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Lignin and cellulose content differences in roots of different cotton cultivars associated with different levels of Fusarium wilt race 4 (FOV4) resistance-response

Lavanya Mendu [a,b], Mauricio Ulloa [c], Paxton Payton [c], Cecilia Monclova-Santana [a,d], Jennifer Chagoya [a,d], Venugopal Mendu [a,b,*]

[a] Department of Plant and Soil Science, Texas Tech University, Lubbock, TX, 79409, USA
[b] Department of Plant Science and Plant Pathology, Montana State University, Bozeman, MT, 59717-3140, USA
[c] Plant Stress and Germplasm Development Research Unit, USDA-ARS, PA, CSRL, 3810 4th Street, Lubbock, TX, 79415, USA
[d] Texas A&M AgriLife Research & Extension Center at Lubbock, TX, 79403, USA

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ABSTRACT

Fusarium wilt disease is caused by fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 4 (FOV4), which enters the plant through the root system for its successful colonization of xylem. Plant cell wall forms the primary barrier against pathogen infection in addition to providing the mechanical support. However, the role of cell walls for developing FOV4 resistance has not been explored. The present study focused on examining the variation in lignin and cellulose contents in root tissue of Pima (*Gossypium barbadense* L.) and Upland (*G. hirsutum* L.) cotton with different levels of FOV4 wilt resistance-response. Traditional cultivar-checks susceptible Pima S-7, resistant Pima S-6, susceptible Upland Stoneville 474, and resistant Upland PSSJ-FRU14 (U77B) were used in the present study. Biochemical differences in root cell walls were investigated first by a rapid visual staining method for both lignin (phloroglucinol-HCL) and cellulose (Congo red) contents of root cross sections at three stages of cotton plant development followed by biochemical estimation of root lignin and cellulose contents. These studies revealed differences between susceptible and resistant cultivars at specific stages visually by rapid staining as well as biochemically in their cellulose and lignin contents within Pima and Upland cultivars. This is the first report in lignin and cellulose content estimation of Pima and Upland resistant and susceptible FOV4 cotton cultivars and paves the way for developing cell wall mediated FOV resistance.

1. Introduction

Cotton (*Gossypium* spp.) is a natural textile fiber crop grown worldwide with more than 70% of production originating from China, India, U.S., and Pakistan. Tetraploid *G. hirsutum* L. (Upland) accounts for 90% of cotton production, while other species such as tetraploid *G. barbadense* L. (Pima) and diploids *G. herbaceum* L., and *G. arboreum* L. accounts for the remaining 10% [1]. Yields of many crops especially the cotton crop were greatly impacted worldwide by the fungus *Fusarium oxysporum.* Strains of this fungus are diverse with many races and genotypes [2,3]. Over the past 18 years [4,5], the fungus *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) W.C. Snyder & H.N. Hansen race 4 (FOV4) has impacted cotton production in the San Joaquin Valley of California, and more recently this pathogen was formally identified in 2017 in El Paso, TX region [6] and in 2019 in New Mexico [7]. This race 4 was identified first in India in 1960 and for the first time in the U.S in California in 2003 [8]. The best diagnostic symptom of FOV4 infection is a characteristic brown streak throughout the root tissue inside the root resulting in root necrosis evident by vascular root staining [4,5,9]. FOV4 race does not require the presence of root knot nematode (*Meloidogyne incognita*) that is common for FOV race 1 or FOV1 for successful infection. Agricultural practices like crop rotation or fallowing generally employed in pathogen control were not helpful for fusarium pathogen removal as it can survive on the roots of cotton plants. Therefore, once this race is established, it remains in the soil indefinitely. The best method or management control against FOV4 currently is the development of resistant cultivars. Hence, there is an urgent need to develop FOV4 resistant cotton cultivars and alternative methods of prevention

* Corresponding author. (V.M.); 415, Leon Johnson Hall, Montana State University, Bozeman, MT, 59717-3140, USA.
E-mail address: venugopal.mendu@montana.edu (V. Mendu).

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and management [5,9-11].

Plant disease resistance to pathogens is generally dependent on both constitutive and inducible defensive barriers. The cell wall forms the primary passive barrier that pathogens need to cross to establish/colonize in the plant tissues [11,12]. This dynamic complex network of cell wall triggers both constitutive and inducible defense responses [12]. Additionally, wounding/pathogen attack induces release of plant-signaling molecules by the loss of cell wall integrity [13]. Further, plant cell wall and pathogen interaction studies suggested that changes in the cell wall composition also changes plant pathogen infection [14]. Because of the limited information of FOV4 infection in cotton, there is a critical need to study alternative disease resistance mechanisms that might enhance *Fusarium* resistance in cotton plants. Hence, it is important to investigate whether there is any link between cell wall thicknesses/composition changes of cellulose and lignin content in FOV4 resistant and susceptible cotton cultivars.

Several evidence from various crops suggest the role of cell walls in plant disease resistance. Secondary cell wall composition [15], particularly lignin is associated with the pathogen resistance [16-18]. Mathematical models showed specific correlation between the amounts of specific carbohydrate epitopes and disease resistance/fitness phenotypes in Arabidopsis [19]. The peanut seed coat, which is primarily composed of cell wall was shown to act as a physical and biochemical barrier against *A. flavus* infection [20].Overall, the cell wall offers both physical and biochemical resistance, hence presents an excellent opportunity to explore cell wall mediated resistance against FOV4. In the present study, we investigated the biochemical differences of root cell walls by using two methods with resistant and susceptible cotton cultivars to FOV4. The first method is a rapid staining and visual screening of root tissues using cellulose specific and lignin specific stains. We used three different stages of cotton plant development [cotyledon stage (one-week-old) (I), true leaf stage (two-week-old) (II) and vegetative stage (three-week-old) (III)] to observe differences between the susceptible and resistant cultivars. The second method is an estimation of cellulose and lignin content using plant root tissue biomass of these resistant and susceptible cultivars to provide additional evidence for the visual changes observed in the first method. This study revealed differences between susceptible and resistant cultivars in the first rapid staining method and were further observed by the second biochemical method using the cellulose - Updegraff and lignin - Thioglycolic acid method. The cellulose difference did not follow similar trend in Pima and Upland cultivars. These difference and composition-changes might be stage specific, differ by growing conditions (growth chamber/-greenhouse/field) that might be enhanced by pathogen wounding of the cell wall upon FOV4 infection and might play a role in FOV4 disease resistance response along with other mechanisms. The present study will pave the way for deeper investigation into the cell wall mediated resistance against *Fusarium* infection in cotton and other crop plants, which eventually helps in developing varieties resistant to *Fusarium* infection.

