Quantitative traits according to maize digestibility

Minor thesis Crop Science Analysis

Jo Depandelaere 270189-179-110

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Supervisor: Prof. Dr. Ir. Paul C. Struik

Dr. John W. Cone

Dr. Ing. Norbert C. A. de Ruijter

Preface

This report is written to obtain my Minor in Crop Science Analysis at the University of Wageningen. I chose this subject because it closely resembles my main interest; Animal nutrition. I think it's very important as an Animal nutritionist that you have knowledge about the crops where the feed is coming from. The research I did started in July 2012 and I ended the report in December 2012.
I learned a lot about microscopy and plant sciences. Next to that I gained a lot of lab experience. During the research period I had some difficulties and the results weren't always easy to obtain.
Finally I want to thank some people for their help and contribution. First of all I want to thank Norbert de Ruijter for his help, patience and expertise in very divers fields. Next to that I'm grateful to all the other people from Plant sciences for the nice work environment and useful advice. Furthermore I want a small word of thanks for my brother who helped in correcting my errors in the report and finally I want to thank my supervisors Paul Struik and John Cone for their help and contribution to my research.

Summary

The report starts with a literature research about the subject. Thereafter the materials and methods are explained. Furthermore the results are presented in chapter 4. Next a conclusion is drawn, the results are discussed, and finally there are some considerations about further research

Relevant subjects in the literature study are considered; development of a maize stem, different digestibility traits and lignification in a maize stem. This was background information to answer the main research question:

- Search for visible proves that Ambrosini is less digestible than Aastar.

(This statement was set in the beginning of the research.)

In the materials and methods first all, the different procedures that were executed are described. After that a short description follows about the experimenta. Generally the most experiments were executed using images of fresh sections of a maize stem. These were photographed and subjected to a range of analyzes using Image J.

Vascular bundles and surfaces were measured and ratios were calculated. Eventually the only visible prove that Ambrosini is less digestible than Aastar was the higher vascular bundle density in both pith and rind region.
A lot more research can be done in this subject, using Technovit sections. A lot more detail and precision can be obtained and more measurements can be executed.

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AP	Acid Phloroglucinol
CBB	Coomassie Brilliant Blue
GA	Glutaraldehyde
M	Molair
PFA	Paraformaldehyde
RPM	revolutions per minute
TDN	Total digestible nutrients
WUR	Wageningen University

1. Introduction

Over 161 million hectares of maize is harvested all over the world, of which 70 to 80% is used for animal feed (FAO, 2010). Maize is a primary resource of energy in animal feed. It can contain up to 30 percent protein, 60 percent energy and 90 percent of starch. The Total Digestible Nutrients (TDN) of maize silage is 68.8%. This is due to the good digestible parts of the plant, such as the grain. But also less digestible components are present, thinking about the fibers in the maize stem. Even though the fiber fraction is less digestible, it contains 1/3 of the energy in corn silage for ruminants. An important reason why the fibers are less digestible is the lignifications of the primary cell walls. Rumen bacteria digest cell walls from the lumen side of the cell. If this cell wall is hard to digest, the further digestion process is obstructed.
The extent to which tissues are lignified is negatively correlated with the fiber digestibility (Jung, 1989). However, not all the compounds of lignin are indigestible. Lignin is composed out of different phenolic compounds and acids, some more digestible than others (Borneman et al., 1986).

The goal of this report is to investigate whether there are microscopic differences in two maize cultivars: in theory, Aastar should be better digestible than Ambrosini (Struik, 2012). Several experiments were conducted to establish this statement. This research is supportive to a PhD study about rumen fermentation of the same two maize cultivars and the same internodes. The research is executed at WUR livestock research.

The structure of the report is as followed. First a literature study was carried out in chapter 0. This serves as background information about growth and development of maize, and how this is related to the digestibility of the plant. In chapter 3 the methodology and different experiments are explained. Subsequently, the materials and methods are followed by the results of the experiments. Next to that, the achieved results are discussed and a conclusion is drawn. Finally the report ends with experiences and recommendations for future research.

2. Literature study

Plant tissues consist out of different cell types. Some of these cell types form a cell barrier and provide strength to the plant. Three cell wall types are determined with different functions: primary, secondary and tertiary cell wall. Exact functions are described in Jung Et al, (1993). As plants are growing, they become more rigid and they gain strength. The biggest contribution to that change is the deposition of lignin (Juniper, 1979).

2.1. Stem composition

When a cross-section of a maize stem is cut, the epidermis is the outermost layer. Underneath are several layers of sclerenchyma tissue. This increases the strength and rigidity. On the inside of the epidermis is the rind region. Vascular bundles in the rind are surrounded by several layers of sclerenchyma cells whereas vascular bundles in the pith region only had one layer of sclerenchyma tissue, which stained mild red with phloroglucinol. The arrangement of vascular bundles is complex and they seem to be scattered throughout the parenchyma tissue. The vascular bundles seem to be arranged more loosely in the middle of the stem than at the sides (Esau 1977b). Because of the difference in composition between the rind and pith region, the rind is less degradable than the pith region. The rind region consists of the epidermal and sclerenchyma tissues and many vascular bundles. On the other hand, the pith region has thinner-walled parenchyma tissue and less vascular bundles (Jung, 1998).
As expected, the rind and pith region both decreased in polysaccharide degradability with internode maturity. Next to that the decline was steeper for the rind region. The reason for the steeper decline is because the rind contains more vascular and sclerenchyma tissue than the pith. These tissues undergo a more extensive secondary cell wall thickening and lignifications than the pith region does (Jung, 1998).

2.2. Vascular tissue development

Vascular tissue is originates from the apical meristem. This exists of the protoderm, procambium and the ground meristem. The shoot apical meristem of angiosperms shows histological stratification into the outer and inner cell layers. These are the start of the formation of the central zone and the organogenic zone in the stem (Takacs et al., 2012). Maize is just like most grasses a monocotyledon. This means that they have ‘closed’ vascular bundles. In a monocotyledon, all the procambial cells in a vascular bundle become vascular elements (Phloem, xylem). Therefore no cambium is left and no secondary growth is possible.
Another specification of the vascular tissue in maize is their collateral characteristic. When phloem and xylem are positioned in the same radius of the stem, and phloem and xylem are respectively confined to the outer and inner side of the stem, the vascular bundles are called collateral vascular bundles. The vascular bundles are scattered throughout the whole stem section. This shows an atactostele pattern (Ye, 2002).

The phloem develops from the outer cells of the procambial strand, and the xylem develops from the inner cells. The phloem and the xylem differentiate in opposite directions. The first-formed phloem and xylem elements (protophloem and protoxylem) are stretched and destroyed during elongation of the internode. This results in the formation of a very large space, called a protoxylem lacuna, on the xylem side of the bundle. The mature vascular bundle contains two large metaxylem vessels, and the phloem (metaphloem) is composed of sieve-tube elements and companion cells. The entire bundle is enclosed in a sheath of sclerenchyma cells (Evert and Eichhorn, 2012).

The monocotyledons vascular construction is better known as the *Rhapis* principle, because it was first reported analyzing the small palm, *Rhapis excelsa*. In this construction the vascular bundles describe a shallow helix pattern as they twist up the stem. The bundles incline towards the middle of the stem, but at regular intervals they bend out to form a leaf. When bending they divide into two strands. One half is entering the leaf and provides the ability of axial transport in the midvein. The other half continues as an axial bundle in the periphery of the stem.
Sengbusch (2003) showed that vascular bundles seem to be scattered in the whole stem, but more concentrated in the rind region. If the course of one single vascular bundle is determined, a helical arrangement can be seen. Not one vascular bundle is lying only in the pith or rind region. Depending on the position the section is cut, the particular vascular bundle can be found in the centre part of the stem and sometimes in the periphery. At regular intervals vascular bundles branch to develop leaves. They run, next to axial vascular bundles, for a long time along the stem in the rind region. This is the main reason why a lot of vascular bundles can be seen in the periphery in a transversal section of a maize stem (Figure 1). Part of the vascular bundles are axial strands, part are leaf traces. They are to distinguish from each other in sections.

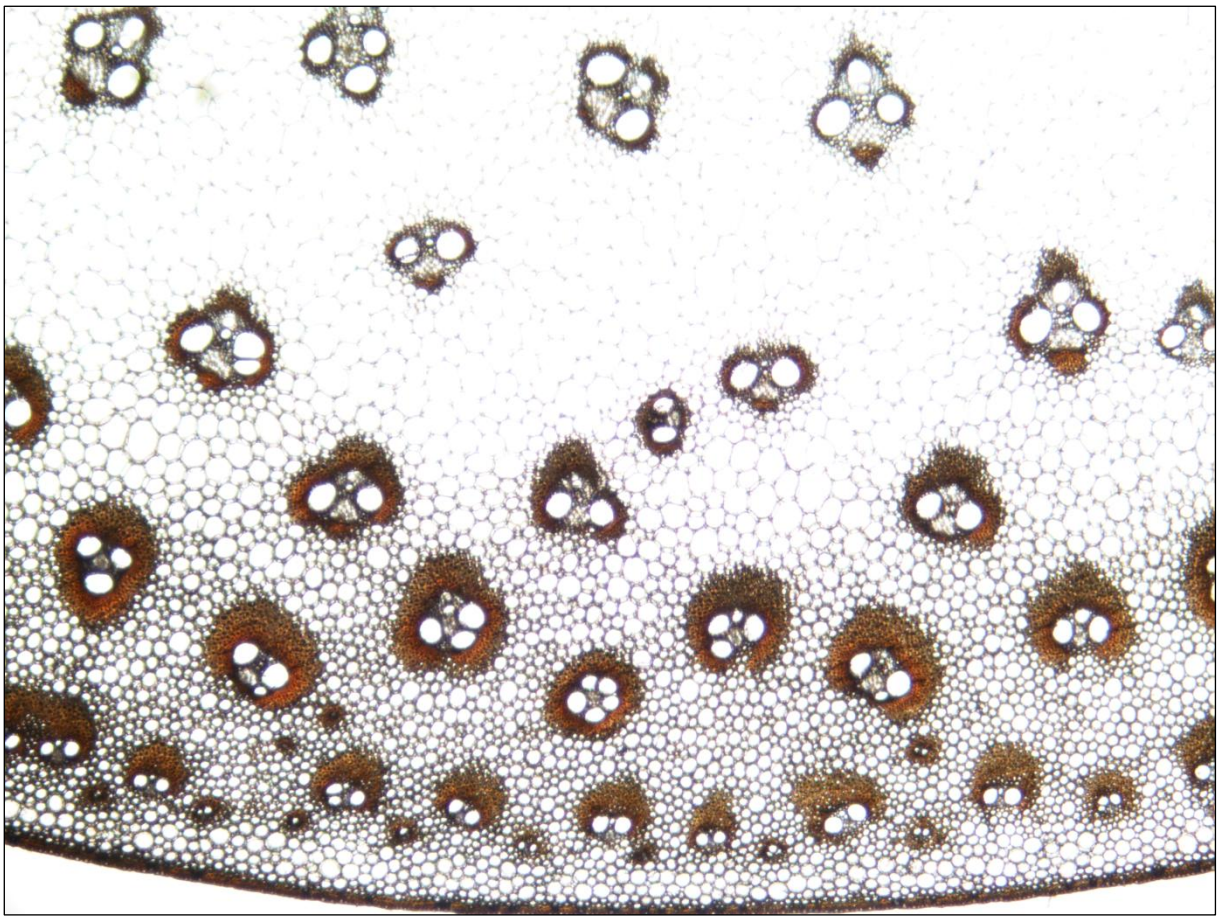


Figure 1: Transversal section of a maize stem: More and more developed vascular bundles are seen in the rind region

Vascular bundles are already formed in the roots. They grow up in the stem, splitting off for the development of new leaves during growth of the plant. No new conducting tissue is formed when the stem ages. The roots develop further during maturation.
In the rind region there are more vascular bundles present because it's the place where leaves start to develop. Some vascular bundles bend to the outside of the stem to become a midvein in a leaf. When sectioning younger internodes the rind region is smaller and the vascular bundles become less numerous. In younger internodes more vascular bundles already bended of for developing leaves. In the rind region the vascular bundles are more developed than in the pith region. A reason for that might be a higher concentration of auxin, as this hormone is one of the key regulators of vascular development. The vascular bundles in the rind region, who are more developed, contribute to the rigidity of the maize stem. The rigidity and strength of the stem is more important in the old and lower internodes of the stem. The older internodes have to carry the weight of the internodes above. That is why more vascular bundles are present and why they are better developed in the older internodes. On the other hand Botha and Cutler (2007) report that the difference in tissue composition between the rind and pith region might be due to the aerial habit of the stem, as well as its mechanical function.

2.3. Morphological traits

Boon et al (2008) investigated the length of internode 7-16 and the internode diameter in two cultivars. He determined that the internode length increased from internode 14 downwards to internode 8. However, internode 7 was shorter than internode 8. The internode near the ear is an exception to this tendency as it's always shorter than the neighboring internodes. A negative correlation was discovered by Struik et al. (1985).
Another trait is the internode diameter: it increases very consistently from the top of the plant to the base. There was a significant difference between the consecutive internodes (Boon et al., 2008). Nevertheless drought can reduce the length and diameter of the internodes below the ears (Espinoza-Paz, 1996). Bennouna et al. (2004) reported that water stress can reduce the stem diameter. The internode volume increased from the top to the base of the stem.

2.4. Anatomical traits

The digestibility can be influenced by anatomical traits. Out of results of experiments with rumen fluid incubation appears that the digestibility of the different tissues of a maize stem varies (Deinum and Struik, 1986; Cone and Engels, 1990). The different tissues are vascular bundles, sclerenchyma, epidermis, chlorenchyma and parenchyma. The degradation of chlorenchyma and parenchyma is almost complete after 24 hours of incubation. On the other hand, the breakdown of vascular bundles, sclerenchyma and epidermis is far from completed, even after long incubation (96h) (Engels and Schuurmans, 1992).
When the composition of a maize stem is taken into consideration, 80% of the weight of the stem are vascular bundles and sclerenchyma. These are the less digestible parts and so the whole plant digestibility can be improved by stimulating the digestibility of these tissues. The biggest part of the vascular bundles and thick-walled sclerenchyma can be found in the rind region. The pith consists mainly of thin-walled parenchyma and small vascular bundles (Boon et al., 2005).

2.4.1. Amount of vascular bundles

The number of vascular bundles per cross section increases from the top to the base of the stem. The amount of vascular tissue in the rind region versus the pith region is analysed by Esau (1977) and Wilson et al. (1993). There is a higher proportion of vascular tissue present in the rind region than in the pith region. The tissue in the rind region also had greater ferulic acid and *p*-coumaric acid levels than the pith region. This was expected because there is more lignin deposition in the rind region to support the conductive and supportive tissues of the internode (Morrison et al., 1998).

2.4.2. Sclerenchyma

The number of layers of sclerenchyma tissue who surround the vascular bundle affect the digestibility. According to Boon et al. (2008), the sclerenchyma at the top of the stem was roughly equally positioned on the abaxial and adaxial side of the vascular bundles. When elder internodes were examined the sclerenchyma was dominant on the adaxial side of the vascular bundle. This trend was also seen when a young vascular bundle was compared with an elder vascular bundle. Boon et al.

