

Combining Fourier-transform infrared spectroscopy and multivariate analysis for chemotyping of cell wall composition in Mungbean (*Vigna radiata* (L.) *Wizcek*).

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Method Article

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Abstract

Background

Dissection of complex plant cell wall structures demands a sensitive and quantitative method. FTIR is used regularly as a screening method to identify specific linkages in cell walls. However, quantification and assigning spectral bands to particular cell wall components is still a major challenge, specifically in crop species. In this study, we addressed these challenges using ATR-FTIR spectroscopy as it is a high throughput, cost-effective and non-destructive approach to understand plant cell wall composition. This method was validated by analysing different varieties of mungbean which is one of the most important legume crop grown widely in Asia.

Results

Using standards and extraction of a specific component of cell wall components, we assigned 1050-1060 cm^{-1} and 1390-1420 cm^{-1} wavenumbers that can be widely used to quantify cellulose and lignin, respectively, in *Arabidopsis*, *Populus*, rice and mungbean. Also, using KBr as a diluent, we established a method which can relatively quantify the cellulose and lignin composition among different tissue types of the above species. We further used this method to quantify cellulose and lignin in field-grown mungbean genotypes. The ATR-FTIR-based study revealed the cellulose content variation ranges from 27.9% to 52.37%, and the lignin content variation ranges from 13.77% to 31.6% in mungbean genotypes.

Conclusion

Cell wall composition in different mungbean genotypes was determined by the developed FT-IR-based method, which was cross-validated using canonical wet-chemistry methods. Overall, our data suggested that ATR-FTIR can be used for the relative quantification of lignin and cellulose in different plant species. This method can be used for rapid screening of cell wall composition in large number of germplasms of different crops including mungbean.

Introduction

The plant cell wall is a complex polymeric network structure that is mainly composed of cellulose, hemicellulose, lignin and pectin. The diversity in plant cell shapes and sizes is because of the distinct physicochemical properties of the cell wall which plays a pivotal role in morphogenesis, providing mechanical support, transporting nutrients and water, and defending against different environmental stresses [1–5]. The plants consist of several types of cells [6] which have distinct and dynamic cell wall compositions and organization. The structural diversity of cells is due to the complex chemical and structural heterogeneity of plant cell wall. Also, in the last couple of decades, the focus has been shifted to understanding the genetic regulation of the cell walls and these efforts have identified many biosynthetic, modifying enzymes and transcription factors [2, 7]. These studies are mainly related to selected species like *Arabidopsis*, *Populus*, rice, *Brachypodium*, and *Eucalyptus*. More focus is now

needed to understand the regulation in plant cell walls of different economically important crop species to identify the genes involved in cell wall biosynthesis by exploiting natural variation in species followed by quantitative trait loci identification and validation using genome editing tools. To attain this, it is necessary to understand the qualitative and quantitative composition of the plant cell wall. Many wet chemistry-based strategies are available for cellulose and lignin quantification [8, 9]. Matrix polysaccharide sugars are quantified by derivatization and detection with GC-MS [10] but these methods are laborious and time-consuming. Biophysical methods such as pyrolysis gas chromatography-mass spectroscopy, Raman spectroscopy, and Fourier transformation infrared spectroscopy (FTIR) can be also used for studying the cell wall composition [11, 12]. The main advantage of the pyrolysis-based approach is that it does not require the pre-treatment of cell wall material. It is a rapid and highly reproducible which requires less sample weight; however, it can relatively quantify only total carbohydrate content and lignin composition [13]. The Raman and FTIR spectroscopy are also non-destructive approaches to study plant cell wall properties [14]. The limitation of both methods is that molecular vibrations of all molecules result in many overlapping bands. Thus, it is also challenging to understand and interpret the acquired spectra. Therefore, it is highly imperative to implicate a rapid and accurate method for plant cell wall analysis. Attenuated total reflectance (ATR)-FTIR has been used for fast cell wall characterization [15]. The ATR-FTIR spectroscopy is widely applicable for the chemical analysis of biological materials. It is relatively high throughput, inexpensive, requires simple sample preparation and less sample size as compared to wet chemistry based or chromatographic methods. In ATR- FTIR wide range of spectra can be generated for either powders, liquids or pastes with a minimum of sample preparation, reducing the analysis time [16–20]. Moreover, the spectral data can be exploited using multivariate statistical techniques for quantitative applications based on the relationship between spectral and reference data obtained by conventional methods. This data can be used to build predictive models and qualitative applications to infer diversity and classify samples according to their spectral characteristics. In ATR-FTIR, the absorption spectra are generated because of molecular vibration which leads to change in dipole moment. The absorption frequency depends on the functional group, which varies between different cell wall molecules. Cell wall composition may be determined by predicted equations based on near-infrared absorbance spectra of ground plant materials. This technique has been successfully used to understand the cell wall composition in *Arabidopsis* [21, 22], forage crops [23–27], rice [28, 29] and *Populus* [30, 31]. The quality of the spectra depends on the selection of sampling methods and the total internal reflectance of ATR-FTIR beam. In this technique, the accuracy of the measurement depends on the direct contact between the sample and the ATR crystal surface [32]. Therefore, the solid or powdered sample, must be clamped using pressure gauges onto the crystal surface that can be either semiliquid or liquid form. The Beer-Lambert law and multivariate chemometric analysis can be performed for fast and accurate quantification using ATR-FTIR. Qualitative and quantitative accuracy of analysis in ATR-FTIR also depends on the homogenization of the sample. However, the homogenization of plant cell wall material is difficult and untreated native cell wall materials do not dissolve in organic solvents. Therefore, the powdered cell wall material can be quantified using two classical methods, KBr pellet method and the Nujol method [33]. The KBr pellet is the most common alkali halide which becomes stiffy when subjected to pressure and forms a transparent sheet that can be used to measure the infrared spectrum

in the 600 to 4000 cm^{-1} wavenumber regions. Generally, the sample is well mixed and pulverized with KBr, which can stabilize the pellet and be subjected to ATR-FTIR.