2. Materials and methods

2.1. Plant material and FOV4 evaluation

Cotton seeds from four cultivars: susceptible Pima S-7 PI 560140 (PS-7) [21], a FOV4 selection line/population resistant Pima S-6 PI 608346 (PS-6) [22], susceptible Upland Stoneville 474 (STV474), and resistant Upland PSSJ-FRU14 (U77B) (Ulloa et al., 2021. Notice of Release of Upland PSSJ-FRU01-PSSJ-FRU17 Germplasm-in review), were used in the present study. At the time of release, the major advantages of Pima S-6 were early maturity and high yield. Later, it was discovered that Pima S-6 also possesses a major gene(s) for FOV4 resistance [5,23,24]. Since S-6 identified as a source of FOV4 resistance in Pima cotton, several cycles of evaluations and selections were performed to increase its uniformity and the level of FOV4 resistance on the 'Pima-S6' used in this study [5].

Using the same seed production/source these four cultivars were evaluated under field conditions in 2020 and in 2021 seasons in El Paso, TX Lower Valley. The cultivars were planted in fields known to be infested and confirmed with FOV4 and exhibited severe root vascular *Fusarium* wilt symptoms. Each cultivar was grown in two-row plots 5-m long with 1-m row spacing in a complete randomized block design with four replications. In addition, susceptible and resistant cultivar-checks (PS-7 and PS-6, respectively) were spatially placed at the same area, too. Five plants were assayed for vascular root necrosis or staining (VRS) per plot in each year of plantings. To determine the level of resistance for each cultivar, plants were assayed from each replicated plot for VRS. A scale 0 to 5 was used in which 0 = no vascular staining evident, 1 = light vascular staining as continuous or intermittent, 2 = more continuous, color staining - covering area equal to between one quarter and one half of stem cross-section (10%–25%), 3 = vascular staining in a band dark and continuous, cross section (25%–45%), 4 = intense vascular staining and darker in color than in No 1 or No 2, and evident across most vascular tissue in stem cross section (50%–70%), and 5 = plant severely damaged, vascular staining evident throughout cross-section (70%–100%) of damaged tissue with no clean tissue [5].

2.2. Histochemical staining of roots

Histochemical staining was performed for both cellulose and lignin content using Congo-red and phloroglucinol-HCL stains, respectively. Cotton seeds from four cultivars: susceptible Pima PS-7, resistant PS-6, and susceptible Upland STV474, resistant Upland U77B, were sterilized in 100% ethanol for 3–5 min, washed three times with sterile water, transferred to a sterile filter paper placed in petri plates in the laminar flow hood. The Petri plates were sealed with parafilm and allowed to germinate seedlings for two days in a growth chamber (Percival Scientific, Perry, Iowa) maintained at 28^0C/26^0C for 12-h day/12-h night cycles. After two days, the germinated seedlings were transferred to pots with soil and sand mixture, labelled, and transferred to growth chambers with 28^0C/26^0C 12-h day/12-h night cycles for four weeks. Plant roots were harvested at each week at cotyledon stage (I), true leaf stage (II), and vegetative stage (III), and root-sectioning data was collected. For cellulose staining, 0.5% (w/v) of Congo-red stain was used, for which 0.5 g of Congo-red was dissolved in 100 mL of distilled water and used for staining root sections [25]. The root sections were incubated in Congo red stain for 5 min, washed with distilled water, mounted using coverslip, and observed under DAPI filter. For lignin staining, 3% of phloroglucinol-HCL was used. The solution was prepared by adding 0.45 g of phloroglucinol in 100% ethanol and HCL solution (10 mL 100% ethanol and concentrated 5 mL HCl) to prepare 3% phloroglucinol-HCL. Root sections were incubated in the phloroglucinol stain for 1 min, washed with water, mounted with coverslip, and observed under the microscope in bright field mode (Olympus Corporation, Model DP80 Light Microscope) to identify staining differences among different cultivars [25,26].

The roots were collected by gently pulling the seedlings from the pots and separating the roots from soil-sand mixture. The pots were cut open from the side, gently separated from soil sand mixture so that the root structure is not disrupted. Then, roots were washed thoroughly with water and placed in a sterile Petri plate with water. Sterile blade was used to make thin sections by hand on the slide by applying gentle pressure and the sections were transferred to a glass plate filled with sterile water. The sections were stained for cellulose or lignin by adding Congo red and phloroglucinol stains to the slide with root sections and incubated for around 5 min. Using tissue paper the stain was gently blotted from the sides of the root sections. Slide was washed with water and gently covered with a cover slip without air bubbles/removed air bubbles to observe under the microscope. Pictures were taken at different magnifications [4X (scale 200 μm), 10X (scale 100 μm) and 40X (scale 50 μm)] using Olympus BH2 fluorescent microscopy in bright

field mode for lignin staining and in DAPI filter for cellulose staining. Primary roots were used for the sectioning of each cultivar for cellulose and lignin staining. Lateral root sections were also stained at True leaf stage as these are the points of pathogen entry. Three biological and three technical replicates were included for each stage and each cultivar.

2.3. Biochemical analysis

2.3.1. Preparation of plant biomass samples for cellulose and lignin estimation

Roots from 60-day old plants were collected from the greenhouse grown four cultivars (susceptible Pima PS-7, resistant Pima PS-6, susceptible Upland STV474, and resistant Upland U77B) planted in 5-gallon pots. The soil in the pots of each sample was loosened, gently flipped, and collected root samples with intact lateral roots. Plant roots were placed in trays filled with water, thoroughly washed, air-dried on labelled paper towels for 2 days at room temperature on the lab bench to prevent any fungal contamination. Three biological replicates were collected for each cultivar. The dried root samples were transferred to labelled containers/aluminum foils and incubated in a temperature-controlled incubator at 49 °C for 7–10 days until samples were completely dried. The incubator-dried tissue was cut into fine pieces using scissors/biomass grinder. The finely cut tissue was loaded into freezing vials and ground into fine powder using a freezer mill filled with liquid N_2. The collected root tissue samples were ground into powder using SPEX sample prep freezer mill 6870 as described [20].

2.3.2. Lignin content estimation using thioglycolic acid method

Twenty mg of the freezer mill ground fine root tissue powder was transferred into the pre-weighed 2 mL microfuge tube and the lignin extraction protocol was followed as described [27]. Briefly, 20 mg tissue powder was washed with water, methanol, and then treated with TGA. Standard curve was generated using industrial bamboo lignin using three technical replicates for each concentration ranging from 0.5 mg to 4.0 mg (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5 mg). The lignin was precipitated under acidic conditions following the TGA treatment, which was measured at 280 nm using a spectrophotometer (Eppendorf Biospectrometer® kinetic) and compared with the standard lignin curve for estimation of sample lignin. We measured the unknown concentration of lignin by using the regression line values m and c of the lignin standard curve and absorbances of extracted samples at 280 nm.