(2005) found that the number of sclerenchyma layers on the central side of the vascular bundle varied, especially at the base of the internode. Cone and Engels (1990) tested the difference between two growth conditions and stated that the diameter of the parenchyma cells was larger and the cell walls of the subepidermal sclerenchyma were thicker in the maize grown in a low temperature environment compared to maize grown in a high temperature environment. Within the internode, the number of sclerenchyma layers increased from top to base.
On the other hand, cell wall thickness of the sclerenchyma tissue decreased from top to base in the internode (Boon et al., 2005). The number of subepidermal sclerenchyma layers was significantly influenced by the factors year, position and cultivar, and by the interactions year x position, year x cultivar and position x cultivar (Boon et al., 2005).
The subepidermal sclerenchyma is also a possible trait for digestibility. It exists out of 1-4 layers. The number of layers are increasing slowly from internode 16 to internode 12. The internodes older than internode 12 tend to have less subepidermal sclerenchyma. The number of layers sclerenchyma tissue adaxial to the vascular bundles in the rind region increases from the top to the base of the stem, leveling off at about internode 8.

2.4.3. Cell wall thickness

As rumen bacteria have to digest the cell walls to go to adjacent cells, the cell wall thickness is very important. The cell wall thickness of the sclerenchyma tissue increases from internode 15 to the base of the stem. This tendency was seen both in the subepidermal layers and in the vascular bundles. Boon et al. (2005) stated that the sclerenchyma tissue at the top of the internode had a thicker cell wall on the central side of the vascular bundles in the rind (4.2-4.7µm) than in the subepidermal layer (3.0-4.0µm). Sclerenchyma cells at the base of the internode had the same thickness on the central side of the vascular bundles in the rind (2.2-2.3µm) and in the subepidermal layer (2.1-2.7 µm). Cone's and Engels' (1990) experiment showed that thin cell walls and small cell diameters can be the cause of intensive cell elongation in plants, grown in a high temperature environment. The rind region contains lots of vascular bundles embedded in small-diameter parenchyma tissue. In contrast to this, the pith region showed few vascular bundles and the parenchyma cells were larger. This caused a lower cell wall concentration in the pith region than in the rind region. The high cell wall concentration at the stage of full development in the rind region was caused by the thicker parenchyma cell walls. Also, more sclerenchyma layers were observed around the vascular bundles than in the pith region (Jung and Casler, 2006)
An experiment with two varieties showed that the thick-walled rind region, which exists mainly out of epidermis, subepidermal sclerenchyma tissue and subsclerenchymal parenchyma with scattered vascular bundles in it, occupies 20% of the cross-sectional area. Nevertheless these tissues contribute to 80% of the dry weight of the stem (Engels, 1992).
The cell wall concentration was investigated by Morrison et al. (1998). From the youngest internode (I 13) to the oldest (I 7), cell wall concentration became denser in the rind tissue. In the pith region the concentration increased until internode 10 and plateaued afterwards.

The area of phloem tissue in the vascular bundle decreases from the top to the base of the stem. This is connected with the reduction in number of vascular bundles per unit area.
As the internodes become older, vascular bundles tend to lie further apart. This means there is more sclerenchyma tissue in between different vascular bundles.

2.5. Lignin

Not all the tissues or cell types lignify to the same extent. In forages the collenchyma and phloem fiber cells do not, or rarely lignify. Even in mature tissues (Wilson, 1993). Also Jung and Casler (2006) found that, at the time of maturity phloem was the only tissue in maize stems that was not lignified. Although some pith-region parenchyma immediately next to a vascular bundle wasn't lignified either.
Jung and Casler (2006) also showed that during elongation only protoxylem vessels stained positively for lignin. After elongation, metaxylem, parenchyma and sclerenchyma lignified but ploem did not. The confinement of lignin deposition to only the protoxylem vessels in elongating internodes is a general feature of forages, as it was also observed in alfalfa. Current data shows limited lignin concentration in the youngest maize internodes and even demonstrates a proportional decline in lignin of the cell wall during the elongation. The cells or tissues that are lignified are less digestible (Jung and Himmelsbach, 1989; Akin et al., 1990). The accessibility of the carbohydrates in their walls to rumen microbes is limited by the composition and structural arrangement of each cell type within a tissue.

2.5.1. Chemical composition

There are different types of lignins. Lignins can be described as three-dimensional networks built up out of phenylpropane units as seen in Figure 2.

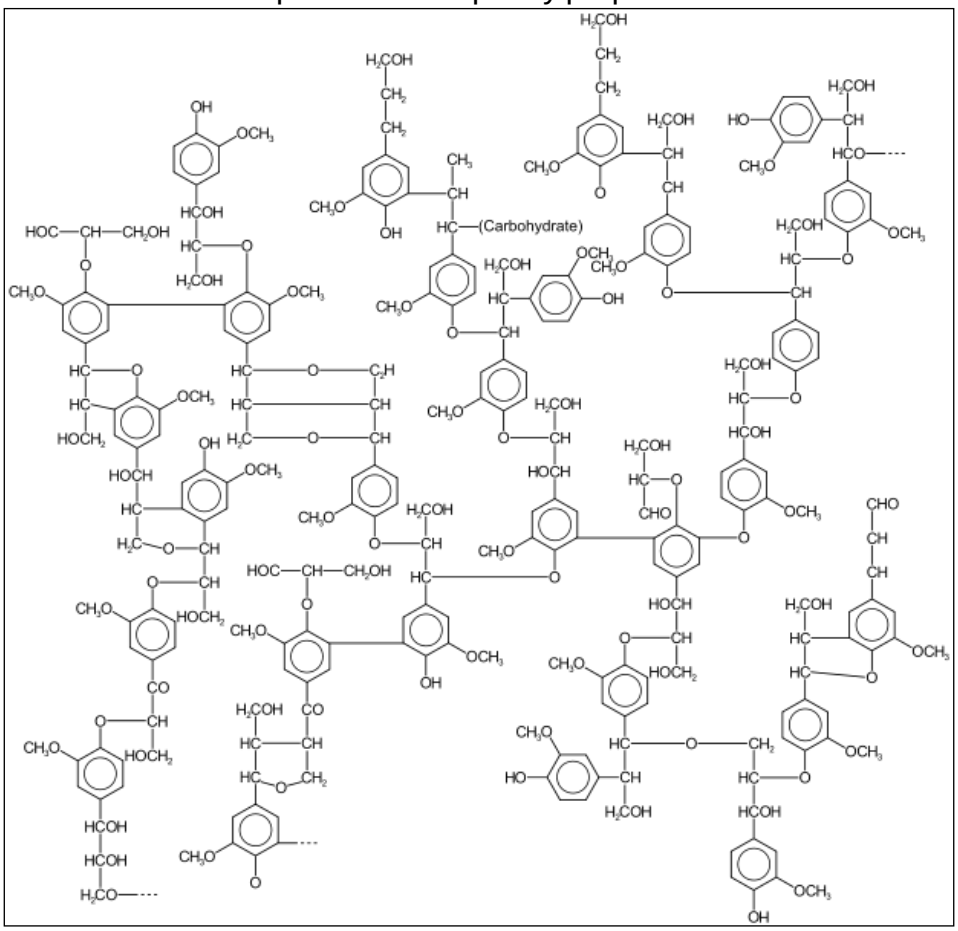


Figure 2: Chemical structure lignin (Sustainable energy, 2012)

Lignin is a complex polymer that mainly consists out of three monomers: coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Boudet, 2000; Humphreys and Chapple, 2002). Coumaryl alcohol is mainly found in lignin from grasses or forages (Yeung, 1998). The three chemical formulas are shown in Figure 3Figure 3

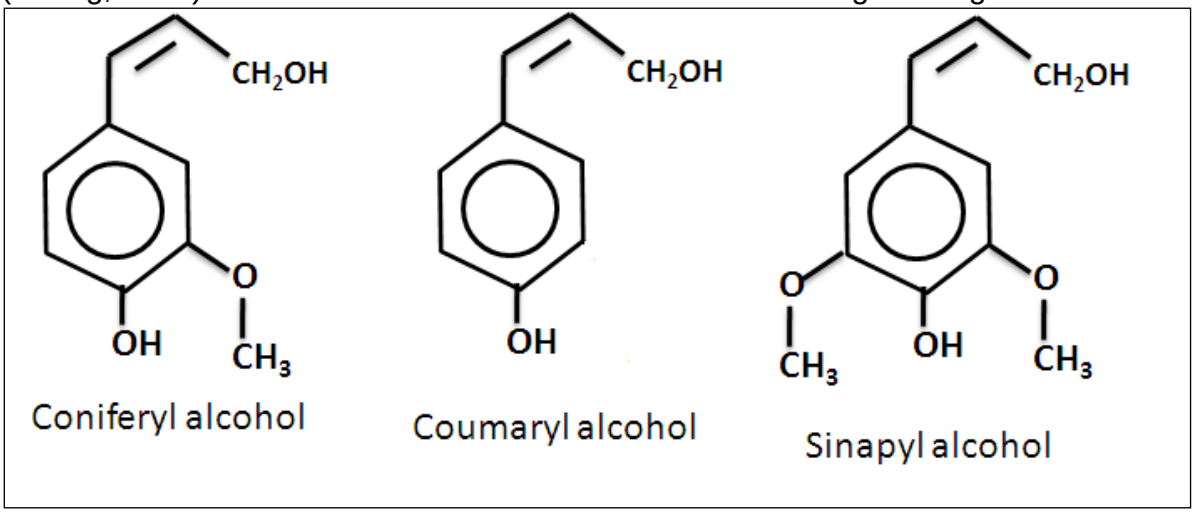


Figure 3: Precursors lignin (Yeung, 1998)

After radical-mediated polymerization these monomers contribute to *p*-hydroxyphenyl, guaiacyl and syringyl. The ratio of these substances in different types of lignin differs among plants. Lignin is bound to the cell wall matrix covalently through ester bonds with uronic acid residues and equally through phenolic acids. These acids contribute to the lignification process in the plant cell wall (MacAdam and Grabber, 2002). The fusion of cellulose and noncellulosic compounds by lignin give mechanical strength and hydrophobicity to the cell walls. On the negative side, it also causes a decreased accessibility for the gastro intestinal enzymes and rumen micro-organisms (Theander and Westerlund, 1993). Jung and Buxton (1994) elucidate that it's not the lignin content that is the major factor to influence the digestibility. Variation in subunit composition is the biggest affection to the chemical and physical properties of the cell wall and also to the digestibility.

An increase of phenolic compounds results in a decrease in digestibility, since they are the building blocks of lignin (Jung and Sahlu, 1986). Two of those phenolic compounds are *p*-coumaric acid and ferulic acid. Primary tissues of grass cell walls are relatively rich in ferulic acid and yet they are highly digestible nonetheless (Harvey and Hartley, 1976; Akin and Chesson, 1989). Next to that, *p*-coumaric acid is present in secondary cell walls and these tissues are less digestible (Burrit et al., 1984; Akin and Chesson, 1989; Jung and Casler, 1990). Morrison et al, (1998) showed that the rind region of a cross section, which lignified earlier and to a greater extent than pith tissue, had significantly higher levels of ferulic acid and *p*-coumaric acid. Harris and Hartley (1976) investigated the relation between fluorescence / staining and digestibility. They related fluorescence in digestible walls of immature grasses to the presence of ferulic acid. When the tissues are treated with a hydroxide. the fluorescence is reduced. The fluorescence is mainly caused by indigestible walls that contain phenolic compounds. Xylem vessels for example show high fluorescence. Delignification of the tissue resulted in reduced fluorescence and staining (Akin et al., 1990). Digestible secondary cell walls are fluorescence and Acid Phloroglucinol (AP) positive. However, they become AP negative when treated with permanganate. On the other hand, the primary cell walls are still AP positive but fluorescence negative after permanganate treatment. This indicates that staining and fluorescence only indicate that phenolics are present and not whether the cell wall is digestible or not (Akin et al., 1990). Another experiment with sclerenchyma tissue in maize also showed that the secondary cell walls are biodegradable although they were intensively stained with lignin detecting dyes (Engels, 1989; Cone and Engels, 1990).

Jung and Deetz (1993) stated that ferulic acid is only esterified to the polysaccharide components of the cell walls in the developing primary cell wall and not in the secondary wall. Ferulic acid also provides ether bond sites for monolignol in the primary cell wall. This is an explanation why lignification starts in the primary cell wall region and proceeds only later into the secondary cell wall.

All the secondary thickened cell walls showed an intense positive staining with phloroglucinol (Mulder et al., 1992). The chemical structure of phloroglucinol is seen in Figure 4. Occasionally the middle lamella and primary cell wall were more intensively stained. A red color after staining with phloroglucinol was correlated with a high lignin content. This also means that the tissue is more resistant to digestion (Akin and Burdick, 1981; Akin and Chesson, 1989).

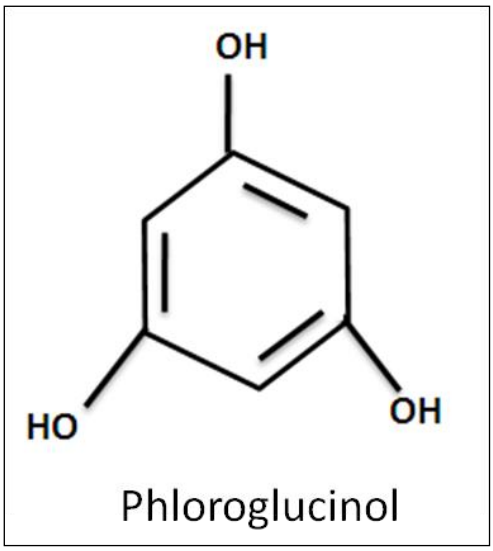


Figure 4: Phloroglucinol

Lignin can also be divided on the basis of their susceptibility to hydrolysis. Non-core lignin consists out of low molecular weight phenolics. These phenolics are quickly released from the cell walls by hydrolysis (Hartley and Ford, 1989). Core lignin is highly condensed and is largely resistant to mild degradation processes, as opposed to non-core lignin. The two types are important because according to Jung, 1989. the forage nutritional value is not only influenced by the amount of lignin but also by the type of lignin.

Lignification is correlated with a decreased digestibility for over fifty years. Nevertheless the exact mechanism is not determined yet. Plant tissues are always a composition of different cell types. The walls of the different cell types vary greatly in digestion characteristics and accessibility (Akin, 1989). The factor that influences the cell wall concentration and composition of forages most of all is the plant maturity (Jung, 2011).

2.5.2. Lignin development

Secondary wall deposition and lignifications do not begin until complete growth of the plant and elongation starts. Once secondary cell wall thickening begins, the primary cell wall is the first region to lignify. Next to the primary cell wall also the secondary cell wall lignifies (Terashima et al., 1993). The final concentration of lignin in the primary cell wall is higher than in the secondary cell wall but since the secondary wall is larger, the total amount of lignin is the highest in the secondary cell wall (Terashima et al.,1993). During the deposition of lignin in the cell walls water is removed, and this causes it to become hydrophobic (Inomana et al., 1992). The extent to which plant tissues lignify is genetically determined. It can however be influenced by environmental factors such as temperature. Lignin is a highly necessary component of the plant. It inter alia protects the cells from pathogens, protects the whole plant against climatic influences and provides the plant's structural strength and rigidity (Boon et al., 2005).

2.5.3. Lignification and digestion

Non-lignified tissues are always completely digestible. However, if a tissue starts to deposite lignin, the digestibility decreases tremendously. In theory a piece of plant tissue with nothing but primary cell walls will be reduced in its digestibility from 100% to zero when secondary cell wall deposition occurs. Even though the secondary cell walls are highly digestible, the primary cell walls will act as a perfect barrier for bacteria unless mechanically ruptured (Engels, 1992). Any tissue with lignified cell walls is never completely digestible. A thin strongly lignified residual will always remain, even after very long rumen incubation times (Engels, 1989). This indigestible residual is what is left of the original, but intensely lignified primary cell wall. Because of the high lignin content the primary cell wall forms an impenetrable barrier for rumen bacteria. Rumen bacteria digest cell walls from the interior of the cell and proceed to other cells by attacking the lumen side of the secondary cell wall and, finally, the primary cell wall (Engels, 1989). Since the bacteria cannot digest the highly lignified primary cell wall, they cannot move on to the adjoining cells and so digestion comes to a hold (Chesson, 1993). In non-lignified tissues bacteria move to other cells by digesting the intermediary cell wall (Engels and Jung, 2005).

