Assessing the effect of artificially reduced lignin content in jute with regard to its use as textile fiber, biofuel and raw material for paper-pulp industry

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by

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Declaration

I hereby declare that the thesis entitled **"Assessing the effect of artificially reduced lignin content in jute with regard to its use as textile fiber, biofuel and raw material for paper-pulp industry"**, submitted by me under the supervision and guidance of **Dr. Haseena Khan,** Professor, Department of Biochemistry & Molecular Biology, University of Dhaka. This is original research work carried out by me during the period 2016-2017 for partial fulfillment of the requirements for the award of the degree of Masters of Philosophy (M. Phil).

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ABSTRACT

Jute (*Corchorus* sp.), a lignocellulosic plant grown abundantly in Bangladesh, is known for its fiber quality. Agriculture-based, renewable and biodegradable nature together with low-cost availability puts jute at an advantage. Potential high yield of cellulosic biomass per acre is another reason for increased global interest on jute. Textile and paper industries are interested in its potential as an important ingredient for producing paper and fine textiles. As with other lingocellulosic plants, the drawback of jute when used as a source for such purpose, is the abundance of lignin polymer that renders the plant material almost inaccessible for downstream processes. Previously, a project on down regulation of lignin biosynthesis related genes in jute using RNAi technology had resulted in significant decrease in the lignin content. In this backdrop, the current study has been performed with a view to evaluating the practical application of the effect of reduced lignin content in industrially important applications of jute. After down-regulation of the lignin biosynthetic genes, the decrease in lignin content was estimated by Klason method (Tanmoy et al. 2015) in transgenic lines and the lignin content was compared with the wild type. The result exhibited ~16–25% reduction in acid insoluble lignin for the whole stem and ~13–14% reduction in fiber lignin content compared to the control lines. The increase in cellulose content 3-6% (Updegraff et al. 1969), as well as increase in glucose release after enzymatic saccharification are considered as subsequent consequences of lignin down regulation. The morphological features of jute fiber was observed under a scanning electron microscope (SEM). This showed an exposure of underlying fibrils in transgenic lines whereas fibrils were found to be hidden underneath the lignin layer in control jute fiber. Furthermore, an analysis of the mechanical properties show enriched fiber quality in all transgenic lines when compared to the wild type controls. No compensation in growth which could be linked to lignin reduction was found in transgenic jute. It can be concluded that, the application of these environment friendly high quality jute fiber in textile and paper industries, eco-friendly production of biofuel from its increased cellulosic biomass and effective increase in quality of fiber will lead to sustainability of these jute lines.

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1 INTRODUCTION

Jute, one of the best natural bast fibers, is gaining importance because of an increasing demand of bio-polymers. Economic and environmental factors favor the adoption of lignocellulosic bioenergy crops like jute for the production of liquid transportation fuels (Dixon, Reddy et al. 2014). However, lignocellulosic biomass is recalcitrant to saccharification (sugar release from cell walls), and this is, at least in part, due to the presence of the phenylpropanoid-derived cell wall polymer lignin (Lewis and Yamamoto 1990; Vanholme, Demedts et al. 2010).

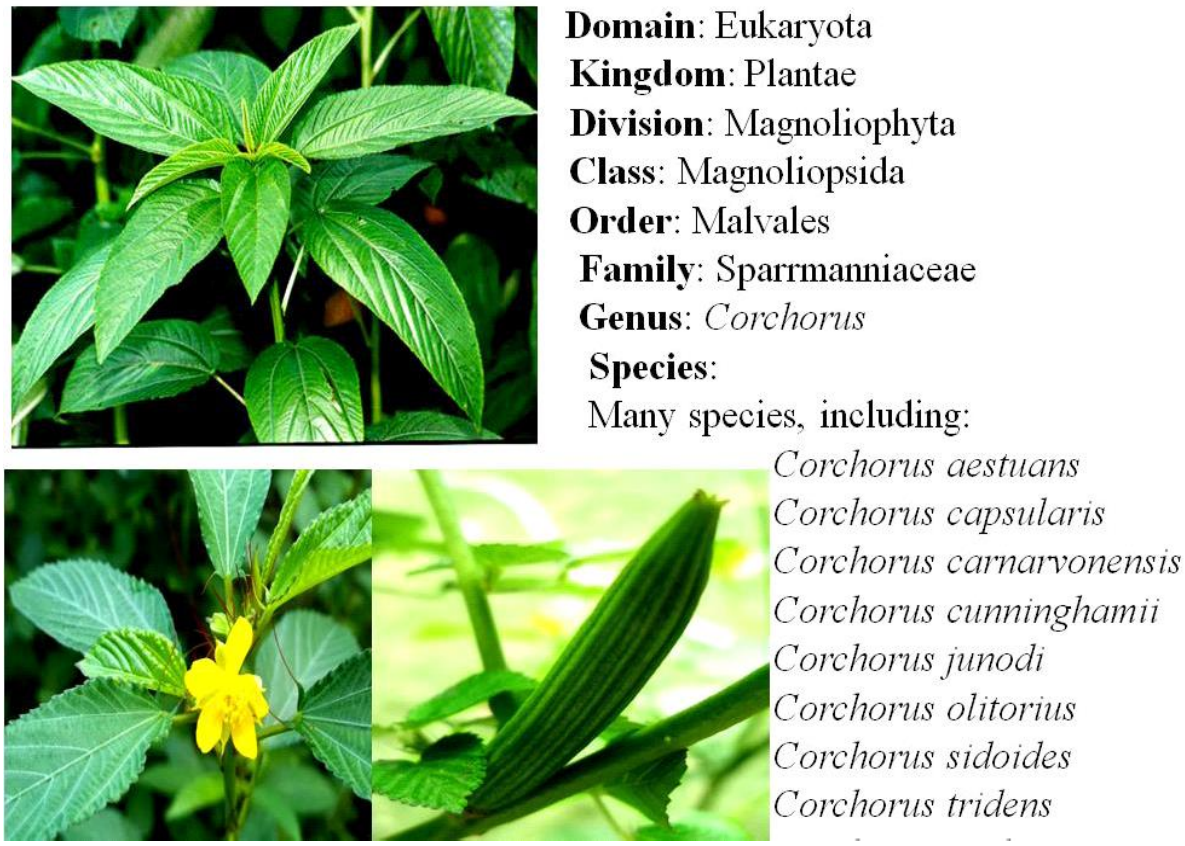

Figure 1:1 Jute plant with taxonomic classification

Constituent	***Percentage (%)***
Cellulose	59-61
Pentose	15-17
Lignin	12.5-13.5
Polyuronide	4.8-5.2
Acetyl value	2.8-3.5
Fat and Wax	0.9-1.4
Nitrogenous matter	1.56-1.87
Mineral Substances	0.5-0.79

Table 1-1 Chemical composition of jute fiber

Jute fibers are composed primarily of the plant materials, cellulose (major component of plant fiber), and lignin (major components wood fiber) (Giwa and Akwu 2007). It is thus a lingo-cellulosic fiber that is partially a textile fiber and partially wood. Jute (*Corchorus* spp. 2n=14, Genome size =400 Mb) is one of the best affordable vegetable fiber producing crops which is naturally rich in lignin. The two species of the genus *Corchorus* (family: Sparrmanniaceae), which are cultivated as jute crop include *Corchorus capsularis* L. (white jute) and *C. olitorius* L. (dark/tossa jute), although 50 - 60 species are widely known and 170 names are described under the genus *Corchorus* in index Kewensis (Jackson 1908; Edmonds 1990). The two major species are distinct in their growth and, branching habits and characteristics relating to leaf, flower, fruit, bast fiber and photosensitivity (Basu, Ghosh *et al.* 2004). Generally, the bast fibers of two plant species, *Corchorus capsularis* and *Corchorus olitorius* are known as jute (Islam, M., Saito, J., Emdad, E. *et al.*, (2017). Bast fiber develops in the phloem or bast region of the plant stem. These fibers are mainly sclerenchyma cells with copious secondary wall thickening (Sengupta and Palit 2004).

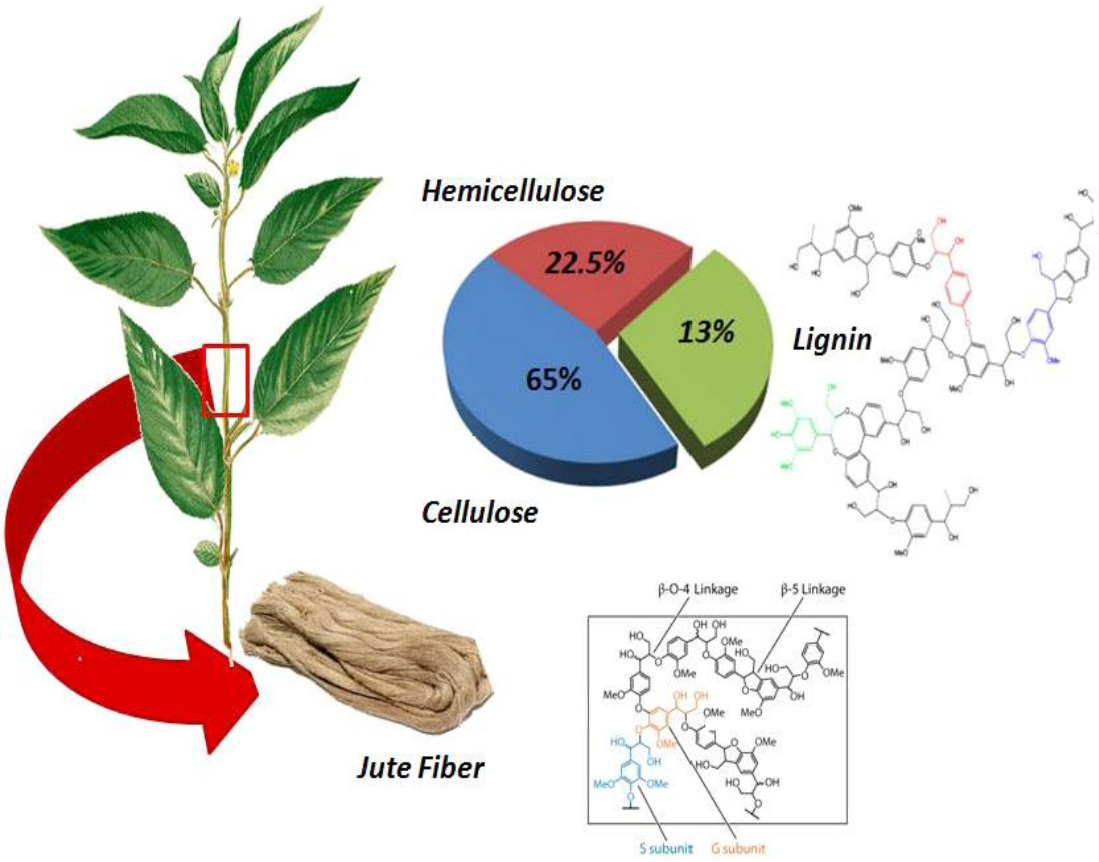

Figure 1:2 Chemical constituents of jute fiber

Although there is a strong demand for jute in both local and international markets, there are problems in increasing the productivity and profitability of jute. Natural fibers like jute have intrinsic properties – mechanical strength, low weight and low cost – that have made them particularly attractive to the automobile industry (Saha, Manna *et al.* 2010). Being cheap and easily available in large quantities, it is an ideal lingo-cellulosic substrate for producing industrial raw materials of much higher values (Barbieri, Somigliana *et al.* 2007; Karmakar, Hazra *et al.* 2008). Developing jute varieties having low lignin content would result in improved fiber quality and consequently better profitability (Bogoeva-Gaceva, Avella *et al.* 2007; Del Rio 2010, Rencoret *et al.* 2009).

In terms of usage, production and global consumption, jute is second only to cotton (Roy, Bandyopadhyay *et al.* 2006). It is environmentally friendly as well as one of the most affordable fibers; jute plants are easy to grow, have a high yield per acre and unlike cotton, have little need for pesticides and fertilizers. It is considered as the "Fiber of the Future".

Jute is also used as herbal medicine to control or prevent dysentery, worm and constipation etc. (Oboh, Raddatz *et al.* 2009). Jute leaves are used as health-food in Japan. Jute leaves contain anti-tumor agents as phytol and monogalactosyl-diacylglycerol. It may reduce the risk of cancer (Islam, 2013).