2.4. Crystalline cellulose content estimation by Updegraff method

Twenty mg of the above prepared plant biomass material were used and transferred into a pre-weighed 2 mL microfuge tubes. All centrifugation steps were carried out at 15,000 rpm for 10 min at room temperature (RT) in the preparation of plant cell walls and cellulose was estimated using Updegraff method [28]. Briefly, the tissue powder was washed with 1 mL of protein solubilization buffer (PSB) [50 mM of Tris hydrochloride (HCl) buffer pH 8.8 with 0.5 mM EDTA and 10% sodium dodecyl sulfate (SDS)] for 2 to 3 times to solubilize proteins. Following the solubilization, the samples were washed with water, 70% ethanol, 100% methanol, 1:1 prepared mixture of chloroform and methanol, and acetone. The dried pellet represents the crude cell wall, 5 mg of which was used for estimation of crystalline cellulose content. Each tube with 5 mg of sample was treated with Updegraff reagent (acetic and nitric acid) to remove non-cellulosic components. Three biological replicates of each sample along with three replicates for positive control were included in the study. Following the Updegraff treatment, pellet was washed with acetone, and dissolved in 67% sulfuric acid. These samples were measured using the UV spectrophotometer (Beckman coulter DTX 880 multimode detector spectrophotometer) at 620 nm for the glucose content. Glucose standard curve was prepared using different concentrations of glucose and regression line was generated for cellulose content estimation.

2.5. Statistical data analyses

Differences observed among four cultivars in FOV4 infection response was determined by vascular root necrosis or staining (VRS) within Pima or Upland and among cultivars were evaluated for each experiment using PROC GLM (SAS, ver. 9.4, SAS Institute, Cary, NC, USA). If significant differences ($p < 0.05$) were observed from the analysis of variance, then mean separation was performed for examinations of main effects using the Waller-Duncan k-ratio procedure [29]. Student t-test was used to test if the identified lignin and cellulose differences were significant or not ($p > 0.05$) in both Pima and Upland resistant cultivars against their susceptible cultivars.

3. Results

3.1. FOV4 evaluation of selected cultivars

Pima cultivars, susceptible PS-7, and resistant PS-6 since their identification for their infection response to FOV4 in early 2000s have been used in multiple-year studies [4,5,30,31] as control/check cultivars (Fig. 1). More recently, a set of germplasm cultivars with improved or higher levels of FOV4 resistance compared to any available commercial Upland cultivar were developed and publicly released (Notice of Release of Upland PSSJ-FRU01 – PSSJ-FRU17 Germplasm, USDA-ARS, Lubbock, TX). From this set, the Upland resistant U77B provided the base-cultivar difference of infection response or susceptible versus resistance response to FOV4 within Upland cotton (Fig. 1A) to perform this study.

Pima cultivars susceptible to FOV4 show more defined/clear symptoms such as stand loss, stunting, and vascular root necrosis than any Acala and non-Acala Upland cottons. Plant FOV4 response to infection of these cultivars was performed in a field confirmed to have FOV4 since 2017. FOV4 inoculum varied throughout the field, and disease pressure occurred in the infested field based on tested accessions and observations of susceptible cultivar-checks, indicating moderate to severe inoculum levels with the site from 2017 through 2020. Under field conditions, vascular root necrosis, or vascular root staining (VRS) usually occurs in the taproot and in the lower part of the stem approximately 2–5 cm above the soil level when infection is most severe (Fig. 1B). In the field, plants start to die after germination. In the greenhouse evaluation studies using artificial FOV4 inoculation (data not shown), clear VRS symptoms in the xylem appears at about 8 days of inoculation and some plants start to die about 2–4 weeks after inoculation (Figs. 1C and 6). In this study using the same seed production/source for staining and biochemical assays, we observed significant differences ($p < 0.05$) in comparisons of Pima resistant, PS-6 vs Pima susceptible, PS-7 as well as resistant Upland, U77B vs susceptible Upland, STV474 cultivars. These cultivars and their response to FOV4 were evaluated during 2020 and 2021 under infested FOV4 field conditions at a site in the lower Valley of El Paso, TX region.

3.2. Histochemical analysis and biochemical estimation showed difference in the lignin content among the cultivars

At each stage (stage I, II and III) with three biological replicates, Pima roots were collected, sectioned, stained, and observed under microscope at 200 μm and 100 μm (Fig. 2). Visual analysis of phloroglucinol staining showed higher lignin content in resistant roots Pima cultivar PS-6 at true leaf stage (II) when compared to susceptible Pima PS-7 cultivar (Fig. 2B).

Results of phloroglucinol staining showed higher lignin content in resistant roots, then lateral roots were also sectioned for visual lignin content estimation. Lateral roots were observed at 100 μm (10X) and 50 μm (20X) scale under the microscope. Results showed that resistant cultivar, PS-6 lateral roots showed visually higher lignin content than in susceptible cultivar, PS-7 lateral roots (Fig. 2C). Both primary and