Knowing that rumen bacteria move through the digesta by penetrating lignified primary cell walls, there are big amounts of potentially digestible cell wall polysaccharides that are unavailable to rumen digestion because cells in the feed particles have not been physically ruptured by either chewing or mechanical grinding (Wilson and Mertens, 1995). An average feed particle in the rumen exists out of many hundreds of cells and more of 1/3rd of these cells will not be digested due to inaccessibility. Non-ruptured cells will be inaccessible because the surrounding lignified primary cell walls cannot be digested. This theory shows that lignified primary cell walls of non-ruptured cells are an impenetrable barrier for rumen bacteria. It also supports the statement that potentially digestible cell wall material is inaccessible when it's surrounded by non-ruptured cells with lignified cell walls in a larger feed particle (Jung, 2011). The hypothesis of Wilson and Mertens (1995) was analysed on grasses, but the concept also holds true for legumes.

2.6. Rainfall during growth season

The water need of the maize during the growth season is not constant. The biggest need for water is at the time of anthesis, when the maize is tasselling and the kernel is formed. This is seen by the curve in Figure 5.

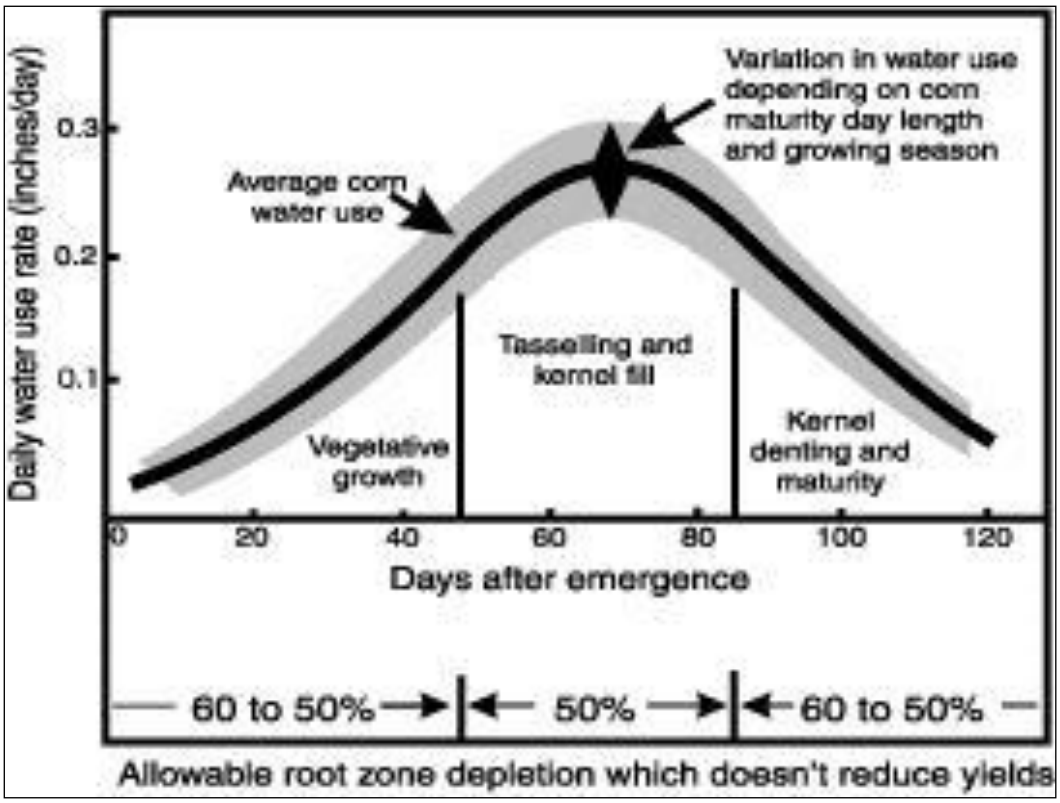


Figure 5: Water need for maize growth

In Figure 6 the rainfall data are shown from Wageningen during the period the maize was grown. It is observed that the period when the maize is in need of the most water at times of anthesis. This time corresponds with 1067,5 GDD (14/08/2012). When the rainfall data of Wageningen are observed, it seems that around 14th of august not much rainfall was registered. This might have influences on the composition of the maize stem. As the tasselling and kernel fill needs a lot of water, water from the stem will be repositioned to the kernel.

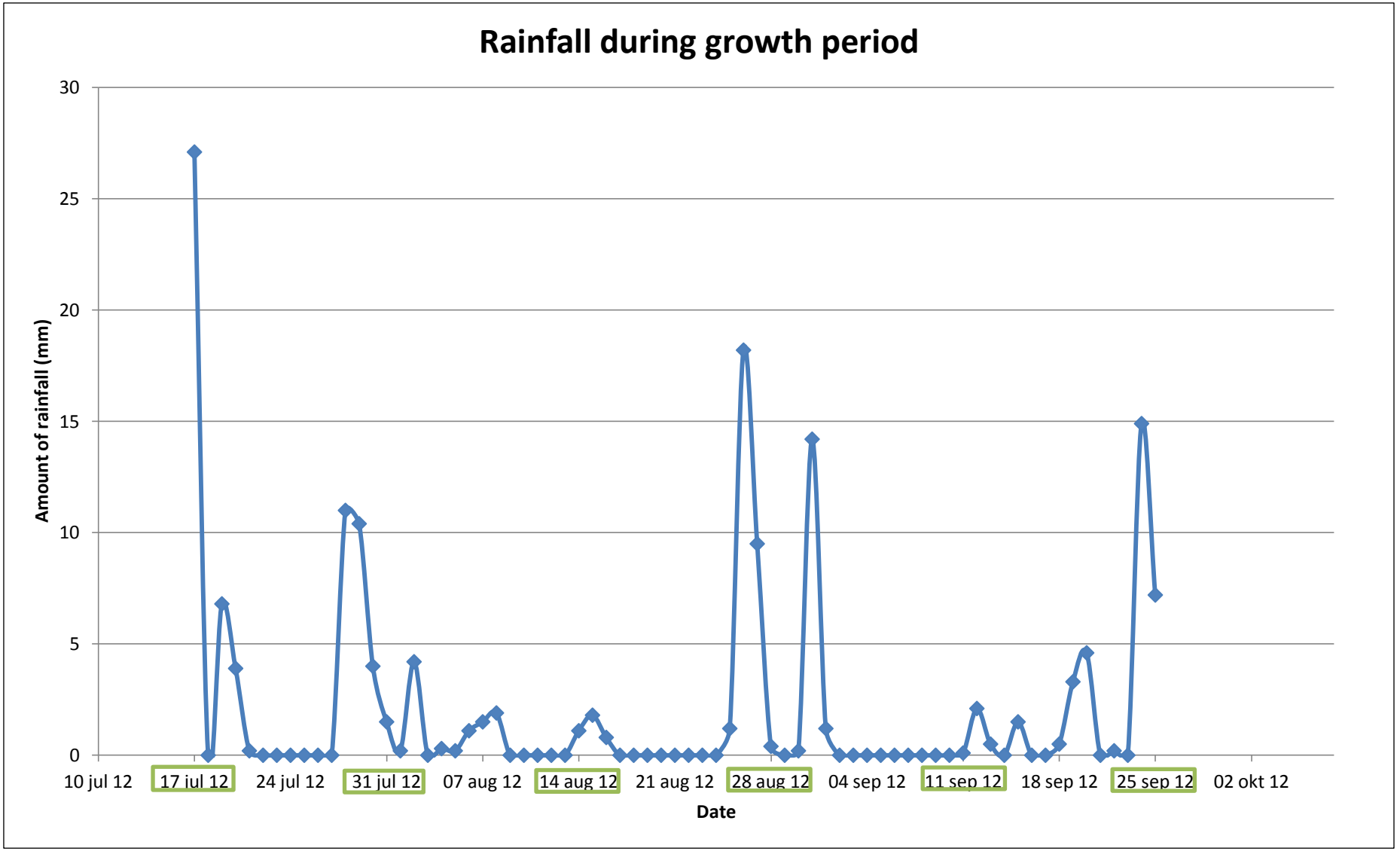


Figure 6: Rainfall in Wageningen during the periode the maize was grown (KNIM, 2012)

3. Materials and methods

3.1. Procedures

3.1.1. Maize growth

The maize was sown on the 7th of May on a sandy soil in Wageningen (Nl). Two varieties were grown in a randomized block design. The trail field existed of 2 cultivars and 4 replications, therefore there were 8 plots (Figure 7). Each plot contained 6 rows of plants. The outer sides of the field were surrounded by two border rows. The whole field was located in a bigger maize field to minimize the environmental influences on the growth.

Plot 5: Aastar	Plot 6: Ambrosini	Plot 7: Ambrosini	Plot 8: Aastar
Plot 1: Ambrosini	Plot 2: Aastar	Plot 3: Aastar	Plot 4: Ambrosini

Figure 7: Set up trail field

The harvest of the maize started on 17th of July. Due to the weather conditions the maize had 701,5 growing degree days (GDD) during the period previous to the first harvest. From that moment on the harvest took place every 14 days. The harvest days were converted into GDD to take the temperature into account. This influences the growth of the maize. The formula to translate data into GDD is shown below.

$$GDD = \left[\frac{(Tmax + Tmin)}{2}\right] - Tbase$$

Where Tmax and Tmin are the daily maximum and minimum air temperature, respectively, and Tbase is the base temperature. Tbase is the minimum threshold which is set at 10°C for maize. In theory, development does not occur unless temperatures exceed the Tbase.

In Table 1 the harvest dates are translated into GDD. During the experiment there were 6 sampling dates. The anthesis was observed on 14th of August. And that moment one whole plant was sampled of both cultivars. All the internodes were fixated and stored.

Harvest date	GDD
17/jul	701,5
31/jul	880,5
14/aug	1067,5
29/aug	1327,5
11/sep	1462,5
25/sep	1523

Table 1: Harvest dates converted to GDD

Each plot existed out of 6 rows. To randomize the harvested plants in each row 3 plants were harvested, consequently 3 plants were left in the field. For this project 5 plants were harvested in each plot and for a rumen incubation experiment 10 plants were harvested. Therefore 3 plants were surplus.

After the harvest, the plants were taken to a fresh lab in Unifarm (WUR). There, the leaf length of the 7th internode was measured. The recorded data are shown in Appendix A. The leaf length was mainly measured as control for the internode. The average leaf length of the 7th internode, of Ambrosini and Aastar should be around 60-65 cm (Struik, 2012). The results of the leaf length data are shown in the results in chapter 4.1.1. and 4.1.2.
Finally the 7th internode was cut out of the whole maize plant to prevent that the molecular labs should be entered with five whole maize plants. During cutting out the internodes, the latter were stored in a cold room. Immediately after the internodes were separated from the whole plant they were taken to the molecular lab in Radix (WUR).

3.1.2. Fixation

Fixation is a procedure to preserve cells of a small sample. After fixation the samples can be stored for a longer time than fresh samples. The preservation is not absolute; sometimes the samples can be affected by the storage. The formula of the fixate is as followed.

$$2\%\ PFA + 0.1\%\ GA + 0.05\ MP40 + 10\%\ 0.5M\ Pipes$$

Paraformaldehyde (PFA) is a necessary component of the fixate. Its main goal is fast penetration of the fixate in the tissue (Baum, 2008). The procedure for making a paraformaldehyde solution can be found in Appendix C. This initiates a structural stabilization of the tissue. Glutaraldehyde (GA) on the other hand is making sure of thorough cross-linking by more slowly penetrating (Baum, 2008). MP40 is a surfactant to ensure a good penetration of the fixate in the tissue. It lowers the surface tension of the fixation liquid. Finally the solution is buffered by 0,5M pipes, which is a standard buffer solution. More information about the pipes buffer solution is found in Appendix B.

The maize internodes were first cut with a sharp razor blade to get a piece of 1,5-2 cm thick. Next to that a tangential section of ¼ of the diameter was made. This to make sure a good penetration of the fixate. These pieces of maize were intended to make fresh sections. Thereafter pieces of 1 cm were cut from the maize internodes.

These were consecutively cut in half. These pieces were made with the intention to do plastic imbedding later.
The samples were fixated in 100 ml bottles per plot. First 70 ml of fixate was poured into the bottles. Next to that the samples were cut and immediately put into the bottles. This to minimize the drying of the samples. If the samples starts to dry the penetration of the fixate later decreases. If all the samples were cut they were put into a vacuum bowl. Subsequently a vacuum pump was connected to the bowl. The pressure was reduced to approximately 150 Torr.
Torr is a unit of pressure with the ratio of 760 to 1 atmosphere. The range of the vacuum pump was 150-760 Torr. The first time the bowl came under pressure a lot of air was pulled out of the maize samples. When the pressure was released the fixate was penetrated in the samples. This procedure was done a few times after each other, until no air bubbles were observed. Finally the vacuum bowl was left under pressure overnight.
The day after, the remaining pressure was released and the fixate was diluted in a ratio 1:1 with water. After that the samples were stored in a fridge (4°C).

3.1.3. Sectioning (fresh)

The samples stored in the fridge, first were washed in water to get rid of traces of fixate. Subsequently the maize piece was fixed in the Reichert sledge microtome (Reichert, 2012). Slices were cut of 100-120 µm. The sections were picked up using a paint brush and put in water, preventing from drying out. Finally the sections were placed in a well-plate with a drop of water.
For imaging on lower magnification sections were made of 250µm.

3.1.4. Staining

For the staining procedure, first the remaining water had to be removed from the wells. One drop of coloring agent was added in the well (1% Phloroglucinol and 20% HCl in water). At first a small experiment was executed to determine the staining time. Therefore four sections were made and four different staining times were used; 15, 30, 45 and 60 minutes. There was no on-sight difference so the shortest staining time (15 minutes) was used during the following experiments. The results of this experiment are shown in chapter 4.1.3.
After the 15 minutes staining time the coloring agent was removed from the wells. The sections were again washed in water and placed on an microscopic slide. After that a drop of water was added and the section was enclosed by a cover slip. Lastly the remaining water was removed by pushing the microscopic slide onto a paper towel.

3.1.5. Imaging

To prevent the stained sections from fading, they were always photographed within 15 minutes after staining. The pictures were made with a Nikon 80i (Nikon, 2012) microscope. The magnification range was from 20x to 1000x. For imaging on lower magnification to determine the amount of vascular bundles per surface unit a Zeiss stereo discovery microscope was used. The magnification range is 8x to 100x. As the whole sections were sometimes too big to capture at once, a method was searched to standardize the measurements. One picture was taken of arithmetic paper on 8x magnification. This way the pixels per millimeter could be determined and Image J could be calibrated.

3.1.6. Analyzing

For analyzing the results Excell was used. To help determine whether significant differences were present also IBM SPSS statistics 19 was used. For analyzing the pictures NIS elements and NIS viewer were used. To measure surfaces or count vascular bundles Image J was used.

