Salinas, California, USA.

Inoculation and sampling

Plants were grown in a greenhouse in sterilized soil mixture (2:1 ratio of sand to potting mix) at 20–25 °C during the day and 15–17 °C at night. Inoculation with *S. minor* mycelia followed a previously published protocol [14]. Two independent experiments were performed using a completely randomized design, with two replications and one plant per accession and replicate. For inoculation, ten *S. minor*-infested rye seeds were placed a few millimeters under the soil surface next to the plant's basal stem. To ensure that the accessions were compared at the same developmental stage, inoculation took place when plants reached approximately 50% flowering. For analyses of cell wall composition, plants were sampled at three stages: at the time of inoculation (control, healthy plants), six to seven days post-inoculation (early phase of disease development), and 14 to 20 days post-inoculation (late sampling at an advanced phase of disease development). This sampling approach was designed to mimic the two-phase infection model proposed for a closely related *S. sclerotiorum*, in which the pathogen suppresses defense reactions in the early phase and induces host necrosis and cell wall degradation in the late phase [23].

Stem sampling involved cutting each plant at the stem base, and 8.5 cm of the stem above the base was harvested. The sampling of 8.5 cm of stem tissue (from the base to 8.5 cm above) was chosen because this area represents the stem region in contact with the pathogen and thus is the primary site of infection. This sampling length is based on our experience with the pathogen infecting lettuce in both field and greenhouse conditions. For sampling underground parts, the entire root system was removed from the pot, rinsed under running water to remove any cultivation media, and then placed into 50 mL tubes. Samples were frozen in liquid nitrogen, lyophilized, and stored at -20 °C until processing.

Cell wall composition analysis

Lyophilized stem and root samples were ground using a Thomas Wiley Mini-Mill, 40 mesh (Thomas Scientific, Swedesboro, New Jersey, USA). A 100–200 mg of ground sample was submitted for analysis. Sixty mg aliquots of the ground, dried biomass were ball milled with the iWall grinding and feeding robot [48] for primary (cellulose and hemicellulose) and secondary (lignin) cell wall composition analyses [49, 50]. The milled material was used to prepare the alcohol insoluble residue (AIR) by sequential extraction with water, 70% ethanol, and 1:1 chloroform:

methanol to remove the soluble components, such as sugars, proteins, lipids, pigments, DNA, and RNA. The AIR was then treated with amylase and pullulanase in a 37 °C rotisserie incubator with end-over-end rotation for approximately 12 h and washed with water to remove the starch content, yielding isolated lignocellulosic material for cell wall analysis.

The isolated lignocellulosic cell wall material was dried and weighed into three 2 mg technical replicates for matrix polysaccharide (hemicellulose) composition and crystalline cellulose assays. Polysaccharide composition was analyzed via GC-MS (Agilent 7890A GC / 5975C MS, Agilent Technologies, Santa Clara, California, USA) after 2 M trifluoroacetic acid (TFA) hydrolysis and subsequent alditol acetate derivatization of neutral monosaccharides present in the hydrolysate. Crystalline cellulose was isolated and purified from the insoluble residue remaining from the TFA hydrolysis and then hydrolyzed in 72% sulfuric acid. The crystalline cellulose content of the lignocellulosic material was determined using the colorimetric anthrone assay.

For lignin content analysis, the cell wall material was weighed into three 2 mg technical replicates and assayed using the acetyl bromide soluble lignin (ABSL) method [49]. The ABSL method involved treating cell wall material with a 25% (v/v) solution of acetyl bromide in glacial acetic acid at 50 °C for 3 h to solubilize the lignin matrix. The solubilized lignin was diluted with glacial acetic acid and assayed using a photospectrometer at 280 nm (Spectramax 384 plus, Molecular Devices, San Jose, California, USA). The lignin content was calculated using a molar extinction coefficient of $18.21 \text{ g}^{-1} \text{Lcm}^{-1}$ [49].

Lignin monomers composition analysis was performed using the thioacidolysis method, which detects the *p*-hydroxyphenyl, guaiacyl, and syringyl monomers incorporated in the lignin matrix via β -O-4 ether linkages. Dried and isolated lignocellulosic material was weighted onto three 2 mg replicates, to which a mixture of 87.5% (v/v) dioxane, 10% (v/v) ethanethiol, and 2.5% (v/v) boron trifluoride diethyl etherate was added and heated at 100 °C for 4 h to liberate the lignin monomers. The extracted thioether derivatized monomers were derivatized with *N*, *O*-bis[trimethylsilyl]acetamide (BSA) and quantitated using GC-MS analysis (Agilent 7890A GC / 5975C MS, Agilent Technologies, Santa Clara, California, USA) according to the published procedures [51].

The composition analyses provided information about the concentrations of crystalline cellulose (term cellulose used henceforth), seven neutral monosaccharides (arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose), soluble lignin (acetyl bromide soluble lignin, ABSL) and lignin monomers composition (*p*-hydroxyphenyl – term hydroxyphenyl used henceforth, guaiacyl, and syringyl). Yields of all compounds were reported in

µg per mg cell wall unit. Samples of *S. minor* mycelium and sclerotia, and wood chip were included as controls in the composition analysis; samples of *Populus trichocarpa* (black cottonwood) were included in the ABSL analysis only. The *S. minor* sclerotia submitted for cell wall composition analysis were produced as previously described [13] but on rye seeds.