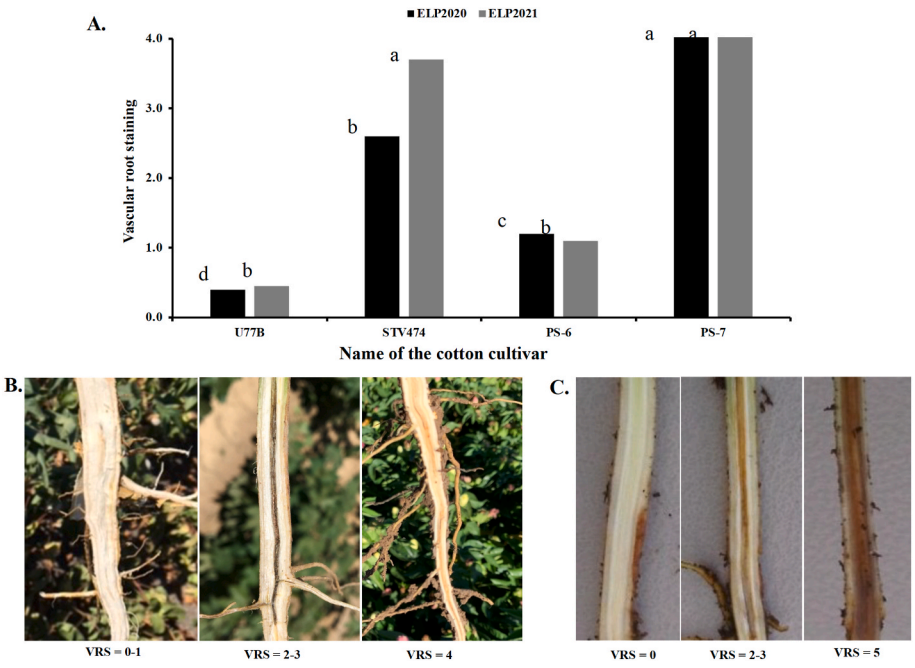

Fig. 1. A. Vascular root staining (VRS) of cultivars: resistant Pima S-6 (PS-6), susceptible Pima S-7 (PS-7), susceptible Upland Stoneville 474 (STV474), and Upland PSSJ-FRU14 (U77B) evaluated under Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*) race 4 (FOV4) infested field at the lower Valley, El Paso, TX in 2020 (ELP2020) and 2021 (ELP2021) seasons. B. VRS of infected cotton plants by FOV4 under naturally infested soil field conditions showing a scale of 0 (no symptoms) to 5 (dead plants). The VRS = 2–3 is only showing the infection in a section of the root and the picture was taken closer to the root. VRS = 4 showing the infection on an older root from the top to the bottom tip-root. C. VRS of infected cotton plants by artificial FOV4 inoculations under greenhouse conditions showed symptoms on a scale of 0 (no symptoms) to 5 (dead plants).

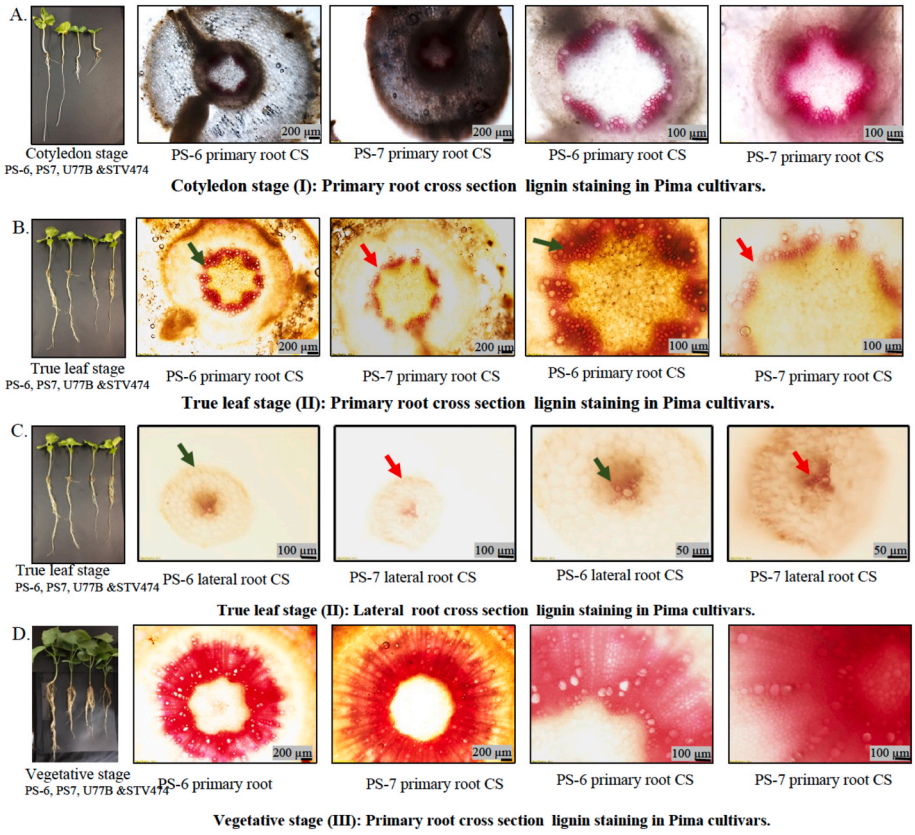

Fig. 2. Lignin staining of Pima root sections using pholoroglucinol-HCL. Pima resistant cultivar, PS-6 root cross section (CS) staining (0.3% of phloroglucinol) for lignin visually showed higher lignin content in primary root and lateral roots at the true leaf stage when compared to PS-7. (A) Primary root CS of PS6 and PS7 at cotyledon stage at 200 and 100 µm (4X and 10X) (B) primary root CS of PS6 and PS7 at true leaf stage (II) at 200 and 100 µm (4X and 10X) (C) Lateral root cross section of PS6 and PS7 at true leaf stage at 100 and 50 µm (10X and 20X) (D) primary root CS of PS6 and PS7 at vegetative stage (III) at 200 and 100 µm (4X and 10X). Green and red arrows represent higher and lower lignin content changes. Plant stages represented in this picture are cotyledon (stage I), true leaf (stage II), and vegetative stage (stage III) were presented in the order of Pima resistant (PS-6), Pima susceptible (PS-7), Upland resistant (U77B), and Upland susceptible (STV474). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lateral roots showed similar staining pattern in Pima cultivars. To investigate such lignin content differences in Upland cultivars like Pima cultivars, Upland cultivars (resistant U77B and susceptible STV474) were also subjected to a rapid visual root staining lignin assay by phloroglucinol-HCL at three developmental stages [cotyledon (I), true leaf (II), and vegetative stage (III)]. Root cross sections were observed under microscope at 200 µm and 100 µm (4X and 10X) (Fig. 3). Results from primary root cross-sections showed higher lignin staining in resistant Upland, U77B when compared to susceptible Upland, STV474 cultivar root cross sections at true leaf (II) and vegetative stages (III) (Fig. 3B and D).