3.1.7. Imbedding

To preserve the maize samples for long time and to make rotary microtome sections, the sample materials should be imbedded in Technovit. The imbedding is split up into two procedures.
First the maize tissue is imbedded in a polymer. Next the polymerized blocks are attached to a holder. This holder can be placed in the rotary microtome and sections can be cut. The detailed procedure of Technovit imbedding is found in Appendix D.

3.1.8. Microtome sectioning

For Technovit sectioning a Microm HM 340 rotary microtome (Thermo Scientific, 2012) was used. Before fixing the Technovit block in the microtome it was first prepared with a precision saw and a small file to make a smooth intersection. Sections were made of 0,5 – 3µm.
After the sections were cut they were immediately put in water with a small paint brush to prevent folding. Afterwards they were put on a microscopic slide and dried out. Also longitudinal section were made.

3.1.9. Staining

The Technovit sections were first stained with phloroglucinol (1% Phloroglucinol and 20% HCl in water). As no staining was seen after a long incubation time another phloroglucinol solution was tried. The second time a 1% phloroglucinol was dissolved in a solution methanol: HCl 1:1.
Furthermore Technovit sections were stained with a series of other coloring agents:

- Toluidine blue
- Safranin O
- Sudan 4
- Methyl red
- Acid Fushine
- Chloor zink jodium
- Coomassie Brilliant Blue (CBB)

During staining the slides were stored in a storage box. The different slides were incubated for at least 30 minutes. After that they were washed and enclosed with a drop of glycerol.

3.1.10. Imaging

Taking pictures of the sections happened with the same microscope as the fresh sections. Thanks to the thinner section the magnification could be higher and sharper pictures could be captured.

3.1.11. Weather influences on the growth of the maize

The harvest dates are corrected for the temperature by translating the harvest dates into GDD as shown in chapter 3.1.1. Also the rainfall influences the growth of the

maize. The water that maize needs to grow changes during the growth season. The rainfall data are shown in chapter 2.6.

3.2. Expermenta

3.2.1. Growth of leaves in time

By measuring the leaf length of the leaf linked to the 7th internode, a tool was used to determine the 7th internode. As the leaf length of the 7th internode should vary around 60 cm for Aastar and around 65 cm for Ambrosini (Struik, 2012). By measuring the leaf length also variations in growth can be shown. At anthesis one plant of each cultivar was harvested. The leaf lengths were measured for both cultivars and the growth of the leaves was shown within the plant.

3.2.2. Staining time

To determine the staining time, an experiment was carried out to compare the reaction of the coloring agent in time. Four different staining times were chosen; 15, 30, 45 and 60 minutes. Afterwards pictures were taken from sections of one internode and a vascular bundle 600µm from the epidermis. This way the staining intensity could be compared. The results are shown in chapter 4.1.3. The used samples were from the Ambrosini cultivar, the 5th internode and harvested after a growing period of 701,5 GDD.

3.2.3. Quantify red color

The fresh section were stained with phloroglucinol (1% phloroglucinol and 20% HCl). The duration was determined by a previous experiment and was standardized to 15 minutes. Thereafter pictures were taken of several sections and several vascular bundles. The quantification was tried to perform with Image J.

3.2.4. Number of sclerenchyma layers surrounding a vascular bundle

As was observed during the imaging of the maize stem sections, in the rind region a lot more sclerenchyma layers were present than in the pith region. The number of layers was determined on the phloem side of several vascular bundles in a range of distances from the epidermis. This was executed on high magnification (100x) images by NIS elements

3.2.5. Cell wall thickness of sclerenchyma tissue surrounding a vascular bundle

Cell wall thickness was measured by imaging on high magnification (400x) using fresh stained sections. Next the thickness of the cell wall was determined by NIS elements. This number should be divided by two because the cell wall is mutual for two adjacent cells.

3.2.6. Cell wall concentration in sclerenchyma sheath

Using Image J it was tried to manipulate a picture of a vascular bundle to a black/white picture. This way the cell wall was black and the cell lumen white. Having this separation the amount of white and black could have been determined and it was possible to calculate the cell wall concentration of the sclerenchyma sheath. To

become a black and white image a threshold should be set. In this attempt the Otsu method is used (Morse, 2000).

3.2.7. Incubation of sections in rumen fluid

To visualize the digestion of maize by rumen fluid, an experiment was set up. Fresh sections of 120µm were cut and attached to a microscopic slide with double sided tape. The sections were incubated in buffered rumen solution for 48h at 37°C and with a shaking frequency of 30 RPM/min. The intention was to take a picture before incubation and take a picture after incubation. This way it could be observed which tissues are easily digestible and which tissues it took longer to digest.

3.2.8. Development of vascular bundles from epidermis to pith

For this experiment pictures were taken from internode 5, 9, 13 and 17 of a maize stem of the Ambrosini cultivar. Several pictures are taken from random vascular bundles starting of close to the epidermis and ending in the middle of the maize stem. The exact distances of the vascular bundles in the pictures can be found in Appendix E. The pictures are merged into one image with the picture the closest to the epidermis positioned in the top left corner, subsequently the top right corner and bottom left corner and finally the picture from the middle of the maize stem in the bottom right corner. The difference in staining can be seen between the pictures.

3.2.9. Total surface of a stem section

This experiment was executed by cutting thick cross sections of a maize stem of both cultivars (250µm). These were photographed on a low magnification (8x). A segment of $1/4^{th}$ of the section was measured by Image J and multiplied by four to become the actual surface. The experiment was performed with a range of internodes (14, 11, 8, 5) so the trend from young to old within the plant at anthesis was visible. On the other hand three harvest times were investigated (701,5, 1067,5 and 1462,5 GDD). These are three growth periods where the trend is showed in time. Always the 7^{th} internode was examined.

3.2.10. Amount of vascular bundles in a cross section of a maize stem

The pictures on low magnification (8x) were used to count the vascular bundles of a total maize section. Therefore a Tally clicker counter was used, as seen in Figure 8.

Figure 8: Tally clicker counter

The vascular bundles were counted in a 1/4th segment of the cross-section and multiplied by four to become the total amount in the whole section. The experiment was again executed on sections of internode 14, 11, 8 and 5 from one maize plant harvested at anthesis and on sections of the 7th internode from three different plants harvested after three growing periods (701,5, 1067,5, 1462,5 GDD). The experiment was conducted on the two cultivars.

3.2.11. Surface of the pith and rind region

Using the picture on a low magnification (8x), a visual separation was made between the pith and rind region of the maize stem. The separation was made where the thick-walled sclerenchyma tissue became thin-walled parenchyma (Jung and Casler, 2006). The measurement was conducted using Image J.
First the experiment was carried out for the pith region of both cultivars and again in the same setup as the previous experiments (four different internodes within the plant at anthesis and the 7th internode of three plants with a different growing period). Subsequently the same was done for the rind region of Aastar and Ambrosini.

3.2.12. Amount of vascular bundles in the rind and pith region per surface

Following the number of vascular bundles per square centimeter was determined. This was achieved by dividing the calculated number of vascular bundles by the total surface of the stem sections. The experiment was performed on the 7th internode of three maize stems with different growth period to show the trend in time and on four different internodes of maize stems harvested at anthesis to show the trend within the plant. The comparison was made between two cultivars and both rind and pith region were treated.

3.2.13. Ratio of the surface of the rind and pith region

In this experiment there was determined which part of the total surface of a cross-section of a maize stem is pith region and which part is rind region. This ratio is defined both in time and within the plant for two cultivars. To achieve this, a small

calculation was done. The surface of the rind and pith were divided by the total stem surface and multiplied by hundred.

3.2.14. Ratio of the number of vascular bundles in the rind and pith region

In the different internodes within the plant the division of vascular bundles between rind and pith region was determined. This was realized by dividing the vascular bundles in the pith and rind region by the total vascular bundles in a cross-section. The ratios of both cultivars were compared to each other.

3.2.15. Correlation of a vascular bundle between the distance from the epidermis and the surface of the sclerenchyma sheath.

Images on high magnification (100x) were used to check whether there is a correlation between the distance a vascular bundle lies from the epidermis and the surface of the sclerenchyma sheath of that vascular bundle. The surface of the sclerenchyma sheat was measured using Image J. Lines were drawn by hand between the sclerenchyma tissue and the inside of the vascular bundles (vascular elements). The surface of the inside of the vascular bundles was subtracted from the total surface of the vascular bundle, resulting in the surface of the sclerenchyma sheath. As the distance from the epidermis was noted for every picture, Excell was used to calculate a possible correlation.

4. Results

4.1.1. Leaf length variation within the plant at anthesis

Leaf length within the plant at anthesis was measured to analyse the growth of the plant. The results are shown in Figure 9.

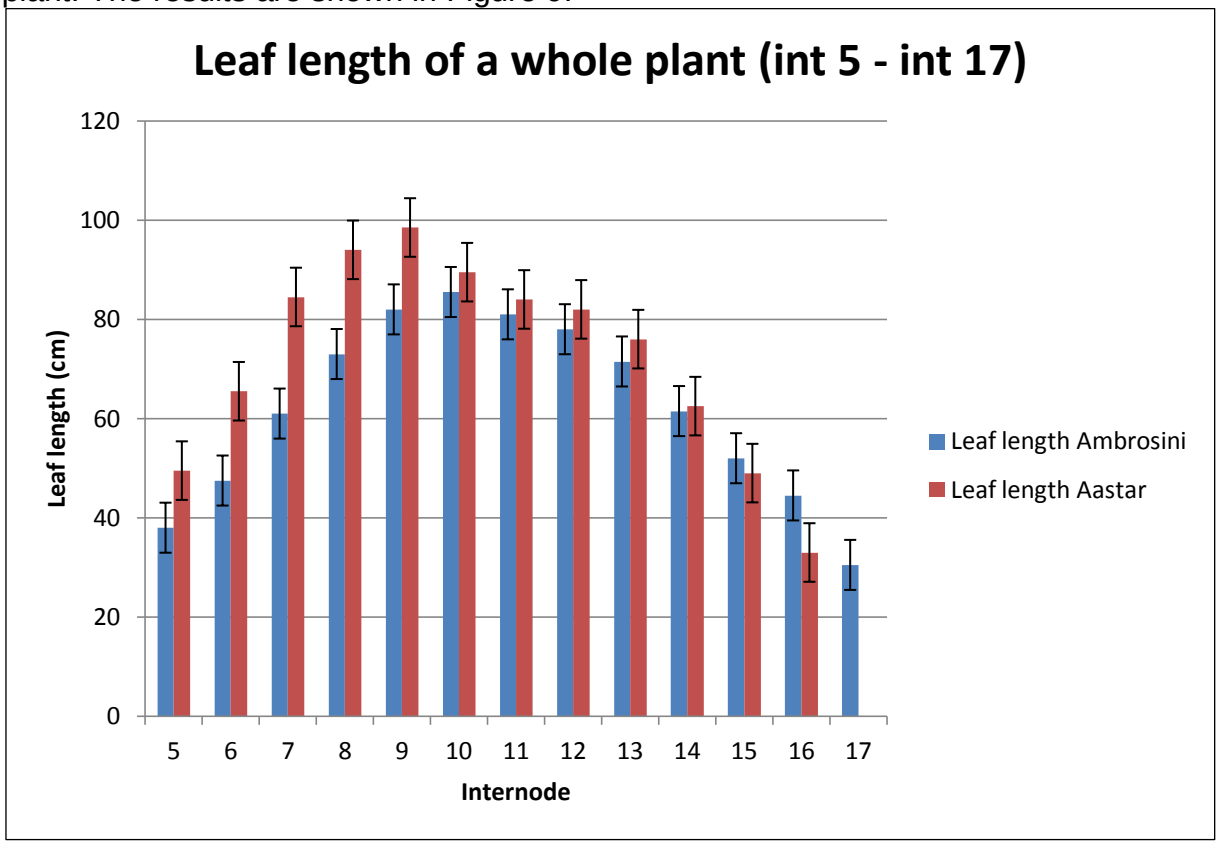


Figure 9: The variation of the leaf length, at time of anthesis, within the plant of two cultivars

In Figure 9 can be seen that the leaves of Aastar are longer than the leaves of Ambrosini. From internode 15 this trend changes and Ambrosini gets longer leaves. This shows that Aastar develops faster than Ambrosini. Another remark is that the maximum leaf length of Aastar is higher than the maximum leaf length of Ambrosini.

4.1.2. Leaf length of the 7th leaf of two cultivars in time

The leaf length was measured every harvest to get confirmation about the number of the internode. Per harvest 60 plants of each cultivar were harvested and analyzed. The results in Figure 10. show that the differences are very small and not significant. This suggests that the leaves were already fully grown at the first harvest. It seems that the average leaf length of both cultivars is not unambiguously 60 cm for Aastar and 65 cm for Ambrosini. They are more alike.

The data were collected by two different persons. One rounded the numbers to the integers and the other person rounded to one decimal. This way the minimum standard error was set on 1 cm and no significant differences were found.

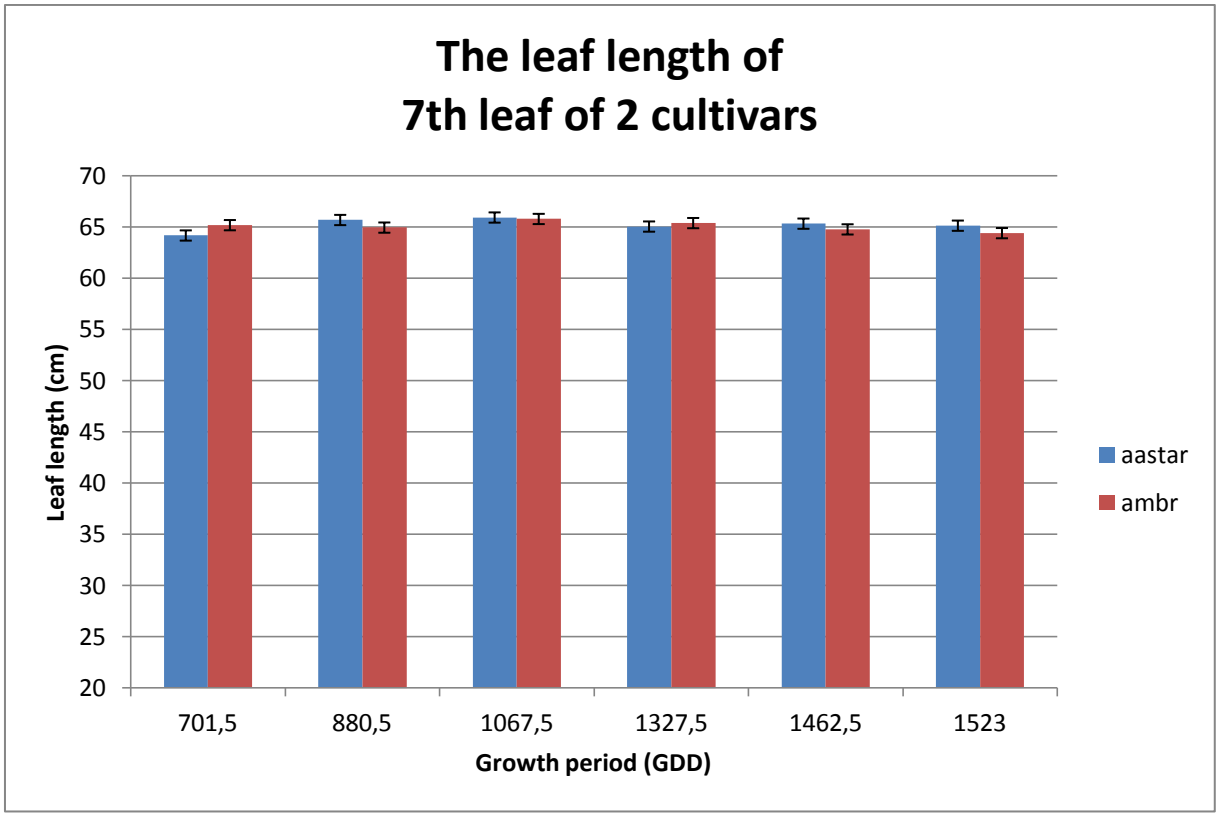


Figure 10: The leaf length of the 7th leaf in time of two cultivars

4.1.3. Staining time

The results of this experiments are shown in a merged picture in order to make the difference in staining visible. Four different staining times are seen in Figure 11