Figure 1:3 Jute and jute fiber

Paper industries at present employ chemical delignification, a process that requires extensive chemicals and energy which leads to damage of polysaccharide components of wood and is also associated with the release of toxic pollutants into the environment (Dwivedi et al., 1994; Rastogi and Dwivedi, 2006, 2008; Whetten and Sederoff, 1991). To circumvent these problems, lignin genetic engineering provides a much more economical approach to chemical delignification. By reducing lignin content and altering lignin composition in woody plants,

the quality and efficiency of pulping was found to be improved with an increase in wood extractability and a reduction in mill effluents (Grabber et al., 2008) Genetic manipulation is thus being used successfully in improving alkali extractability and pulping properties of wood, for example, reduced Klason lignin in saponified residue, alkali labile conjugated phenolics ratio, augmented cellulose content, enhanced bleachability and increased levels of paper brightness (Halpin et al., 1994; O'Connell et al., 1998; Pilateet al., 2002; Wei et al., 2008).

Synthetic fibers used nowadays are non-renewable sources of fiber and their production lead to environmental pollution. Consequently, there is renewed interest in the use of plant fibers, for example, cotton, jute and flax, in the textile industry (Jhala et al., 2009; Stephens and Halpin, 2007). Flax fibers are lignocellulosic in nature that contain ~70% cellulose, with hemicellulose, pectin and lignin and are routinely used in textile industry. The presence of lignin gives mechanical strength to the fiber and plant on one hand, but its presence confers poor elastic properties to fibers as compared to non-lignocellulosic fibers, for example, cotton fibers (Kwiatkowska et al., 2007). Thus, by producing low-lignin flax, fibers with improved elastic properties may be obtained. This has been successfully achieved by CAD down-regulation using RNAi technology (Kwiatkowska et al., 2007). Besides their utility in textile industry, bio-fibers also find applications in biomedical science and automotive industries. Bio-fibers have the potential to replace petroleum based synthetic polymers owing to their low production costs, biodegradable nature, physical properties and environmental friendliness. It has been suggested that these transgenic flax plants producing both components of the flax / β-hydroxy butyrate composites (i.e. fibers and thermo plastic matrix in the same plant organ) could serve as a source of an attractive and environmentally safe material for industry and medicine.

Figure 1:4 Uses of jute fiber

The intimate association of both core (i.e., highly condensed polymeric matrices) and non-core lignins (i.e., low molecular weight phenolic monomers) with cell wall polysaccharides, such as cellulose and hemicellulose, through covalent bonding physically separates digestive hydrolytic enzymes from cell wall polysaccharides, consequently limiting their digestibility by ruminants and decreasing energy yields (Dixon, 2004; Jackson et al., 2008; Jung et al., 2012a; Rastogi and Dwivedi, 2008; Shadle et al., 2007; Vailhe et al., 2000.

In a different note, depleting resources of fossil fuel supply and their hiking prices, generate a need to supplement them with renewable energy sources, for example, bio-based fuels. The stored solar energy in plant biomass can be released by burning, thermo-chemical conversion to syngas, or biological conversion using microorganisms and enzymes to generate biofuels including alcohols and biogas (Johnson et al., 2007; Lin and Tanaka, 2006; Vertès et al., 2008; Vogel and Jung, 2001; Wenget al., 2008; Yuan et al., 2008). An emerging effort has been expended to increase the use of plant lignocellulosic biomass for the production of bioethanol (Carroll and Somerville, 2009; Grabber et al., 2010; Lee et al., 2011).

Lignocellulose is, however, recalcitrant in nature due to its molecular structure and heterogeneity, where lignin is found to resist enzymatic degradation to convert plant cell wall polysaccharides into fermentable sugars for biofuel production (M. Li et al., 2012;Vanholme et al., 2010c; Vega-Sanchez and Ronald, 2010; Weng et al.,2008). Consequently, the conversion of biomass to biofuel requires costly and harsh pretreatments to degrade lignin and allow access to polysaccharides for saccharification (Grabber et al., 2010; Simmons et al., 2010). The plants can thus be rendered more amenable to bioprocessing by genetic modification of lignocellulosic matter. The highly photosynthetic efficient C4 grasses, such as switchgrass, Miscanthus, sorghum and maize are expected to provide abundant and sustainable resources of lignocellulosic biomass for the production of biofuels (Bosch and Hazen, 2013; Carpita and McCann, 2008; Cesarinoet al., 2012; Weijde et al., 2013).

Lignin modification is also a promising path for improving the access of cellulases to degrade cellulose (Rodriguezet al., 2000; Sticklen, 2008). Additional research has revealed the coregulation of lignin and cellulose biosynthesis in several studies (Hu et al., 1999; Rastogi et al., 2006, 2008). Repressing a single lignin biosynthetic pathway gene, for example, 4-coumarate CoA-ligase (4CL), O-methyltransferase (OMT) has been shown to result in a reduction in lignin content with a concomitant increase in cellulose content (Hu et al., 1999; Rastogi and Dwivedi, 2006, 2008).

Jute (a naturally rich lingo-cellulosic fiber yielding crop) delineates a new promising sphere with respect to lignin manipulation. Like other bast fibers, jute has a fair amount of lignin, which makes it the strongest of them all (Del Rio, Rencoret *et al.* 2009; Stevens et al., 2010). In spite of the importance of lignin in plant growth and development, it is evident that reduction in lignin content would result in superior fiber quality for industrial purposes and consequently better profitability (Mandal and Srimani 1987). A method for lowering lignin content can be viewed as an attempt that would improve the jute fiber so that it may be used in paper industry, as a potential biofuel source and as a finer source of thread for garment factories. The most effective of such approaches is to down-regulate the enzymes involved in lignin biosynthesis, in other words silencing the expression of genes involved in the

synthesis of lignin (Ralph, Akiyama et al. 2006; Chapple, Ladisch et al. 2007; Chen and Dixon 2007).

As part of an effort to increase alternative utilization of jute, the golden crop of Bangladesh, reducing lignin content in jute using state-of-the-art RNAi techniques (artificial microRNA and hairpin RNA) for fine-tuned alteration of lignin biosynthetic pathway was done for four different genes namely, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamate 4-hydroxylase (C4H) successfully in jute (Shafrin et al. 2015).

These genes are found not to overlap with the defense mechanism of a plant (Ladisch et al. 2007; Li, Weng et al. 2008). The reason behind using RNAi technique is simple. RNA interference (RNAi) is based on homology sequence dependent degradation of cognate RNA that turns out the formation of double-stranded RNA (dsRNA) followed by transcript depletion of homologous target gene (Baulcombe 2004; Brodersen, Sakvarelidze-Achard et al. 2008). With the advent of functional genomics, RNAi vector mediated gene silencing has emerged as an efficient approach for genetic manipulation in plants because of its ease of application and the possibilities for genome-wide reverse genetics overcoming the drawbacks of insertional mutagenesis or knockout method (Travella, Klimm et al. 2006). hpRNAi (gene constructs encoding intron spliced RNA with a self complementary hairpin structure) have been found to show post-transcriptional gene silencing with almost 100% efficiency for endogenous genes and transgenes (Smith, Singh et al. 2000; Travella, Klimm et al. 2006; Travella and Keller 2009). Like hpRNA, the approach of artificial microRNA appends a new dimension in the gene silencing epoch.

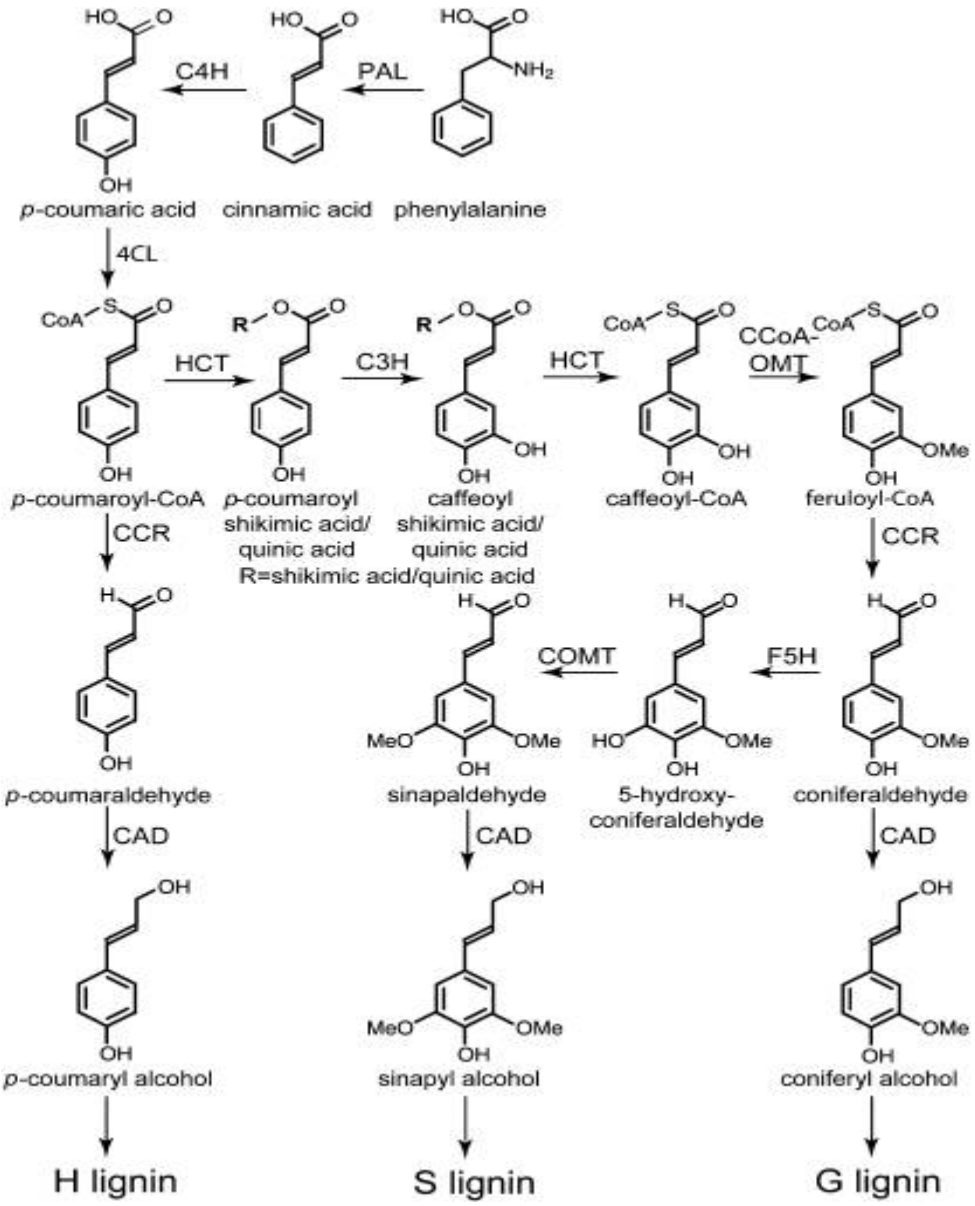

Figure 1:5 The main biosynthetic route toward the monolignols *p*-coumaryl, coniferyl, and sinapyl alcohol (Boerjan et al., 2003). (PAL-Phenylalanine ammonia-lyase; C4H-Cinnamate 4 hydroxylase; 4CL-4 Coumarate:CoA ligase; C3H-*p*-Coumarate 3-hydroxylase; HCT-*p*-Hydroxy cinnamoyl-CoA:quinate/shikimate *p*-Hydroxy cinnamoyl transferase; Ccoaomt, Caffeoyl-Coa *O*-Methyltransferase; Ccr, Cinnamoyl-Coa Reductase; F5h, Ferulate 5-Hydroxylase; Comt, Caffeic Acid *O*-Methyltransferase; Cad, Cinnamyl Alcohol Dehydrogenase).

Stable transgenesis is a critical issue while genetic manipulation is being carried out. These transgenic plants showed in each successive lines, stable incorporation of hp-RNA/amiRNA constructs. RNA interference (RNAi) is based on homology sequence dependent degradation of cognate RNA that turns out the formation of double-stranded RNA (dsRNA) followed by transcript depletion of homologous target gene (Sakvarelidze-Achard et al. 2008). Owing to certain attributes like exceptionality, efficacy and precision, gene silencing through hpRNAi and amiRNAs is considered a second generation RNAi technology, and has been conveniently applied in many studies (Travella and Keller 2009; Chen et al. 2008). This was examined by genomic PCR and Southern blot analyses which confirmed successful RNAi transgenesis in jute.