Using KBr based pellet method, we have established a non-destructive high-throughput phenotyping method to analyse cell wall composition using ATR-FTIR spectroscopy. This method can be used for qualitative and quantitative analysis of solid cell wall material which is not dissolved in organic solvent. The cellulose and lignin standard were prepared in KBr and standard curve was used for quantification above components in different species. The cell wall composition was further cross-validated using wet-chemistry based method in different plant and tissue types. Besides, cellulose and lignin content were measured using a developed ATR-FTIR-based approach and validated chemically among a set of 48 genotypes of mungbean which suggested that this method can be used for quantitative analysis in high throughput manner.

Material and Method

Plant growing, tissue collection and processing

In this study, the leaf and stem of crop species like rice (*Oryza sativa* subsp. *Indica*), *Populus* (*Populus trichocarpa* L.), mungbean (*Vigna radiata* (L.) R. Wilczek) and Arabidopsis (*Arabidopsis thaliana*) have been analyzed. Arabidopsis was grown under 16 h light/8 h dark cycle conditions at 22°C. Rice was grown under 14 h light/10 h dark at 26°C with 85% relative humidity. Mungbean was grown under 16 h light/8 h dark at 32°C. Mature leaf tissues of rice, mungbean and Arabidopsis were harvested from fully grown healthy plants grown in well managed field at Regional Centre for Biotechnology, Faridabad (Latitude: 28°4052' N; Longitude: 77.2532° E). The *Populus* stem and leaf tissues were collected from Forest Research Institute (FRI), Dehradun, India (Latitude 30.343769° N; Longitude is 77.999559° E). The fresh leaf and stem tissues were harvested and ground into fine powder using QIAGEN Tissue Lyser (TissueLyser III, Cat. No. 9003240, Germany).

Preparation of Alcohol Insoluble Residues (AIR) to isolate cell wall material.

100 mg of crude powder was incubated with 5 mL ethanol (80%) containing 4.0 mM HEPES buffer (pH 7.5) at 70°C for 30 min, cooled on ice, and centrifuged (10,000 rpm; 15 min). The pellet was washed with 5 ml of 70% ethanol, and further treated with 5 ml chloroform: methanol (1:1). The remaining pellet was washed with 5 mL of acetone, pellet obtained after centrifugation was dried in the desiccator and this AIR sample was used for further analysis [34].

ATR- FTIR spectroscopy

The AIR, along with KBr, was used for preparing the FT-IR standards. The ATR-FTIR spectra of the powdered sample was measured, and the mixture was subjected to a Tensor FTIR spectrometer (Bruker Optics) equipped with a single-reflectance horizontal ATR cell (ZnSe Optical Crystal, Bruker Optics). Between scanning range from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} . A pressure applicator with

a torque knob ensured that the same pressure was applied for all measurements. For each sample, 16 scans were acquired, averaged with a background scanning and correction of 15–20 min regular intervals. The standard deviations of spectra of the subsamples were obtained by the OPUS 5.5 software (Bruker Optics, <http://www.brukeroptics.com/>). The standard deviations of the different biological samples were used to create an overall standard deviation using the multi-evaluation tool in the OPUS software.

Estimation of cellulose content using the Updegraff method

2 mg of AIR was incubated at 100° C for 30 min in Updegraff reagent, and the pellet obtained after centrifugation was washed with water and acetone. The dried pellet was treated with concentrated sulphuric acid and glucose was quantified by anthrone assay [35].

Acetyl Bromide Soluble Lignin content (ABSL)

2 mg of AIR was incubated in freshly prepared 25% acetyl bromide (prepared in acetic acid) in screw cap tubes at 50° C for 2 h. The solubilized lignin was mixed with 400 µl of 2M sodium hydroxide, 70 µl of 0.5M hydroxylamine hydrochloride and diluted with 1430 µl of acetic acid. 85 µl of this reaction mixture was mixed with 85 µl of acetic acid into a Corning® UV-transparent microplates (Corning, CLS3635, USA) and absorbance was taken at 280 nm using spectrophotometer [36].

Total sugar estimation

The AIR samples were mixed in deionized water at a concentration of 0.5 mg/mL 100 µl of this AIR suspension sample was mixed with equal volume of 5% (v/v) phenol. Then, 500 µl of concentrated sulfuric acid was added and incubated for 20 min. 250 µl of each reaction mixture was transferred into a Costar 3598 ELISA plate. The colour development in the reactions was measured using an ELISA plate reader by reading the OD at 490 nm. A standard curve of glucose was used to calculate glucose content in AIR sample.

Extraction of lignin

The AIR sample was treated with 1 mL of 4M KOH containing 1.0% (w/v) sodium borohydride and incubated for 24 h with constant shaking [37]. After centrifugation, the pellet was washed with water. The dried pellet was treated with CTec2 enzyme blend (SAE0020, Sigma-Aldrich, Country) at 45°C for 24 h to remove cellulose and the pellet mainly containing lignin was dried and used for FT-IR analysis as a standard.

Results

Quantitative analysis using ATR-FTIR spectroscopy

In this study, leaf and stem tissues from *Populus*, rice, mungbean and Arabidopsis were used for cell wall compositional analysis using ATR-FTIR. In ATR-FTIR, the infrared spectrum from sample is generated

due to superposition of light absorbance by functional groups of cell wall polymer (chemical information) and light scatter (physical information). ATR-FTIR spectra can be categorized into different regions, including the X-H stretching region ($4000-2500\text{ cm}^{-1}$), the triple-bond region ($2500-2000\text{ cm}^{-1}$), the double-bond region ($2000-1500\text{ cm}^{-1}$) and the fingerprint region ($1500-600\text{ cm}^{-1}$) [38,39]. The spectral region $600-2000\text{ cm}^{-1}$ was used to analyze linkages by determining functional groups which corresponds to specific cell wall components [40–42].