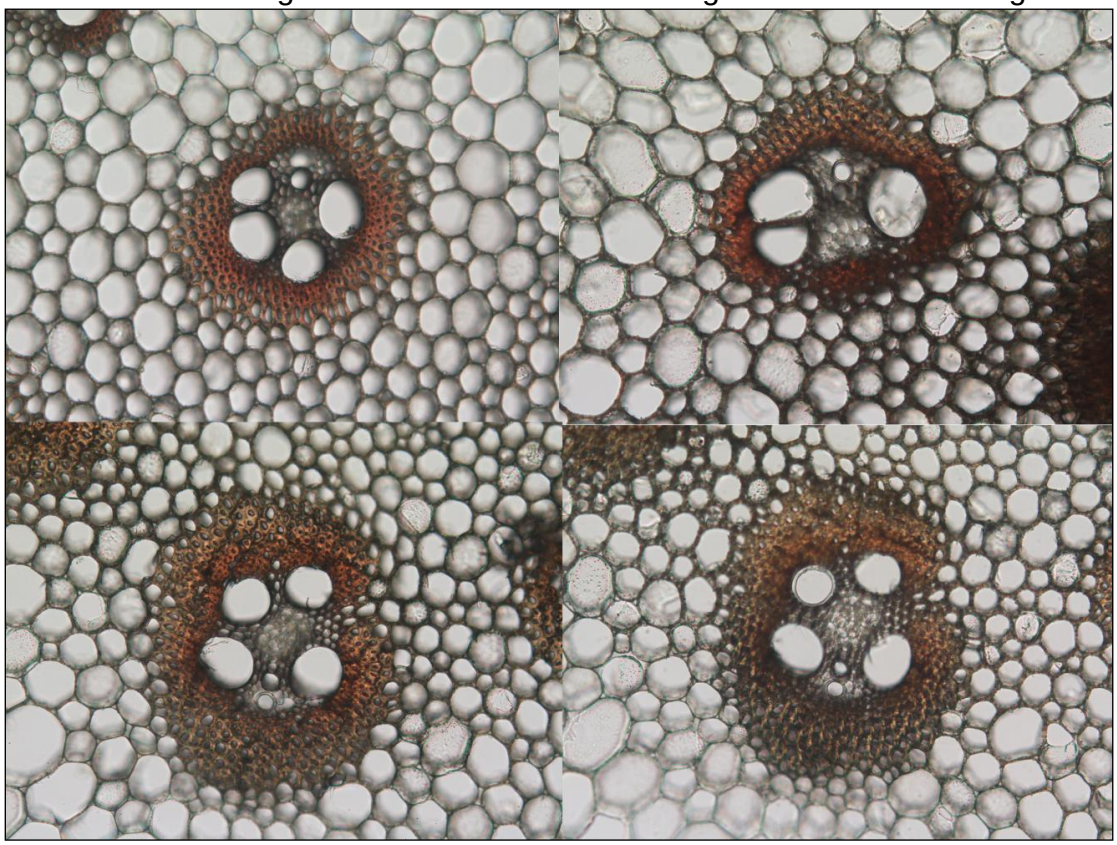


Figure 11: Staining intensity after four different staining times

The four different staining times are arranged in the picture as followed; Top left is 15 minutes, top right is 30 minutes, bottom left is 45 minutes and bottom right was stained for 60 minutes. As a result is concluded that no difference in staining intensity can be observed. For the other experiments 15 minutes of staining time was used.

4.1.4. Development of vascular bundles from epidermis to pith

As stated in the literature in chapter 2.4.2. the vascular bundles in the pith region have less sclerenchyma tissue surrounding than in the rind region. Another main difference is that the tissue surrounding the vascular bundle is changing from thick-walled sclerenchyma in the older internodes and rind region, to thin-walled parenchyma in the younger internodes and pith region. Furthermore, the sclerenchyma sheath surrounding the vascular bundles is smaller in the younger internodes than in the elder.
In the picture of internode 17 (Figure 15) is seen that the rind region of the stem section is only few cell layers thick. On the other hand in the sections of internode 5 the rind region is much bigger (Figure 12). When the intensity of the staining is investigated, it is seen that the color in Internode 5 is much brighter than the other internodes. This shows that there is more lignification in internode 5 than in the upper internodes. Which is stated by the literature in chapter 2.5.2.
Finally, from the pictures is observed that the vascular bundles in the younger internodes are smaller than in the older internodes.

For internode 17 only three pictures were made, because the internode was a lot smaller than the upper internodes.

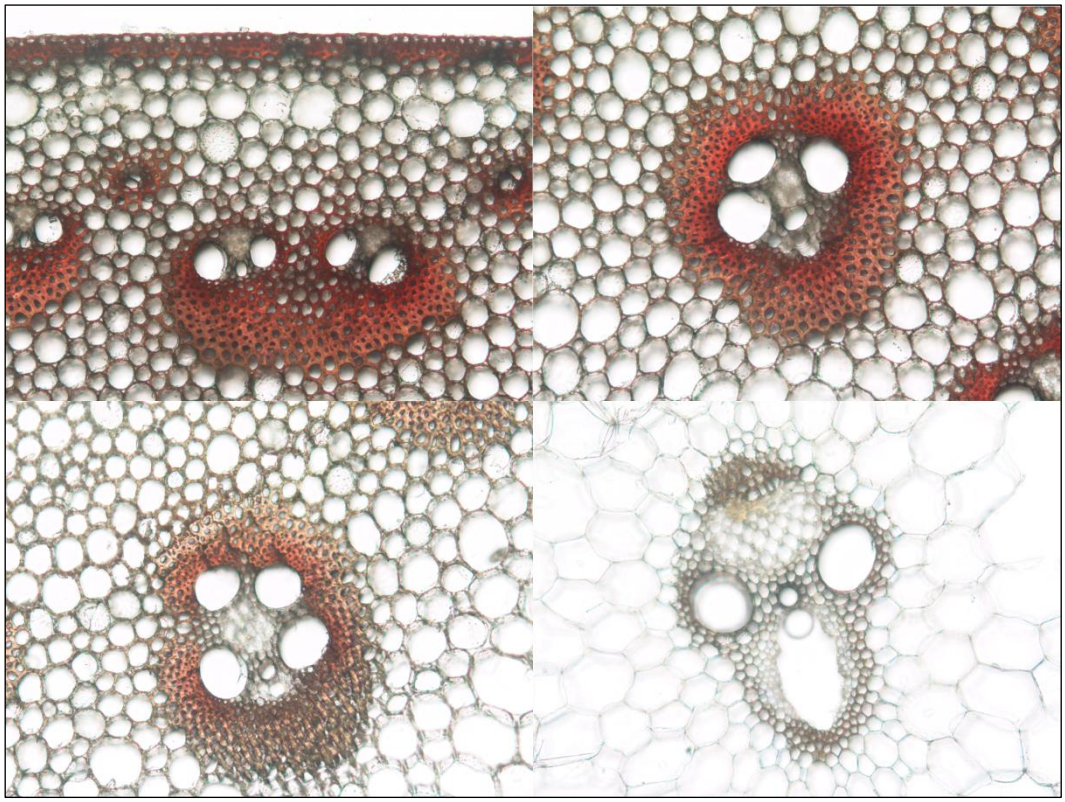


Figure 12: Development of vascular bundles in Internode 5 (ranging from epidermis to cortex from top left to bottom right)

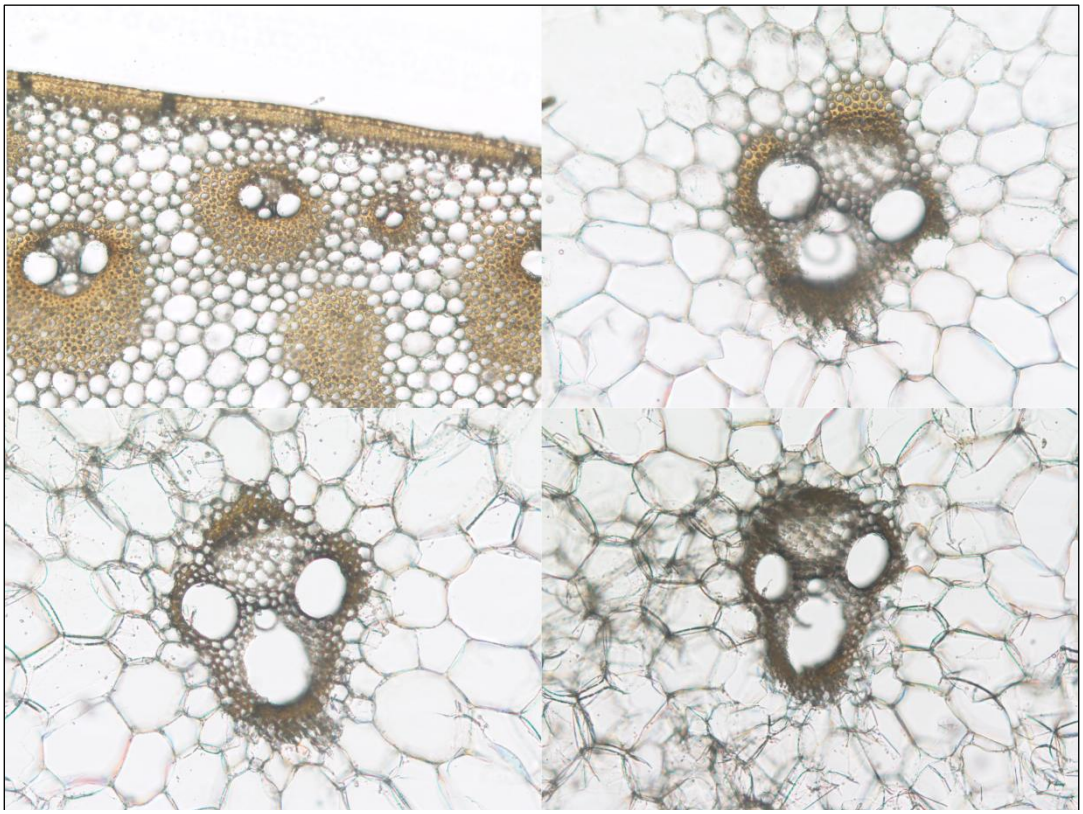


Figure 13: Development of vascular bundles in Internode 9 (ranging from epidermis to cortex from top left to bottom right)

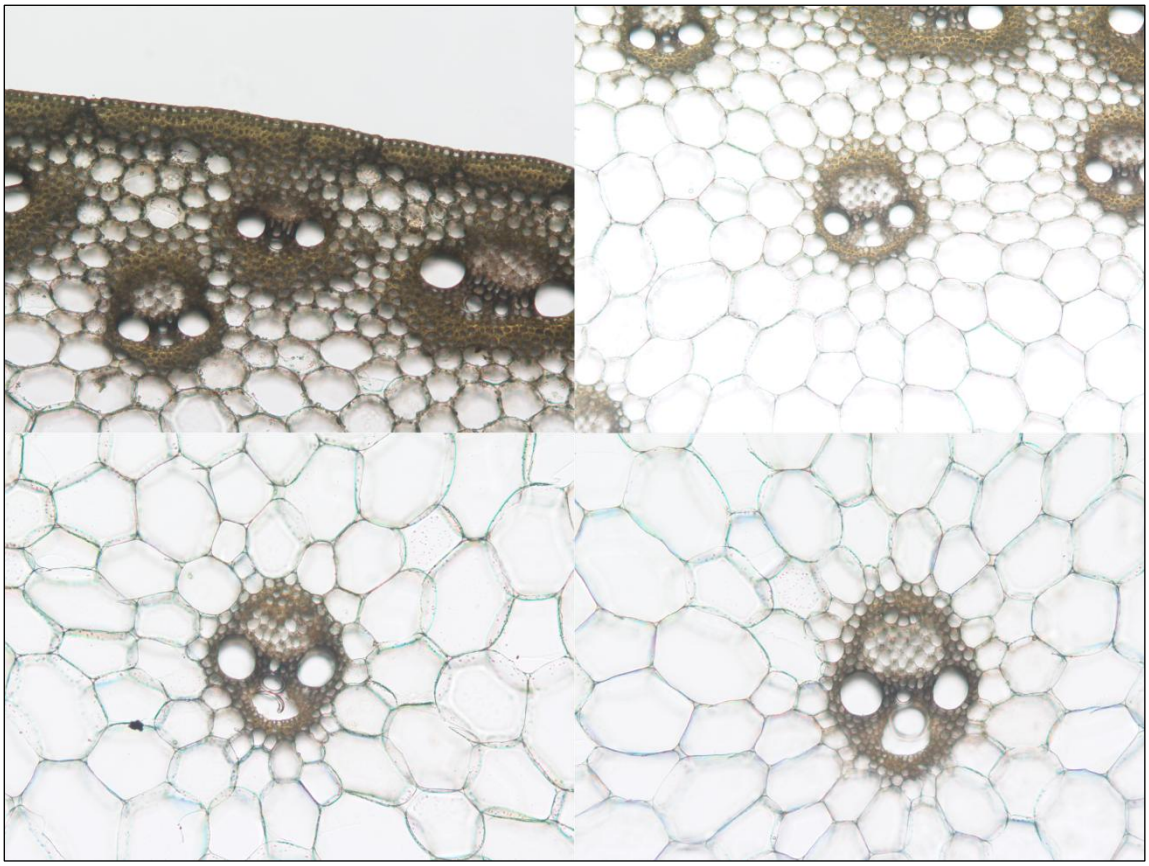


Figure 14: Development of vascular bundles in Internode 13 (ranging from epidermis to cortex from top left to bottom right)

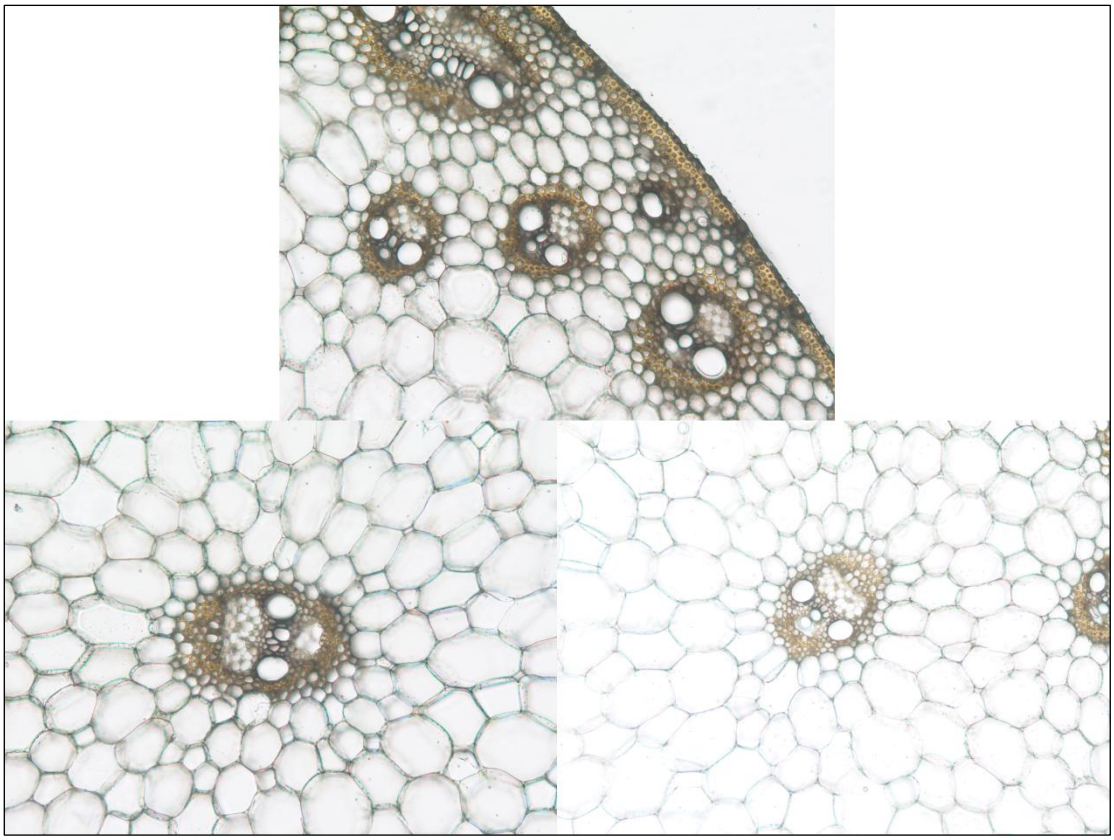


Figure 15: Development of vascular bundles in Internode 17 (ranging from epidermis to cortex from top left to bottom right)

4.1.5. Total surface of a stem section

In Figure 16 the trend is shown within the whole plants, as four different internodes are analyzed. There is an increasing trend, the surface of cross-section is increasing from young to old and becomes constant around internode 8. There is no significant difference observed between the two cultivars.

