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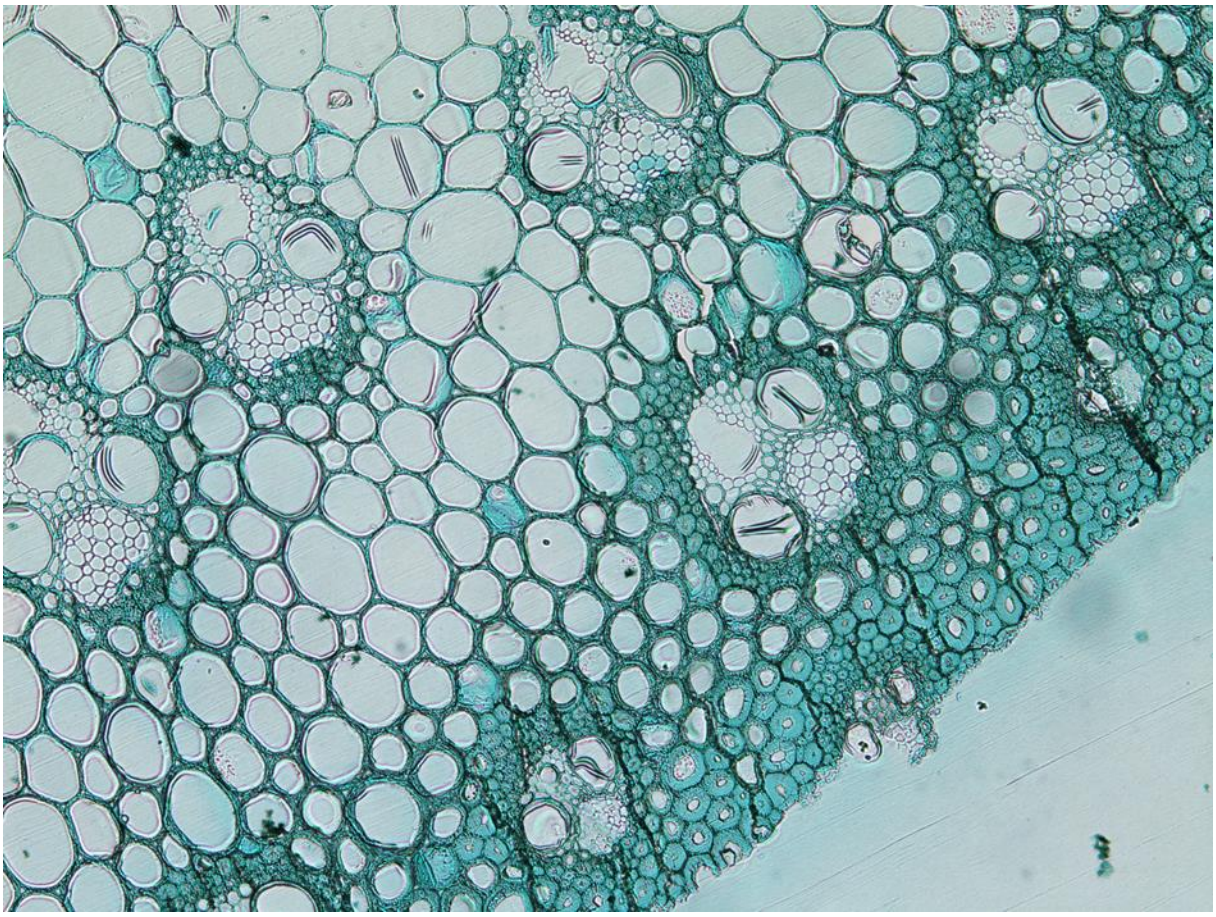

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ABSTRACT

Miscanthus sinensis is a high yielding perennial grass species with great biomass potential that can promote bioenergy production. The digestibility of stems in this species is highly variable because of genetic variation within cell walls. This variation is often associated with differences in stem anatomy and in chemical composition of the various tissues in the cell wall. Four diverse M. sinensis genotypes stem cross-sectional cuttings were analyzed to understand the impact of stem anatomy of different tissues on cell wall digestibility. Results showed variation in stem anatomical structures $p < .001$ between genotypes. There were no significant differences between anatomical structures, internode section and internode type. Amount of cellulose after saccharification was correlated with different stem anatomical traits and there were both positive and negative correlations reported. The positive correlations were reported between amount of cellulose produced and pith area ($r = 0.97$). These findings imply that breeding for improved biomass productivity can be done based targeted breeding for stem anatomical traits and cell wall components that promote biofuel production.

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1. Introduction

Fossil fuels are vital for meeting global energy demands. These contribute 88% of the world's energy with crude oil contributing 35%, coal 29 % and gas 24% of this total (Lee et al., 2014). Nevertheless, these fossil fuels are getting rapidly depleted due to their increased demand brought about by escalation in world population (Robson et al., 2013) and accelerating economic growth of many countries (stern, 2011). Economic growth influences the use of energy for production in facilities like industries among others yet this energy (oil, coal and natural gas) used is captured from the environment with implied environmental disruption. With the increased dependence on fossil fuels, the world now faces a problem of climate change which has been induced by many factors, among which are the extraction and combustion of fossil fuels (Kruger, 2006; Nel & Cooper, 2009; Ong et al.,2011). The consumption of fossil fuels is not a clean process as it brings new carbondioxide into the atmosphere thus it adds to the greenhouse gas effect. Greenhouse gas emissions are responsible for climate change and many studies have confirmed that more than 60% of these emissions (Robson et al., 2013, Lee et al., 2014, Zhao et al., 2016) are instigated by using fossil fuels energy. Due to increased uncertainty surrounding future supplies of fossil fuels, factors like; threats of depletion, political instability in key oil producing areas and increased environmental pollution through emission of air pollutants and greenhouse gasses caused by combustion of these fuels has induced the search for alternative energy sources. Numerous efforts have been dedicated into finding alternative energy sources to implement reduction of greenhouse gas emissions as stated in the Kyoto protocol (Reilly et al., 1999) to mitigate climate change and its detrimental effects.

Renewable energy sources of limitless duration and reduced negative impacts on the environment have been discovered in recent years (Höök & Tang, 2013). Plant biomass has great potential to meet the world's increasing energy/fuel demands in a sustainable manner since cell walls represent the largest source of renewable energy (Burton& Fincher,2014). Plant biomass can replace petrochemicals and directly deliver energy as heat, liquid transport fuels or chemical feedstocks. This form of renewable energy provides a potential route to the requisitioning of carbon in soil that opens the possibility of energy production with negative carbon balances (Robson et al., 2013) hence making this type of energy a clean alternative. Biomass for biofuels can be produced by different classes of energy crops that are being targeted by researchers and these include; short rotation energy crops, agricultural energy crops, aquatics, grasses and non-woody crops among others (Koçar & Civaş, 2013). Energy crops with the C4 photosynthetic route have greater potential for biomass production than the C3 plants because of their increased energy conversion efficiency. C4 plants generally have low compensation points that ensure continuous photosynthesis at high light intensity even when low carbon dioxide concentrations are available. Besides, the application of carbon dioxide relative to oxygen in C4 plant cells that are responsible for photosynthesis is much higher hence making the rate of photorespiration lower. Such C4 plants include maize, sweet sorghum, artichoke and miscanthus (Gissén et al., 2014; Koçar & Civaş, 2013; Meehan et al., 2013; Rahman et al.,2014). Miscanthus, switchgrass and sweet sorghum can produce high biomass quantities even under poor conditions like poor soils which makes them good energy crops without creating competition for food like in the case of maize. In all the perennial C4 grasses, miscanthus has been identified as the ideal energy crop since it's easy to grow, harvest but also has a high dry matter yield that promotes biofuel production (Gissén et al., 2014; Rahman et al.,2014).

Studies on these different energy crops have determined their high yielding biomass potential (maximum production of dry matter per hectare), low energy inputs requirements and low production costs which makes them a sustainable option for biofuel production (Valentine et al., 2012). Even with the mentioned characteristics that make energy crops a suitable option for biofuel production and breeding of such crops is envisioned to lead to the development of new varieties that can perform well in diverse environments hence improve the production of biofuel potential. Breeding of these energy crops is still at infant stages (Clifton-Brown et al., 2015) compared to food crop species but if implemented, this could change the future of biofuel production.