The spectral data was obtained on the alcohol extracted fraction (AIR) of different species which led to the specific absorption characteristics because of distinct composition (**Fig. 1**). The distinct peaks are commonly used to identify a particular functional group of a specific cell wall component [39]. The spectra exhibited high absorbance at specific wavenumbers characteristic of cell-wall polysaccharides, which resulted in a peak which was assigned to a particular functional group of a specific cell wall polysaccharide. In this study, different absorbance patterns or peak characteristics have been observed for different samples. According to previous studies, cellulose peaks were assigned to $1060-1072\text{ cm}^{-1}$ wave number for which (CO), (CC) and (OCH) ring in Arabidopsis and *Populus* [40,43]. For aspen and acetobacter $1099-1115\text{ cm}^{-1}$ wavenumber was assigned to O-H, (CO) and (CC) ring of cellulose [42,44]. Therefore, based on these reports and cellulose standards, we selected the range $1050-1060\text{ cm}^{-1}$ wavenumber for further analysis. Previously, lignin peaks were detected in different ranges of wavenumber. In Arabidopsis, aspen, barley and bamboo region $1502-1520\text{ cm}^{-1}$ wavenumber was used to detect aromatic C=C stretch of lignin [43,45–48]. In another study, $1456-1465\text{ cm}^{-1}$ wavenumber was reported for the detection of CH_3 asymmetrical bending of lignin [49,50]. In this study, a prominent peak, $1390-1420\text{ cm}^{-1}$ wavenumber was observed for C-H deformation aromatic skeletal, which was assigned to lignin. This peak was reported in flax, *Populus* and hardwood maple [49,51] (**Table 1**). Based on these peaks or region, we compared cell wall composition in different species and tissue types.

The absorbance at different wavenumber for different cell wall components clearly differentiated tissue of different plant samples (**Fig. 1**). In the case of cellulose, within $1050-1060\text{ cm}^{-1}$ wavenumber higher absorbance was observed for *Populus* stem (0.20 ± 0.0025), rice stem (0.20 ± 0.0206), rice leaf (0.21 ± 0.0125) and mung stem (0.19 ± 0.0081). Leaf tissues reflected lower absorbance as compared to stem tissues. Among leaf tissues, *Populus* leaf (0.15 ± 0.0205) showed higher absorbance, followed by mungbean (0.12 ± 0.003) and Arabidopsis leaf (0.11 ± 0.002) for cellulose specific peak. Interestingly, higher absorbance at the cellulosic region was observed for rice leaf. This may be due to the presence of higher hemicellulose (xylan) in rice leaf [52]. For lignin, within $1390-1420\text{ cm}^{-1}$ wavenumber, higher absorbance was observed for leaf samples than stem samples, which was further used to compare lignin content in different samples. Mungbean leaf lignin peak showed higher absorbance (0.085 ± 0.031) followed by rice leaf (0.05 ± 0.00165), Arabidopsis leaf (0.05 ± 0.00346), mungbean stem (0.048 ± 0.00145), *Populus* leaf (0.045 ± 0.00275), Arabidopsis stem (0.045 ± 0.00010), rice stem (0.041 ± 0.0015) and *Populus* stem (0.033 ± 0.00005). All these data correlated with existing knowledge of plant

cell wall composition in different tissues or species, suggesting these wavenumber regions can be used for analysing cellulose (1050-1060 cm^{-1}) and lignin (1390-1420 cm^{-1}) across monocot, dicot and tree species.

Analysis of the differential composition of plant cell wall through principal component analysis (PCA)

PCA has been successfully used to analyze FT-IR generated spectral data to understand the differences and clustering in different groups [53,54]. In this study, PCA was performed raw data obtained from different spectral ranges and samples as explained earlier (Fig.1). The most preferable spectral region for cell wall component analysis, i.e. 800-2000 cm^{-1} was used for PCA analysis. The score plot of principal component was generated to identify differences in samples and model predictability. The scatter plot represented two principal components; PC1 and PC2, which together explain maximum variability in PCA within 800-2000 cm^{-1} range (**Fig. 2a, Fig. S1a**). Q2 value was also significant, suggesting the PCA model was predictive (**Fig. 2b**). While comparing cell wall composition across different species and tissues, PC1 and PC2 showed 73% of variability. The grouping effect was observed across both the axes. PC1 contributed to maximum variability (44.7%) as compared to PC2 (28.3%) (**Fig. S1a, Fig. 2a**). The grouping of the samples along the PC1 axis majorly represented stem samples of Arabidopsis, mungbean, *Populus* and rice. PC2 represented the leaf samples of Arabidopsis, mung bean and *Populus*. The PC1 grouping of rice leaves can be explained by the fact that rice leaves contain more cellulose and hemicellulose than other studied crops. This was well documented and validated through absorption spectral analysis, which was higher for rice leaf within 800-2000 cm^{-1} range [55,56].

To specifically understand the variation in cellulose content in different species, PCA was used within the 1000-1050 cm^{-1} range (**Fig. 2c, Fig. 2d, Fig. S1b**). As expected, stems of *Populus*, Arabidopsis and mungbean clustered together, separated by PC2 component with 31.3% of the variability. These groups were separated from leaf of mungbean, Arabidopsis and *Populus* and rice stem, which were clustered together. Rice leaf was grouped separately from above tissue types. This data was further validated by K-mean clustering (**Fig. S2**). These data indicated that cellulose of leaf and stem of monocot and dicot species have distinct cellulose structures. The PCA within region 1390-1420 cm^{-1} revealed lignin characteristics for different tissues and plant samples. PC1 and PC2 both contributed around 89% of the variation. PC1 exhibited highest variability of 57.8% (**Fig. 2e, Fig. S1c**). Also, significant Q2 value represented predictive ability of the model (**Fig. 2f**). Rice stem, rice leaf and *Populus* stem clustered together and well separated from other tissues. The study suggests that multivariate analysis using 800-2000 cm^{-1} , 1000-1050 cm^{-1} and 1390-1420 cm^{-1} wave number ranges clearly distinguished different tissues of different plant samples based on total cell wall composition, cellulose and lignin content, respectively.