The lateral roots of the true leaf stage were harvested, sectioned, stained with phloroglucinol, and observed under the microscope at 100 (10X) and 50 µm (20X). Visual observations of the lateral root cross

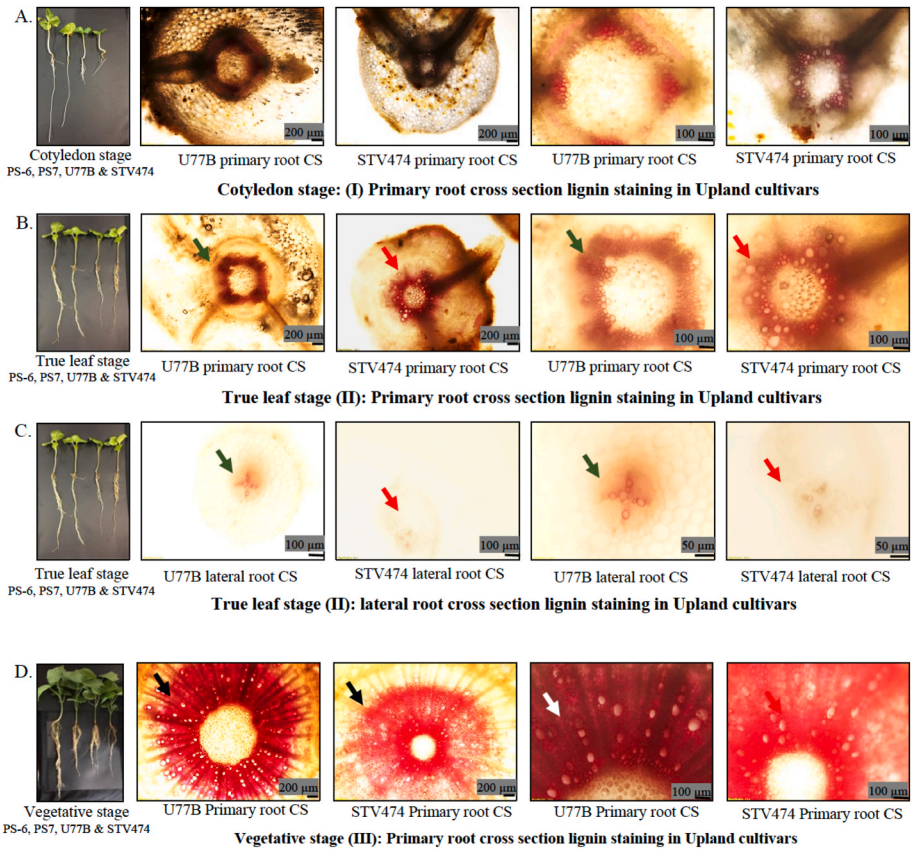

Fig. 3. Lignin staining of Upland root sections using phloroglucinol-HCL. Upland resistant cultivar, results of U77B root cross section staining (0.3% phloroglucinol) for lignin content showed visually higher lignin content in primary root at true leaf stage (II) at 200 and 100 μm (4X and 10X magnification) and vegetative stage (III) at 200 and 100 μm (4X and 10X) and lateral roots at true leaf stage at 100 and 50 μm (10X and 20X). (A) Primary root CS at cotyledon stage of U77B and STV474 (I) (B) primary root CS at true leaf stage (II) of U77B and STV474 at 200 and 100 μm (4X and 10X) (C) Lateral root cross section at true leaf stage of U77B and STV474 at 200 and 100 μm (4X and 10X) (D) primary root CS at vegetative stage (III) of U77B and STV474 at 200 and 100 μm (4X and 10X). Green and red arrows represent higher and lower lignin content changes. Plant stages represented in the picture at three developmental stages: cotyledon (stage I), true leaf (stage II), and vegetative stage (stage III) were presented in the order of Pima resistant (PS-6), Pima susceptible (PS-7), Upland resistant (U77B) and Upland susceptible (STV474). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sections showed that Upland resistant cultivar, U77B has more lignin content relative to Upland susceptible cultivar, STV474 (Fig. 3C). Both primary and lateral roots showed similar staining pattern differences in Upland cultivars. Preliminary data of histochemical staining of root sections (stages I-III) suggested higher lignin content in Pima resistant, PS-6 cultivar when compared to Pima susceptible, PS-7 cultivar roots at the true leaf stage. To confirm the visual observations, the lignin content was estimated in the root tissues of Pima cultivars. Both Pima resistant and susceptible cultivars (PS-6, PS-7) were grown in the greenhouse (approximately 60 days) and harvested roots from 2-month-old cotton plants for the root biomass assay. Lignin content was estimated by using thioglycolic acid method, and lignin content changes were estimated by biochemical analysis using lignin standard curve regression line developed from commercially available industrial bamboo lignin. This analysis showed that PS-6 roots have 11.64% of lignin content while PS-7 cultivars have 10.2% of lignin content (Fig. 4A). Further, analysis by student t-test suggested that these differences between resistant and susceptible cultivars were not statistically significant ($p > 0.05$).

Lignin content differences were estimated by TGA method. This method showed that the lignin content was 10.3% in U77B and 9.08% in STV474 (Fig. 4B). Though, differences were observed in resistant and susceptible cultivars of Upland cultivars, application of student t-test statistic showed that these lignin content differences were not statistically significant ($p > 0.05$).

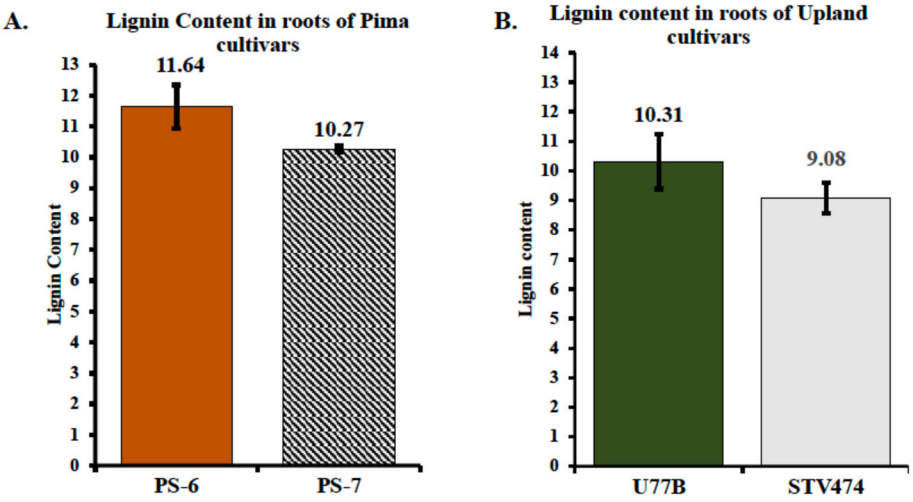

Fig. 4. Lignin estimation of Pima and Upland root biomass. Lignin content estimation by TGA method showed that Pima resistant cultivar and Upland resistant cultivar has higher lignin content in the primary roots at mature stage when compared with their susceptible cultivars. A. Lignin content differences between Pima Fusarium resistant (PS-6) relative to Fusarium susceptible cultivar (PS-7). B. Lignin content differences in Upland resistant cultivar U77B against Upland susceptible cultivar STV474.