The goal in this study was to analyse if the down-regulation of lignin biosynthetic genes in all four transgenic lines (available generations- COMT T2-T7; C4H T2-T7; C3H T2-T5; F5H T2-T5) of jute plants had effectively reduced the lignin content. The study also aimed to see if the cellulose content had increased by decreasing recalcitrance to glucose release and if it led to an increase in the quality of the fiber and if it became more suitable for use in biofuel, paper, pulp, and other industries.

The key objectives of the present study were-

- Advancement of all four transgenic lines up to seventh generation for stable transgenesis and field trial.
- Determination of lignin reduction (due to lignin biosynthetic gene downregulation) in all transgenic jute plants compared to the wildtype controls.
- Determination of the increase in cellulose content (as a consequence of lignin down regulation) in transgenic plants compared to wildtype.
- Measurement of glucose release (quantitatively) after enzymatic digestion of the transgenic lines and comparison with the control plants.

- Microscopic analysis for lignin reduction as well as increase in cellulose content in the transgenic lines.
- Analysis for the tensile properties of all transgenic as well as wildtype jute fibers.
- Observation for growth compensation in the transgenic lines due to lignin reduction.

Altogether, current proposal sought to explore the beneficial effects of lignin reduction in jute with a goal to produce improved quality of jute fiber as well as better utilization of this cash crop which would help to serve the welfare of our economy and the environment.

Commercial production of any transgenic crop is the culmination of a four step process (Stewart 2002). These are-

- In the first step, scientists investigate potential beneficial traits, identify genes and execute genetic transformations in government or private research laboratories and in greenhouses.
- If these results are successful, the plant is then advanced to the second step that is open field trials in a real life environment.
- The third step is securing regulatory approval in the country where the plant is being grown, and its products consumed by humans or animals.
- The fourth and final step is market acceptance and large scale production.

It has been considered obligatory by biosafety regulations of individual countries to test the feasibility of transformed varieties in contained and controlled environments for any potential risks to both human health and environment (Prakash et al., 2011). Biological safety (i.e., biosafety) means the mechanism developed through policy and procedures to ensure the environmentally safe application of biotechnology which results in developing genetically modified organisms (Zerbini 2014). It is the protected status of human and animal health and the environment from the possible adverse effects of the products of modern biological techniques and consortium of precautionary approached for its assessment and assurance

(Kimman et al 2008). Several pieces of information would be necessary for successful risk assessment prior to release of the transgenic varieties (Smith et al 2010). These are-

- Molecular characteristics of the modified varieties with detailed information on genetic changes in the size and sequence
- Details of the technology used to effect the genetic changes
- Details of the genes and their properties that have been introduced and the possible effects of any other genetic change brought about in the organism
- Growth characteristics of the transformed varieties in comparison with host unmodified varieties.
- Nutrient, soil, climatic, and other requirements
- Gene flows from the transgenic varieties under normal ecological conditions and its impact on ecology in controlled field trials

In order to evaluate whether a transgenic plant is safe for environment, most varieties are reproduce and grow in the environment after successful transgenesis (Prakash et al 2011). The first transgenic plants were generated in the early 1980s, and they have been commercialized since 1994. In 1998, over 40 million acres of transgenic crops were grown around the globe (Sharma et al., 2002). Twenty eight countries have adopted the planting of transgenic crops, including twenty developing countries and eight industrialized countries (James, 2012).

This study dealt with low lignin containing transgenic jute plants where RNAi-based gene silencing strategy was used for the successful reduction of lignin without apparently compromising with the growth and defense mechanism of jute. Stable transgenesis is a critical issue while genetic manipulation is being carried out (Ferry et al. 2006). Our transgenic plants showed stable incorporation of hp-RNA/amiRNA constructs in each successive line (T0 to T7). The genetic manipulation of lignin biosynthesis pathway enzymes particularly the four genes of this study has been specifically reported to reduce the need for pretreatment processes for better industrial purposes. Independent down-regulation of genes

encoding C4H (cinnamate 4-hydroxylase), C3H (4-hydroxycinnamate 3- hydroxylase), F5H (ferulate 5-hydroxylase) and COMT (caffeate Omethyltransferase) have been shown to reduce the recalcitrance of alfalfa and thereby improve the release of fermentable sugars during enzymatic hydrolysis eliminating the prior chemical pretreatment in the production of fermentable sugars (Templer et al. 2006, Ladisch et al. 2007, Sticklen 2007, Chen et al. 2009).

By using RNAi (hp-RNA and amiRNA) strategy, our total project can be regarded as the first successful attempt on jute to reduce its lignin content without any negative effects on the morphological features of the plant. With the results obtain, we can expect to develop a jute variety with low lignin content in near future, which will certainly boost its commercial usability and have an impact on the economic acceleration of Bangladesh. So, after completion of our successful analysis, it is necessary to apply for approval from Bangladesh National Committee on Biosafety (NCB), the policy and decision making regulatory body for biosafety in Bangladesh, to make these new transformed jute varieties commercially available for its practical applications. After getting approval from Bangladesh National Committee on Biosafety (NCB), commercial release in field sector and application of these environment friendly high quality jute fiber in textile and paper industries and eco-friendly production of biofuel from its increased cellulosic biomass will lead to sustainability of these jute lines.

2 MATERIALS AND METHODS

2.1. Growth condition and Advancement of Plant Material

Down-regulation of four different lignin biosynthetic pathway genes namely, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamante 4-hydroxylase (C4H) were performed successfully in jute (*Corchorus olitorius* (var. 0-9897)), (Shafrin et al. 2015), (Shafrin et al. 2017) using state-of-the-art RNAi techniques (artificial microRNA and hairpin RNA). Each of the four transgenic lines were advanced to several generations after successful transformation.

To advance the transgenic lines, seeds from the first transformed generation (T_1) of all four transgenic lines were grown repeatedly. COMT and C4H transgenic lines were advanced to seventh generation (T_7) whereas C3H and F5H transgenic lines were advanced to fifth transformed generation (T_5). Jute seeds of both wildtype (var. 0-9897) and transgenic lines were germinated in the field under sterile conditions with nutrient rich soil in a greenhouse condition at 25°C to 35°C. The jute plants were harvested at the end of the vegetation period (September 2018) in the almost-mature stage of growth (16th week of growth). At this stage plants were fully developed, seed capsules were shaped but plants were still green. The stalks (the middle part of the stem, without seed capsules, flowers, or roots) were collected for expression study and other metabolite analysis.

The jute fiber were collected from the stem and ribbon (outer skin) of the jute plant. At first, the fibers were extracted by retting where jute stems were bundled together and immersed in water for approximately 15 days. After completion of the retting process, non-fibrous matter was scraped off. Then the fibers were stripped from the jute stalk (Ali et al, 2015). Thus the fibers of all four transgenic lines and wildtype jute plant were collected for quality analysis. Seeds were collected after three to four months of growth until the green color around the mature seed pod tanned and cracked. Collected seeds from first generation of all four transgenic lines were grown repeatedly to advance up to several generations under same greenhouse environment with nutrient rich soil. Thus up to seventh-generation of transgenic

jute lines of COMT and C4H, fifth-generation of transgenic jute lines of C3H and F5H were available for analysis using wild type jute plant as control (line O-9897).

2.2. Lignin Content

Lignin content of whole stem and fiber for both transgenic and wild type jute plants were measured to determine the amount of acid insoluble lignin. A modified Klason lignin estimation method was used to estimate the acid insoluble lignin according to the protocol described by (Tanmoy et al., 2015) with slight modifications. Plant stems and fiber were first collected and dried overnight at 105° C to get the dry weight (Ehrman 1994) and about 0.5 gm of sample was taken for the experiments, which was denoted as *W1*. The samples were hydrolyzed with 72% H_2SO_4 at room temperature, followed by 4% H_2SO_4 hydrolysis at boiling temperature. The hydrolyzed sample solutions were vacuum-filtered by glass crucibles with silica filter. After filtration, the crucibles with the residual content were heated at 105° C in an oven. Then, the weight was taken for the crucibles with the dried residual sample and denoted as *W2*. The residual sample in the crucibles was then heated at 575 ± 25° C in a muffle furnace. The crucibles were cooled in a desiccator, the weight was taken and designated as *W3*. With all these different weights, the percentage of acid-insoluble lignin was calculated using the formula given below (David Templeton *et al*, 1995):

$$\textit{Acid Insoluble Lignin}\ (\%) = \{(W3 - W2)/W1\} \times 100$$

2.3. Cellulose Content

The cellulose content was determined for both transgenic and wildtype plants using the colorimetric method with the reagent anthrone, as described by Updegraff et al. (1969). Samples from the whole stem were incubated with a mixture of acetic acid and nitric acid (8:1 v/v) for 1 h at 100 ºC and then centrifuged at 14000rpm for 5min. The pellet was washed with water and then re-suspended in 1ml of 72% H_2SO_4 (v/v). After mixing the samples, cold anthrone reagent was added and the amount of cellulose in these samples was measured

spectrophotometrically at 620 nm. After hydrolysis, commercially available cellulose was used for generating the calibration curve.

2.4. Enzymatic saccharification

At first, Neutral Detergent Fiber (NDF) was prepared for enzymatic saccharification (sugar release per unit of biomass) using an analytical technique describe by Soest et al., 1991. The fiber was then incubated with commercial cellulase (Sigma, *A. niger*) for 24 hours at 37 °C at 80 FPU/g NDF and reduced sugars was measured by dinitrosalicylic (DNS) acid (Miller *et al*, 1959). Here, 3ml of DNS was added to the sample solutions and the mixtures were heated for 5 minutes in a boiling water bath and then cooled in ice water. The color intensities were measured in a spectrophotometer at 540 nm.

2.5. Histochemistry and fluorescence microscopy

Histochemical analysis was performed using phloroglucinol, calcofluor in order to analyse the lignin and cellulose structure and content of all four transgenic and wildtype jute lines. Stem samples of all the jute lines were collected after 45 days of growth. Stems were cut 10 cm above the root and cross sectioned (25 μm) using a microtome (Thermo Scientific MICROM HM 430) and sections were stained separately with phloroglucinol and calcofluor and visualized under a light microscope (Nikon ECLIPSE Ci) as well as fluorescence microscope (EVOS® FL; Catalog Number 6500-FL). Histochemical analysis was performed both in the Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka and in the Bangladesh Jute Research Institute (BJRI).

2.5.1 Phloroglucinol staining

Histochemical analysis of lignin was done using phloroglucinol staining. Mounted sections of plant stems were stained with phloroglucinol (1% in 95% alcohol, 25% HCl) for 2mins

and observed under a light microscope (Nikon ECLIPSE Ci). Lignified walls appeared as a pinkish violet color.

2.5.2 Calcofluor staining

Further histochemical analysis was carried out using calcofluor to analyse the cellulose content of all four transgenic and control jute plants. Sample collection and cross-sectioning procedures were the same as described for the light microscopic analysis section. The samples were first mounted in 0.01% calcofluor white solution with a drop of 1N NaOH. After 5 minutes, samples were washed with distilled water to rinse out the calcofluor white solution and observed under a fluorescence microscope (EVOS® FL Imaging System).

2.6. Scanning Electron Microscope

Scanning electron microscope (SEM) was used to observe the morphological features of the transgenic and non-transgenic jute fibers. Scanning electron microscope produces images by scanning the sample with a high energy beam of electrons. As the electrons interact with the sample, they produce secondary electrons, backscattered electrons, and characteristic X-rays (Mohammed et al 2018). In order to form an image the receiving signals produced from the electron-sample interactions are detected with different types of detectors depending on the mode of SEM being used and displayed on the computer screen. The maximum resolution found in an SEM depends on multiple factors, like the electron spot size and interaction volume of the electron beam with the sample (Sampath et al 2013). Here, the sample specimens to be observed were single elementary fiber and observed using voltage of 10 kV.

2.7. Mechanical properties of jute fiber

For the analysis of tensile properties of all transgenic and control jute fibers, individual elementary fibers were separated manually from the fiber bundles. Jaw separation of 50 mm

and a maximum load cell capacity of 5000 N were used for assessment. The tests were carried out with a speed of 5 mm/min. Tensile testing was performed at room temperature. For each sample, 20 individual fibers were studied and the data represented as average.

2.8. Statistical analysis

Morphological studies were carried out using five randomly selected plants of each generation. For chemical analysis such as lignin, cellulose content and enzymatic glucose release, independently selected two biological replicates and technical triplicates were used for each generation of jute lines. For mechanical analysis, 20 samples were taken into account and average data were presented for all transgenic and control fibers. Analysed data were presented as mean±standard error.