Fungal DNA quantification to study effect of stem base microenvironment on *Sclerotinia* biomass

Twenty-five to thirty plants of two susceptible accessions (Da Ye Wo Sun and Reine des Glaces) and two resistant accessions (11G-99 and PI 251246) were grown in the greenhouse as described above. Plants were prepared for inoculation at approximately 50% flowering, following a completely randomized design with three replications (biological samples) for each treatment. After inoculation with rye seeds infested with *S. minor* mycelia, basal stems were either left uncovered ('uncovered' group), wrapped with the plant's own leaves ('leaves' group), or wrapped with clear plastic wrap (Saran Wrap, SC Johnson, Mt. Pleasant, Wisconsin, USA) ('plastic' group). Three groups of uninoculated plants were also grown from each accession to serve as controls for the 'uncovered', 'leaves', and 'plastic' groups, respectively. The experiment was conducted twice following the inoculation procedure outlined previously [14]. Five to six days post-inoculation, 8.5 cm of the basal stem tissues from inoculated and uninoculated plants were harvested, frozen in liquid nitrogen, lyophilized, and stored at -20°C until processing.

Sample preparation for quantification of fungal DNA [52] were performed using a standard SDS-based genomic DNA extraction protocol. Lyophilized tissue was ground to a fine powder and genomic DNA was extracted using an extraction buffer containing Tris, NaCl, EDTA, and SDS [53]. The extracted DNA was precipitated with isopropanol, washed with ethanol, and resuspended in TE buffer. The DNA was then cleaned using the Zymo Genomic DNA Clean & Concentrator-25 kit (Zymo Research, Orange, California, USA), and quantitated using the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). *S. minor* gDNA from mycelial culture used for plant inoculation served as a positive control.

Duplicate 20-µl qPCR reactions were performed with 10 ng gDNA, 2× TaqMan Fast Universal PCR master mix (Applied Biosystems, Waltham, Massachusetts, USA), 18 µM primers (SMLcc2-1 F: 5'-CGGTTGAGA ACTCCACTATAACC-3' and SMLcc2-1 R: 5'-AAGC TTCCCTTCTGACGAATAC-3'), and 5 µM of probe (SMLcc2-1 5'-(FAM)-TCCGATAGCGCACCGAATCT CAAA-(TAMRA)-p-3'). The 285 bp amplicon targeted *S. minor* laccase CDS. Reactions were run on a Roche

LightCycler 480 (Roche Diagnostics, Indianapolis, Indiana, USA) with 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 60 °C for 30 s. Standard curves were generated using 0.001 to 100 ng *S. minor* DNA with a 10-fold increase (Additional file S1). Specificity was tested against non-target pathogens (*Verticillium dahliae* isolate VdLs17, *Botrytis cinerea*, and *Aspergillus niger*) and lettuce accessions. Water controls were used to confirm no contamination. The pathogen DNA was quantified from mean Ct values of duplicates, subtracting traces of fungal biomass detected in uninoculated controls.

Statistical analyses

Statistical analyses were conducted using JMP Pro 17 software (SAS Institute, Cary, North Carolina, USA). The methods included analysis of variance (ANOVA) with *post-hoc* Tukey's honestly significant difference (Tukey's HSD) test, *t*-test, Pearson linear correlation, and principal component analysis (PCA). Differences in compound content were analyzed using a mixed-effect ANOVA model, with resistant and susceptible groups as fixed effects and accessions as random effects. The number of days to reach five disease progress stages (mycelium emergence, lower leaf discoloration, leaf wilting, shoot wilting, and plant mortality), previously published [14], was converted into the area under the disease progress stairs (AUDPS) [54]. AUDPS values were then correlated with stem and root cell wall composition data collected at the control (healthy plants), early, and late phases of disease development. *P*-values for correlations were adjusted for multiple comparisons using false discovery rate (FDR) method implemented in JMP Pro 17 software. PCA was performed using resistance or composition data from all evaluated plants of six accessions.

To assess the effects of microenvironmental conditions and nutrient availability on fungal DNA quantity (pathogen growth/development), partial eta squared (η^2) values were calculated from ANOVA to estimate effect sizes. A simplified model included three main factors: resistance phenotype (susceptible or resistant), microenvironmental conditions (wrapped or uncovered), and nutrient availability (leaves or no leaves). The 'plastic' and 'leaves' wrapping treatments were assumed to provide similar microenvironmental conditions but differed in nutrient availability due to the presence of leaf tissue. The 'uncovered' treatment was assumed to have similar nutrient availability to the 'plastic' treatment but with different microenvironmental conditions. However, this simplified model was incomplete as no treatment provided the same microenvironmental conditions as the 'uncovered' treatment while having nutrient availability similar to the 'leaves' treatment.

Table 1 Cell wall composition of lettuce stems and roots, *S. minor* mycelium and sclerotia, and wood chips

Compound ^a	Lettuce stem	Lettuce root	<i>S. minor</i> mycelium	<i>S. minor</i> sclerotia ^b	Wood chips ^c
Rhamnose	9.70±0.72	4.77±0.52	0.38	0.93	1.79
Fucose	1.28±0.09	0.80±0.08	0.01	1.00	0.54
Arabinose	17.72±1.93	9.21±1.04	2.02	0.76	9.39
Xylose	128.08±8.37	48.38±7.11	2.12	1.28	21.46
Mannose	8.20±0.38	5.77±0.53	70.55	48.79	13.16
Galactose	14.82±1.02	8.77±0.91	18.23	45.79	5.77
Glucose	30.08±2.37	18.93±2.85	258.01	264.23	10.17
Cellulose	363.09±9.88	146.39±15.19	0.00	305.90	163.23
ABSL	127.94±5.71	94.24±7.48	56.51	12.74	135.13
Syringyl	30.33±2.93	7.69±1.51	0.14	0.33	0.86
Guaiacyl	15.77±1.11	9.22±1.14	0.19	0.17	10.28
Hydroxyphenyl	0.09±0.01	0.10±0.01	0.00	0.00	0.73