Quantitative analysis of cellulose and lignin using ATR-FTIR

ATR-FTIR can efficiently be used for quantitative analysis of liquid samples but the homogenization is the major constraints for quantification of powdered samples. Most of the cell wall material usually does

not dissolve in organic solvent. Therefore, in this study emphasis was made on quantifying cell wall composition in solid form using ATR-FTIR by mixing commercially available standards (cellulose and lignin) with KBr in different concentrations. Wavenumber number regions $1050-1060\text{ cm}^{-1}$ and $1390-1420\text{ cm}^{-1}$ were used to detect cellulose and lignin, respectively. An absorbance gradient of 0.10, 0.12, 0.14 and 0.175 was observed for 10%, 20%, 30% and 40% cellulose respectively (**Fig. 3a**). Similarly, 0.026, 0.036 and 0.05 was observed for 10%, 15% and 20% lignin, respectively (**Fig. 3b**). The standard curve was prepared for both to quantify cellulose and lignin in different tissue types. Based on the standard curve, the stem of *Populus*, rice, Arabidopsis and mungbean contained 48.89%, 46.23%, 39.2% and 42.6% of cellulose, respectively. The leaves of *Populus*, rice, Arabidopsis and mungbean composed of 34.07%, 52.47%, 12.1% and 18.1% of cellulose, respectively (**Table 2**). It was observed that rice leaf contains a high amount of cellulose, which was evident from multivariate analysis (**Fig. 2b, Fig. S1b**). The stem of *Populus*, rice, Arabidopsis and mungbean contained 16.39%, 23.4 % 19.63% and 22.32% of lignin respectively. The leaf of *Populus*, rice, Arabidopsis and mungbean contained 20.83%, 23.4%, 24.16% and 34.78% of lignin respectively. Generally, leaf tissue contains less lignin, and we hypothesized that lignin peaks probably interfere with other sugars or phenolics. To test this, AIR was treated with a high concentration of NaOH followed by digestion with cellulase to remove cellulose, and the remaining pellet mostly contained lignin, which was used for FT-IR analysis. However, the lignin content was higher in mungbean leaf followed by *Populus* stem, Arabidopsis stem and rice leaf; which was consistent with our previous observation. This data again confirmed that the selected region can be used to quantify the lignin content (**Fig. 4**).

Validation of FT-IR results with wet-chemistry methods

To check whether cell wall content calculated using FT-IR is correlating with wet-chemistry methods, the cellulose and lignin content were analyzed by standard Updegraff and acetyl bromide soluble lignin method respectively. The result revealed that the cellulose content of Arabidopsis leaf, Arabidopsis stem, mungbean leaf, mungbean stem, *Populus* leaf, *Populus* stem, rice leaf and rice stem was 10.84%, 29.53%, 6.50%, 28.69%, 11.27%, 39.72%, 21.07% and 17.91% respectively (**Fig. 5a**). The total sugar analysis was performed using phenol-sulfuric acid with slight modification and standardization [37]. This experiment validated the outcome of multivariate analysis and cellulose content among different tissues of different crop plants. The result suggested that Arabidopsis leaf, Arabidopsis stem, mungbean leaf, mungbean stem, *Populus* leaf, *Populus* stem, rice leaf, rice stem contain 8.84%, 53.33%, 18.92%, 52.43%, 26.29%, 61.42%, 44.63% and 43.97% total sugar (**Fig. S3**). It was evident that *Populus* stem contain highest total sugar as compared to other species. However, rice leaf and stem contain comparatively equal amounts of total sugar, which correlated with cellulose content analysis. Whereas, the lignin content of Arabidopsis leaf, Arabidopsis stem, mung leaf, mung stem, *Populus* leaf, *Populus* stem, rice leaf and rice stem were found to be 6.60%, 7.57%, 8.58%, 8.15%, 5.27%, 10.26%, 7.53% and 7.18% respectively (**Fig. 5b**). The analysis using both the method revealed that stem cellulose content is higher as compared to leaf in all the plant sample, which is also validated using total sugar analysis. In case of lignin, the wet-chemistry analysis revealed that the stem lignin content is higher as compared to leaf

lignin. However, the FTIR analysis revealed that leaf lignin content is higher as compared to leaf lignin measured using the wet-chemistry method.

Chemotyping of mungbean accessions for stem cellulose and lignin content using ATR-FTIR and wet-chemistry-based approach.

Our results suggested that ATR-FTIR can be used for quantification of cellulose and lignin. Therefore, we analysed and validated the cell wall composition of different mungbean accessions as the large-scale qualitative and quantitative analysis its composition was never performed earlier considering it as important legume crop. Therefore, the main stem from field-grown mungbean accessions was collected and subjected to ATR-FTIR and wet-chemistry-based analysis of cell wall composition. The percentage of cellulose content determined by the ATR-FTIR-based method was 10-15% higher as compared to the percentage determined by the Updegraff method (**Fig. 6a; Table S1**).

Chemical analysis revealed that the total cellulose content variation ranges from 23.26% (KM 11-40) to 39.55% (ML 1451) with a mean value of 31.22%. The comparatively lower cellulose containing genotypes were KM 11-40 (23.26%), IPM 02-17 (23.43%), IC 282094 (23.67%), LGG 460 (24.45%), KM 2241 (25.79%), IPM 288 (25.97%), HUM 6 (26.78%), CHINA MUNG (27.37%), M 875 (27.84%) and M 880 (28.02%). The higher cellulose-containing genotypes are GANGA 1 (35.95%), M 565 (36.51%), KM 16-75 (36.81%), TM 9725 (37.12%), MH 934 (37.26%), PUSA 1331 (37.93%), IPM 409-4 (38.33%), PLM 167 (38.57%), EC 550851(38.67%) and ML 1451 (39.55%). The ATR-FTIR-based study revealed the cellulose content variation ranges from 27.9% (KM 11-40) to 52.37% (M 565) with a mean value of 41.81% (**Fig. 6a**). In the case of the ATR-FTIR-based spectroscopic approach, the comparatively lower cellulose-containing genotypes were KM 11-40 (27.9%), M 499 (29.75%), IC 282094 (32.63%), CHINA MUNG (33.75%), PLM 167 (34.505%), EC 3988891 (34.76%), M 1400 (35.05%), YM 2 (35.165%), EC 520026 (37.625%), PUSA 1441 (38.04%). The higher cellulose-containing genotypes were PUSA 1131 (45.73%), KM 16-58 (45.87%), M 1053 (46.805%), KM 16-75 (47.395%), MH 934 (47.47%), OLRM 4 (49.40%), ML 1451 (50.48%), EC 550851 (50.87%), TM 9725 (51.035%) and M 565 (52.37%) (**Fig. 6a, Table S1**). The cellulose content was lower in KM 11-40, IC 282094 and CHINA MUNG using wet-chemistry and ATR-FTIR method. We also found that M 565, KM 16-75, TM 9725, MH 934, EC 550851 and ML 1451 had higher cellulose content using both approaches.