To evaluate the statistical significance of obtained data, one way ANOVA and Tukey's test were carried out through R program. For experimental data analysis, '***' indicates P value <0.001, '**' indicates P value <0.01, '*' indicates P value <0.05; are considered as statistically significant.

3 RESULTS AND DISCUSSIONS

3.1. Results

Jute, one of the best natural bast fibers, is losing its diversification because of an abundance of lignin polymer that renders the plant material nearly inaccessible for downstream processes. In this regard a previous study had successfully carried out the downregulation of four lignin biosynthetic genes of jute (Shafrin et al., 2015; Shafrin et al., 2017). In a continuation, the current study was designed to assess if the lowered lignin content in the same transgenic plants had been effective in enhancing the quality of the fiber by increasing the cellulose content as well as decreasing the recalcitrance to glucose release. Such changes would allow jute to be suitable for biofuel production, paper and pulp industries as well as other industrial purposes. The analysis was performed to visualize the effect of individual down-regulation of four different lignin biosynthetic genes across all the advanced generations (2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 7^{th}) of transgenic plants in terms of lignin and cellulose content, enzymatic glucose release and fiber strength.

3.1.1 Morphological study

To determine phenotypic variations if any, the delignified transgenic generations were analysed all through the growth period. All transgenic and control plants were grown under the same environmental field conditions for a comparable appraisal. Besides, morphological evaluations were measured and compared to conclude if any changes in transgenic generations had resulted from lignin reduction. The analysis as reflected in Table 3-1 shows that plant height and width, pod length and number remained almost the same in each transgenic generation as in the control plants. This evidently indicates that there was no distinct variation in vegetative and reproductive growth characteristics due to lignin downregulation. For statistical analysis, plant samples were arbitrarily chosen ensuring two biological duplicates with three technical triplicates from individual generations.

3.1.2 Histochemical analysis

3.1.2.1 Phloroglucinol staining

The presence of lignin in stem cross-sections of control and transgenic generations were observed under a light microscope with 20X magnification using phloroglucinol-HCl. Phloroglucinol, which specifically binds with hydroxy-cinnamaldehyde end-groups of lignin develops a purple or reddish chromophore with the color intensity reflecting the amount of lignin present (Blaschek et al., 2020). Comparatively, the intensities of the purple coloration detected in fiber and xylem regions of the transgenic stems were lower as displayed in Figure 3-1.

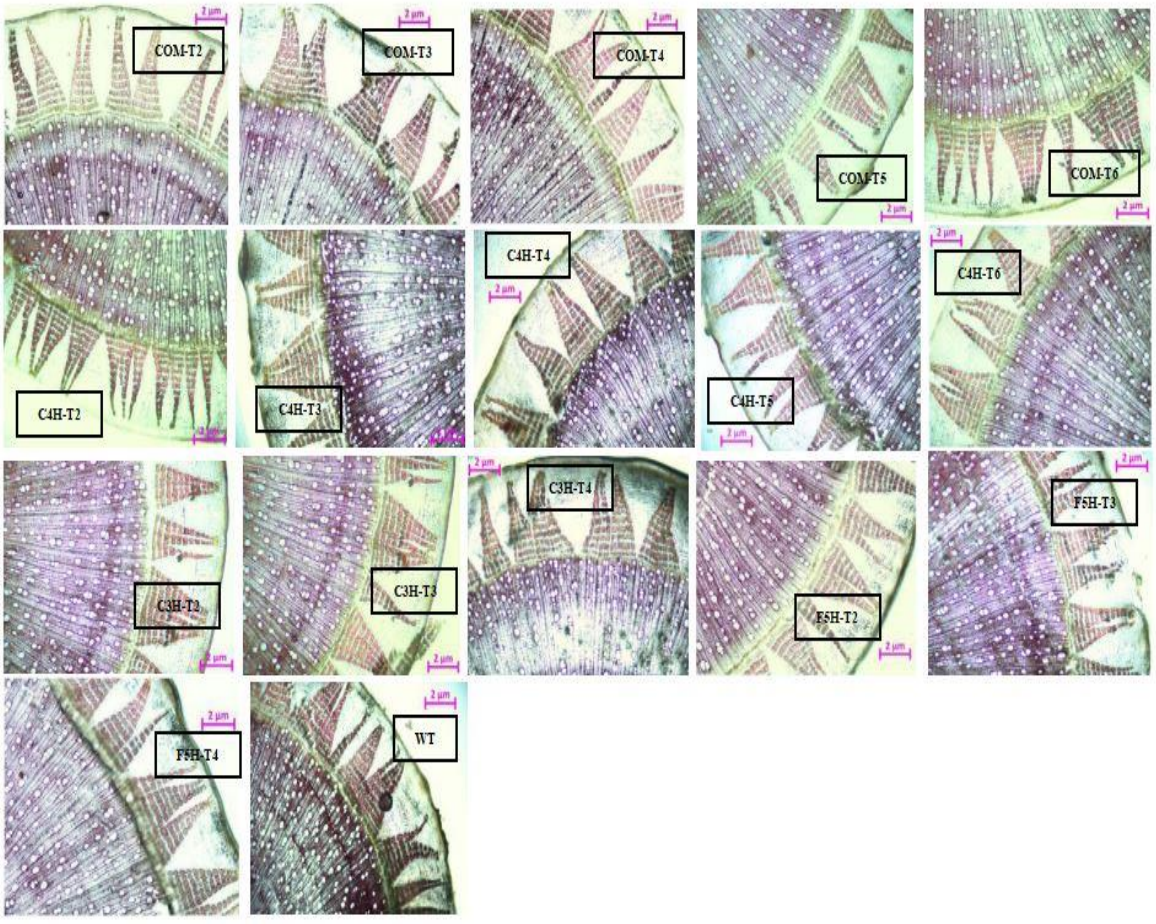

Figure 3-1 Phloroglucinol-HCl staining of all available generations of transgenic lines and wild type jute stem cross-sections.

Parameters		Plant height (cm)	Plant width (cm)	Pod number	Pod length (cm)
Control plant		350.72±1.66[a]	1.20±0.006[a]	60.67±1.20[a]	5.00±0.06[a]
COMT lines	**T2**	350.74±1.47[a]	1.21±0.01[a]	62.33±0.88[a]	4.97±0.12[a]
	T3	351.97±0.51[a]	1.20±0.003[a]	61.00±1.15[a]	4.93±0.12[a]
	T4	350.01±1.28[a]	1.19±0.008[a]	60.67±0.88[a]	5.03±0.09[a]
	T5	349.56±0.46[a]	1.20±0.005[a]	60.00±1.15[a]	5.00±0.06[a]
	T6	349.81±1.48[a]	1.18±0.003[a]	58.67±1.20[a]	4.97±0.12[a]
	T7	351.64±0.67[a]	1.20±0.005[a]	60.33±1.86[a]	5.07±0.15[a]
C4H lines	**T2**	351.63±0.63[a]	1.20±0.005[a]	60.33±1.20[a]	5.00±0.06[a]
	T3	350.90±0.82[a]	1.18±0.003[a]	60.00±1.15[a]	5.00±0.06[a]
	T4	351.40±0.84[a]	1.21±0.008[a]	59.00±1.15[a]	5.07±0.12[a]
	T5	350.76±1.24[a]	1.19±0.005[a]	60.67±1.86[a]	5.07±0.09[a]
	T6	349.99±0.99[a]	1.20±0.005[a]	60.67±1.76[a]	5.00±0.12[a]
	T7	351.10±0.65[a]	1.19±0.005[a]	60.33±1.76[a]	5.00±0.12[a]
C3H lines	**T2**	351.71±0.46[a]	1.19±0.005[a]	59.67±0.88[a]	5.07±0.12[a]
	T3	350.84±0.87[a]	1.20±0.005[a]	61.00±1.53[a]	4.90±0.12[a]
	T4	351.04±0.95[a]	1.18±0.008[a]	60.00±1.53[a]	4.87±0.09[a]
	T5	351.40±0.79[a]	1.20±0.005[a]	59.33±0.88[a]	5.07±0.09[a]
F5H lines	**T2**	350.28±0.31[a]	1.20±0.003[a]	59.67±1.76[a]	4.93±0.09[a]
	T3	350.34±0.56[a]	1.19±0.005[a]	59.00±1.73[a]	5.00±0.12[a]
	T4	349.66±0.87[a]	1.21±0.003[a]	60.33±0.88[a]	5.00±0.06[a]
	T5	350.62±0.76[a]	1.19±0.008[a]	60.67±0.88[a]	5.00±0.06[a]

Results are shown as means ± standard error. Statistical analyses were done by one-way ANOVA and Tukey's test, P value < 0.001 considered as highly significant where '***' indicates P <0.001, '**' indicates P <0.01, '*' indicates P <0.05. For every single parameter, averages that do not share a letter are significantly different. (cm = centimeter; Control = Wild type plant)

Table 3-1 Measurement of growth and yield parameters of control and transgenic lines of jute plants

This indicates significantly lower deposition of lignin in these locations as compared to control (WT). The epidermis, cortex and vascular cambium portions of all stem sections did no stain due to an absence of lignin. For experimental analysis, 90 day of old jute plants were used in this study.

3.1.2.2 Calcofluor staining

To look for variations in the cellulose content in transgenic and control lines, a further histochemical analysis was performed with 45-day old stem cross-sections using calcofluor white. Stem cross-sections were analysed at 10X magnification under a fluorescence microscope. Calcofluor is a specific stain for cellulose which binds with cellulose and is visualized under UV light. This yields cyan-green color under fluorescence light. The epidermis, cortex, fiber cell and the xylem contain cellulose as the major polysaccharide polymer in their cell walls. Here, more intense bluish green color was observed in all transgenic lines of jute stem compared to the wild type plant (Figure 3-2). This was a clear indication of the presence of more cellulose in all the transgenic lines. This increase in cellulose content is expected to happen in order to compensate for the reduced lignin content in all transgenic lines in comparison to the wild type plants (Wen-Jing Hu et al. 1999).

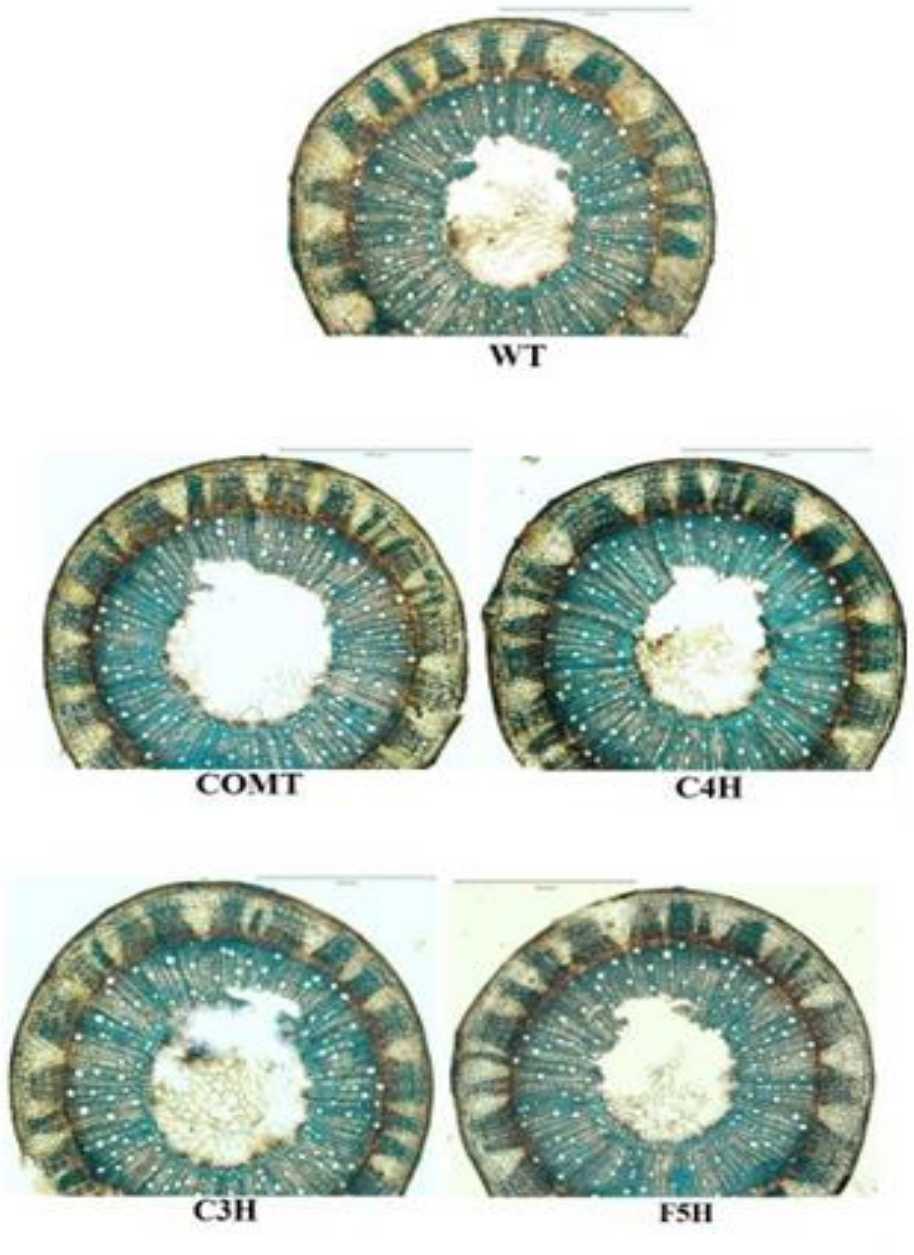

Figure 3-2 Calcofluor staining of wild-type (WT) and sixth generation COMT-T6, C4H-T6 and fourth generation of C3H-T4,F5H-T4 of all four transgenic lines of jute. Increase in color intensities indicate increased cellulose in the stem cross-sections of all transgenic lines compared to the wild type plant. (Scale bars: 1000 μm.)