1.1 Miscanthus as a C4 bioenergy model crop

The genus miscanthus comprises a series of C4 perennial grasses that are believed to have originated from Eastern Asia although they can also be found throughout some parts of Africa. Miscanthus belongs to the family Poaceae and has about twenty different rhizomatous species grown in different parts of the world. The most economically popular species are *Miscanthus sinensis*, *M. sacchariflorous* and *M. x giganteus(*hybrid) (Zhao et al., 2016). The adaptability of miscanthus to a wide range of environments has made it a suitable crop for distribution in European and North American climatic conditions. This adaptive characteristic gives Miscanthus an advantage of withstanding cold weather during early and late growth hence having a long growing season that allows it to accumulate large quantities of biomass which is not the case with other energy crops like sorghum. In Europe, miscanthus has become a crop of economic value because it has traits that enable it to grow in areas that are marginal and cannot be used for food crop production. These areas include; those areas with insufficient soil depth to ensure reliable yields in dry years, areas with stones that damage machinery during cultivation, awkward field shapes that cannot be accessed by machinery and areas infested with recalcitrant weeds like black grass (*Alopercurus myosuroides*) mainly in the United Kingdom (Lovett et al., 2015). Therefore, miscanthus is an economic option for areas that are not arable and reduces competition between food production and biofuel production like in maize.

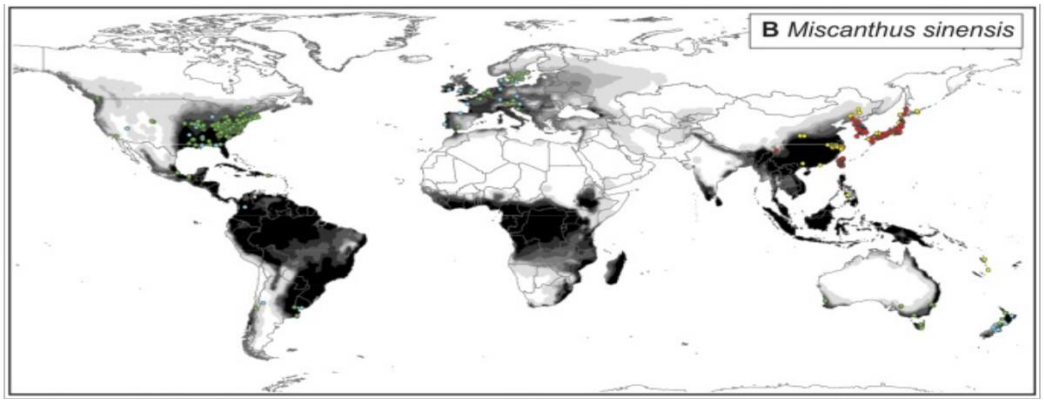

Figure 8: Map showing the distribution of Miscanthus sinensis in the world (Hager et al.,2014)

Circles show the observed plant distributions (red means native, yellow means native inferred, green means introduced and blue means introduced inferred), shaded area means modelled native and introduced plant distribution.

1.2 Genetic variation in the Miscanthus gene pool

The genus Miscanthus s.s (sensu stricto) comprises around 10-12 species native to regions of eastern Asia. M. sacchariflorus, M. sinensis and interspecific hybrids between these two species are considered to have the highest potential for biomass production in temperature climates. Ploidy levels vary amongst species although all species are characterized by a basic chromosome number 19 (Adati&Shiotani,1962, Clifton-Brown et al., 2008). Miscanthus genotypes that originate from the primary centre of diversity(China) are nearly always diploid (2n=2x=38). These include M. sacchariflorus and M. sinensis. Those from secondary centres of diversity have allopolyploid genome.

1.3 Breeding for Miscanthus

The manipulation of genetic variability through breeding is anticipated to lead to the development of new Miscanthus varieties that can perform well in different environments, are pliable to seed-propagation and are compositionally tailored for efficient bioconversion into bio products. Despite efforts to promote improved Miscanthus varieties, breeding of this species is still at infant stages compared to food crops like maize and sorghum .There are different approaches that are being used to breed for improved Miscanthus varieties and these include; genetic modification to introduce new variability in existing germplasm, genetic improvement of M.sinensis through classical population improvement to produce synthetic varieties and development of highly productive allotriploid hybrids(Sacks et al.,2013). Conventional breeding is an option for Miscanthus but is slow due to multi-year field trails involved making the process time consuming. Therefore, the application of marker assisted selection could substantially increase the efficiency of breeding Miscanthus rather than using multi-field year trails in breeding. Traits that are being targeted for improvement through breeding include; resistance to biotic and abiotic stress, early flowering time, biomass quality(composition), propagation (seeds not rhizomes) among others (Clark et al., 2014). Therefore, using different breeding techniques the benefits of increasing harvestable biomass in miscanthus can be achieved.

1.4 Miscanthus cell walls and biofuel production

Miscanthus cell walls are very crucial in the production of biofuels yet research on cell wall composition indicates that there is variation within genotypes and depending on the plant development stage (De Souza et al., 2015). These cell walls are made up of both strong flexible and non-flexible polymers (Henrissat et al., 1998; Hodgson, Lister et al., 2010) that ensure both structural integrity and rigidity of the cell. Most plant cell walls are composed of cellulose, lignin and hemicellulose polysaccharides (Zhang et al., 2014; De Souza et al., 2015) with the primary cell wall laid outside the cell membrane as a layered structure while the secondary cell wall emerges between the primary cell wall and the cell membrane. The primary cell wall allows new growth and cell extension to occur since it's thin and very flexible.

Cellulose is one of the cell wall components that is relevant in the creation of biofuels since it can be deconstructed into fermentable sugars (Lygin et al., 2011). It is a homopolymer that comprises 50-60% of the biomass hence providing rigidity and support to plant cells. Cellulose can be exceedingly long and is formed from many semi- crystalline chains composed of D-glucose monomers that are joined linearly by β1-4 linkages (Domon et al., 2013). These long chain cellulose polymers are held together by hydrogen and Van der Waals bonds that cause cellulose to be packed into microfibrils. It's also resistant to degradation due to its insolubility in water, crystallinity in addition to its interaction with other cell components like lignin and hemicellulose.

Hemicelluloses are heterogeneous polysaccharide groups that have β-(1-4)-linked backbones of glucose and xylose which can be converted into fermentable sugars. Different *M. sinensis* genotypes have different hemicellulose composition but their main role is to strengthen the cell walls by interacting with cellulose and lignin. Lignin is primarily made up of three components: hydroxycinnamyl, alcohol and monolignol monomers. Many breeders aim to reduce lignin composition in the cell wall since it's associated with recalcitrance that affects enzymatic saccharification efficiency (Fockink et al., 2016;

Wang et al., 2013). Although reducing lignin content in the miscanthus stem can improve the production of biofuels, this comes with reduction in structural integrity since lignin determines mechanical strength (Ye, 2002). Other studies have showed that lignin composition in plants is not the only factor that affects enzymatic saccharification (Chae et al., 2013; De Souza et al., 2015; Lionetti et al., 2010; Weijde et al., 2013) but the architecture (deposition and composition) of cell wall components (Arantes & Saddler, 2010; Lionetti et al., 2010; Tavares, De Souza, & Buckeridge, 2015) interfere with cell wall hydrolysis hence causing recalcitrance. Scientists have now developed pretreatment methods that easily convert hemicelluloses and cellulose into their respective monomers by loosening the lignin bonds. Lignin is known to greatly hinder the accessibility of cellulose and hemicelluloses which affects the formation of fermentable sugars that play an important role in the formation of biofuels from plant materials(biomass).