^a Content of all compounds is expressed in µg per mg. Lettuce composition data are pooled from across four susceptible and two resistant accessions. Data for individual accessions and three disease progression stages can be found at Table S2

^b *S. minor* sclerotia were produced on rye seeds; therefore, the composition may be affected by debris coming from the seeds. The cellulose assay was validated for plant cell wall analysis solely; fungal composition is provided as a reference

^c Wood chips were from the potting mix used for growing plants and originated from an undetermined tree species. In addition, black cottonwood (*Populus trichocarpa*) chips were also added as control to analyses of acetyl bromide soluble lignin (ABSL). Two tested samples contained 24.48 and 24.49 µg/mg ABSL.

Results

Significant differences among lettuce accessions were observed in disease progression, stem and root

degradation after inoculation with *S. minor* in a greenhouse [14]. Stem mechanical strength was correlated with the outcome of *S. minor* infections [14] (Fig. S1), prompting detailed analyses of stem and root cell wall composition to identify compounds related to delayed disease symptoms, reduced degradation, and higher stem strength observed in 11-G99 and PI 251246 (Fig. S2).

Analysis of monosaccharides determined that stem cell walls were primarily composed of xylose, followed by glucose, arabinose, galactose, rhamnose, mannose, and fucose (Table 1). Root cell walls exhibited a similar composition with xylose being the most abundant (Table 1). Among the three monolignols quantified in stems, syringyl had the highest content, followed by guaiacyl, and hydroxyphenyl. Guaiacyl was the most abundant in root cell walls, followed by syringyl, and hydroxyphenyl (Table 1).

Three-way ANOVA revealed significant differences ($p \leq 0.05$) among accessions in the content of all compounds except for mannose and glucose. Differences between stem and root composition were significant for all compounds except for hydroxyphenyl, and significant changes occurred in the content of all compounds as the disease progressed (Table S1, S2).

In stems, disease progressions led to a significant decrease in arabinose, fucose, galactose, and rhamnose, and a significant increase in glucose and guaiacyl (Fig. 1). In roots, disease progression was accompanied by a significant increase in glucose, mannose, xylose, cellulose, ABSL, hydroxyphenyl, guaiacyl, and syringyl (Fig. 1).

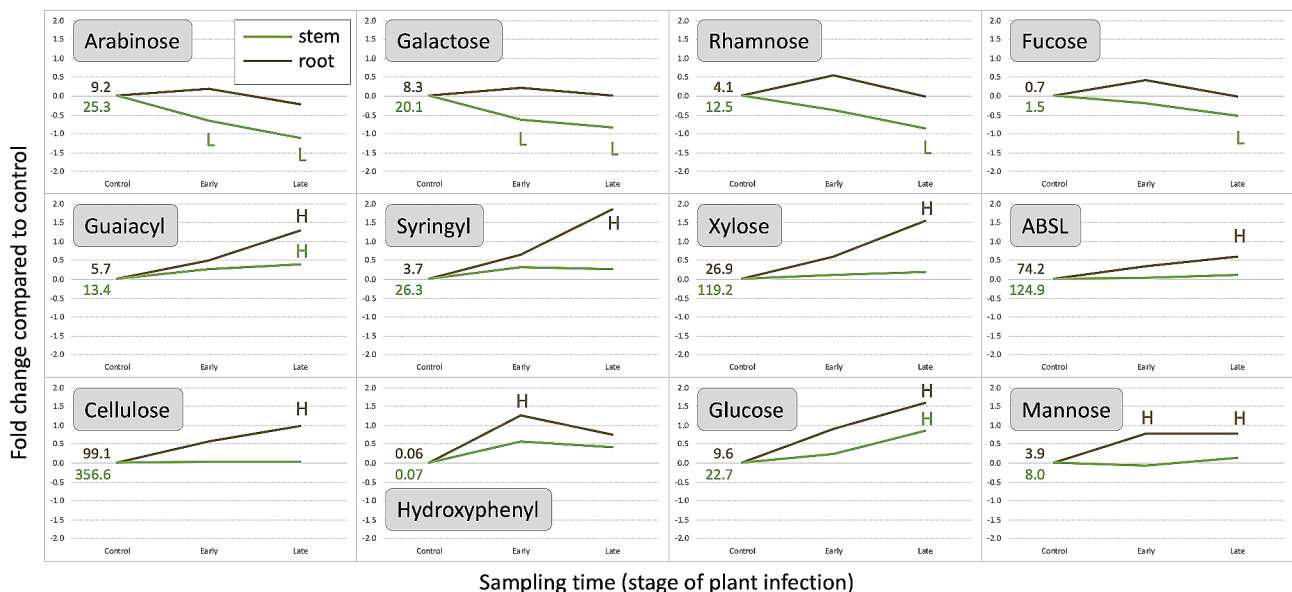


Fig. 1 Relative changes in cell wall composition across all accessions during disease progression. Stem and root samples were collected before inoculation (control) and at early and late disease stages after inoculation with *S. minor*. Logarithmic scales show changes compared to the control. Numbers represent the average content of compounds (µg per mg) in stems (green) and roots (brown) of healthy plants. ‘H’ and ‘L’ indicate values significantly ($p \leq 0.05$) higher or lower than the control, respectively

Correlation analyses performed on both tissue types from all accessions sampled at the three disease progression stages confirmed these trends (Fig. S3).