3.3. Histochemical staining for cellulose

3.4.1. Histochemical analysis of Pima resistant cultivars primary root showed higher cellulose content at true leaf and vegetative stages: Rapid staining of primary root cross section by Congo red stain showed higher cellulose content in Pima resistant, PS-6 and Pima susceptible, PS-7 cultivars at the cotyledon stage (I) and vegetative stage (III) (Fig. 5A and D). Visual microscope observations of the primary root at cotyledon stage showed that cellulose content was stained with no gaps/thicker cellulose content in resistant Pima cultivars, PS-6, while there were gaps in the susceptible cultivar PS-7 (Fig. 5A, gaps were pointed by arrow) suggesting reduction in cellulose content deposition at the cotyledon stage suggesting stage specific differences in resistant and susceptible cultivars.

3.3.1. Lateral root showed higher cellulose content

Lateral roots were collected from true leaf stage, sectioned, stained, and observed under microscope at 100 and 50 μm (10X and 20X). Thinness was observed in susceptible Pima cultivar (Fig. 5C).

3.3.2. Histochemical analysis of upland resistant cultivars primary root for cellulose content showed higher cellulose content at the true leaf stage

Similar to Pima cultivars, Upland cultivars (resistant U77B and susceptible STV474) were subjected to a rapid visual root staining cellulose assay with Congo red and observed under microscope at 200 and 100 μm (4X and 10X) at three developmental stages that are a week apart from each stage [cotyledon (I), true leaf (II), and vegetative stage (III)] and root sections were observed under microscope at 200 μm (4X) and 100 μm (10X) (Fig. 3). Visual differences were observed between Upland resistant U77B and susceptible STV474 cultivars at the true leaf stage (II) (Fig. 6B).

3.3.3. Histochemical analysis of upland resistant cultivars lateral root showed higher cellulose content at true leaf stage

Lateral root cross sections of Upland cultivars at true leaf stage were analyzed for visual cellulose content differences. Visual observation showed more lignin content in U77B than in STV474 cultivars at 100 and 50 μm (10X and 20X) (Fig. 6C).

3.4. Cellulose content is higher in resistant cultivars compared to susceptible cultivars

The estimation of cellulose content showed that resistant cultivars contain relatively higher cellulose content compared to susceptible cultivars, especially in the Pima cultivar. To confirm whether rapid staining differences really existed between resistant and susceptible cultivars, Updegraff method was used to estimate the crystalline cellulose content from both Pima and Upland cotton plants biomass material. Cellulose content was estimated using regression line m and c values of the developed glucose standard curve. The cellulose content difference was higher in roots of Pima resistant PS-6 (40%) compared to Pima susceptible cultivar PS-7 (22.8%) (Fig. 7A). Similarly, crystalline cellulose content in roots of Upland resistant cultivar, U77B is higher compared to Upland susceptible cultivar, STV474, however, the difference was less compared to Pima lines (39.2% in U77B and 34.5% in STV474) (Fig. 7B). The cellulose content differences were significant for both Pima and Upland cotton between resistant and susceptible cultivars ($p > 0.05$). The difference in cellulose content was higher in Pima than in Upland, again suggesting a more complex defense response to pathogen infection.

4. Discussion

Plant cell wall forms the primary barrier that pathogen needs to cross for successful colonization in the plant system [12]. cell walls,

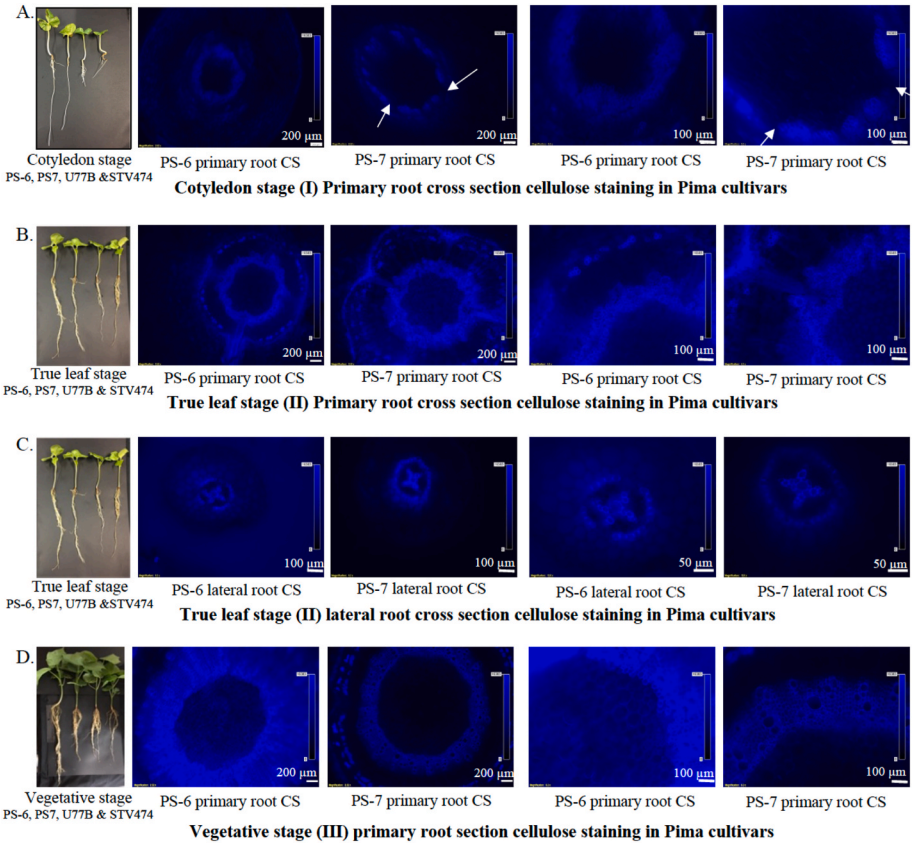

Fig. 5. Cellulose staining of Pima root sections using Congo red. Pima resistant cultivar, PS-6 root cross section staining (0.5% Congo red) for cellulose showed relatively higher cellulose content in primary roots at cotyledon stage (I) and at vegetative stage (II). (A) Primary root CS at cotyledon stage (I) of PS6 and PS7 at 200 μm and 100 μm (4X and 10X). Highlighted gaps in susceptible cultivar using a white arrow. (B) primary root CS at true leaf stage of PS6 and PS7 (II) at 200 μm and 100 μm (4X and 10X) (C) Lateral root cross section at true leaf stage of PS6 and PS7 at 100 μm and 50 μm (10X and 20X) (D) primary root CS at vegetative stage (III) at 200 μm and 100 μm (4X and 10X) showed thin layer of cellulose in susceptible cultivar, PS7. Plant stages represented in this picture at three stages: cotyledon (stage I), true leaf (stage II), and vegetative stage (stage III) were presented in the order of Pima resistant (PS-6), Pima susceptible (PS-7), Upland resistant (U77B), and Upland susceptible (STV474). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