In a separate set of 48 accessions, lignin content was determined using ATR-FTIR-based and acetyl bromide soluble lignin (ABSL) measurement approach. The lignin content determined by the ATR-FTIR-based method was 5-10 % higher as compared to the percentage determined by the ABSL method (**Fig. 6b and Table S2**). Chemical analysis revealed that the total lignin content variation ranges from 9.47% (EC 520029) to 20.2% (M 313) with a mean value of 13.91% (**Fig. 6b**). The lower lignin content genotypes are EC 520029 (9.47%), IC 282094 (9.49%), M 880 (9.56%), SML 668 (10.42%), MUSKAN (10.70%), KM 11-40 (10.9%), PUSA 1131 (11.01%), M 1053 (11.11%), PUSA VISHAL (11.31%) and M 1400 (11.48%). The higher lignin content genotypes were IPM 288 (16.83%), M 906 (16.96%), V 1153 (17.33%), KM 7-134 (17.36%), HUM 1 (17.9%), PUSA 0971 (17.63%), MH 96-1 (17.72%), IPM 02-19 (18.89%) M 1370 (19.97%)

and M 313 (20.21). The ATR-FTIR-based study revealed the lignin content variation ranges from 13.77% (M 875) to 31.6% (M 313) with a mean value of 22.21%. The lower lignin content genotypes using ATR-FTIR approach were M 875 (13.77%), PUSA 0971 (15.73%), EC 520029 (16.91%) M 1400 (17.25%), M 1032 (17.26%), IC 282094 (17.59%), PUSA 1441 (18.88%), PUSA VISHAL (18.27%), MH 96-1 (18.49%) and TM 96-25 (18.77%). The higher lignin content genotypes were HUM 1 (25.15%), PUSA 1342 (25.17%), M 831 (25.28%), TM 96-2 (26.77%), V 1153 (27.27%), IPM 02-19 (27.77%), IPM 02-17 (29.05%), KM 7-134 (30.085%), M 1370 (31.036%), M 313 (31.6%) (**Table S2**). The genotypes, EC 520029, IC 282094, PUSA VISHAL and M 1400 had the comparatively lower lignin content using both the ATR-FTIR and wet-chemistry based methods. The genotypes V 1153, KM 7-134, HUM 1, IPM 02-19, M 1370 and M 313 contained comparatively higher lignin in both methods. The reason where FT-IR data did not correlate with wet chemistry is because of overlapping peaks corresponding to different function group of cell wall components. Overall, this data revealed that ATR-FTIR-based quantification can be used high throughput analysis of cellulose and lignin.

Discussion

The samples from diverse plant species were selected to cover the variability of cell wall composition for qualitative and quantitative estimation of its composition. Leaf and stem tissues from *Populus*, rice, mungbean and Arabidopsis were used for cell wall compositional analysis for the following reasons. *Populus* is highly desirable as a feedstock for biofuels as compared to other woody crops. It grows fast and produces a significant amount of biomass within a short period. Moreover, the stem of *Populus* is a source of high cellulose (45–50%), hemicellulose (20–25%) and lignin (25–30%) [57–59]. The biomass of mature rice cell walls is usually composed of 40–50% cellulose, 20–25% hemicellulose and 20–25% lignin, and most rice residue (straw and husk) biomass is underutilized [60], which can be effectively used for bioenergy feedstock. Mungbean is an important pulse crop with a very short growing season. Its mature stem cell wall biomass is composed of around 35–45% cellulose, 20–25% hemicellulose and 15–20% lignin [61]. Arabidopsis is a plant model system wherein plant cell wall is extensively studied. Arabidopsis stem cell wall biomass is reported to be composed of 40–45% cellulose, 15–20% hemicellulose and 10–15% lignin [62, 63]. Several studies revealed that the cell wall composition is dynamic across different plant species [2]. Moreover, most of these cell wall components are water-insoluble. Thus, it is challenging to quantify the cell wall composition using ATR-FTIR in powder form. Therefore, in this study, efforts have been put forward for quantitative and qualitative analysis of plant cell wall composition in powder form. Specific regions were identified for analysis of total cell wall composition (800–2000 cm^{-1}), cellulose (1000–1050 cm^{-1}) and lignin (1390–1420 cm^{-1}). Multivariate analysis revealed clear grouping of leaf and stem samples in these regions suggesting our method is able to capture variation in cell wall composition (Fig. 2). This data concluded that the selected wavenumber region can be used to compare cellulose and lignin content with different spectral characteristics. Further, quantitative analysis using ATR-FTIR indicated that stem cellulose content of all the plant species is higher as compared to leaf. However, it has been observed that leaf lignin content is slightly higher as compared to stem lignin content. In general, leaf tissue contains less lignin, and we

hypothesized that lignin-specific region probably interfere with other sugars or phenolics. Therefore, extraction of lignin rich fraction was performed from AIR to analyse using FT-IR (Fig. 4). We found that the lignin content was higher in mungbean leaf followed by *Populus* stem, Arabidopsis stem and rice leaf; validating that the selected region can be used to quantify the lignin content (Fig. 4). Further, the result of FTIR-based quantification method was validated by using the canonical wet chemistry method. The wet-chemistry-based approach revealed that rice leaf, stem and mung leaf contain comparatively less cellulose than ATR-FTIR data, probably because of overlapping peaks from other matrix polysaccharide components [53]. Moreover, the large-scale quantitative analysis of cell wall composition in different accessions of mungbean was performed. The percentage of cellulose content determined by the ATR-FTIR-based method was 10–15% higher as compared to the percentage determined by the Updegraff method (Fig. 6). This variation can be explained by the presence of non-crystalline cellulose or from other hemicellulosic polysaccharide that can be measured using the ATR-FTIR-based method but not by the Updegraff method [64, 65]. In general, plant cell wall consists of around 10–20% amorphous cellulose depending on plant and tissue types [66, 67]. During cell wall material preparation, amorphous cellulose was removed, and the remaining pellet was used for Updegraff analysis. This non-crystalline form of cellulose can only be detected using a spectroscopic approach but not using the Updegraff method since it is removed during the extraction process [68]. The lignin content determined by the ATR-FTIR-based method was found to be 5–10 % higher as compared to the percentage determined by the ABSL method. In our study, lignin has been detected within $1390\text{--}1420\text{ cm}^{-1}$ wavenumber for C-H deformation of aromatic skeletal vibrations. Lignin monomers are synthesized through the phenylpropanoid pathway [69]. Therefore, it is possible that within this range of wavenumber, some derivative compounds having C-H deformation of aromatic skeletal vibrations can be overlapping. The reason for miscorrelation between the two methods could be the same wavenumber can represent peaks for different components. The ideal way would be to perform sequential extraction of varying cell wall components and analyze their composition using FT-IR and wet chemistry methods. Overall, the combined effort of ATR-FTIR-based and chemical methods led to the determination of stem cellulose and lignin content of several mungbean accessions. To best of our knowledge, there are no reports on characterization of polysaccharide in mungbean. Few reports are based on isolation of soluble polysaccharides, characterization of mono or oligosaccharides and their role in inducing the immunomodulatory properties [70]. Mungbean stem and leaf tissues are repertoire of complex polysaccharides, but their characterization was done before. In our study, we found that average stem cellulose content is around 32% (Updegraff method) and 42% (ATR-FTIR) in mungbean. The stem lignin content is found to be around 14% (Updegraff method) and 22% (ATR-FTIR) (Fig. 6). This approach also leads to relatively discriminating stem cellulose and lignin content among different mungbean accessions. This data suggested that ATR-FTIR can be used for the relative quantification of lignin and cellulose in mungbean and different crop species.