1.5 Pretreatment of cell walls

Many plants have approximately 90% of their dry weight stored in the form of cellulose, hemicellulose, lignin and pectin (De Souza et al., 2015). The presence of lignin in plant cells creates a protective barrier that hinders cell destruction by different living organisms. But for conversion of biomass into biofuels to occur, celluloses and hemicelluloses must be converted into their corresponding monomers which enables micro-organisms or enzymes to utilize them hence leading to the production of biofuels. This process is not as easy as it's theoretically stated because plant cell walls have many physicochemical, structural and compositional factors that hinder the hydrolysis of cellulose and other cell components into biofuels and other usable products from biomass. To make the hydrolysis and access of cellulose in addition to other cell components for conversion into biofuel possible, various pretreatment methods are employed. These include; physical, chemical, physical-chemical and to same extent biological pre-treatments. Before a suitable pretreatment procedure is chosen, it must be in position to;(1) disrupt the hydrogen bonds found in crystalline cellulose, (2) break down the cross-linked matrix of hemicellulose and lignin (3) give highly digestible pre-treated solid, (4) avoid the formation of inhibitory toxic by-products and finally (5) raise the porosity and surface area of cellulose for enzymatic hydrolysis (Alvira, P. et al., 2010). The goal of pretreatment is to break down the lignin structure and to interrupt the crystalline arrangement of cellulose so that enzymes can easily access and hydrolyse cellulose into usable products like biofuels. Therefore, pretreatment changes the structure of lignocellulosic biomass hence preparing it for enzymatic saccharification. This makes pretreatment very crucial in conversion of lignocellulosic biomass but also very costly. Despite the importance of pretreatment in conversion of lignocellulosic biomass into usable products, pre-treatments may also affect the composition and interaction between biomolecules in a way that is not advantageous to this process of converting lignocellulosic biomass into usable products. So, it's important to have the right pretreatment conditions to ensure successful conversion of plant biomass into usable products like biofuels.

1.6 Enzymatic saccharification

Enzymatic saccharification is a process that involves breaking complex polysaccharides into monosaccharide components. It entails converting native or pre-treated lignocellulosic biomass to glucose, cellobiose and xylose to determine comparative digestibility or efficacy of enzymes (Binod et al., 2012). Enzymatic saccharification is a very complex process and hydrolysis of all polysaccharides requires a range of numerous hydrolytic enzymes. The enzymes that are commonly used to hydrolyse cell wall components include Novozyme (celluelast, Novozyme 188 etc.) and Genencor (spezyme, Accelarase 1500 etc.). To achieve maximum biomass conversion, the reaction conditions for enzymatic hydrolysis should be at their optimum levels. Such conditions include pH, temperature, interaction of the enzyme with substrates and enzyme dosage. The impact of enzyme concentration/dosage is affected by the degree of polymerization, crystallinity, accessible area and the presence of lignin (Zhang et al., 2004). When all the required conditions for hydrolysis have been met, the mechanism of cellulose hydrolysis involves physical disruption of insoluble cellulose in addition to endo-and exo- acting enzymes. This disruption leads to enlargement, splitting up and destratification of cellulose, a process called amorphogenesis (Binod et al., 2012, Zhang et al., 2004, Vieille & Zeikus, 2001). This physical disruption

enhances enzymatic hydrolysis and renders the crystalline cellulose more accessible to enzymes hence promoting formation of fermentable sugars that can be converted into usable products like biofuels.

1.7 stem anatomy and its influence on cell wall digestibility

Many studies regarding biomass conversion to biofuels rotate around the plants of interest but not the anatomy of their stems and its influence on cell wall digestibility. Stem digestibility varies between different plant species and this is due to genetic variation in cell walls. This variation is related with alterations in anatomy and in chemical composition of the various tissues. An example is a maize stem internode that is made up of vascular bundles, sclerenchyma, chlorenchyma to mention but a few. All these tissues have differences in digestibility as it has been shown in studies done by Chen et al., 2002, Matos et al., 2013 and King et al., 2014. Stem digestibility is mainly determined by the digestibility of its cell walls which declines as the plant grows older. The distribution pattern of cell wall components like lignin and cellulose is strongly influenced by how different cell wall tissues are distributed in the stem. Cell wall tissues like the rind have been shown to hinder cell wall digestibility since they contain large concentrations of lignin (De Souza et al., 2015) while vascular bundles found in the pith area contain more cellulose.

Although with its growing potential to change breeding for crucial cell wall components that favor improved conversion of plant biomass into biofuels, plant stem anatomy remains one of the least researched on areas in lignocellulosic biofuel production. Such studies on anatomy are poorly investigated in large populations because they are laborious and time consuming. Despite the downfalls of anatomical studies, this type of research could be the future of plant breeding. This is because such research will enable the investigation of correlative patterns between cell wall biochemical development at specific anatomical spheres hence will enable targeted breeding of cell wall components that promote biofuel production from biomass.

1.4 Objectives

This research seeks to develop histochemical and image-analysis techniques that enable the targeted identification and quantification of major cell wall polymers (i.e. cellulose and lignin) across stem transections of promising C4 grasses.

1.5 Specific objectives

1. To optimize an effective methodology for creating cross-sectional cuts and staining of lignin and cellulose in M. sinensis.

2.To compare different image-analysis tools for identification and quantification of the major cell wall polymers, lignin and cellulose, across stem cross-sectional cuts of M. sinensis.

3.To study the deposition of the major cell wall polymers (lignin and cellulose) across different: internodes, sections of the same internode and genotypes of M. sinensis.

4. To perform a preliminary study of the relationship between the deposition of the major cell wall polymers, lignin and cellulose, across stem cross-sections and cell wall digestibility characteristics in M. sinensis.

2.0 Methodology

2.1 Plant materials

Four (4) different M. sinensis genotypes were manually harvested at the fall of 2016 from a collection of M. sinensis under the miscanthus breeding program at Wageningen university. Because miscanthus has tillers, for each genotype selected four plants located in the middle of the tillers were selected. The selection of genotypes was based on data previously collected on contrasting characteristics of the miscanthus cell wall. The leaves were removed, the fifth internode and the best middle internode from all the genotypes was taken to make stem cross sectional cuts. Two different whole internodes were taken because there was no information about which internode is representative of the different cell wall components that influence biomass production.

Table 1: Variation in cell wall composition of M. sinensis genotypes

Entry	pLIG%	Hem%	Cel%
H0198	10.73	28.25	44.32
H0201	15.29	32.38	33.73
H0241	15.49	29.37	39.88
H0245	17.36	26.76	37.42

Source: Wageningen university miscanthus breeding program

2.2 Stem cross-sectional cuts

Internodes from the harvest were manually cut longitudinally into circular pieces using single edge razor blades (GEM scientific) made by Fisher scientific. Three different positions were cut from the internodes, at the top, in the middle and at the bottom. Three different positions on the internode were studied to understand the variation cellulose and lignin deposition across the internode influences cell wall digestibility.

2.3 Sectioning

The cross-sectional cuttings of miscanthus stem internodes were fixed in 5% glutaraldehyde with a 0.1M phosphate buffer(w/w). This buffer was made up of 0.1M potassium dihydrogen phosphate and 0.1M Di sodium hydrogen phosphate dihydrate. The cross-sectional cuttings were fixed for forty-eight (48) hours in this buffer with the aid of a vacuum pump. The sections were then dehydrated through a series of 10%, 30%,50%,70%,96% and100% ethanol for two hours at each dehydration step at room temperature. The tissue samples were later infiltrated with Technovit A solution (from Heraeus Kulzer) made up of 100ml of Technovit liquid for twenty-four (24) hours and 1ml Hardener II to harden them off. After embedding, slides of 3µm were made using a sliding microtome (Leica Rijswijk ZH) using Knife D at an angle of 70^0. The cuttings were then placed in water to ensure that they fully open before being placed on slides. The slides were placed on a slide warmer to ensure that the cuttings firmly got attached to the slides.