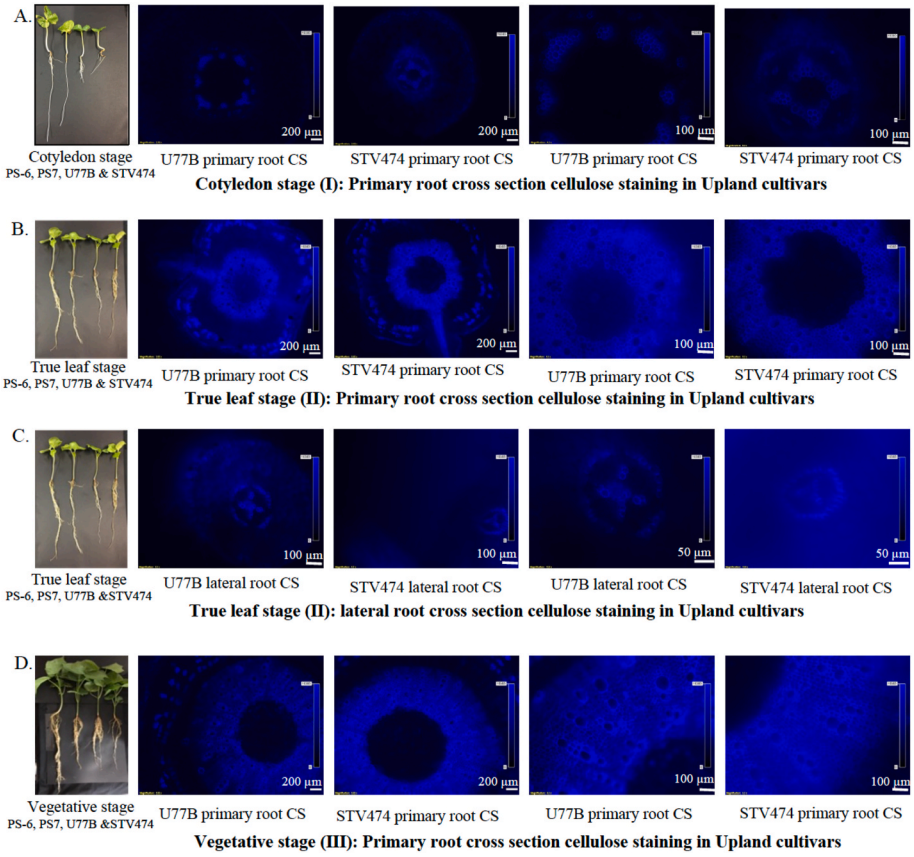

Fig. 6. Cellulose staining of Upland root sections using Congo red. Upland resistant cultivar root cross section cellulose staining (0.5% Congo red) showed relatively higher cellulose content in the resistant cultivar, U77B lateral roots at the true leaf stage. (A) Primary root CS at cotyledon stage at 200 and 100 µm (4X and 10X) (I) (B) primary root CS at true leaf stage (II) at 200 and 100 µm (4X and 10X) (C) Lateral root cross section at true leaf stage (D) primary root CS at vegetative stage (III) at 100 and 50 µm (10X and 20X). Plant stages represented in the picture at three stages: cotyledon (stage I), true leaf (stage II), and vegetative stage (stage III) were presented in the order of Pima resistant (PS6), Pima susceptible (PS7), Upland resistant (U77B), and Upland susceptible (STV474). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

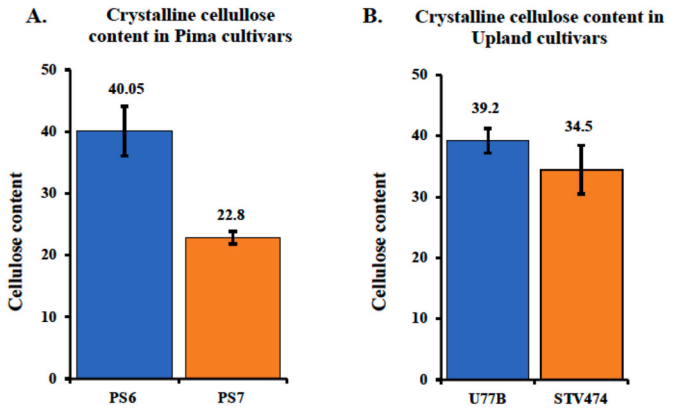

Fig. 7. Cellulose content estimation by Updegraff method. Cellulose estimation showed higher cellulose content in Pima resistant and Upland resistant cultivars when compared with their susceptible cultivars. A. Crystalline cellulose content differences between Pima Fusarium resistant (PS-6) relative to PS-7 Fusarium susceptible cultivar B. Crystalline cellulose content differences in Upland resistant (U77B) against Upland susceptible cultivar STV474.

particularly secondary cell walls were reported to play critical role in plant disease resistance due to the presence of lignin and phenolic compounds. Primary cell walls are made up of cellulose, hemicellulose, and pectin, whereas secondary cell walls contain lignin in addition to cellulose, hemicellulose, and pectin [32,33]. To investigate such cell wall mediated mechanism in disease resistance against fusarium, we examined lignin and cellulose content in root tissue of Upland and Pima cotton with different levels of Fusarium wilt [*Fusarium oxysporum* f. sp. V*asinfectum* (FOV) W.C. Snyder & H.N. Hansen] race 4 (FOV4) resistance-response. Results from this study showed differences in lignin and cellulose contents in cotton cultivars (FOV4 Pima susceptible, PS-7 and resistant, PS-6, and Upland susceptible, STV474 and resistant, PSSJ-FRU14 - U77B), suggesting a variation in cell wall composition in these different cultivars which can be exploited to develop FOV4 resistant lines. The higher amounts of cellulose or lignin content may provide resistance, or the increased amounts could be further enhanced by the presence of the fungus and a possible mode of disease resistance mechanism in resistant cultivars.