Comparing resistant (11-G99 and PI 251246) and susceptible (Da Ye Wo Sun, Eruption, Reine des Glaces, and Salinas) accessions, significant differences in cell wall composition were observed in stems and roots and at all disease progression stages (Figs. 2, 3 and 4). Before inoculation, healthy stems of resistant accessions had higher levels of syringyl (274%), guaiacyl (199%), xylose (193%), ABSL (142%), and cellulose (125%), but lower levels of arabinose (43%) and fucose (48%) (Figs. 2, 3 and 4). In the early stage of disease development, significant differences were still observed for syringyl, xylose, guaiacyl, and fucose, while at a later stage for syringyl and xylose (Fig. 3). In roots, differences in cell wall composition were observed only in the early stage of disease development for syringyl (462%), xylose (265%), guaiacyl (214%), cellulose (183%), and ABSL (159%) (Figs. 3 and 4). Similar trends were observed in the late stage of disease progression, but the differences were not significant.

Correlation analysis between disease progression (AUDPS values) and cell wall composition revealed significant ($p \leq 0.05$) associations in stems for fucose, xylose, ABSL, guaiacyl, and syringyl at different disease progression stages (Fig. 5). Higher levels of xylose, ABSL,

guaiacyl, and syringyl were associated with slower disease progression, while higher levels of fucose were associated with more rapid disease progression.

Since composition data from uninoculated, control plants hold the most practical use for plant breeders, the content of ABSL, syringyl, guaiacyl, xylose, and fucose was used in principal component analysis. The PCA results using composition data from healthy plants (Fig. 6) closely resembled results obtained from disease resistance and stem strength data (Fig. S1), indicating a close match between the results.

Quantification of fungal DNA showed that susceptible accessions consistently had higher amounts of *S. minor* DNA than resistant accessions across all treatments (Fig. 7). The effect size was significant for the phenotypic groups (susceptible vs. resistant, $\eta^2 = 0.185$, $p < 0.001$) and microenvironmental conditions (wrapped vs. uncovered, $\eta^2 = 0.065$, $p < 0.05$), but not for additional nutrient availability (leaves vs. no leaves, $\eta^2 = 0.008$).

Discussion

Several hundred lettuce accessions were tested for resistance to *S. minor*, with substantial resistance observed in the oil-seed accession PI 251246 [13, 17, 55]. Contrary to initial hypotheses of resistance mechanism being related to pathogen avoidance [12], recent studies suggest that

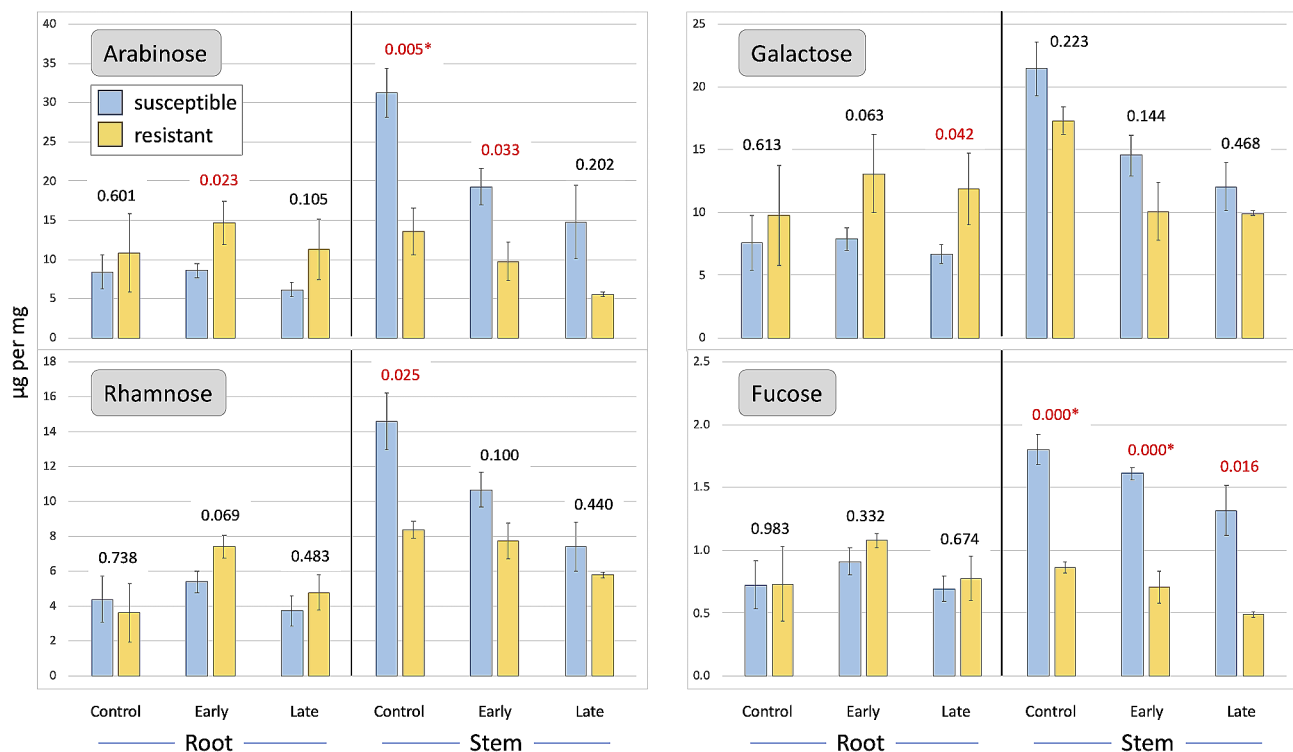


Fig. 2 Changes in arabinose, galactose, rhamnose, and fucose content in cell walls. Samples from susceptible and partially resistant groups were collected at three disease progression stages (control, early, late) after inoculation with *S. minor*. Numbers above bars are p -values calculated between two phenotypic groups differing in resistance ($p < 0.0004$ is shown as 0.000). Values in red are significant at $p \leq 0.05$, asterisks indicate values significant after false discovery rate adjustment