2.4 Preparation of slides

Stem cross-sections of different *Miscanthus sinensis* genotypes were immersed in water, stained for the two cell wall components under study and analysed under light microscope

2.5 Histological staining

2.5.1 Histological staining for lignin

For lignin staining, toluidine blue and safranin O staining techniques were used. For toluidine blue, cross-sectional cuts were incubated in 0.05% solution for 30 seconds and rinsed with water (O'Brien et al., 1964). 0.25% Safranin O (w/v) from Sigma-Aldrich Nederland was diluted in 10ml of 95% ethanol and this solution was dissolved in 90ml of distilled water (Lux et al., 2005). The cross-sectional cuttings were stained with safranin O for five minutes and then washed with distilled water for five minutes too. For positive lignin staining, Toluidine blue stained the samples blue while safranin O stained them red.

2.5.2 Histological staining for cellulose

Cellulose staining was done using Alcian Blue 8GX from MP Biomedicals .1g of Alcian Blue powder was mixed with 100ml of 1.5%(v/v) acetic acid. 15ml of the Alcian blue solution was dissolved in 85ml of distilled water to make the staining solution as indicated in the MP Biomedicals protocol. Positive staining of cellulose showed a light blue colour.

2.6 Microscopy

Light microscope Zeiss Axiophot and a stereo microscope from Germany which is equipped for bright field, phase contrasts and reflected polarization among others was used. An Axiocam ICc5 megapixel colour camera was attached to the microscope that enabled capturing images on the computer. The sensor of this camera was a CCD (Sony ICX655) with a pixel size of 3.45µm in addition to the RGB Bayer colour filter with a ROI that is adjustable.

2.7 Image software analysis

Image J 1.5 1 by wayne Rasband from the National institute of health USA is a Java image processing and analysing program based on NIH image for Macintosh. It was used to measure the area by setting a threshold using a thresholding tool around the region of interest (ROI). This software was used to measure different anatomical traits of the Miscanthus genotypes. Such traits include; cross sectional area, pith area, rind thickness and colour quantification of the stained slides.

2.7.1 Measuring anatomical traits

The image is uploaded into image J software and using the tool bar, the Analyse icon was clicked, then set measurements and here area was selected as a parameter of interest. A scale of 500 pixels per mm was set and using the area irregular selection tool, the area of interest was selected and measured. This was done for all anatomical traits. To study the intensity of staining for both lignin and cellulose, a color picker plugin was used. This enabled quantification of a colour of interest from the microscopic images by using pixel intensity as a base for measurement

2.8 Data analysis

2.8.1 Statistical analysis

General analyses of variance (ANOVA) were performed to determine the significance of genotypic differences ($p<0.05$) in stem anatomical traits. Correlation analyses were performed to identify the significance, strength and directions of interrelationships between traits using Pearson's correlation coefficients. All these statistical analyses were completed using Genstat for windows ,18th edition software package (VSN international, Hemel Hempstead, UK).

3. Results

To preliminarily understand the diversity in anatomical constructions in plant species, four genotypes of *Miscanthus sinensis* were analyzed. Stem internode sections of the different genotypes were embedded and cut into 3µm cross-sections which were later stained for both lignin and cellulose. These stained sections were then viewed under a microscope; images were obtained and analyzed using image J. Images of different genotypes showed differences in miscanthus stem anatomy. Two biological replicates were used for this experiment to acquire data for phenotyping and this was done with the aim of improving the statistical power of the experiment. Both quantitative and qualitative phenotyping techniques were used.

In summary, four genotypes were studied using two internodes types (middle or sixth) that were divided into three sub-sections (top, middle and bottom). Data from this study was used to observe how cell wall components are deposited at an anatomical mesoscale and how these differences in cell wall deposition between genotypes could affect biomass deconstruction for biofuel production.

3.1 Quantitative differences between genotypes

when quantifying anatomical differences between genotypes H0198, H0201, H0241 and H0245, significant variations were detected. Stem anatomy regarding cross sectional area was significantly different between genotypes H0198, H0201, H0241 and H0245. Genotype H0198 had 21% while H0201 had 26% of the detected variation in the mean cross sectional area of the genotypes. Like genotype H0201, genotype H0241 had a mean cross sectional area of 26% which also varied from genotype H0245 with 27%. Interestingly, genotype H0245 that had the highest cross sectional area had 23% of the total variation in rind thickness which was the same with genotype H0241. Genotype H0201 had 27% of the total variation in rind compared to 20% that was showed by genotype H0198.

Regarding number of vascular bundles, genotype H0198 had 20% of all the detected differences between genotypes while genotype H0201 had 33%. In contrast, genotype H0241 had 23% of all the number of vascular bundles while genotype H0245 had 24% of these differences. The vascular bundle density between genotypes was significantly different as seen in table 2. Genotype H0198 had a 26% of the total variation in vascular bundle density while genotype H0201 had a 31% of this variation in its stem anatomy. This means that despite genotype H0198 having a smaller cross sectional area and fewer vascular bundles compared to other genotypes, it has more vascular bundles compacted with in its cross-sectional area compared to genotype H0241 that had 20% of the total variation in vascular bundle density while genotype H0245 had 23%.

To understand the impact of stem anatomy about cross sectional area and vascular bundles, vascular bundle ratio was calculated which showed that genotype H0201 had a 32% of the total variation in vascular bundle ratio while genotype H0241 had 22% of this variation. Genotype H0245 had 21% variation in vascular bundle ratio while genotype H0198 had 25%. About pith area, genotype H0198 had a 29% variation in mean pith while genotype H0201 had 24% of this variation which was the same as genotype H0241. Finally genotype H0245 had a 23% variation in its mean pith compared to other genotypes.

To predict the amount of lignin and cellulose in the above-mentioned stem anatomical traits, color pickers were used to measure the intensity of a given color based on the RGB value. Cellulose color picker showed that genotype H0198 had a 24% variation with the blue color compared to genotypes H0201 and H0245 that had 25% of this variation while genotype H0241 had 26%. Interestingly for the lignin color picker, both genotype H0198 and H0245 had a 25% variation in mean compared to genotypes H0201 and H0241 that had 26% and 24% variation respectively.

Therefore, genotype H0201had the highest variation regarding the rind thickness, vascular bundle density, vascular bundle ratio and finally the lignin color picker compared to the other three genotypes. Despite genotype H0241 and H0245 having similar stem anatomical characteristics regarding rind thickness and vascular bundles, they both had differences in area, vascular bundle density, cellulose and lignin color intensity. Finally genotype H0198 had a constant pattern regarding stem anatomy except for the pith area which was higher compared to the other genotypes.