3.1.3 Lignin content of whole stem and fiber

To check if the lignin reduction is stable across all generations of the four transgenic lines, an optimized lignin estimation method for jute (Tanmoy et al. 2015) was used. Lignin reduction was analysed across all transformed generations of the four transgenic lines as an effect of the downregulation of lignin genes. To measure lignin reduction, up to T_7 of two lines (COMT and C4H) and T_5 of the other two lines (F5H and C3H) were taken into consideration and compared to the control. A significant decrease in the lignin content was obtained for all transgenic types in contrast to the control. As Table 3-2 illustrates, the wildtype jute plants have an average of 30.56% lignin on the whole stem where 23.15% - 24.63% decrease in the lignin content was observed for the whole stem of all generations of four transgenic lines. In an analysis for the fiber lignin content, the amount of lignin was found to be about 14.09% for wildtype plant and approximately 11.43% - 12.73% reduction in fiber lignin were obtained for COMT, C4H, F5H and C3H lines when compared with control data as shown in Table 3-2. Graphical representations also show that all four transgenic lines have a significant reduction in the amount of lignin for whole stem (Figure 3-3) and fiber (Figure 3-4) in comparison to the WT jute lines.

Parameters		% Klason lignin (whole stem)	% Lignin reduction (whole	% Klason lignin (fiber)	% Lignin reduction (fiber)	% Cellulose (whole stem)	% Increase in cellulose content	Amount of glucose released (unit/hour of
Control plant		30.56±0.51 ^{a}		14.09±0.14^{a}		31.28±0.31^{b}		
COMT lines	**T2**	23.74±0.54^{b}***	19.40	11.87±0.16^{def}***	15.76	32.88±0.47^{a}*	5.11	2.41±0.14^{ab}
	T3	24.63±0.34^{b}***	21.93	12.36±0.26^{bcd}***	12.28	32.84±0.36^{a}*	5.01	2.77±0.18^{ab}
	T4	23.86±0.67^{b}***	22.80	11.65±0.15^{ef}***	17.32	33.03±0.22^{a}**	5.59	2.96±0.14^{a}
	T5	23.59±0.44^{b}***	21.15	12.07±0.11^{cde}***	14.34	32.56±0.36^{a}	4.09	2.40±0.46^{ab}
	T6	24.09±0.60^{b}***	21.09	11.66±0.21^{ef}***	17.25	33.32±0.11^{a}***	6.52	2.32±0.09^{ab}
	T7	24.11±0.55^{b}***	22.31	11.82±0.22^{def}***	16.11	33.29±0.18^{a}**	6.44	2.75±0.12^{ab}
C4H lines	**T2**	23.59±0.44^{b}***	22.80	11.43±0.10^{f}***	18.88	32.74±0.32^{a}	4.69	2.37±0.19^{ab}
	T3	24.29±0.35^{b}***	20.52	12.25±0.24^{bcde}***	13.06	32.83±0.36^{a}*	4.98	2.42±0.29^{ab}
	T4	24.18±0.40^{b}***	20.86	11.70±0.05^{ef}***	16.96	32.81±0.28^{a}*	4.91	2.78±0.19^{ab}
	T5	23.85±0.69^{b}***	21.96	11.99±0.22^{def}***	14.90	32.56±0.27^{a}	4.09	2.80±0.24^{ab}
	T6	23.26±0.43^{b}***	23.87	11.81±0.13^{def}***	16.18	33.18±0.28^{a}**	6.09	2.28±0.19^{ab}
	T7	23.70±0.21^{b}***	22.44	11.73±0.34^{ef}***	16.75	33.22±0.18^{a}**	6.18	2.73±0.12^{ab}
C3H lines	**T2**	23.97±0.62^{b}***	21.56	12.19±0.08^{bcde}***	13.48	32.69±0.43$^{a.}$	4.51	2.39±0.47^{ab}
	T3	23.77±0.32^{b}***	22.22	11.73±0.06^{ef}***	16.75	32.94±0.29^{a}*	5.30	2.18±0.33^{ab}
	T4	23.91±0.50^{b}***	21.74	12.03±0.23^{cde}***	14.62	33.38±0.06^{a}***	6.72	1.95±0.30^{b}
	T5	24.10±0.32^{b}***	21.13	12.10±0.14^{cde}***	14.12	33.27±0.08^{a}**	6.37	2.53±0.14^{ab}
F5H lines	**T2**	23.15±0.08^{b}***	24.25	12.14±0.25^{cde}***	13.84	31.53±0.30^{b}	0.81	2.58±0.16^{ab}
	T3	24.17±0.19^{b}***	20.91	12.73±0.06^{b}***	9.65	32.74±0.31^{a}	4.68	2.33±0.28^{ab}
	T4	23.70±0.18^{b}***	22.43	12.59±0.04^{bc}***	10.65	33.38±0.01^{a}***	6.72	2.12±0.25^{ab}
	T5	23.44±0.50^{b}***	23.29	12.07±0.15^{cde}***	14.34	33.45±0.08^{a}***	6.93	2.60±0.14^{ab}

Results are given as means ± standard error. Statistical analyses were done by one-way ANOVA and Tukey's test, P value < 0.001 considered as highly significant where '***' indicates P <0.001, '**' indicates P <0.01, '*' indicates P <0.05. For every single parameter, averages that do not share a letter are significantly different. (Control = Wild type plant)

Table 3-2 Summary of lignin content, cellulose content and enzymatic glucose release of control and transgenic jute plants

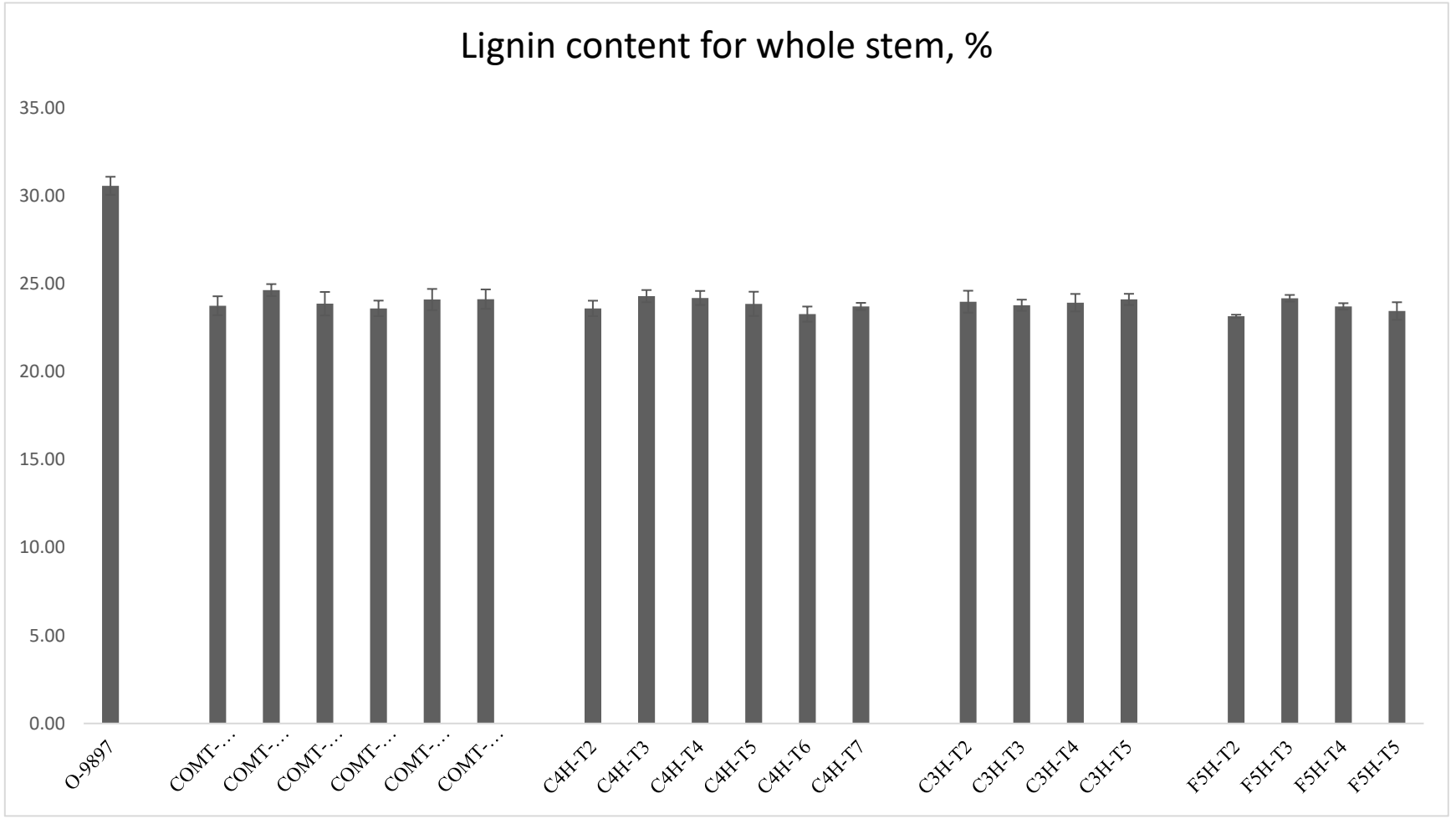

Figure 3-3 Graphical illustration of Klason lignin estimation values of whole stem for WT and transgenic lines of the four targeted genes.