Plant cell walls always maintain the strength by feedback/compensation mechanism where defect in one cell wall component is compensated by depositing some other component(s) [34,35]. These alternations in cell wall composition have consequences in plant biotic and abiotic stress responses. Characterization of cellulose mutants in *Arabidopsis* revealed that irregular xylem mutants, like *irx5, irx3, irx1* with less crystalline cellulose content were reported to have high resistance to different necrotrophic fungi [36], while cellulose mutants such as CESA4, CESA7 and CESA8, were reported to be more resistant to necrotrophic fungi [37]. Additional research studies in *Arabidopsis* reported *Cellulose synthase A7* (*CesA7*) mutation in pectin side chain composition changes in primary cell walls in the cells adjacent to the vasculature [38]. Similarly, mutation in *CesA1* or *CesA3* reported ectopic lignification [39,40] while mutation in cellulose associated genes such as *korrigan* or *cobra* resulted in enhanced lignin and/or pectin contents [41-43]. Cellulose deficient mutant is also known to induce callose, a defensive responsive cell wall component. Mutation in *kobito1* (*kob1*) results in increased pectin content and also ectopic deposition of callose and lignin in *Arabidopsis* seedlings grown under dark conditions [44]. These resistance responses could be attributed to ectopic lignin or other components due to defects in cellulose deposition. Pathogen infection in *Arabidopsis* also showed increased lignin content by inducing specific lignin biosynthetic genes as a defense response to bacteria [45] and enhanced lignin content or composition is known to confer biotic stress resistance. Research studies in *Arabidopsis* have targeted upregulation of

lignin biosynthetic genes by using hormones such as jasmonic acid, salicylic acid, and abscisic acid to increase the lignin content [46-48]. Similar studies in wheat roots suggested cell wall changes upon its interaction with beneficial fungi Trichoderma. These fungal plant interactions particularly resulted in lignin deposition, pectin reconstruction and presence of arabinogalactan proteins in the root cell walls which might benefit plants in plant defense and potential use of these beneficial fungi in enhancing plant disease resistance [49].

Similar studies in cotton identified the role of lignin component of cell wall against *Verticillium dahliae*, a fungal pathogen which causes vascular disease known as verticillium wilt [50]. Enhanced levels of lignin content in different cotton cultivars were reported to have positive effects on disease resistance against *V. dahlia* in cotton [51]. Recent studies also reported that a laccase (LAC) gene *GhLAC4* involved in lignin biosynthesis plays significant role in lignin accumulation and in protection against *V. dahliae* colonization in cotton [52]. Similar studies on LAC genes also reported role of *GhLAC15* in Verticillium wilt resistance and silencing of this LAC gene increased susceptibility of cotton to vascular wilts [53]. Further, studies also showed that glycoprotein GhGLP2 plays key role in verticillium, fusarium resistance by callose and lignin depositions at the sites of infections [54].

In order to break down this barrier of cell wall, pathogens also release enzymes and biochemicals for successful colonization in plant cells. Research studies on necrotrophic fungi and nematodes showed the use of toxins and cell wall degrading enzymes to colonize and or establish feeding structure in plants [55,56]. Studies suggests that *Fusarium oxysporum* (Fo) enters roots through cell walls, alters cell walls, and leads to colonization [57]. Hence, the successful infection of FOV4 strain might depend on the cotton root and FOV4 interactions at cell walls. Root cell wall assays and biochemical analyses suggest that there are cell wall variations, mostly higher contents exist in resistant cultivars that might be natural or induced by the presence of pathogen. Therefore, FOV4 resistant cultivars might have mechanisms to thicken cell walls as resistant mechanism against FOV4 pathogen infection.

Fusarium is a soil borne semi biotrophic fungi that causes root infections in cotton, tomato, cabbage, and banana crop, which results in severe economic losses in those crops [58]. For vascular pathogens like *Fusarium oxysporum*, cell wall forms primary barrier [59]. The first step is the penetration of Fo through root cell epidermis at weaker locations of the cell wall by modifying host structures and releasing effector proteins. Such effectors proteins were reported in tomato plant by pathogen *Fusarium oxysporum* f. sp. *lycopersicon*, where pathogen releases six effector proteins in the xylem sap [60]. After penetration, hyphae of fungi start growing in the direction of xylem, which further prevents plant from getting water and nutrients leading to plant wilting and subsequent death. Studies in this direction in *Arabidopsis* revealed that *Fusarium oxysporum* Fo5176 primarily targets reduction of primary cell wall synthesis [59]. Cellulose deficient mutants with enhanced lignin content in response to Fo5176 infection showed enhanced resistance to Fo5176, while lignin deficient mutants were reported to be equally susceptible to Fo5176 infection as wild type plants [59]. Root pathogen interaction and the precise mechanism of Fo colonization was poorly studied in *Fusarium* cotton root infection process. The present study provides the first report on lignin and cellulose content and revealed the visual and biochemical differences between susceptible and resistant cultivars and lays foundation for future studies in cell wall mediated FOV4 resistance. However, results showed that these differences were higher in both Pima and Upland resistant cultivars compared to susceptible cultivars. Difference and composition-changes might be enhanced by pathogen wounding of the cell wall upon FOV4 infection, and these differences might play a role in FOV4 disease resistance response along with other mechanisms such as alteration in other cell wall components. Future studies involving mapping populations and comprehensive analysis of other cell wall components such as pectin, hemicellulose will provide deeper insights of such FOV4 specific cell wall mediated resistance. Analysis of cell wall composition with and without infection are required for understanding the natural or induced changes in these cultivars.

5. Conclusions

Overall, the present study is the first step in understanding the cell wall mediated FOV4 resistance and provides an excellent opportunity to develop cell wall mediated resistance using molecular, genetic, and breeding methods. The study was undertaken with the hypothesis that the resistant and susceptible lines might show differences in their cellulose and lignin contents. As expected, visual and biochemical analysis showed differences in the cellulose and lignin contents. This is the first step in this direction and investigation on the four lines planted under infected and uninfected fields are required for comprehensively understand the differences. Further, studies are needed to establish the direct relationship, however, presence of visual differences in cell wall composition and biochemical difference in cotton roots of different cultivars presents an excellent indication on resistance and provides basis for further studies. These studies also help researchers to investigate in this field and find novel cell wall mediated FOV4 resistant lines. Since, cell wall is a complex mixture of various polysaccharide and phenolic polymers, genetic engineering of plants with altered composition will help in developing FOV4 resistant cotton and other crops.

Author contributions

Conceptualization, V.M. and M.U.; methodology, L.M. and J.C.; data curation, V.M., M.U. and L.M.; writing-original draft preparation, L.M.; writing-review and editing, L.M., M.U., P.P., J.C., C.M. and V.M.; visualization, L.M.; supervision, V.M., U.M. and C.M.; project administration, V.M., M.U. and C.M.; funding acquisition, M.U., L.M., V.M. and P.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Venugopal Mendu reports financial support, administrative support, article publishing charges, and equipment, drugs, or supplies were provided by Montana State University and Texas Tech University. Authors have no relationship with the journal. Lavanya Mendu is spouse of Venugopal Mendu. Both are faculty at Montana state university.

Data availability

Data will be made available on request.

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