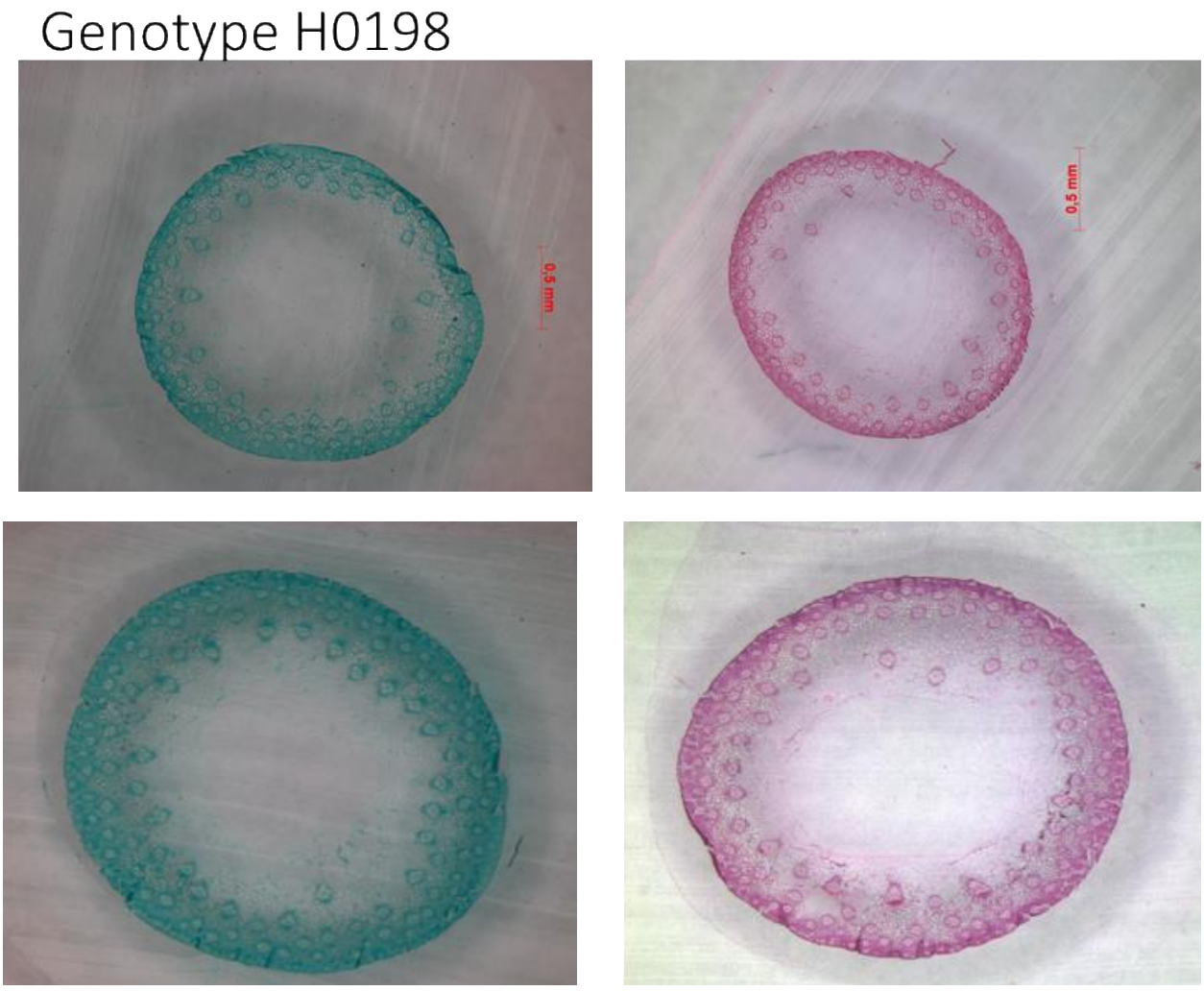

Figure 9 : Showing stem anatomy of genotype H0198, the second set of images at the bottom are magnified(2x). To the left is Alcian blue and right is safranin o

Genotype H0201

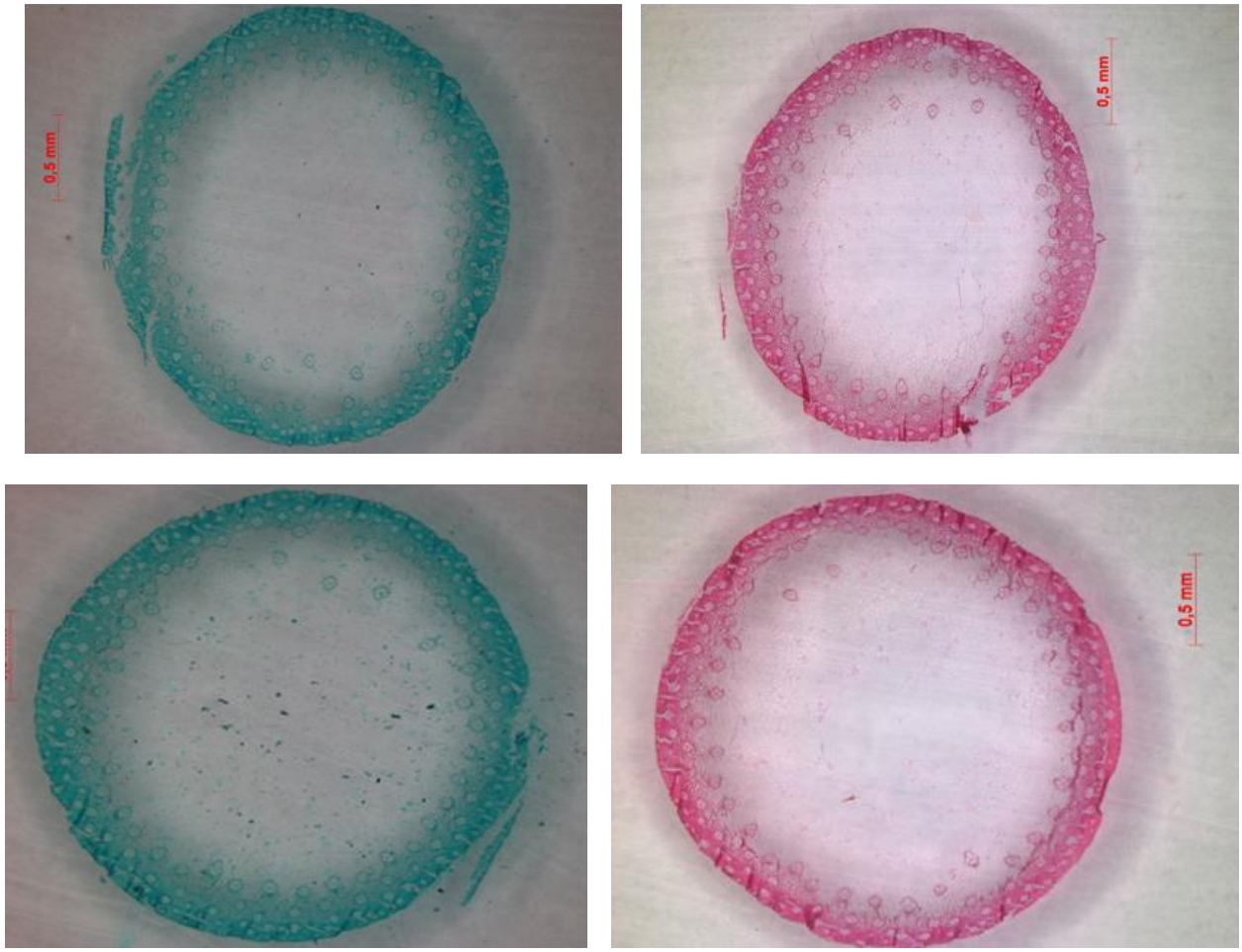

Figure 10 : showing stem anatomy of genotype H0201, the second set of images at the bottom are magnified(2x). To the right is Alcian blue and left safranin