Conclusion

ATR-FTIR is conveniently applicable with simple chemometric and multivariate analysis to predict the cell wall composition in different plant species. In this study, a high-throughput, non-destructive, powerful approach has been developed for qualitative and quantitative analysis of solid cell wall material which is not dissolved in organic solvent. This method has been used to determine the cellulose and lignin content among different mungbean accessions. Further, the wet-chemistry analyses have been performed to validate the outcome of the ATR-FTIR-based method. This approach can be used for large-scale chemotyping of cell wall composition among different plant species. Thus, it could reduce cost, time and labour in breeding programs. The method and strategy presented in this study can accelerate large-scale cell wall compositional analysis among thousands of accessions. Therefore, it has immense potential in molecular breeding, such as QTL-mapping, genome or candidate gene-based association mapping and thus, could enhance the pace of genomics-assisted breeding.

Declarations

Availability of data and materials

Not applicable

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Contributions

SD and PM-AP designed and conceptualized the research. SD, VB and AG performed all the experimental and data analysis. BPD performed total sugar analysis experiment. SD and PM-AP wrote the manuscript. HKD and GPM provided mungbean accession and suggestions during manuscript preparation. All authors have read and agreed to publish the manuscript.

Corresponding author

Ethics declarations

Ethics approval and consent to participate

No specific permit was required for the samples analyzed in this study. The authors comply with relevant institutional, national, and international guidelines and legislation for plant studies.

Consent for publication

Not applicable.

Conflict of statement

Authors declare no conflict of interest

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Tables

Table 1 Specific characteristics of absorbance spectra for cellulose and lignin

| Compound | Band position (cm ⁻¹) | Assignments | Source and reference |
|-----------|-----------------------------------|---|----------------------|
| Cellulose | 1050-1060 | (CO), (CC), (OCH) ring | [71,72,73] |
| Lignin | 1390-1420 | C-H deformation Aromatic skeletal vibrations | [74,75,76,77] |

References

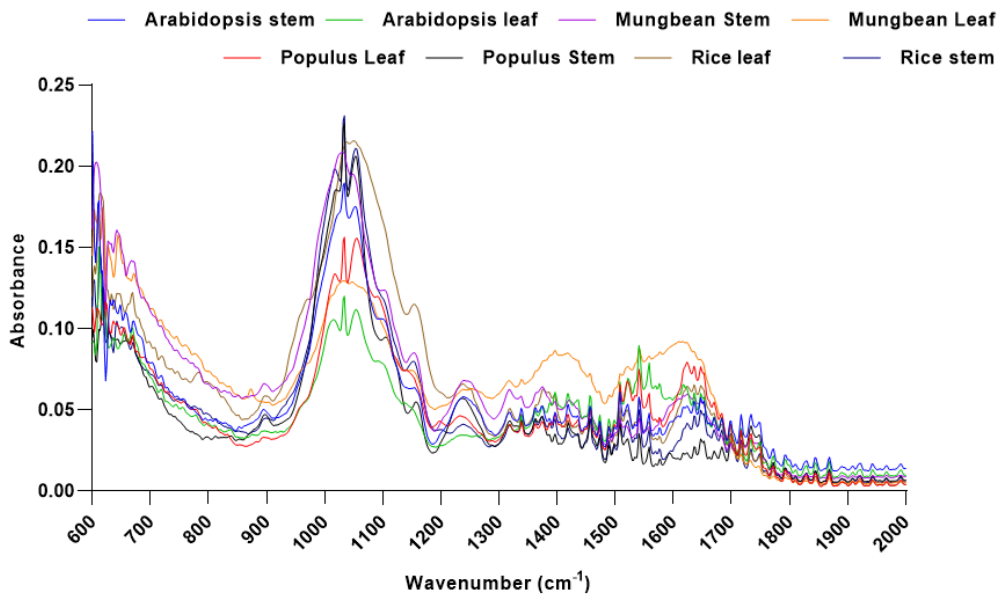
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Table 2: Cellulose and Lignin content of different plant samples measured using ATR-FTIR and wet-chemistry method.

| Species and tissue types | ATR-FTIR-based method | | Wet-chemistry-based method | |
|--------------------------|-----------------------|------------|----------------------------|------------|
| | Cellulose (%) | Lignin (%) | Cellulose (%) | Lignin (%) |
| <i>Populus</i> stem | 48.89 | 16.39 | 39.72 | 10.26 |
| Rice Stem | 46.23 | 23.4 | 17.91 | 7.18 |
| Mungbean stem | 42.6 | 22.32 | 28.69 | 8.151 |
| Arabidopsis stem | 39.2 | 19.63 | 29.53 | 7.56 |
| <i>Populus</i> leaf | 34.07 | 20.83 | 11.27 | 5.26 |
| Rice leaf | 52.47 | 23.4 | 21.07 | 7.52 |
| Mungbean leaf | 18.1 | 34.78 | 6.50 | 8.58 |
| Arabidopsis leaf | 12.1 | 24.16 | 10.84 | 6.59 |