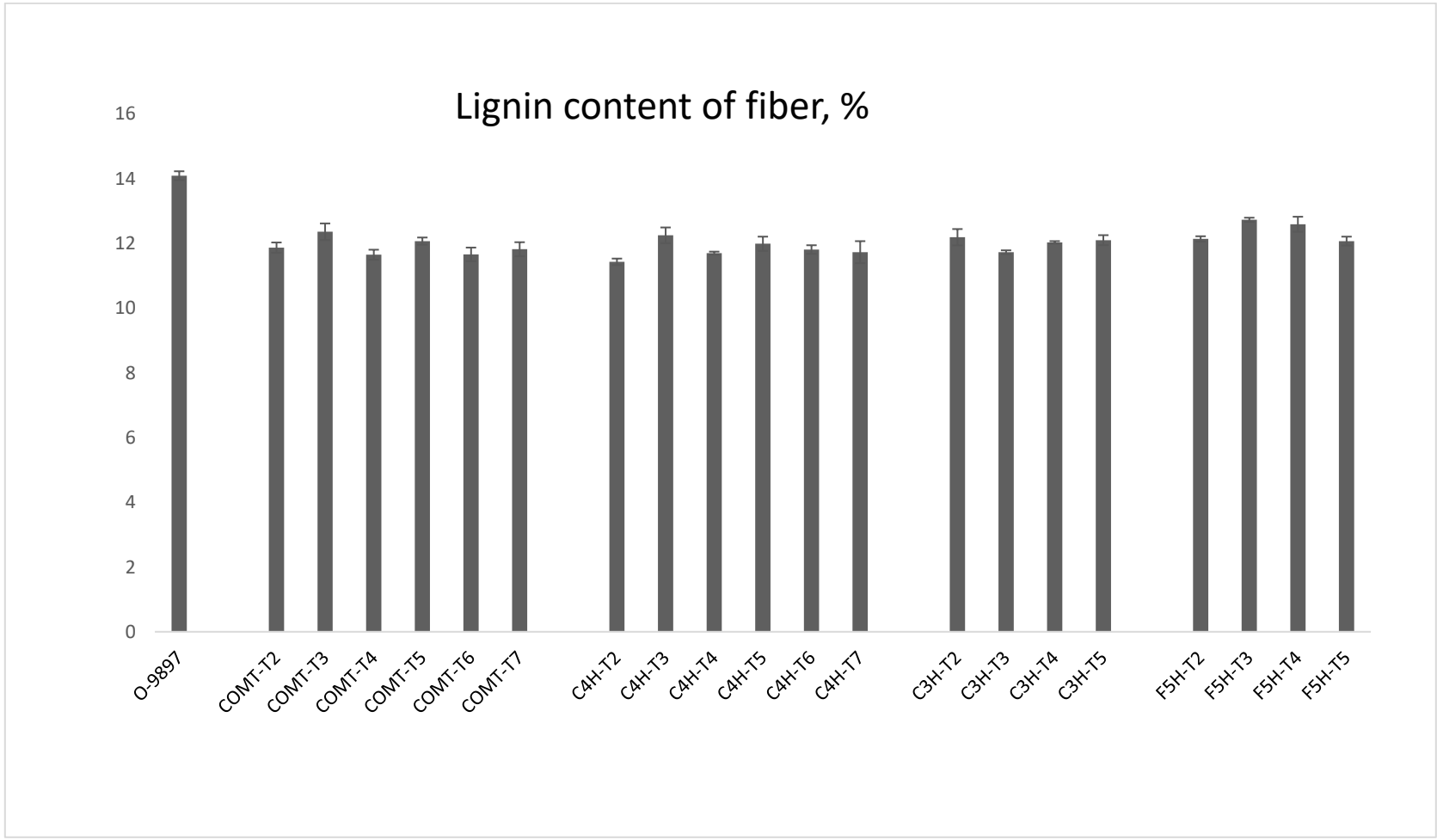

Figure 3-4 Graphical illustration of Klason lignin estimation value of fiber of WT and transgenic lines of four genes.

All samples used here to analyse the lignin content were randomly selected. Three biological replicates with technical triplicates of T2 – T7 (COMT T2-T7 and C4H T2-T7) and T2 – T5 (C3H T2-T5 and F5H T2-T5) were used in all experiments. Comparative analysis points that F5H lines resulted in more reduction in lignin content for the whole stem whereas for C4H lines there was more reduction in the fiber lignin content than others. Klason lignin value of all transgenic generations of jute was statistically analysed using ANOVA which showed significant reduction compared to the control.

3.1.4 Effect on cellulose content

As shown earlier lignin reduction has been found to be compensated by an increase in the cellulose content. Due to compensatory measures, the total lignin–cellulose mass remains unchanged which may contribute to metabolic flexibility and a growth advantage to sustain the long-term structural integrity of plants (Wen-Jing Hu et al. 1999). In this study, the total amount of cellulose has been found to increase in all the four transgenic lines compared to control jute plants. The amount of cellulose was about 31.3% in wildtype plant and in COMT, C4H, F5H and C3H transgenic lines, cellulose content on an average was augmented by 5.28%, 4.95%, 4.06% and 5.50 % respectively as represented in Table 3-2. This increase in cellulose content was in the range of 4.09-6.93% and it was constant for all generations of transgenic lines (Table 3-2). Furthermore, C3H gene downregulated transgenic plants showed better cellulose accumulation because of more lignin reduction compared to other transgenic lines. All the three biological replicates with technical triplicates of the samples that were used here were randomly selected. Graphical illustrations also show that all four transgenic lines have a significant increase in the amount of cellulose content for the whole stem (Figure 3-5) in comparison to the WT jute lines.

3.1.5 Enzymatic glucose release

Usually, a decrease in recalcitrance by lignin reduction is denoted as an increase in enzymatic saccharification rate. The rise in sugar release due to increased saccharification rate was found in delignified sugarcane under a demarcated pre-treatment condition (Bewg et al., 2016). Likewise, in our case, a slight improvement was observed in the amount of

glucose release for all transgenic lines than the control jute plants as illustrated in Table 2. Graphical illustrations show that all four transgenic lines have little increase in the amount of released glucose (Figure 3-6) in comparison to the WT jute lines.

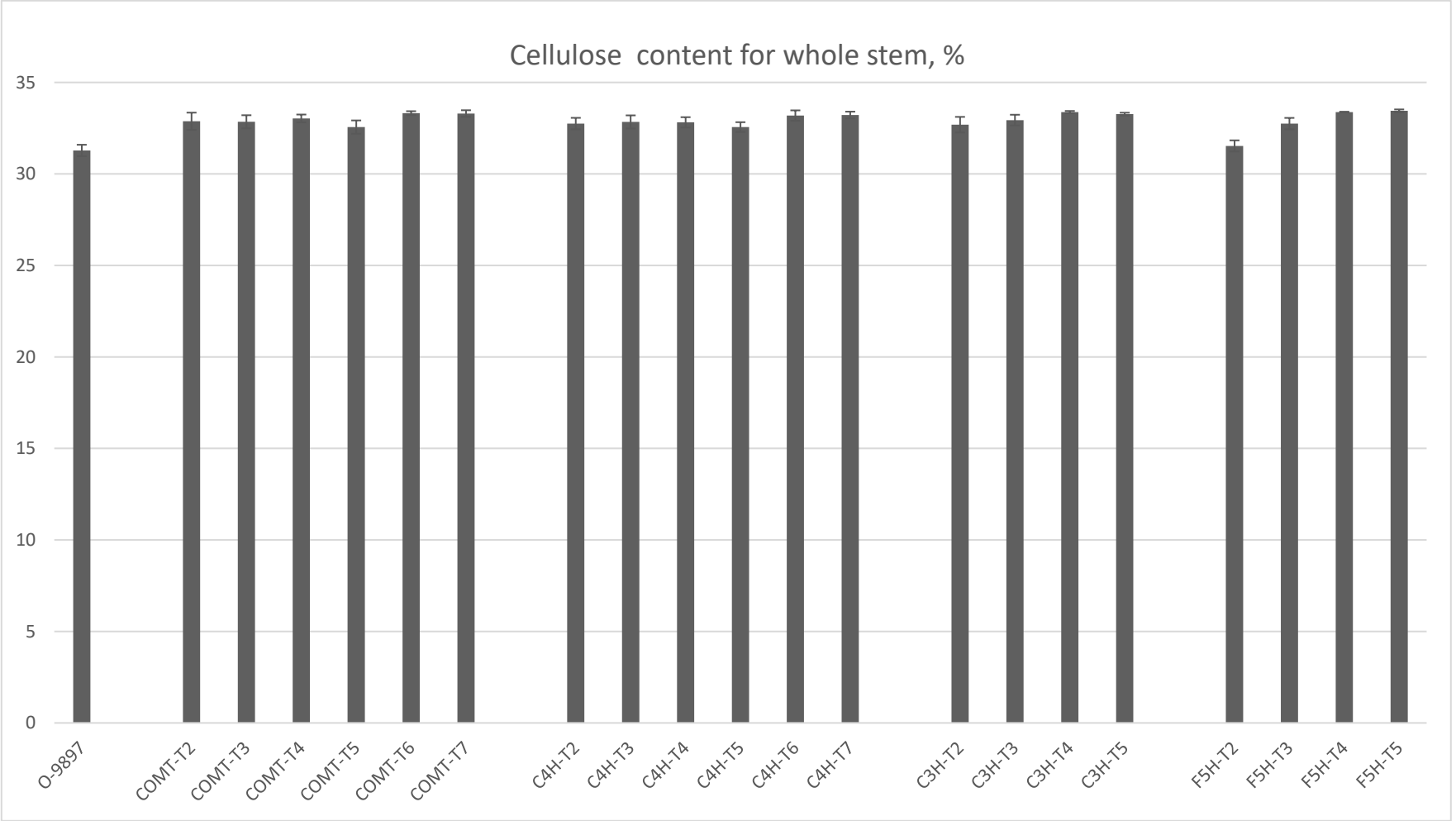

Figure 3-5 Graphical representation cellulose content estimation value of whole stem for WT and transgenic lines of all four targeted genes.

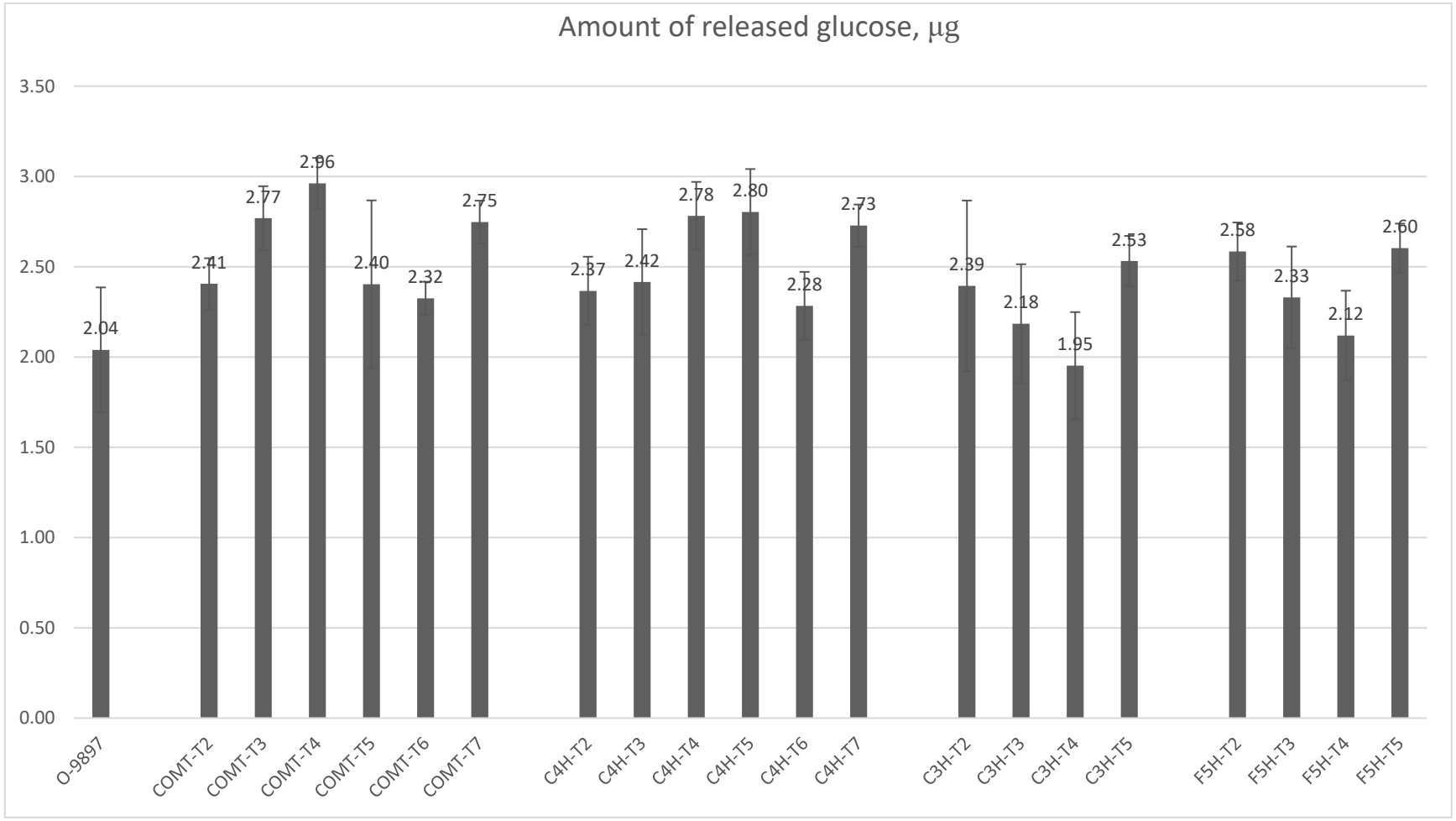

Figure 3-6 Graphical representation of the amount of glucose released for WT and transgenic lines of four genes.