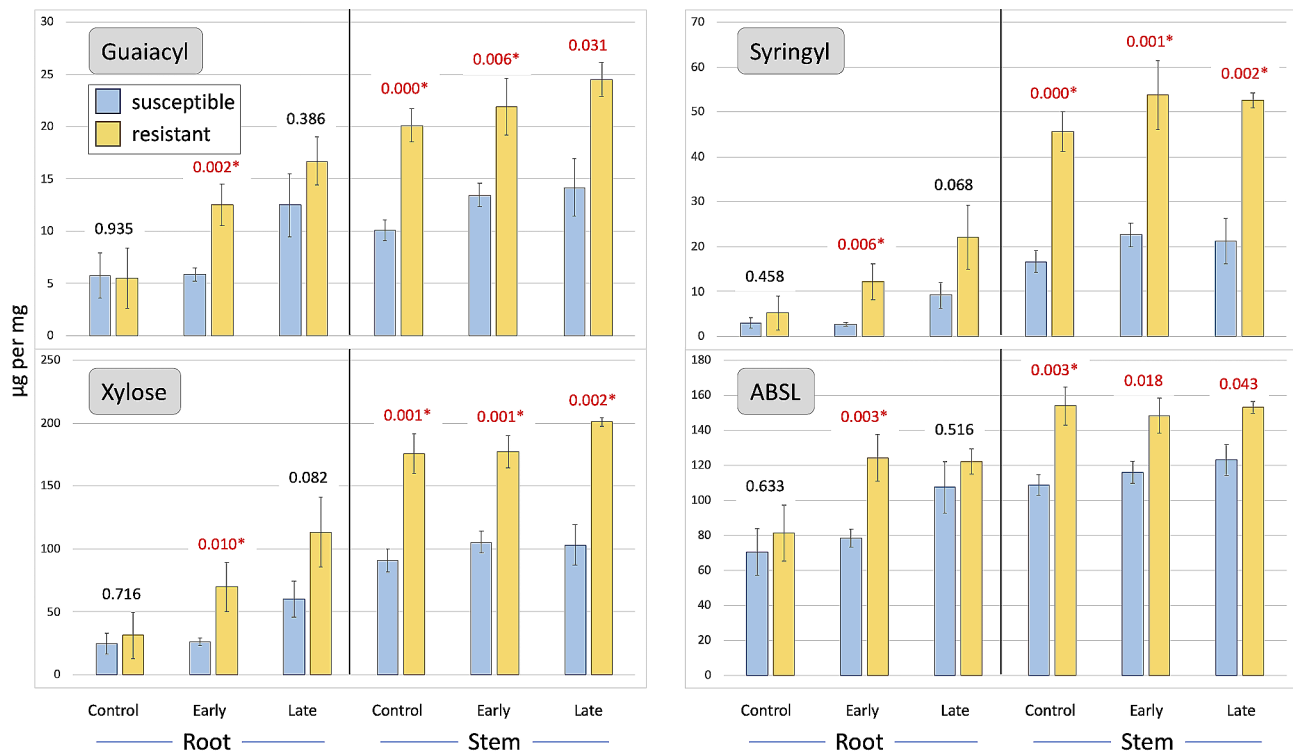


Fig. 3 Changes in guaiacyl, syringyl, xylose, and ABSL content in cell walls. Samples from susceptible and partially resistant groups were collected at three disease progression stages (control, early, late) after inoculation with *S. minor*. Numbers above bars are *p*-values calculated between two phenotypic groups differing in resistance ($p < 0.0004$ is shown as 0.000). Values in red are significant at $p \leq 0.05$, asterisks indicate values significant after false discovery rate adjustment

resistance in PI 251246 and 11-G99 is linked to stem mechanical strength rather than pathogen avoidance [14]. In this study, we investigated the difference in stem and root composition between accessions with rapid basal stem degradation (Da Ye Wo Sun, Eruption, Reine des Glaces, and Salinas) and slow basal stem degradation (11-G99 and PI 251246) following inoculations with *S. minor* under greenhouse conditions.

We found that syringyl and guaiacyl play vital roles in lettuce constitutive resistance to *S. minor*, as both lignin monomers were significantly higher in cell walls of stems of healthy plants from resistant accessions compared to susceptible accessions (Figs. 3 and 5). This finding is consistent with previous studies that demonstrated stem cell wall lignification enhances host resistance to *Sclerotinia* spp. [40, 56–60]. Additionally, lignification plays a role in induced components of lettuce resistance, as *de novo* synthesis of both lignin monomers in stems and roots occurred after inoculation with the pathogen (Fig. 1). However, some previous studies suggested lower stem lignin content may be linked to higher resistance against *Sclerotinia* spp. [44, 46], while also concluding that stem lignin content alone is not a reliable indicator of resistance [45]. Therefore, it has been proposed that rather than just the total lignin content, lignin intermediates

such as caffeic and ferulic acids may play a significant role in the resistance response [47].