Figures

Figure 1

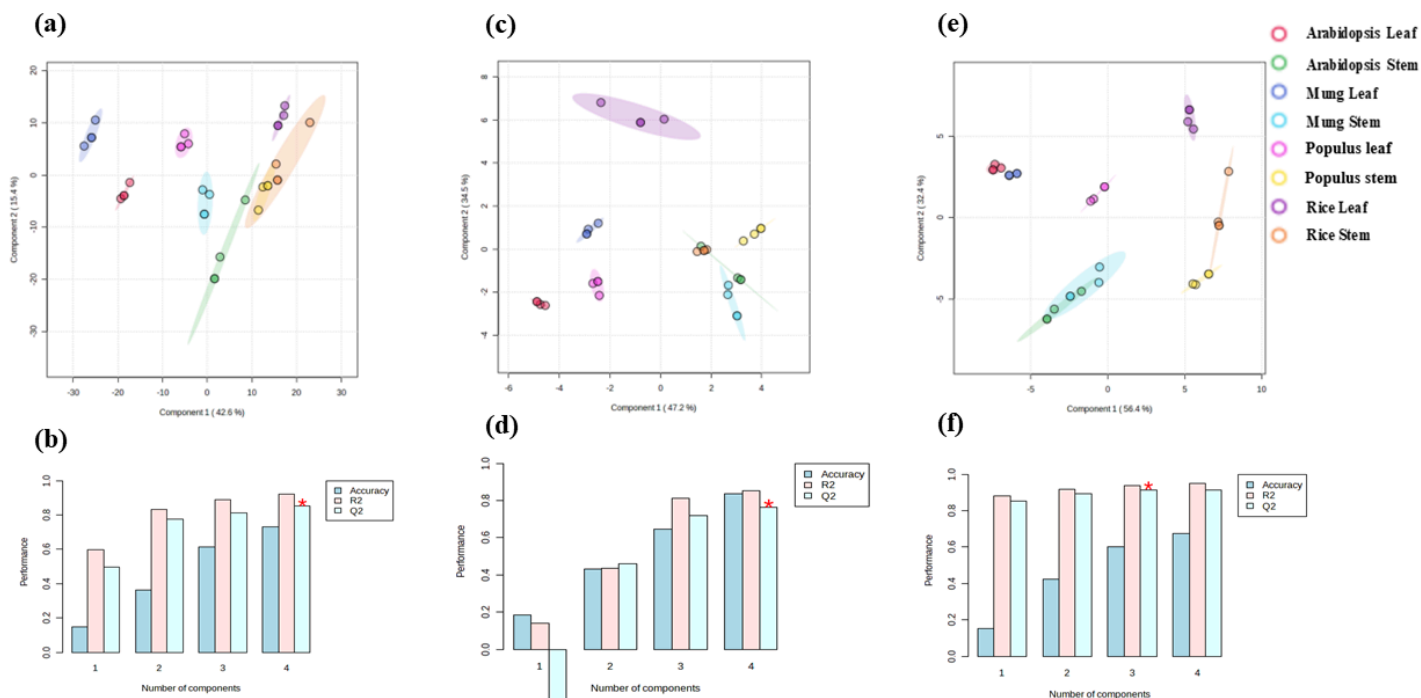


Absorption spectral characteristics for different tissues of different plant samples using ATR-FTIR spectroscopy. The spectral mean was calculated from two biological replicates.

Figure 1

See image above for figure legend

Figure 2

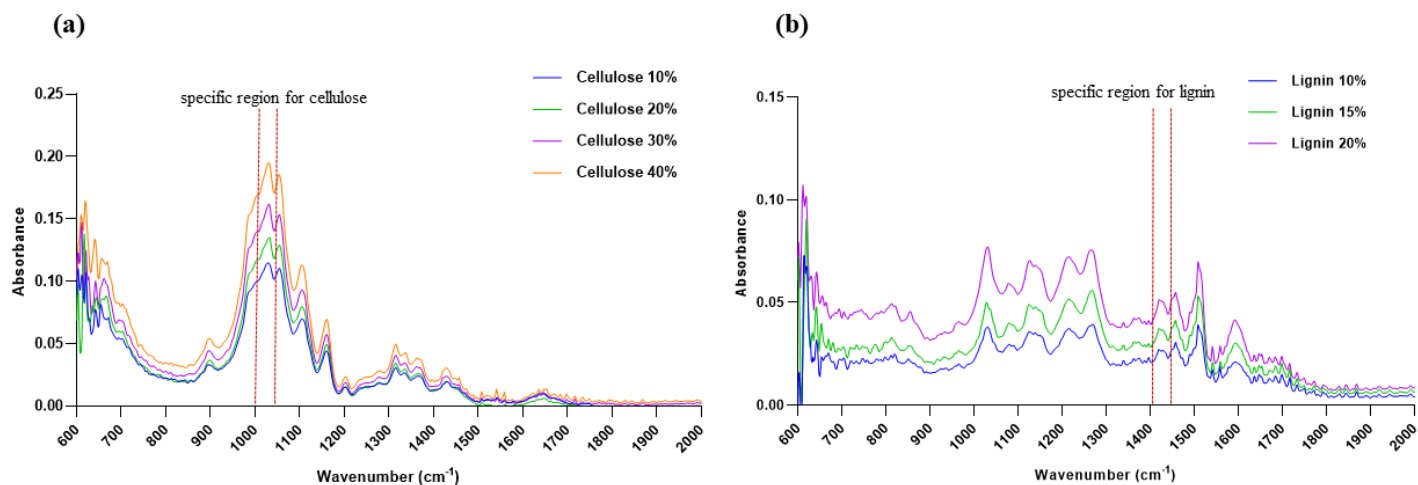


Partial Least Squares Discriminant Analysis (PLS-DA) of absorption spectra and cross-validation using Q-square value within (a,b) 600-2000 cm⁻¹ (c,d) 1000-1100 cm⁻¹ and (e,f) 1390-1420 cm⁻¹ wavenumber for total sugar, cellulose and lignin respectively in ATR-FTIR. These graphs were prepared using online tool - <https://www.metaboanalyst.ca/>