3.1.6 Scanning Electron Microscope analysis of jute fiber

Scanning electron microscopy (SEM) was used for an understanding of the influence of lignin reduction on the stem surface of the transgenic lines. The surface morphological features of the wild type and transgenic fibers (separated single fiber) according to their variation in the quality of fiber was seen under a scanning electron microscope. Figure 3-7 shows the differences between the regions on the control and the transgenic specimens in red circles. It is observed that the presence of lignin on the control is much higher than all the transgenic samples. More specifically, if we consider two regions on the control specimen and the transgenic samples (two red circles), it can be observed that in the control sample, the single fibers are well cemented by the lignin and adhere to each other compactly. No small fibrils are observed in control specimen as they are hidden underneath the lignin layer. On the other hand, on COMT transgenic line within the red circle, the fiber surface appear rough due to the removal of lignin and also the small fibrils are exposed due to the lack of lignin whereas in control samples these fibrils are hidden underneath the lignin layer. Again, from figures of C4H, F5H, C3H transgenic lines, similar features are observed as observed for COMT transgenic line, where a reduction of lignin exposes the hidden fibrils.

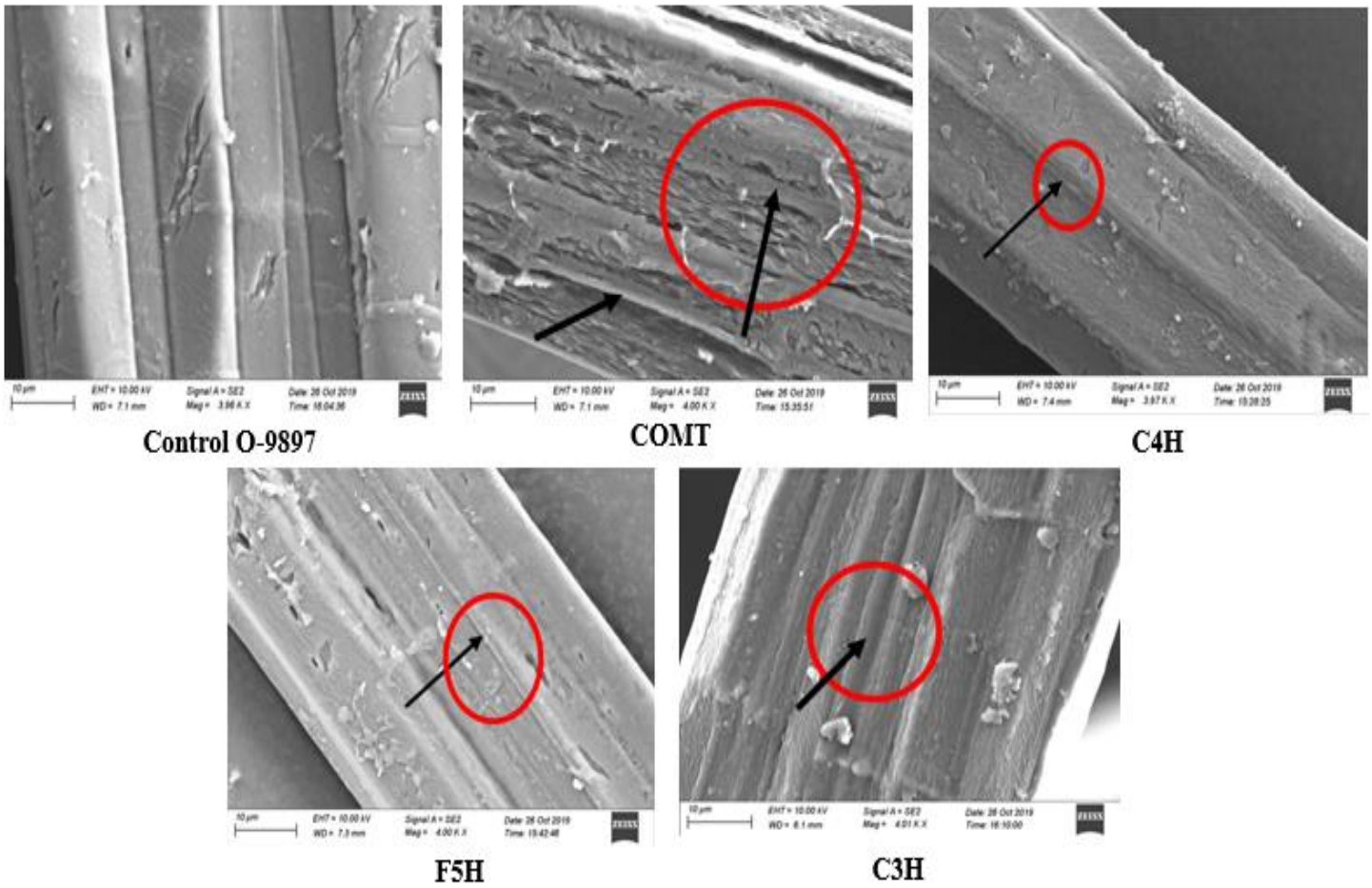

Figure 3-7 Scanning Electron Microscopy (SEM) images of the surface of WT and available most advanced generations of transgenic line of jute plants.

The surface appears much rougher than the control due to the reduction of lignin. So, from these figures, it is confirmed that the presence of lignin on the control jute plants is much higher than all transgenic samples.

3.1.7 Mechanical properties of jute fibers

Different mechanical properties of delignified transgenic and control jute fibers are outlined in Table 3. It is noticed that control jute fiber has an average tensile strength of 493.80 MPa and breaking elongation of 3.25%.

Parameters	Fiber tensile strength (MPa)	Breaking elongation (%)
Control plant	493.80±32.51[a]	3.25±0.13[a]
COMT-T_6 lines	512.50±35.54[a]	3.56±0.11[a]
C4H-T_6 lines	548.77±32.04[a]	3.49±0.17[a]
C3H-T_4 lines	576.13±30.89[a]	3.42±0.09[a]
F5H-T_4 lines	570.97±27.22[a]	3.54±0.12[a]

Table 3-3 Measurement of mechanical properties of control (wild type) and transgenic lines of jute fiber

Comparative analyses indicate that all transgenic jute fibers exhibit a slightly increased tensile strength ranging between 512.50 to 576.13 MPa. The average breaking elongation of individual fibers were found to increase modestly. It ranged between 3.42% and 3.56%. The reason could be the slipping of cellulosic micro-fibrils due to the reduction of lignin content in all transgenic lines (Zhang et al., 2013). Overall, the average tensile strength and breaking elongation of transgenic fibers showed minute increase with the associated lignin reduction. It is worth noting that the tensile strength and breaking elongation of jute fiber exhibited a moderately high standard error. The possible reason could be attributed to the difficulty of extracting uniform fiber samples as the jute fibers are very thin in nature (Sengupta and Palit, 2004).

The pictorial representation as displayed in Figure 3.8 affirms that transgenic jute fibers seem to be softer and enriched appearance than the control fiber which signifies the improved quality of four different varieties of jute fiber and asserts its profitable asset in the commercial market.

Figure 3-8 Image of fiber from WT and available last generations of transgenic line of jute plant.

3.2. Discussion

Jute, the second most common natural bast fiber in the world, is gaining importance because of an increasing demand of bio-polymers. Several advantages including low density, low cost, good specific mechanical properties and biodegradability made jute fiber the focus of intense interest in recent years (Reddy et al. 2015). Like other bast fibers, jute has a fair amount of lignin, which makes it the strongest of them all (Rio et al. 2009). Although, lignin is the major structural component of plant cell walls, it has negative impact on forage digestibility, tree pulping properties, and cellulosic biofuel production. Presence of a high amount of lignin diminishes fiber quality and sometimes restraints the smoothness of fiber and thus, lowers the bio-availability of the raw material. This ultimately lowers the accessibility of fiber (Richard 1996).

Now-a-days, there is revived interest in the use of plant fibers such as cotton, jute and flax, in textile industry because synthetic fibers are non-renewable and their production lead to environmental pollution (Jhala et al., 2009; Stephens and Halpin et al., 2007). Lignin may give mechanical

strength to the fiber but its presence confers poor elastic properties as compared to non-lignocellulosic fibers. Thus, by producing low-lignin fibers with improved elastic properties may be the best choice for the textile industries. This has been successfully achieved by down regulation of lignin biosynthetic gene using RNAi technology (Kwiatkowska et al., 2007).

Biofibers have the properties of bio-degradable nature, low production costs, physical and environmental friendliness. These have the potential to replace petroleum based synthetic polymers. For such applications, transgenic flax with improved mechanical properties owing to an increase in cellulose content, decrease in lignin and pectin contents suggest that these transgenic flax plants could serve as a source of an attractive and environmentally safe material for industry and medicine (Wróbel-Kwiatkowska et al., 2009).

Lignocellulosic biomass is made up of the complex structures of cellulose, hemicellulose and lignin. Such feedstock is highly recalcitrant to bioconversion of its carbohydrates into ethanol compared with starch (Somerville et al 2010). Current biomass fermentation processes for fuels and chemicals have a relatively high cost primarily because of this recalcitrance, which in turn has limited the commercialization of biomass, ethanol (Aden et al. 2009). The conversion of lignocellulosic biomass to ethanol is a three-step process that involves pretreatment followed by polysaccharide hydrolysis to simple sugars followed by sugar fermentation to ethanol (Mielenz JR 2001). Down-regulation of caffeic acid 3-*O*-methyltransferase (*COMT*) gene in switchgrass plants results in reduced lignin content, improved forage quality and most importantly increases the ethanol yield by up to 38% using conventional biomass fermentation processes. These delignified transgenic plants require less severe pretreatment and much lower cellulase dosages to obtain ethanol yields equivalent to yields in control plant (Chunxiang et al 2011).

Chemical delignification for high quality paper requires extensive chemicals and energy which leads to damage of polysaccharide components of wood and is also associated with the release of toxic pollutants into the environment (Dwivedi et al., 2006, 2008; Whetten and Sederoff, 1991). To resolve these problems, more economical and environment friendly approaches to downregulate lignin biosynthetic pathway are required. Delignified plants have been found to enhance the quality and efficiency of pulping with an increase in wood extractability and a reduction in mill effluents (Grabber et al., 2008). It is reported that, reduction of lignin content and alteration of

lignin composition by down regulation of OMT activity in woody tree *Leucaena leucocephala* causes better pulping than that of control plants (Rastogi et al., 2006). Long term field trials of delignified transgenic poplar plant also showed constant improvement in forage digestibility and pulping properties (Hongzhi Wang et al., 2012).

The intimate association of both core (i.e., highly condensed polymeric matrices) and non-core lignins (i.e., low molecular weight phenolic monomers) with cell wall polysaccharides, such as cellulose and hemicellulose, through covalent bonding physically separates digestive hydrolytic enzymes from cell wall polysaccharides, consequently limiting their digestibility by ruminants and decreasing energy yields (Jackson et al., 2008; Jung et al., 2012). Lignin modification can be a promising path for improving the access of cellulases to degrade cellulose (Rodriguezet al., 2000; Sticklen, 2008). Repressing a single lignin biosynthetic pathway gene, for example, 4CL or OMT has been shown to result in a reduction in lignin content with a concomitant increase in the amount of cellulose which led to improvement in saccharification efficiency (Hu et al., 1999; Rastogi and Dwivedi, 2006, 2008).

Due to depleting resources of fossil fuel supply and their hiking prices, there is a need to supplement them with renewable energy sources such as bio-based fuels. The stored solar energy in plant biomass can be released by burning, biological conversion using microorganisms and enzymes to generate biofuels including alcohols and biogas (Johnson et al., 2007; Yuan et al., 2008). Lignocellulosic biomass is an abundant, domestic, renewable feedstock source that can be converted to liquid transportation fuels and other chemicals by fermentation. Cellulosic ethanol is a promising technological option to reduce transportation sector greenhouse gas emissions (Lynd et al. 1996). To achieve sustainable energy production, it is necessary to overcome the chemical and structural properties of biomass that inhibit its deconstruction in dedicated bioenergy crops (Himmel et al. 2007). The presence of lignin in cell walls negatively impacts the conversion steps to fuel production (Keating et al 2006; Li X et al.2008). It is already reported in alfalfa, switchgrass, canary grass, and sorghum that decreased lignin levels improve *in vitro* enzyme hydrolysis while leading to an increase in fuel production (Dien et al.2006).