Genotype H0241

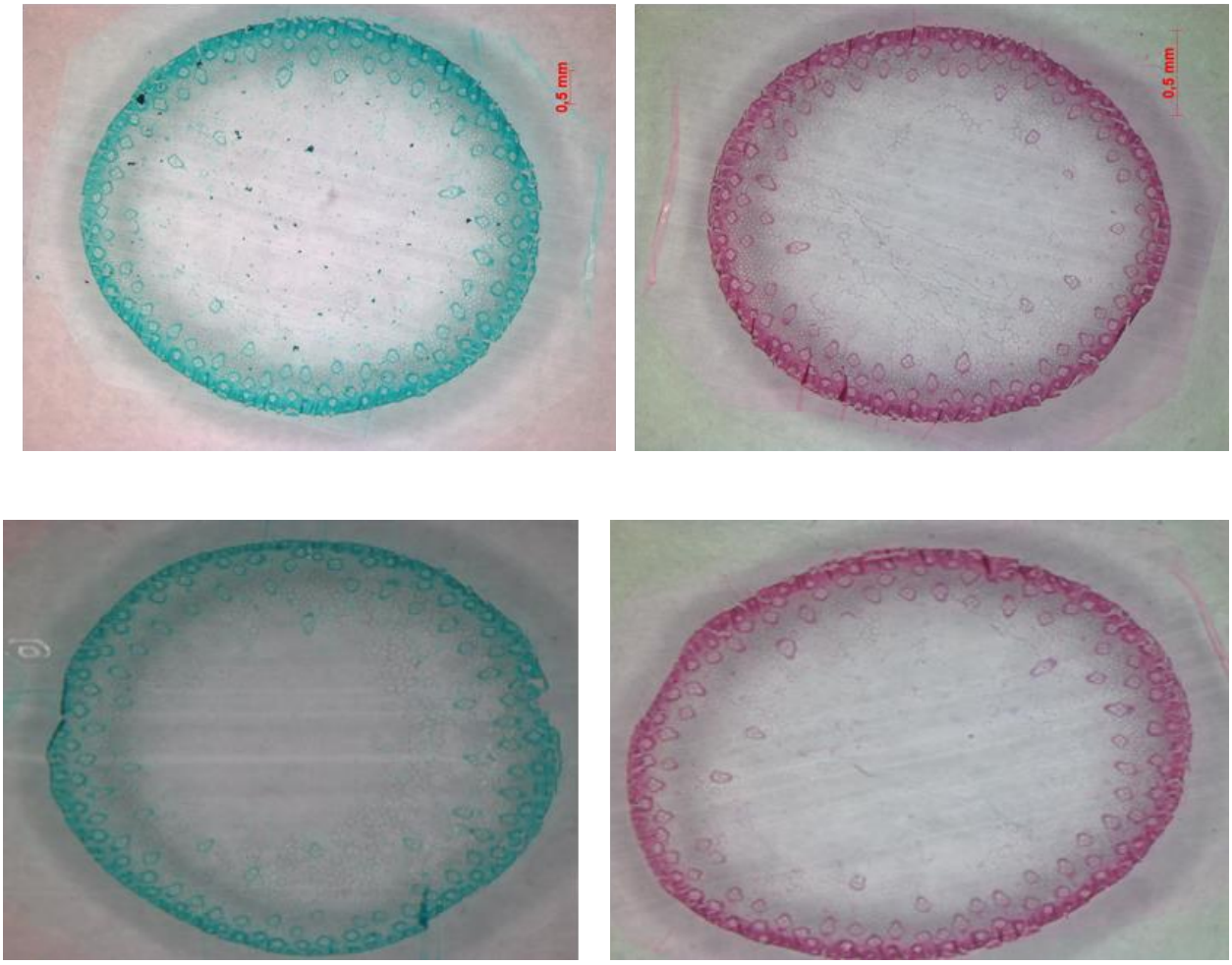

Figure 11 : Showing stem anatomy of genotype H0241, second set of images at the bottom are magnified(2x) To the right is Alcian blue and left safranin

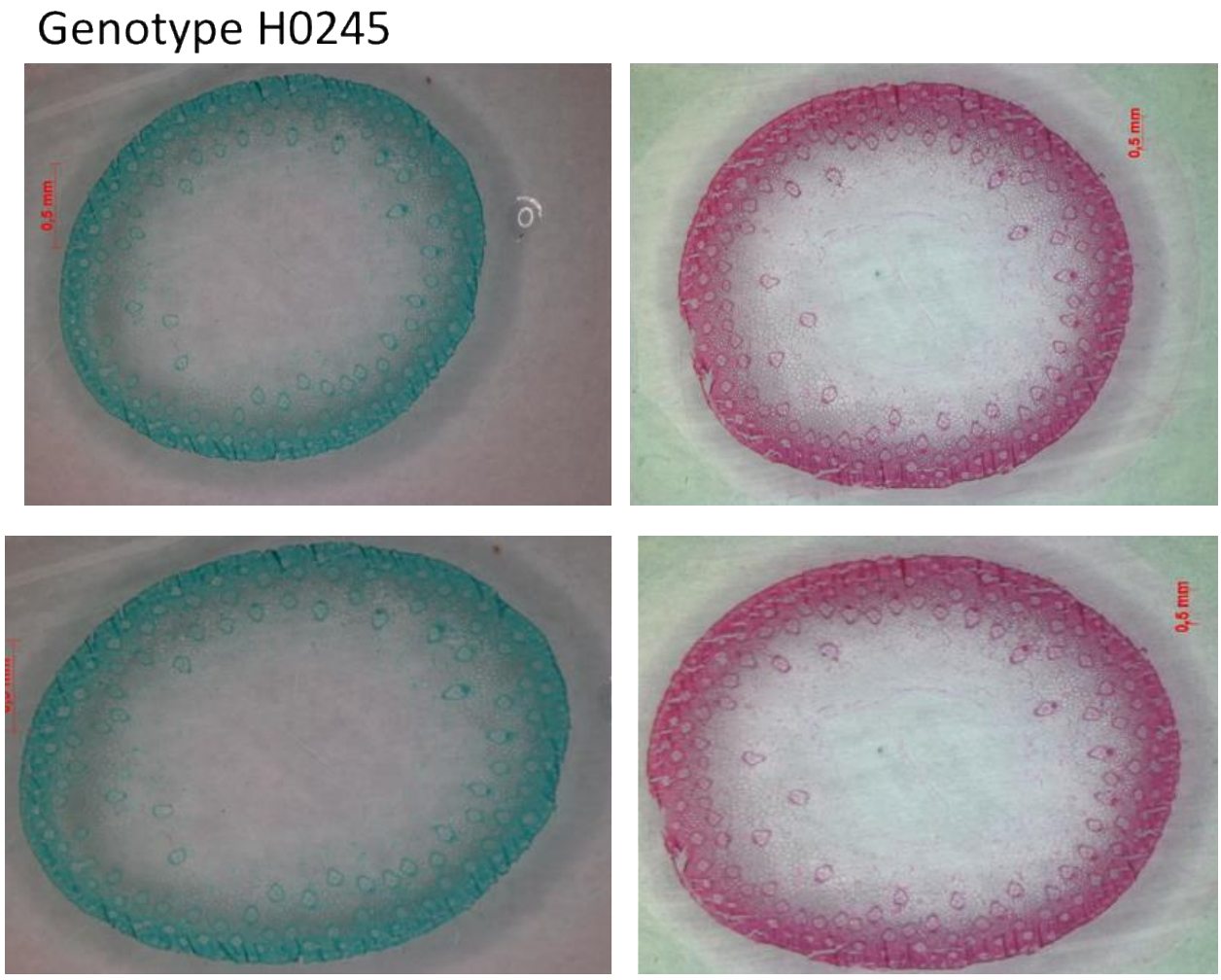

Figure 12 : showing stem anatomy of genotype H0245, second set of images at the bottom are magnified(2x). To the right is Alcian blue and left safranin

3.2 Anatomical differences between miscanthus sinensis genotypes

To further understand miscanthus stem anatomy, an ANOVA- test was performed to test the multiple stem anatomical related traits between genotypes, with in internode sections (top, middle and bottom) and in internode types (middle or the sixth). As shown in table 2, there are significant differences between; number of vascular bundles ($p \leq .001$), cross sectional area($p \leq .001$), rind thickness ($p \leq .001$), vascular bundle density($p \leq .001$), vascular bundle ratio($p \leq .001$) and lignin intensity color picker $p = 0.018$ in the different genotypes. For the pith area and the cellulose color picker, there were no detectable differences in genotypes. There were no noticeable differences between the analysed anatomical traits and the internode section except for the vascular bundle density with $p=0.011$. Furthermore, there were no detectable differences between the internode type and the anatomical traits.

Table 2: P-values for factor effects and anatomical related traits(n=432). P-values for genotype, internode section and internode type.

Traits	Main factors		
	Genotype	Internode section	Internode type
No. vascular bundles	<.001	0.568	0.427
Cross-sectional area(mm)	<.001	0.328	0.963
Pith area(mm)	0.863	0.314	0.324
Rind thickness(mm)	<.001	0.466	0.292
Vascularbundle density	<.001	0.011	0.436
Vascularbundle ratio	<.001	0.443	0.423
Cellulosecolor picker	0.227	0.152	0.632
Lignin color picker	0.018	0.610	0.127

To evaluate if differences existed between genotypes regarding stem anatomy Tukey's test $\alpha=0.05$ was performed. Differences in trait means were compared and if there were differences, different alphabetic letters were used as illustrations. These differences were a measure of different stem anatomical traits within and between genotypes. Regarding number of vascular bundles, there were hardly any differences observed between genotypes H0241 and H024 whereas the number of vascular bundles varied between genotypes H0198, and H0201.These differences in number of vascular bundles were small. The other differences between stem anatomical traits in genotypes followed a similar trend like in vascular bundles as shown in figure 6.