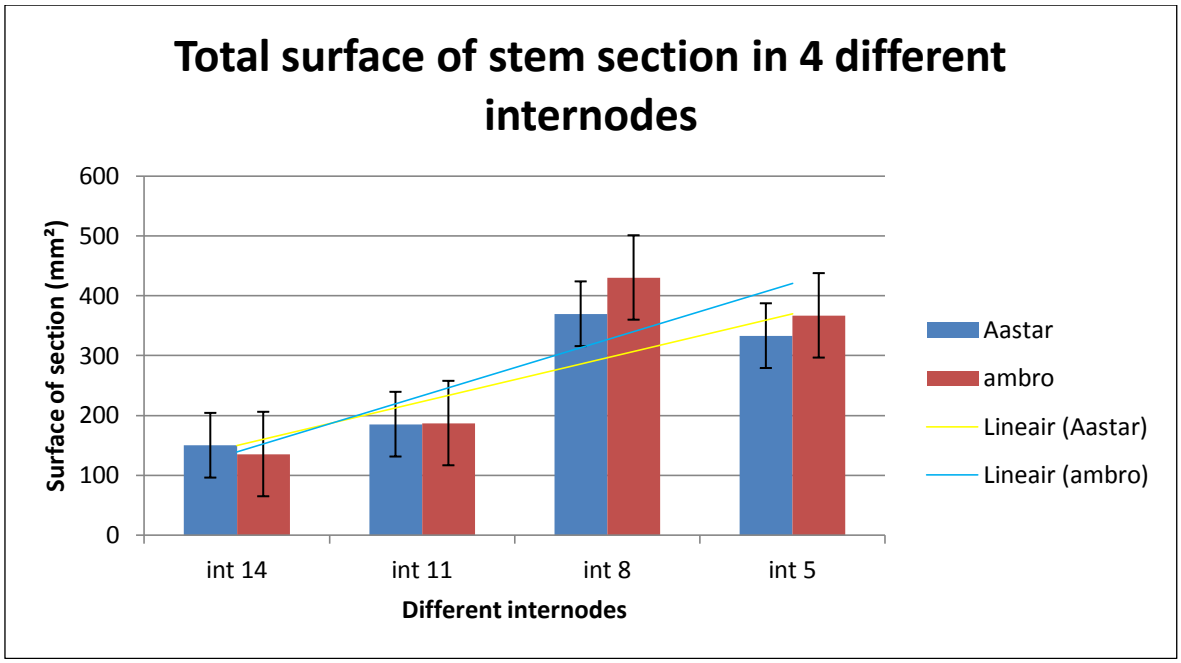
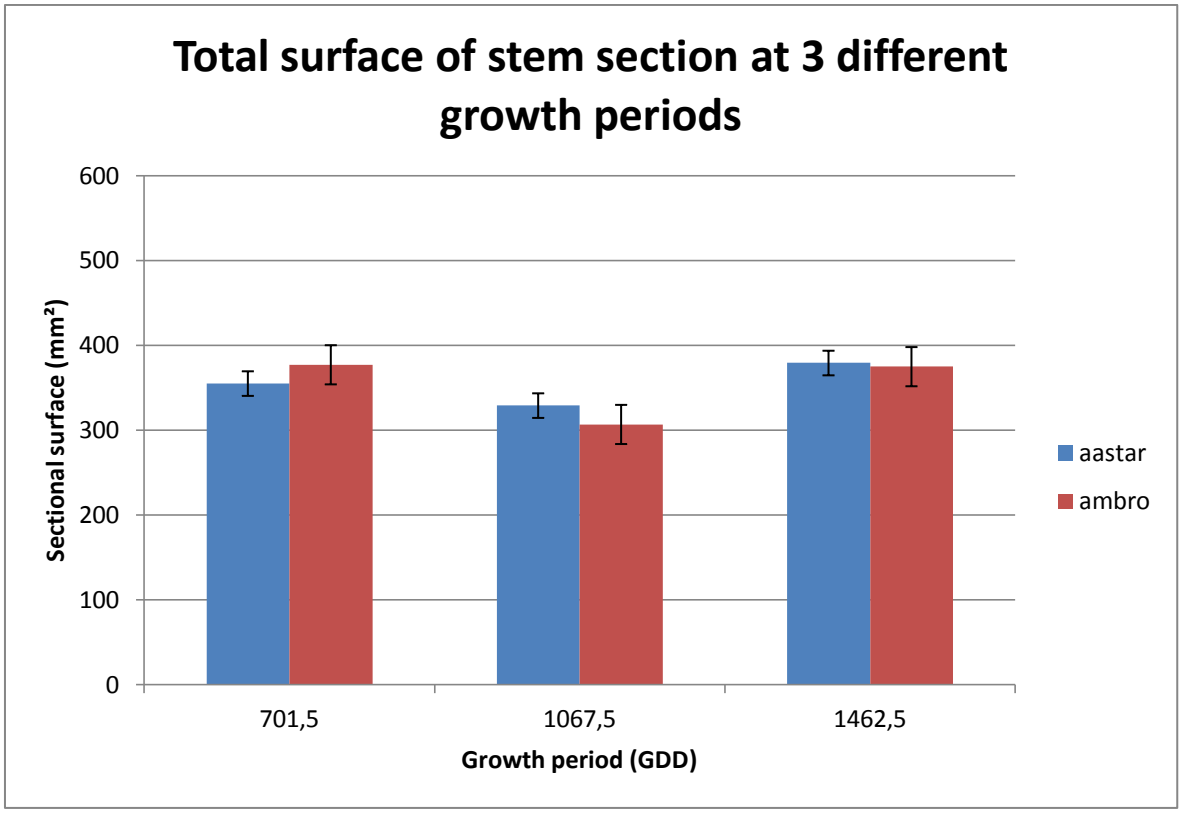


Figure 16: The trend of total surface of a stem section within a maize stem in two cultivars

The trend seen in the surface of the stem section is similar to the trend seen in the leaf length in chapter 4.1.1.

The data in Figure 17 shows that the surface is more or less constant with a small decrease around 1067,5 GDD. Again there is no significant difference between the two observed cultivars.



The surface of a stem section in internode 7 is around 375 mm² in the observed growth periods. This is stated by the trend seen within a plant, because the value also stagnates around 375 mm².

4.1.6. Amount of vascular bundles in a cross section of a maize stem

In Figure 17, first the trend is shown within one plant, harvested at time of anthesis. The internodes ranging from young to old show an increase in number of vascular bundles. This is a logical result, as the internode is getting thicker. A steep increase is seen between internode 11 and internode 8. This means that the maize stem has more strength and rigidity from internode 8 downwards. No significant increase is seen after internode 8. No significant differences are discovered between the two cultivars. The value levels at about 630 vascular bundles per stem section.

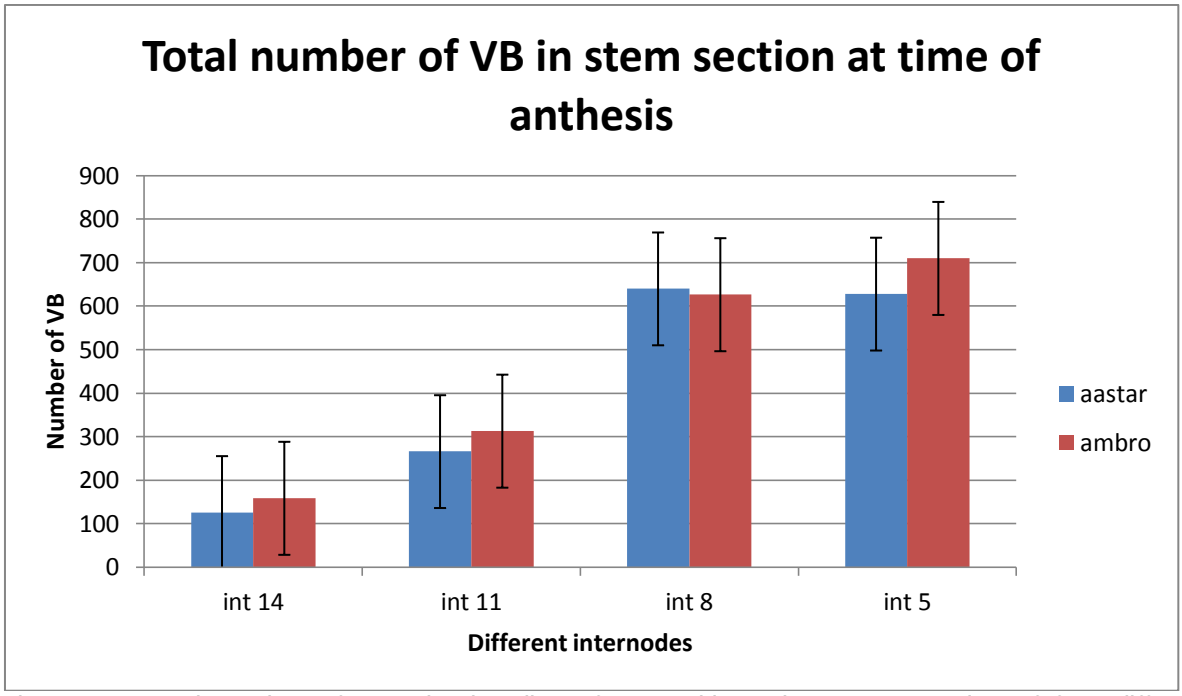


Figure 17: Total number of vascular bundles of two cultivars in a stem section of four different internodes

In the next graph, Figure 18 the total number of vascular bundles in a stem section are shown of three different harvest times. The value is approximately constant around 500 vascular bundles. Again a slight decrease is seen in the maize harvested after a growing period of 1067,5 GDD. Ambrosini has in all case more vascular bundles but the difference is only significant on the last harvest time. The difference between the cultivars is the smallest in the youngest harvest. When the maize is aging the difference between the two cultivars increases.

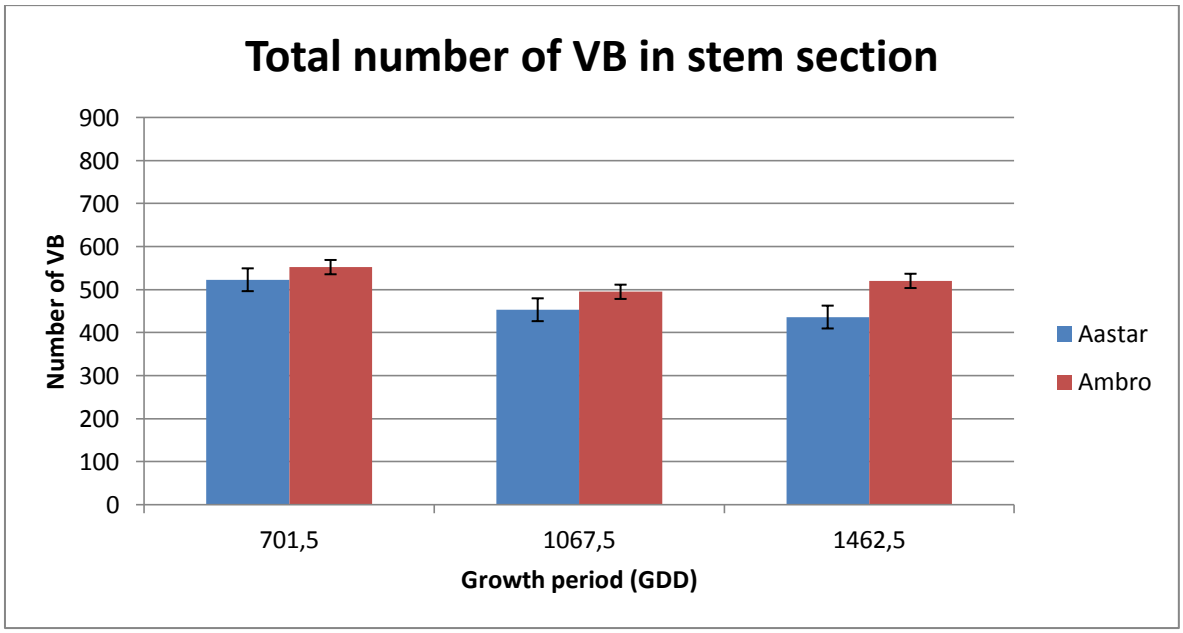


Figure 18: The total number of vascular bundles in a stem section in two cultivars harvested after three different harvest periods

4.1.7. Surface of the pith and rind region

4.1.7.1. Pith region

In Figure 19**Fout! Verwijzingsbron niet gevonden.** the surface of the pith region in four different internodes within a maize plant, harvested at time of anthesis, was reported. A clear increasing trend is seen as the internode is situated lower in the plant. This is due to the increasing diameter of the maize stem in the concerning internodes.