The conversion of biomass to biofuel requires costly and harsh pretreatments to degrade lignin and further allow the access to polysaccharides for saccharification (Grabber et al., 2010). Lignin

modification could bypass the need for these acid pretreatments and the plants can thus be rendered more amenable to bioprocessing as well as facilitate bio-process consolidation. It has been observed in alfalfa that lignin reduction resulted in greater proportions of cellulose or hemicelluloses within cell walls which led to increased saccharification efficiency for low lignin xylem (Chen and Dixon, 2007).

Global gene expression analysis of rice revealed that the SHN (SHINE transcription factor) regulatory network coordinated down regulation of lignin biosynthesis and upregulation of cellulose and other cell wall biosynthesis pathway genes (Ambavaram et al., 2011). The results supported the development of non-food crops and crop wastes with increased cellulose and low lignin with good agronomic performance that could improve the economic viability of lignocellulosic crop utilization for biofuels.

So, from these above discussions, it is clear that, lignin is the most recalcitrant component of the plant cell wall (Angelidaki and Ahring 2000) which needs to be reduced. One of the most promising solutions to the lignin problem would be to decrease its biosynthesis in the plant itself, as this approach might be more efficient and cost effective than removing it at the bio-refinery (Poovaiah, Nageswara-Rao et al. 2014). Total lignin content can vary from 15% to 40% among all plants (Sarkanen et al. 1971). Lignin deposition in cell wall is a crucial step in the adaptation of plants where plants can tolerate up to 40% reduction in lignin without major adverse effects on normal plant growth and development in greenhouse conditions (Zhong et al. 2000).

With the advancement of science, the perceptive of the lignin biosynthetic pathway alternation is relentlessly and rapidly pursued. Lignin engineering can improve the fiber quality (Vanholme, Morreel et al. 2012) of jute and improved fiber is expected to have an impact on the enhancement of its profitable use. The permutation of classical biochemical approaches; allied with the use of transgenic plants to investigate the pathway in vivo has greatly contributed in this research area. RNAi technology, a means of reducing lignin-related problems which embraces a promising horizon for the genetic improvement of lignified crop like jute is an imperative strategy to accelerate commercial usability and monetary enhancement.

As part of an effort to increase utilization of jute, state-of-the-art RNAi technique (artificial microRNA and hairpin RNA) was previously used for fine-tuned alteration of lignin biosynthetic

pathway by down-regulating four different genes namely, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamante 4-hydroxylase (C4H) successfully (Shafrin et al. 2015; Shafrin et al. 2017) in our laboratory. This led us to the production of four new varieties of jute (COMT, C4H, C3H, F5H) lines. Out of four, COMT and C4H varieties reached up to the 7^{th} generation and C3H, F5H varieties reached 5^{th} generation. Here, in this study, our goal was to analyse if the down-regulation of lignin biosynthetic genes in all four transgenic lines (available generations- COMT T2-T7; C4H T2-T7; C3H T2-T5; F5H T2-T5) of jute plants was effectively maintained across all the advanced lines. The study was also focused to determine if the cellulose content had increased together with a concomitant, decrease in the recalcitrance to glucose release and finally if the quality of fiber had improved which would make the jute plants suitable for use in biofuel, paper, pulp, and other industries.

At first an optimized chemical method (Tanmoy, Alam et al. 2015) was used for estimating the Klason lignin content in all four transgenic and wildtype jute lines. Down-regulation of the four lignin biosynthetic genes, COMT and C4H genes by hp-RNAi technique; and C3H and F5H genes by amiRNA technique in jute resulted in lignin reduction with no change in plant growth physiology. Reduction in lignin content was persistent across the different generations of each line (T_2-T_7 in COMT and C4H and T_2-T_5 in C3H and F5H) which proved the stability of lignin gene manipulation among all transgenic generations. Previous studies have reported that, down-regulation of COMT gene expression reduced the lignin content in alfalfa, maize, and poplar (Chabbert et al. 1994; Jouanin et al. 2000; Guo et al 2001). In another study, down-regulation of C4H, C3H, or F5H produced alfalfa plants with greatly reduced lignin without significant impact on its composition (Reddy et al.2005). A 25–35% reduction in Klason lignin content was observed in F5H-overexpressing Arabidopsis (Humphreys and Chapple 2002), and tobacco (Franke, Humphreys *et al.* 2002). All reports mentioned above consistently support our analysis. We observed that the percent reduction of lignin in all four jute transgenic lines (COMT, C4H, F5H, C3H) did not exceed 25% for whole stem and 15% for fiber (Table 3-2). Lignin plays a crucial role in the mechanical support and plant defense, thus lowering lignin always imposes a risk on plant physiology. As mentioned earlier more than 40% of lignin reduction is detrimental for plant physiology and the plants become susceptible to diseases (Franke, McMichael *et al.* 2000; Reddy, Chen *et al.* 2005). But as in our study, the reduction of lignin was less than 25%. It was expected that such a reduction would not have any impact on fiber strength.

Reduction of lignin was found to be compensated by an increase in cellulose content in transgenic aspen (*Populus tremuloides* Michx.) plants. Lignin and cellulose deposition regulate in a compensatory fashion and thus total lignin–cellulose mass remains unchanged (Wen-Jing Hu et al 1999). Monolignol biosynthetic enzyme down regulation reduced lignin content in cell wall and the reduced amount was compensated with higher levels of cellulose and arabinoxylan accumulation in maize (Silvia Fornale et al 2012). The data obtained from our study showed that 25% lignin reduction exhibited up to 6% increase in cellulose accumulation (3-2). As a result, the total lignin–cellulose mass changed very little. An increase in the amount of cellulose to lignin ratio has been reported for most severely lignin-reduced transgenic trees of aspen. This increase in cellulose content was found due to relative locations of the enzymes in the lignin biosynthetic pathway which influence the associated cellulose accumulations. Cellulose synthesis is normally substrate limited and reducing the flow at different stages of the lignin pathway increases the availability of carbon for cellulose deposition (Hu W J et al 1999).

As indicated earlier, lignin is the most recalcitrant component of the plant cell wall which hinders downstream processing of jute and jute fiber (Angelidaki and Ahring 2000). A decrease in recalcitrance by lignin reduction may be referred to as an increase in sugar release after enzymatic saccharification in delignified plants than that of the control samples under a defined pretreatment condition (Li et al 2008). *COMT* down regulation has led to increased saccharification in sorghum (Sattler et al 2012). C3H downregulation in alfalfa also produced greater amounts of sugar. Thus, elimination of pretreatment step by using biomass from low-lignin transgenic plants may reduce the cost of biofuel production (Hiroshi Hisano et al 2009). *C4H* downregulation increases saccharification in eucalyptus (Ziebell et al 2016). Our analysis showed little increase in glucose release after enzymatic saccharification in different transgenic jute lines compared to the control plants (Table 3-2).

Scanning electron microscopic (SEM) provides a wide range of information about the morphological features of cell surface (Tang et al 2014). By SEM fine details of the surfaces of materials, particles, and fibers can be measured and assessed via image analysis to resolve contamination issues, investigate component morphology (Kavitha et al 2016). In our study scanning electron microscopy technique was used to analyse and compare the surface morphology of the fibers of control O-9897 and four lines of transgenic jute plants. From this analysis, it is

observed that small fibrils are exposed due to a reduction of lignin in all transgenic lines whereas in control plants these fibrils are hidden underneath the lignin layer. Here, the reduction of lignin led to an exposure of the hidden fibrils. This comparative analysis of the presence of microfibrils on fiber surface asserts a to lower lignin content in all transgenic fibers which is comparable to a similar observation made by Abraham *et al*. (2011). Another analysis made by Wang *et al*. (2018) identified that untreated poplar fiber pulp had a lower extent of fibrillation than pulp treated for lignin reduction.

The effect of lignin content on the mechanical properties of control and transgenic jute fibers were investigated in terms of their tensile strength and percentage of elongation at break. Comparative results indicate that all the transgenic jute fibers exhibit little variations in tensile strength with the control fiber as illustrated in Table 3-3. This behavior is attributable to the amount of lignin which is responsible for the stiffness or rigidity of the plant but has no significant role in increasing the tensile strength of fibers (Turner and Somerville, 1997). An earlier investigation of jute researchers had made similar observation that fiber strength is not affected by lignin reduction (Sengupta and Palit, 2004). Rather, the enhancement of tensile strength albeit small observed for transgenic fibers can be explained by the rearrangement of cellulose chains resulting from the reduction of lignin leading to an increase in the tensile strength (Zhang et al., 2013). A similar investigation has been reported for palm fiber (Oushabi et al., 2017) in which enhanced tensile strength is observed as a result of effective non-cellulosic lignin removal.

Moreover, according to the results obtained (Table 3-3), increased values of breaking elongation were observed for the delignified transgenic jute lines than the control fiber. Similar analysis of mechanical properties was made by Zhang *et al*., (2013) and Saha *et al*., (2010) with both reporting that the removal of non-cellulosic materials like lignin from the interfibrillar space leads to fiber elongation.

Moreover, the most significant finding is that the fiber of all four transgenic lines show smoother and even surface compared to fibers obtained from control jute plants. We can conclude that due to lignin reduction the fineness of fiber is also improved which is visually evident Figure 3-8.

It has been reported earlier that the presence of lignin in bast fiber makes the same strong (Del Rio, Rencoret et al. 2009; Stevens and Müssig 2010) and that plants can tolerate up to 40% lignin reduction with no major changes in plant morphology (Franke, McMichael *et al.* 2000; Reddy,

Chen *et al.* 2005). Here, in our study, even up to 25% reduction of lignin in all four transgenic lines showed no morphological changes in transgenic jute lines compared to the control jute fiber (Table 3-1).

Genetic manipulation of lignin biosynthesis pathway enzymes in the transgenic lines showed no significant change in the pattern of plant growth, anatomy and physiology of the plant when compared to wild type jute controls (Table 1-1). So, morphologically lignin reduction did not lead to any major growth deformities.

In summary, we observed that lignin biosynthetic pathway genes (COMT, C4H, C3H, F5H) down regulation showed significant reduction of lignin content in an association with a concomitant increase in cellulose content and slight improvement in saccharification. Fluorescence microscopic study of jute stem for lignin and cellulose estimation also support the biochemical analysis of the same.

Lastly, the involvement of RNAi technology for alteration of lignin content and composition has immense potential in the direction of optimal utilization of plant biomass in pulp and paper industry, textile industry for bio-fiber production, easily digestible forage production, bioenergy production and biodegradation. The implementation of these transgenic strategies under field conditions, however, requires appropriate facilities and equipment, permits for testing and approval by the government. Finally, to fully exploit the potential of lignin manipulation, it is necessary to increase the public acceptance of transgenic crops and an enabling regulatory system for the release of transgenic varieties.

Last of all, this would allow the use of jute in paper industry, will have potential to be used as a source of bio-fuel and also as a source of finer thread for the garment factories. This is evident in transgenic plants which show efficient pulping or improved digestibility (Halpin 2004; Zhu, O'Dwyer et al. 2008; Bonawitz and Chapple 2010). It can be claimed that the down-regulation of the four genes used, is going to be highly productive and at the same time will shed light on the gene regulatory network of lignin biosynthesis of this important fiber plant. In conclusion, this study has dealt with jute lignomics, which provides an overview of lignin biosynthesis and structure, emphasizing the development of low lignin containing transgenic jute plants and their benefits in commercial uses.

4 CONCLUSION

In this study, the influence of lignin gene downregulation on different generations of four transgenic lines (COMT, C4H, C3H and F5H) was investigated. The findings indicated a growth in cellulose content, a slight enhancement in saccharification and confirmed the exposure of underlying microfibrils as a result of significant lignin reduction. Microscopic studies of jute stem sections are seen to fully comply with biochemical analysis in terms of lignin and cellulose content. Furthermore, insignificant changes in tensile strength and breaking elongation of transgenic jute fiber were observed. However, no alterations in the morphology and defense mechanisms in transgenic jute traits were observed. Overall, these assert the great potential of down-regulated transgenic jute lines for industrial applications from a commercial point of view. Overall, these high-quality attributes of transgenic jute lines have the potentiality to enforce jute application as a sustainable source of bio-based material for commercial resolutions viz. textile, paper and pulping, biofuel with far-reaching economic acceleration for jute producing countries. From the perspective of practical application, it is mandatory to proceed with field trial and government approval to implement the new jute transgenic lines which will be the future focus of this research.

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