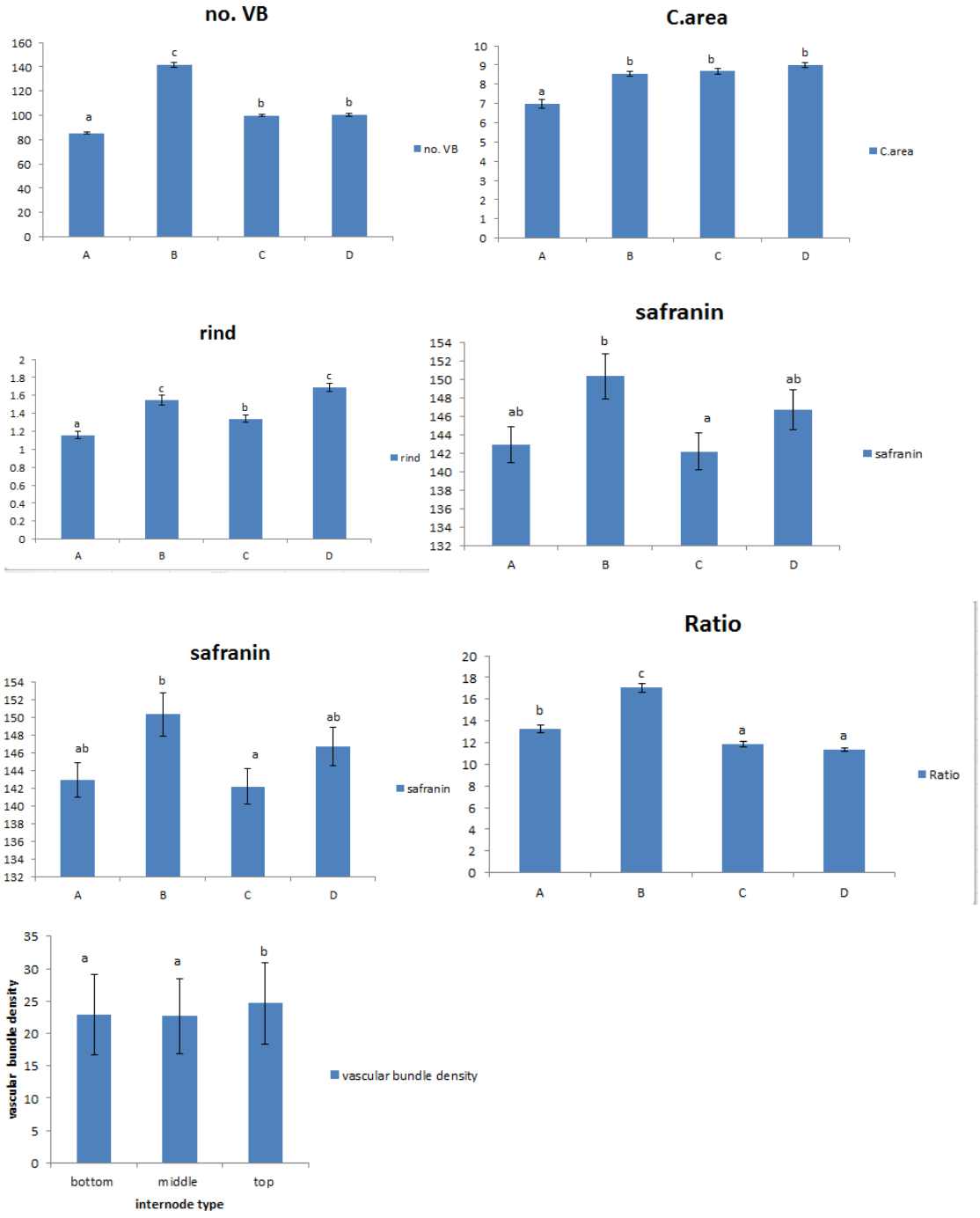

Figure 13 : variation in genotype means of different stem anatomical characteristics. Significant difference between the genotypes and the different anatomical traits is represented by a different alphabetical letter (Tukey's test α=0.05).

To further investigate interrelations between stem anatomy and cell wall deposition patterns cross-sections of different genotypes, a correlation analysis was performed on the whole set of genotypes means for all traits in relation to the amount of cellulose obtained from enzymatic saccharification (results for amount of cellulose were obtained from Rens Bogers' thesis). A few significant associations were observed between amount of cellulose and pith area (0.97). Other significant trait associations but with low coefficients of determination were observed between amount of cellulose and: number of vascular bundles (0.37), vascular bundle density (0.26), vascular bundle ratio (0.219).

Some stem anatomical traits were negatively correlated with the amount of cellulose and these included: cross sectional area (-1), rind thickness (-0.92), safranin intensity color picker (-0.45) and Alcian blue color picker (-0.56).

	VB	*area*	*rind*	*pith area*	*v.B dens*	*v.B ratio*	*safra*	*alcian*
Area	0.45							
rind	0.52	0.86						
Pith	-0.55	-1	-0.89					
v.B den	0.73	-0.2	0.06	0.12				
v.B ratio	0.81	-0.2	0.031	0.04	0.97			
safr	0.87	0.42	0.715	-0.5	0.74	0.704		
alcian	0.31	0.72	0.27	-0.7	-0.4	-0.13	-0.05	
Cel (%)	-0.37	-1	-0.92	0.97	0.26	0.219	-0.45	-0.56

Figure 14: showing the direction of correlations in stem anatomy and cellulose composition. Only Pearson correlation coefficients that differed significantly from zero ($p>0.05$) are reported. Blue values indicate positive correlation coefficients and red values indicate negative correlation coefficients Area is cross sectional area of the stem. Rind is rind thickness, pith is pith area, V.B den is vascular bundle density, V.B ratio is vascular bundle ratio, Safra is safranin color picker, Alcian is alcian blue color picker, Cel (%) is amount of cellulose produced after enzymatic saccharification.

4.Discussion

4.1 Diversity in *Miscanthus sinensis* stem anatomy between genotypes

The extensive diversity in *Miscanthus sinensis* stem anatomy found in the four genotypes analysed in this study indicates that there is a large potential in this species for improvement of biomass traits for different applications by altering different components in the stem anatomy. Particularly, variation between genotypes was found in the number of vascular bundles, rind thickness, stem cross-sectional area, vascular bundle density and vascular bundle ratio which are key factors in determining the anatomy of miscanthus stem sections. The arrangement of these cell wall structures plays a critical role in determining stem characteristics that affect biomass conversion into biofuel (King et al., 2014). Some of the stem characteristics include mechanical strength which has in many studies between related to the distribution of vascular bundles throughout the cross-sectional area of the stem (Akin 1989, Evert, 2006, King et al., 2014). When vascular bundles are scattered throughout the cross-sectional area of the stem mainly around the periphery, the stem becomes mechanically strong hence causing recalcitrance during biofuel production. This recalcitrance dramatically increases the energy cost for releasing polysaccharide components from cell walls during biofuel conversion hence making it costly.

Cross sectional area is also related to the amount of lignin the stem (Engels and Schuurmans, 1992, King et al., 2014). Therefore, the smaller the cross-sectional area, the less lignin deposited within the stem cross section. Within this study, this is evident from figure 2 and table 1were genotype H0198 had the smallest cross section area and the least amount of lignin. This is an important observation, as there is evidence that lignin cross links with other cell wall components affects cell wall digestibility (Grabber et al., 2004, Torres et al., 2014, De Souza et al., 2015). This can also explain why despite genotype H0198 having the smallest cross sectional area compared to other genotypes, it has the highest amount of cellulose (44.32%) produced after enzymatic saccharification.

Furthermore, there was significant variation in rind thickness between the genotypes. Based on figures 3 ,5 and table 1, genotypes H0201 and H0245 have similar rind thickness but also have different composition regarding to the amount of lignin. The rind is a structure in the plant stem that is composed of lignin and is also responsible for recalcitrance. From figures 3 and 5, genotype H0201 has more vascular bundles that are distributed more in the rind compared to the pith while genotype H0245 has more of its vascular bundles distributed evenly with in the rind and pith region. This difference in the distribution of vascular bundles within the rind makes genotype H0245 mechanically stronger than genotype H0201 hence explaining the difference amount of lignin despite the similarity in structure.