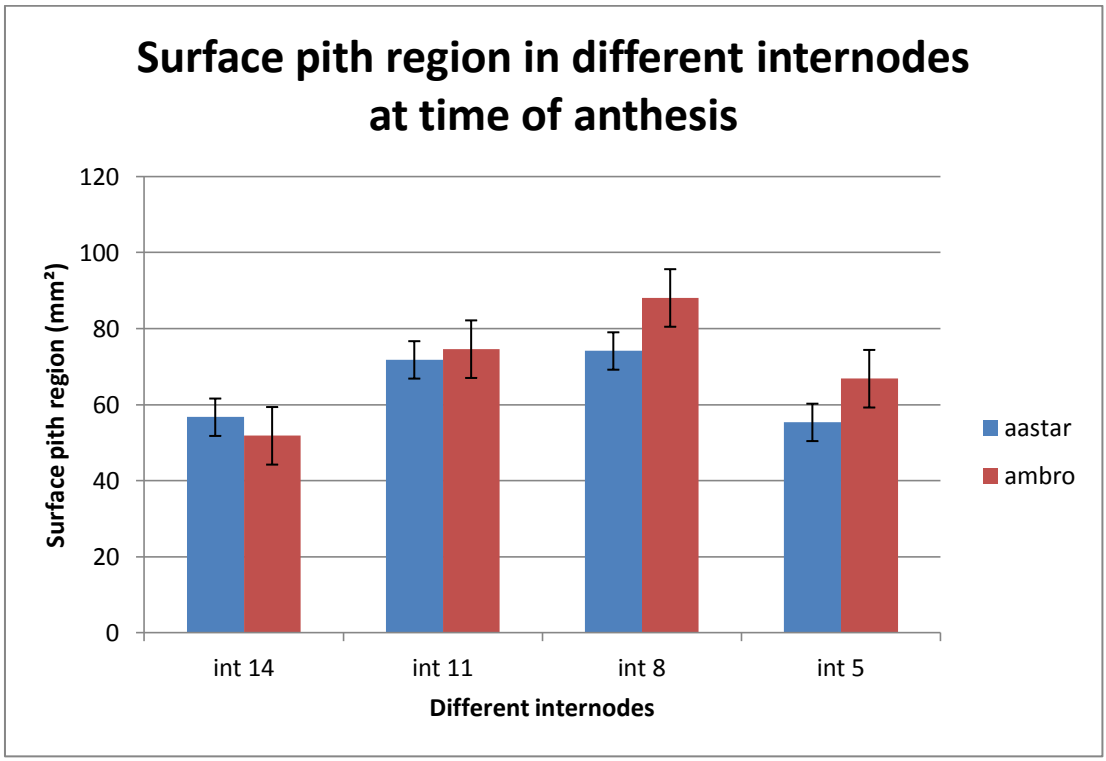


Figure 19: Surface of the pith region in four different internodes in a plant harvest at time of anthesis

From internode 8 to internode 5 a significant decrease is perceived. This is related to the function of the internode.

As the older internodes function as supportive for the whole maize stem, a big pith region would weaken their structure. The surface of the pith region was also measured in time (Figure 20). At three different harvest times a section of the 7th internode was analyzed. The surface of the pith region was around 60 mm². The maize harvested after a growing period of 1067,5 GDD has a slight decrease in pith region surface. No significant differences were observed between the two cultivars.

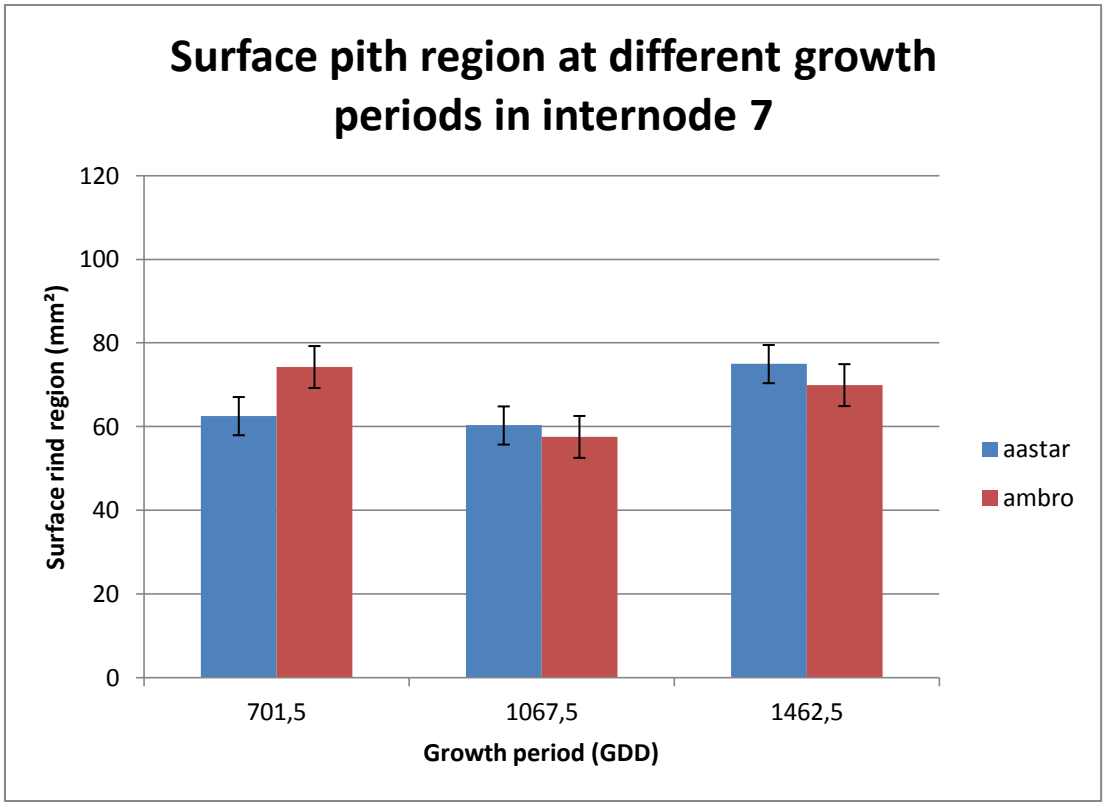


Figure 20: Surface of the pith region of the 7th internode of a maize stem harvested on three different harvest times

4.1.7.2. Rind region

Followed to the pith region, the surface of the rind region was measured. In Figure 21 the trend was shown within the plant and in Figure 22 the rind region was measured in the 7th internode of maize stems harvested on three different points of time.

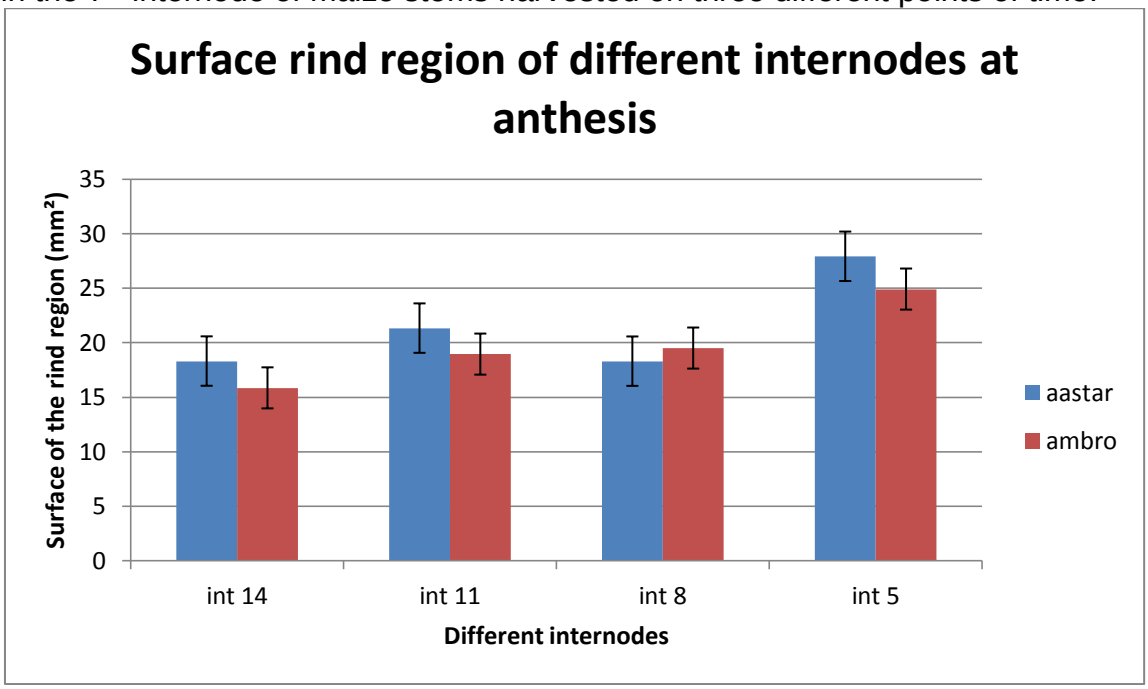


Figure 21: Surface of the rind region of four different internodes of a maize stem harvested at anthesis

The surface of the rind region is increasing from young to older internodes. This is because older internodes needs more strength to carry the maize plant. This is

proven by the big rind region in internode 5. In the young internodes the rind region of Aastar is bigger than Ambrosini. This is due to a faster development of Aastar in comparison with Ambrosini. The faster development of Aastar is also substantiated in Figure 22

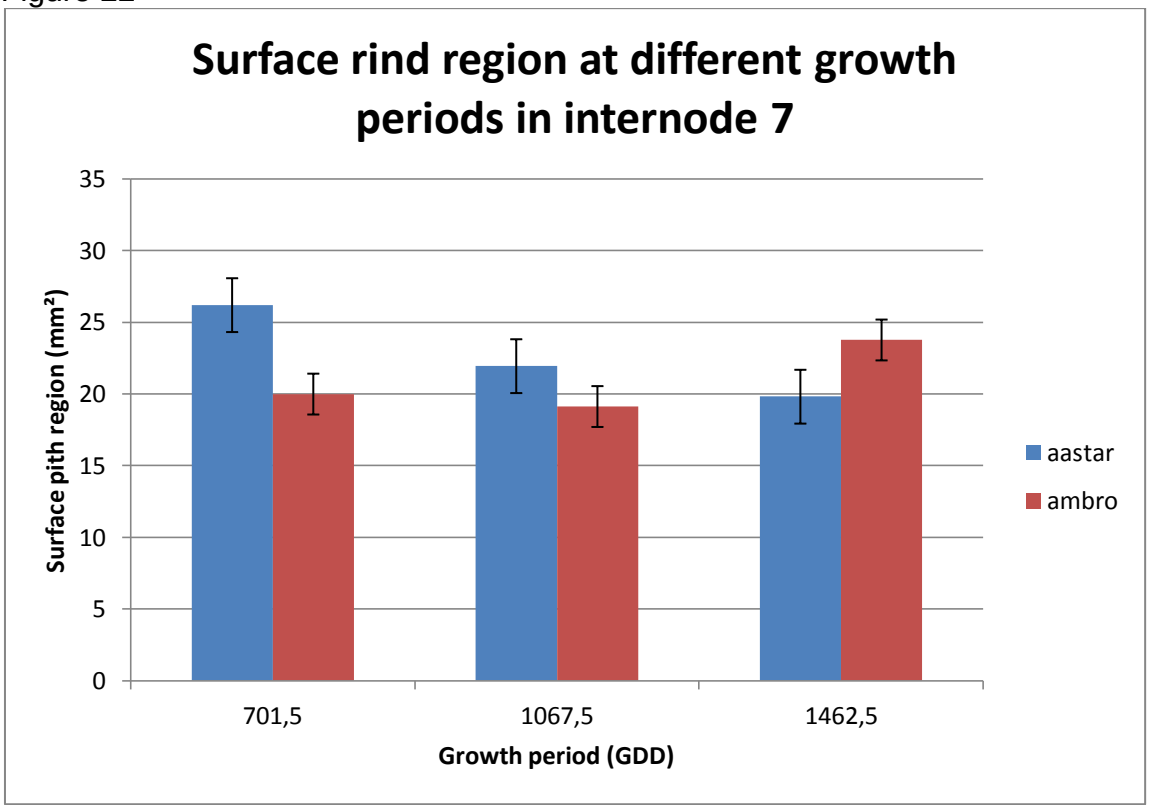


Figure 22: Surface of the rind region of the 7th internode of a maize stem harvested on three different harvest times

The graph above shows that Aastar has a faster development of the rind region in the beginning, but Ambrosini catches up in time and in the latest harvest time Ambrosini has the biggest rind region.

4.1.8. Amount of vascular bundles in the rind and pith region per surface unit

4.1.8.1. Pith region

In Figure 23 the internodes are ranged from young to old. A clear increasing trend is observed. This is stated by the literature in chapter 2.4.1. When going upwards in the plant (observing younger internodes), more vascular bundles are bending to create leaves. Consequently a lower vascular bundle density is observed in younger internodes. Another reason for the higher vascular bundle density is that the lower internodes need more strength to carry the whole maize plant.

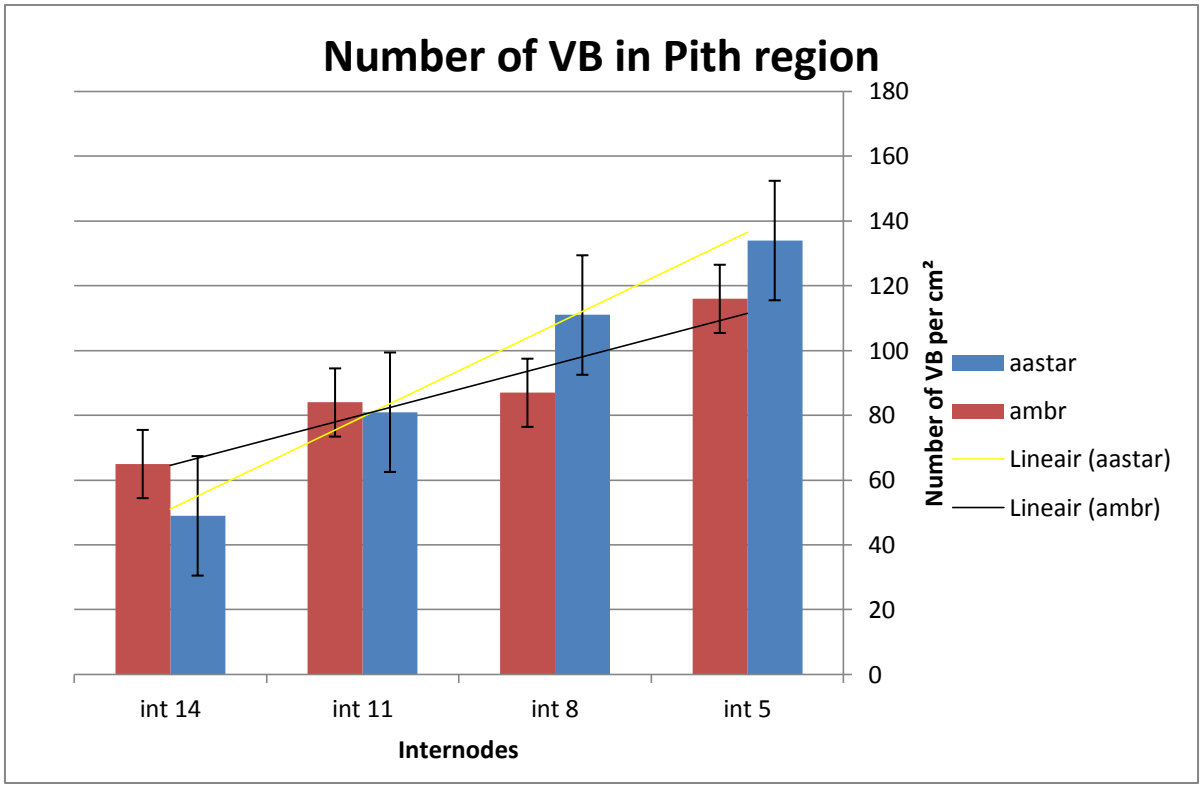


Figure 23: Vascular bundle density in the pith region of four different internodes in a maize plant harvested at anthesis

In Figure 24 it is shown that at every harvest time Ambrosini has a significant higher vascular bundle density in the rind region than Aastar. As a higher vascular bundle density means more thick-walled cells, this suits the statement that Ambrosini is less digestible than Aastar (Struik, 2012).