Figure 2

See image above for figure legend

Figure 3

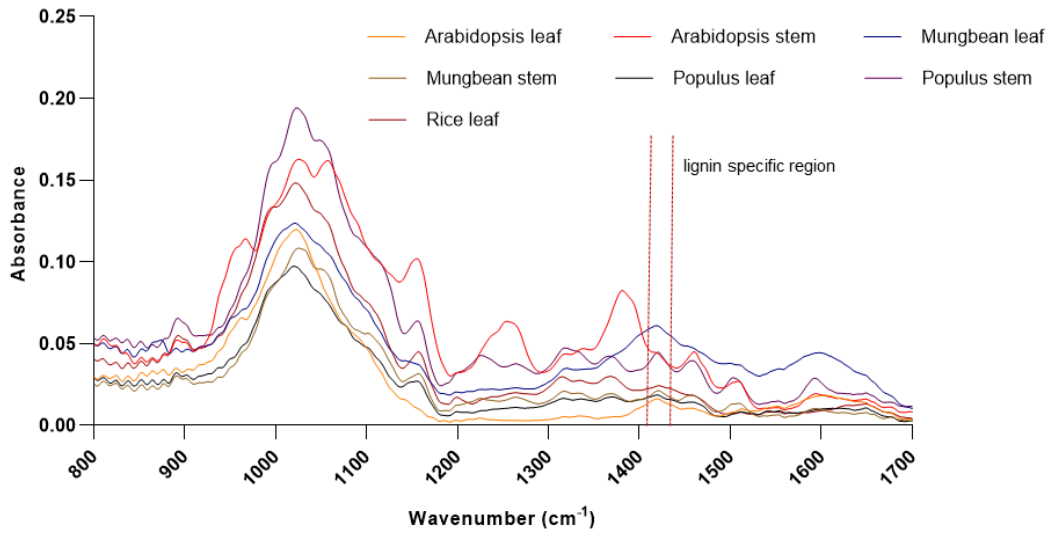


Spectra represent the different concentrations of (a) cellulose and (b) lignin prepared using KBr. The dotted line represents selected region used for quantification. The spectral mean was calculated from two technical replicates.

Figure 3

See image above for figure legend

Figure 4

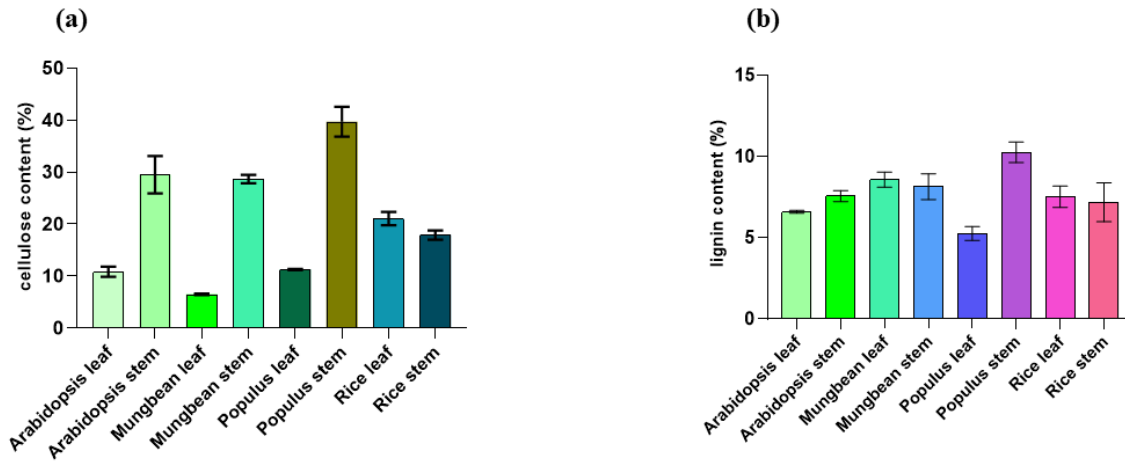


Absorption spectral characteristics of samples after hemicellulose and cellulose removal which is enriched in lignin. Spectral mean was calculated from two different replicates.

Figure 4

See image above for figure legend

Figure 5

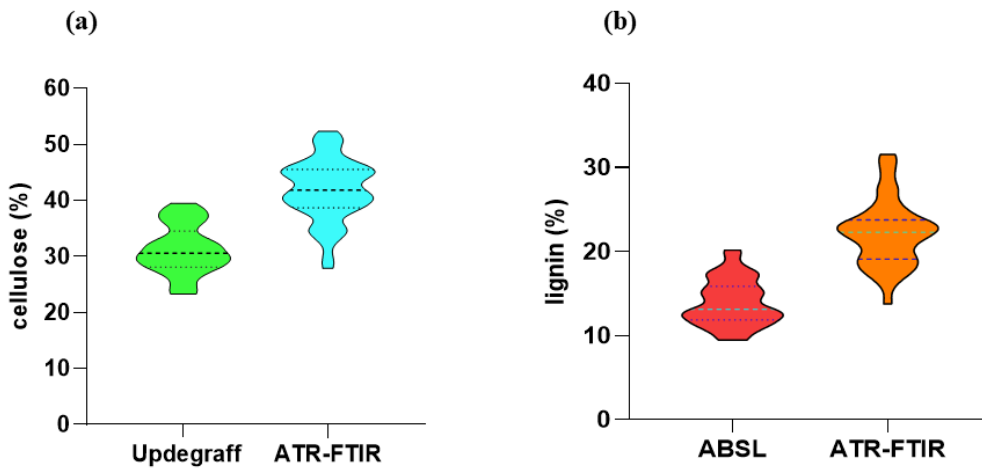


(a) cellulose and (b) lignin determined using Updegraff and acetyl bromide soluble lignin (ABSL) method respectively. Data represents mean \pm SD with $n = 2$.

Figure 5

See image above for figure legend

Figure 6



Distribution of (a) cellulose and (b) lignin content using ATR-FTIR and wet-chemistry methods. Data represents the average from 48 mungbean accessions.

Figure 6

See image above for figure legend

Supplementary Files

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