While previous studies suggested a decreasing syringyl to guaiacyl (S/G) ratio is associated with improved resistance to *Sclerotinia* spp. [40, 56, 60], our results did not show a clear relationship between the S/G ratio and resistance. The S/G ratio was higher in the stem cell walls of healthy resistant plants (2.26) than in susceptible plants (1.61). However, this ratio slightly but consistently decreased in both resistant (2.18) and susceptible (1.43) accessions at the late stage of disease progression. Thus, it is possible that lignin cross-linking, rather than its composition, is directly related to lettuce drop resistance, as syringyl, guaiacyl, and hydroxyphenyl have similar inhibitory effects on cell wall degradability by fungal hydrolases [61, 62]. Additionally, not only the absolute amount of syringyl and guaiacyl or their S/G ratio may influence resistance, but also the location of their deposition. For example, in moderately resistant cultivar of *B. napus* infected by *S. sclerotiorum*, syringyl deposition occurred earlier than in susceptible cultivar [57]. Syringyl lignification was observed in the vascular sclerenchymatic cortex tissue, while guaiacyl was detected in both resistant and susceptible cultivars in the intercellular spaces of sclereids and adjacent phloem cells. Thus, earlier deposition of syringyl appears to be

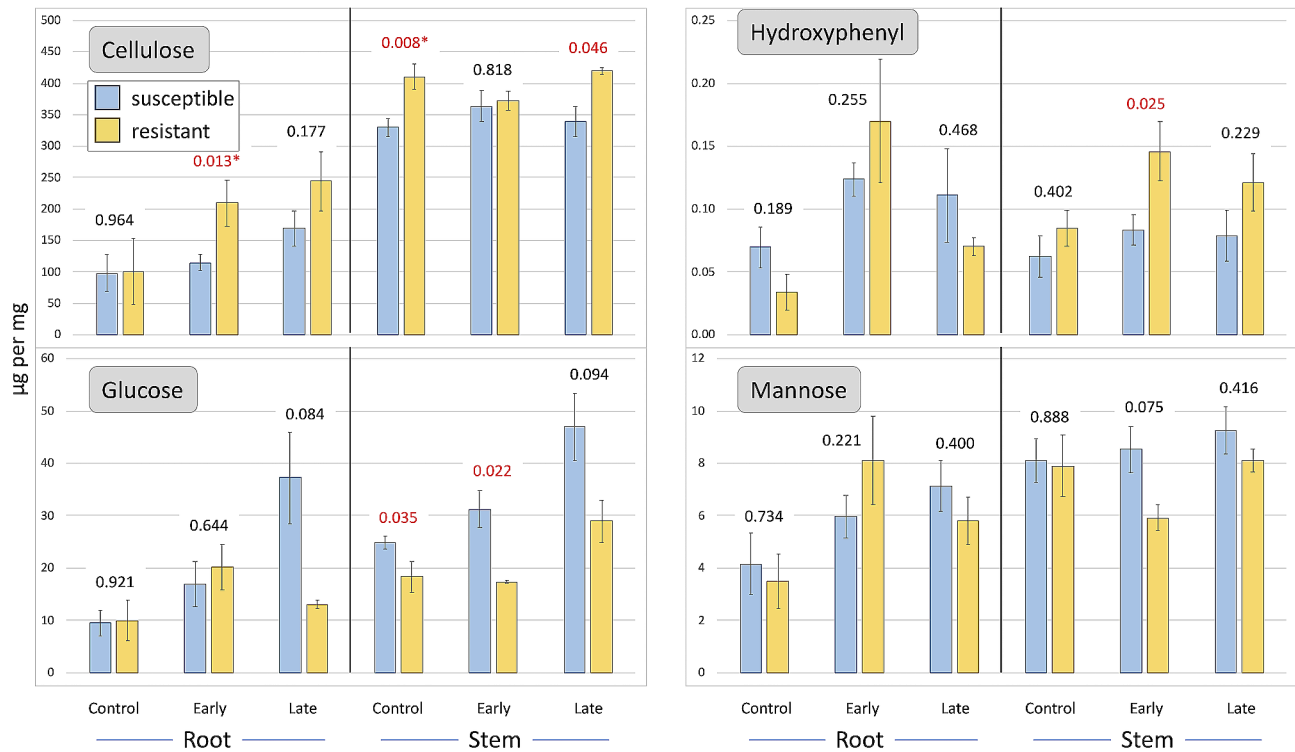


Fig. 4 Changes in cellulose, hydroxyphenyl, glucose, and mannose content in cell walls. Samples from susceptible and partially resistant groups were collected at three disease progression stages (control, early, late) after inoculation with *S. minor*. Numbers above bars are *p*-values calculated between two phenotypic groups differing in resistance (*p* < 0.0004 is shown as 0.000). Values in red are significant at *p* ≤ 0.05, asterisks indicate values significant after false discovery rate adjustment

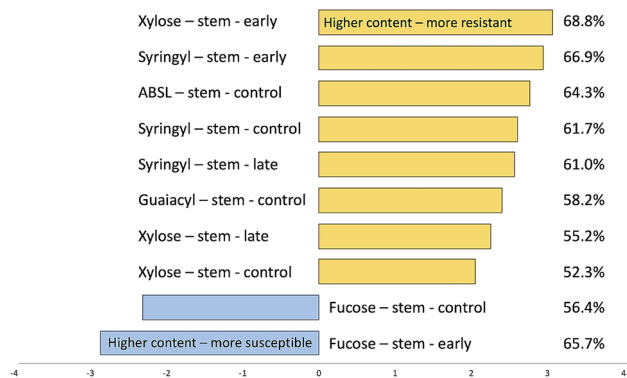


Fig. 5 Compounds significantly correlating with resistance to *S. minor*. AUDPS scores of disease progression were correlated with cell walls composition, and those significant at *p* < 0.05 after false discovery rate adjustment are shown. Values on the right show percent of phenotypic variation in AUDPS explained by the respective compound content

related to the infection response, whereas guaiacyl deposition, which was similar in both resistant and susceptible cultivars, is possibly a part of basal plant defense [57].