Likewise, the difference in the amount of lignin between these genotypes does not explain the variance in cellulose concentration but the distribution of vascular bundles can. Genotype H0245 with a lignin concentration of 17.36% compared to H0201with %15.49 has cellulose concentration of 37.42% which is higher than 33.73% of genotype H0201 (refer to table 1). This difference can be attributed to the difference in vascular bundle location within the stem cross sectional area. Figures 3 and 5 Show that for genotype H0245 has an equal distribution of vascular bundles within the rind and pith regions compared to genotype H0201 that has most of the vascular bundles in the rind. Since the pith is less lignified, presence of vascular bundles in this area explains why genotype H0245 has more cellulose compared to genotype H0201 despite their similarity in structure.

Correspondingly, there was significant variation in vascular bundle density between genotypes with genotypes H0201 and H0198 having more vascular bundles compacted in their stem cross sections. This greatly impacts on cell wall digestibility depending on the location of these vascular bundles. Genotype H0198 has most of its vascular bundles located within the pith area which is known to be less lignified (Zhang et al.,2015) hence explaining why this genotype has more cellulose (refer to table 1) compared to genotype H0201 that has more vascular bundles located within the highly-lignified rind as shown in figure 3. Significant variations were also observed between the lignin intensity color picker and genotypes. This lignin intensity color picker showed that there was a significant difference in the red color intensity between genotype H0201 and H0241. These genotypes interestingly have a similar lignin concentration of 15.29% and 15.49 respectively but the software showed that there was a significant difference in the red color intensity for lignin

Therefore, for many stem anatomical traits that influence cell wall digestibility, significant variations were found in this diverse set of M. sinensis genotypes that can potentially be exploited during breeding of improved varieties that promote biofuel production by altering the anatomy of the stem.

4.2 Diversity in Miscanthus stem anatomy within genotypes

To understand variation in Miscanthus sinensis genotypes as described in chapter 2.1 different internode sections and types on the same stem were analysed. There were no significant differences in stem anatomy within genotypes expect for the vascular bundle density of different internode sections. These differences as shown in figure 6 revealed that there was no significant difference between the bottom and middle internode section but there were differences in the top part of the internode. This can be attributed to maturity levels in the internode since growth of the internode starts from the bottom to the top hence this difference in vascular bundle density between internode sections. This is also supported by Chen et al., 2002, Matos et al., 2013 and King et al., 2014 who showed that growth within the internode begins from the bottom and then proceeds to the top of the internode hence explaining the difference in vascular bundle density.

Similarly, this study shows that there are no significant differences between the middle and sixth internode regarding stem anatomy. This is because at the time of harvest (autumn 2016) miscanthus had reached maturity so there was no growth (Somerville et al., 2010, Weijde et al.,2013).

4.3 Relationship between cellulose concentration after enzymatic saccharification and stem anatomy traits

Amount of cellulose from Miscanthus stems is strongly and negatively impacted by the anatomy of the stem as shown in figure 7. Number of vascular bundles, cross sectional area and rind thickness were strongly and negatively correlated to the amount of cellulose produced after enzymatic saccharification. This can be attributed to the high levels of lignification patterns with in these stem anatomical features which makes it difficult for cellulose to be released from Miscanthus stems. Also in other studies, it's been shown that cellulose release is more negatively affected by presence of lignin (Chen et al., 2009). Despite the negative correlation patterns, there was a strong and positive correlation between pith area and amount of cellulose after saccharification. This is because the pith area is less lignified (Zhang et al.,2015) which makes it possible for cellulose to be released from these stem structures without effects caused by lignin.

5. conclusions and recommendations

This investigation reports high levels in variance in stem anatomy of M. sinensis genotypes and highlights of these differences were evident in genotypes H0201, H0241 and H0245.Genotype H0198 had considerably low stem anatomy traits compared to other genotypes but had the highest concentration of cellulose. Findings from this research imply that future breeding initiatives for improved biofuel production based on alteration in stem anatomy, must focus on breeding varieties with stem anatomical traits like genotype H0198. Therefore, the exploitation of such variations in stem anatomy through breeding will accelerate the realization of biomass derived energy and fuel production as well as many other bio- based applications.

Despite the positive results from this investigation, much more work needs to be done to unveil in detail about:

a. The influence of the time lag between staining time and image acquisition affects the color intensity picked up by different software.
b. Impact of the size of vascular bundles within the rind and pith and its influence on cell wall digestibility.
c. More detailed studies need to be done to determine the effect of pretreatment and enzymatic saccharification on stem anatomy while describing specific effects of these activities on cell wall tissues.

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7. APPENDIX

Appendix1. Protocol for embedding miscanthus stems

Cutting miscanthus

(a) Fresh miscanthus stems of the different genotypes were harvested from the fields.

(b) Samples from the middle of the plot were collected, four plants from the middle of the plot were selected.

(c) Leaves were removed, the fifth and middle internodes from each genotype were taken.

(d) These internodes were cut into three parts (1) at the top of the internode, (2) middle internode and (3) bottom using a STEM blade by hand into pieces that are less than 1cm.

(e) Two biological replicates were made so for each genotype so that makes thirty-six samples per genotype

Embedding miscanthus tissue

This process is divided into three steps: fixation, infiltration and embedding.

Fixation of the tissue

(a) Phosphate buffer was made by mixing 6.8g /500ml of potassium dihydrogen phosphate and 8.9g/500ml of di sodium hydrogen phosphate

(b) The samples were placed in 5% glutaraldehyde with phosphate buffer for forty-eight hours in a vacuum pump.

(c) After fixation, the samples were washed with phosphate buffer four times at room temperature and finally two times with distilled water.

(d) The samples were then dehydrated in a series of 10,30,50,70,98and 100% ethanol for two hours at each step in a vacuum pump.

Infiltration

(a) Solution A was made from 100ml Technovit liquid with one pack of activator.

(b) The samples were infiltrated with 100% ethanol and solution A for two hours.

(c) The last step of infiltration was placing the samples in solution A for twenty-four hours.

Embedding

(a) To making the hardening solution, Technovit and the hardener II were mixed.

(b) The solution was placed in the molding plates, samples added and a cover placed on the molding plates

(c) The samples were left overnight

Appendix2. Cross section cutting of embedded samples

(a) 3µm cross sectional cuttings were made using a sliding microtome.

(b) Knife D was used for cutting and the knife angle was placed at 70^0.

(c) Cross sectional cuttings that were made were placed in water so that they could open.

(d) The cross-sectional cuttings were placed on slides and then placed on a slide warmer to dry.

Appendix3. Staining

Staining for lignin

(a) 1g of safranin O was used to stain for lignin, the staining time was five minutes.

(b) The slides were washed with tap and distilled water for one hour thirty minutes.

Staining for cellulose

(a) 1.5% acetic acid was applied to the samples for three minutes.

(b) 1% Alcian blue was applied for thirty minutes.

(c) 1.5% acetic acid was applied to wash off the excess Alcian blue

(d) The samples were then washed in both tap and distilled water for one hour thirty minutes.

Appendix4: Anatomical traits in Miscanthus sinensis genotypes

Table2: Anatomical characteristics of Miscanthus sinensis genotypes

	Mean	Median	Min.	Max.	range	St.Dev.
No. vascular bundles	106.8	99	65	179	114	25.17
Cross-sectional area(mm)	8.300	8.459	3.051	11.91	8.858	1.840
Pith area(mm)	5.341	4.784	1.865	273	271.1	12.95
Rind thickness(mm)	1.434	1.397	0.346	3.172	2.826	0.497
Vascular bundle density	23.41	22.02	0.355	43.48	43.12	6.177
Vascular bundle ratio	13.39	12.70	8.104	27.39	19.28	3.826
Cellulose color picker	168.8	174	51	235	184	29.78
Lignin color picker	145.5	143	78	224	146	22.64