Among the tested monosaccharides, xylose and fucose content in stem cell walls showed the most consistent results, with higher levels of xylose and lower levels of fucose associated with increased resistance (Figs. 2, 3 and 5). Xylose, the main component of hemicellulose, has

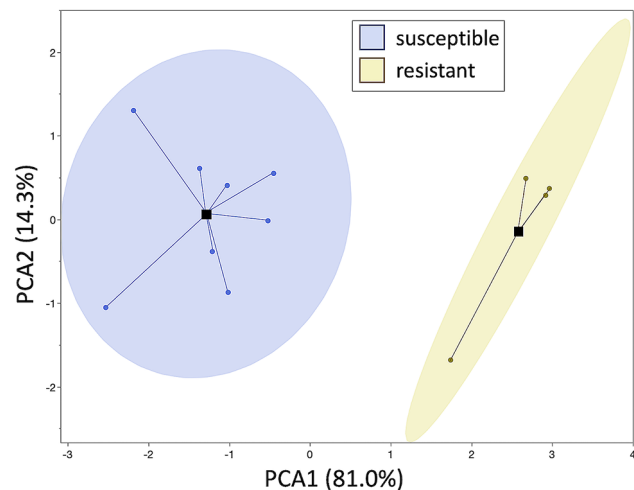


Fig. 6 Principal component analysis based on ABSL, syringyl, guaiacyl, xylose, and fucose content in stem cell walls of healthy, uninoculated plants. The four compounds were selected based on the correlation analysis results shown in Fig. 5. Note that the grouping of plants into two groups is identical to that for disease progression and stem mechanical strength (Fig. S1)

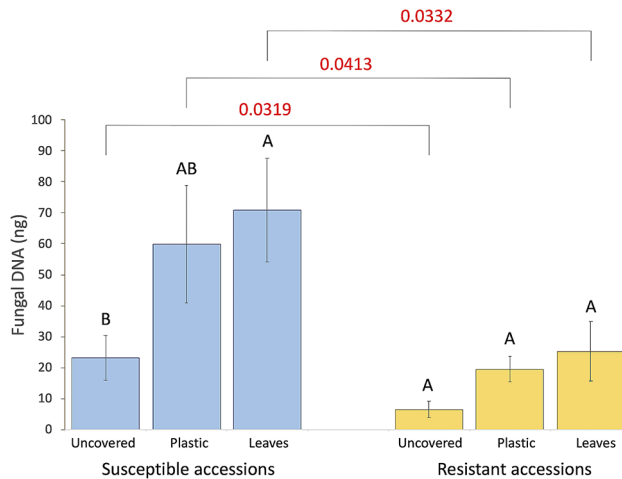


Fig. 7 Fungal biomass accumulation in susceptible and resistant accessions under three treatments. After inoculation with *S. minor* mycelia, basal stems were either left uncovered ('uncovered' group), wrapped with the plant's own leaves ('leaves' group), or wrapped with clear plastic wrap ('plastic' group). Mean values within each group, followed by different letters, are significantly different ($p \leq 0.05$) based on Tukey's HSD test. *P*-values at the top show differences between identical treatments on the two groups of accessions

been implicated in increased resistance of transgenic *B. napus* plants to *S. sclerotiorum* [63] and hypothesized to be one of the determinants controlling *Arabidopsis thaliana* resistance to another necrotrophic fungus, *Plectosphaerella cucumerina* [64].

The association of higher fucose content in lettuce with increased susceptibility to *S. minor* is unexpected, as fucose typically enhances plant resistance by aiding cell wall xyloglucan biosynthesis. For example, *A. thaliana* mutants with higher levels of fucosylated xyloglucans show greater resistance to the necrotrophic fungus *P. cucumerina* [65]. We speculate that the increased fucose in susceptible lettuce may relate to fucosylated polysaccharides in the primary cell wall, serving as targets for pathogen lectins [66]. Proteins structurally similar to fucose-specific lectins have been found in *S. sclerotiorum* [67, 68]. The interaction between fucosylated xyloglucans in lettuce cell walls and pathogen lectins might facilitate pathogen recognition and infection, explaining the unexpected link between higher fucose content and increased susceptibility to *S. minor*.

Similar patterns to that of fucose content were observed for arabinose, galactose, and rhamnose content in stem cell walls (Fig. 2), though the results were not consistently significant. Initially these monosaccharides were higher in susceptible accessions but gradually decreased in both susceptible and resistant accessions as the disease progressed. Sclerotiniaceae lectins have binding specificity towards all these compounds [69].

Conversely, as the disease developed, the glucose content increased, particularly in susceptible accessions

(Fig. 4). Glucose molecules are utilized to produce cellulose and can be incorporated into other cell wall components such as hemicelluloses and pectins. *Sclerotinia* spp. can alter the composition of plant cell walls, including the breakdown of polysaccharides like cellulose and hemicellulose, which may release glucose.

Although the relationship between root composition and resistance was not consistent, root cell wall composition may contribute to the plant's response to *S. minor* infection. Compounds such as syringyl and guaicyl (Fig. 3) showed a pattern similar to that observed in stems. Interestingly, like in stems, the glucose levels in roots of susceptible accessions substantially increased as the disease progressed from 9.5 $\mu\text{g}/\text{mg}$ to 16.9 $\mu\text{g}/\text{mg}$ to 37.2 $\mu\text{g}/\text{mg}$ (Fig. 4), suggesting a potential relationship between glucose levels in cell walls of both stems and roots and resistance to basal stem degradation caused by *S. minor*.

Our results indicate that enhancing lettuce's resistance to *S. minor* could be improved through increased levels of lignin and/or hemicellulose in host tissues. However, it is crucial to implement these changes carefully to prevent negative impacts on plant growth, leaf texture, digestibility, taste, and nutritional value. Therefore, any improvement in cell wall mechanical strength via elevated lignocellulosic compound levels should be specifically targeted to the basal stem. If achieving these modifications naturally proves challenging, tissue-specific gene expression approaches may be considered [70, 71].

It is important to note that cultivar Eruption, previously identified as one of the most resistant accessions to *S. minor* in field trials [11, 13, 55, 72], exhibited elevated disease severity (rapid basal stem degradation) when inoculated with *S. minor* in a greenhouse. This difference in resistance may be related to the anthocyanin content, which decreases under greenhouse conditions compared to field conditions, mostly due to lower ultraviolet (UV) radiation [73]. Loci regulating lettuce anthocyanin content and resistance to *S. minor* frequently co-locate [55], indicating a possible involvement of anthocyanins in resistance to *Sclerotinia* spp. [74]. In soybean, genes encoding for enzymes involved in anthocyanin and phytoalexin biosynthesis were upregulated in resistant plants [47]. Anthocyanins may function as inhibitors of fungal growth [75] and as antioxidants by scavenging reactive oxygen species (ROS), thereby limiting the induction of plant cell death [76]. Alternatively, resistance to *S. minor* in cultivar Eruption could be based on *R*-gene(s), but their expression is modulated by environmental conditions prior to infection [77, 78]. It is also possible that cv. Eruption has a mechanism allowing avoidance of natural *S. minor* infection in the field.

Quantification of fungal DNA revealed that microenvironmental conditions around the stem base substantially

influence disease progression and pathogen development. The lowest quantities of *S. minor* DNA were detected in stems with uncovered bases (Fig. 7). This aligns with previous findings that lettuce plants exhibit higher disease severity in the field under higher relative humidity [14], as humidity is expected to be higher when stem bases are wrapped. In field conditions, when relative humidity ranged from 65 to 95% and temperature from 11 °C to 18 °C near the lettuce stem base, the highest disease rating occurred at temperatures below 11.5 °C or relative humidity above 90% [14]. Small but consistent differences between wrapping stem bases in clear plastic or the plant's own leaves suggest that leaves may also provide additional nutrients to the pathogen. Although our simplified effect size model does not account for all environmental or biological factors, it indicates that creating microenvironmental conditions less hospitable for pathogen proliferation could reduce or delay lettuce drop symptoms in lettuce plants.

Conclusions

Our results suggest that stem cell wall lignification and elevated levels of hemicellulose, as indicated by xylose and arabinose content, contribute to slower degradation of the basal stem after infection with *S. minor*. However, further experiments with larger plant sets are needed to determine the individual effect of each compound and confirm the involvement of fucose in the lettuce-*S. minor* interaction. Future studies will focus on detailed analyses of stem physical properties and cell wall composition in recombinant inbred lines from the Salinas × PI 251246 mapping population, which exhibit considerable variation in these traits and resistance to lettuce drop [12]. These analyses will enhance our understanding of the relationship between resistance to basal stem degradation caused by *S. minor*, stem strength, and cell wall composition. By analyzing mapped quantitative trait loci (QTL) and their positions on the reference genome, we can identify candidate genes involved in resistance, including those in the lignin, hemicellulose, and anthocyanidin biosynthesis pathways. Subsequent steps may include functional analysis of these key genes to elucidate their specific roles in resistance mechanisms.

Our results indicate that targeting hard, lignified stems could allow for the development of lettuce lines with more durable resistance to lettuce drop. The content of lignin, particularly guaiacyl and syringyl, and xylose could serve as biomarkers for selecting lines with enhanced resistance. It is important to reiterate that any increase in lignin content would need to be localized in stems (or even the stem base) and not the leaf tissue consumed. Additionally, improvements in resistance may be achieved by developing lines with a microenvironment

around the stem base that is less hospitable for pathogen proliferation.

Abbreviations

ABSL	Acetyl bromide soluble lignin
ANOVA	Analysis of variance
AUDPS	Area under the disease progress stairs
CDS	Coding sequence
Ct	Cycle threshold
CWDEs	Cell wall-degrading enzymes
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
HSD test	Honestly significant difference test
PAMR	Plant architecture- or stem strength-mediated resistance
PCA	Principal component analysis
qPCR	Quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
S/G ratio	Syringyl to guaiacyl ratio
TE buffer	Tris-EDTA buffer

Supplementary Information

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Supplementary Material 1

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Author contributions

IS, BEM, and KVS designed the experiments; IS and KVS obtained financial support, administered, and supervised the project; BEM assisted in drafting the USDA-AMS grant proposal and conducted greenhouse experiments; CEF conducted cell wall composition analyses; NDA carried out fungal DNA quantification; IS and BEM analyzed the data; IS visualized the results; IS, BEM, CEF, NDA, and KVS wrote, edited, and approved the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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