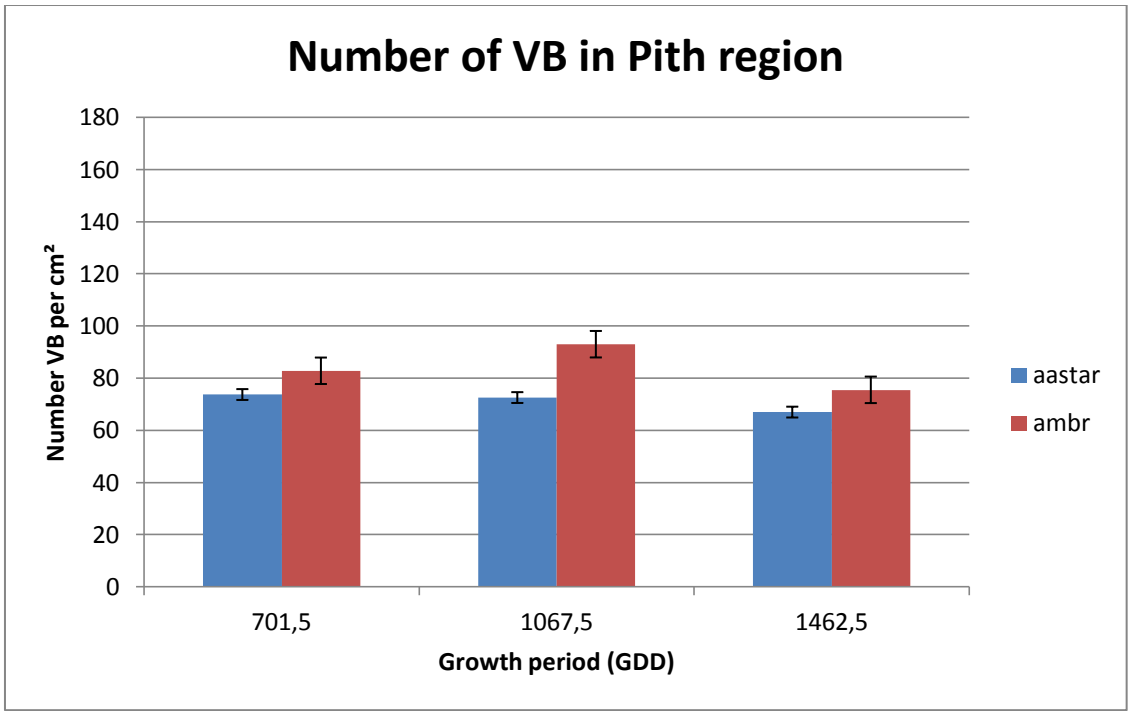


Figure 24: Vascular bundle density in the pith region of the 7th internode of a maize plant harvested on three different harvest dates

4.1.8.2. Rind region

In Figure 25 the vascular bundle density in the rind region is shown. The trend is slightly increasing for Ambrosini and a steeper increase from young to old internodes for Aastar. This means that Ambrosini has a higher vascular bundle density than Aastar in young internodes. This is the same for older internodes but the difference between the two cultivars becomes smaller as the internode is getting older.

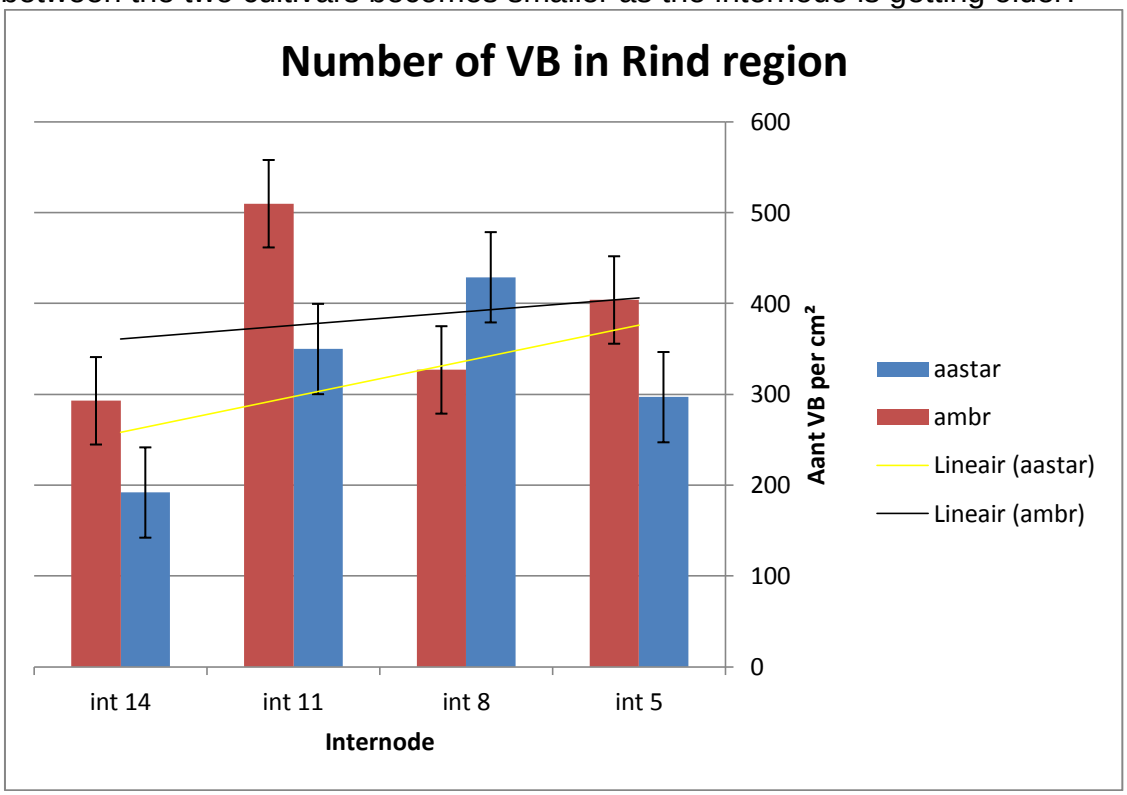


Figure 25: Vascular bundle density in the rind region of four different internodes in a maize plant harvested at anthesis

The vascular bundle density in the rind region varies little in time. Figure 26 shows the density is about stable at 350 vascular bundles per square centimeter for Ambrosini and around 300 for Aastar. There is a significant difference between the two cultivars in vascular bundle density. The vascular bundle density in the rind region stay more or less constant in time for Aastar and shows a little decrease for Ambrosini.

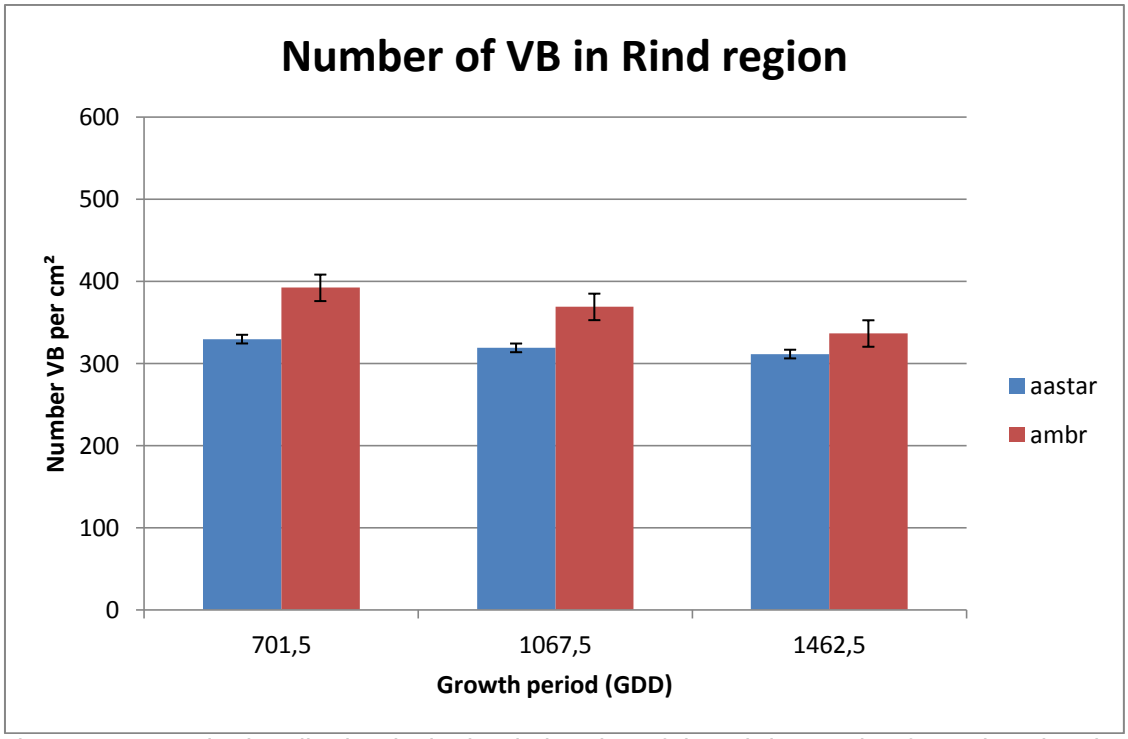


Figure 26: Vascular bundle density in the rind region of the 7th internode of a maize plant harvested on three different harvest dates

4.1.9. Ratio of the surface of the rind and pith region

4.1.9.1. Within a plant

When the ratio of pith and rind region on the total surface of the stem section is visualized it is shown that in general internode 5 has a higher rind region than the younger internodes. There is a clear difference between the ratio rind: pith in the 5th internode of Ambrosini and Aastar. Ambrosini has a significant lower percentage of rind than Aastar. The total cross-section of a maize stem of the 5th internode is in Ambrosini approximately 71% pith and 29% rind. For Aastar, on the other hand the pith and rind are respectively 67% and 33%. The higher ratio rind in the older internodes is due to drying of the tissue. As a result Aastar dries off faster than Ambrosini.

The trend seen in the graph is constant, except when internode 5 is reached, than the stem contains more rind region. This is again for strength reasons.

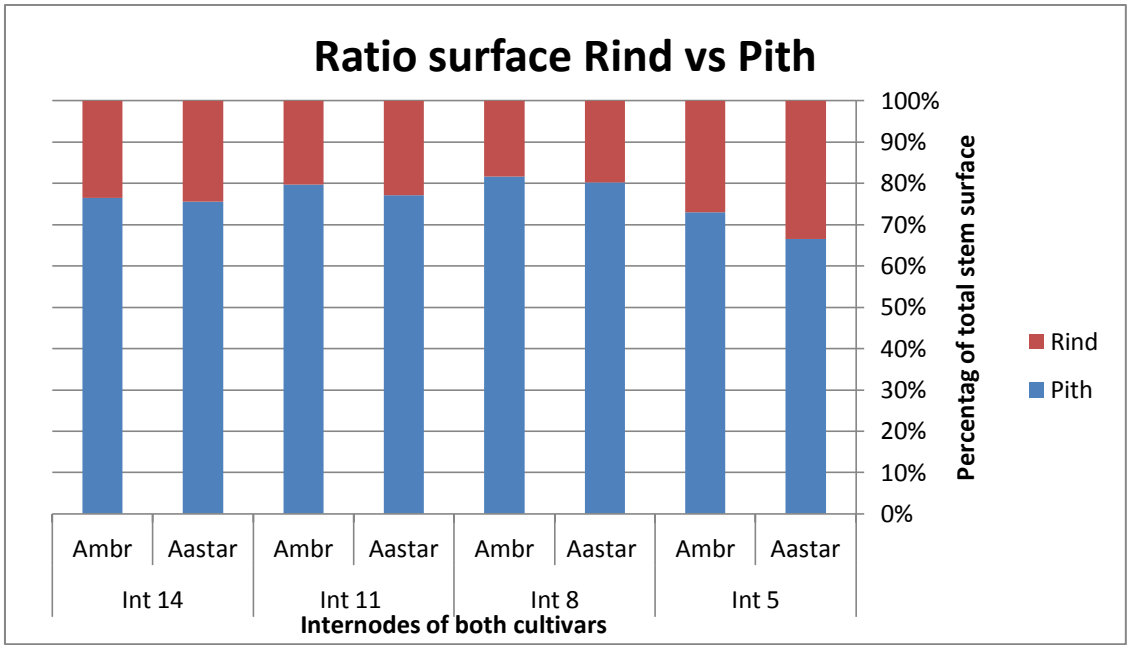


Figure 27: Ratio rind and pith region of the total surface of a stem section. Compared between two cultivars and four different internodes of a maize plant at time of anthesis

4.1.9.2. In time

From Figure 28 appears that Aastar has a bigger percentage rind in the early harvest. At the middle harvest the percentages are approximately the same between the two cultivars and in the oldest harvest Ambrosini has a bigger rind than Aastar. The ratio rind:pith of the maize stem is, as shown in the graph, on average 25:75

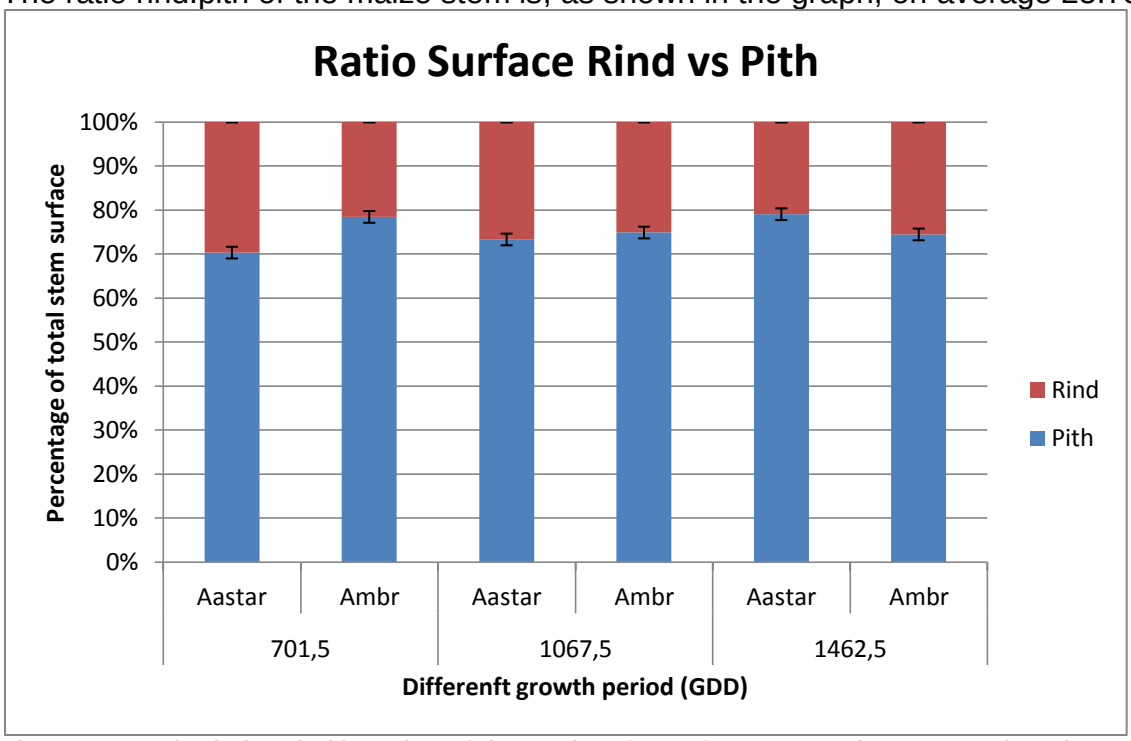


Figure 28: Ratio rind and pith region of the total surface of a stem section. Comparison between the 7th internode of two cultivars and three different harvest times

4.1.10. Ratio of the number of vascular bundles in the rind and pith region

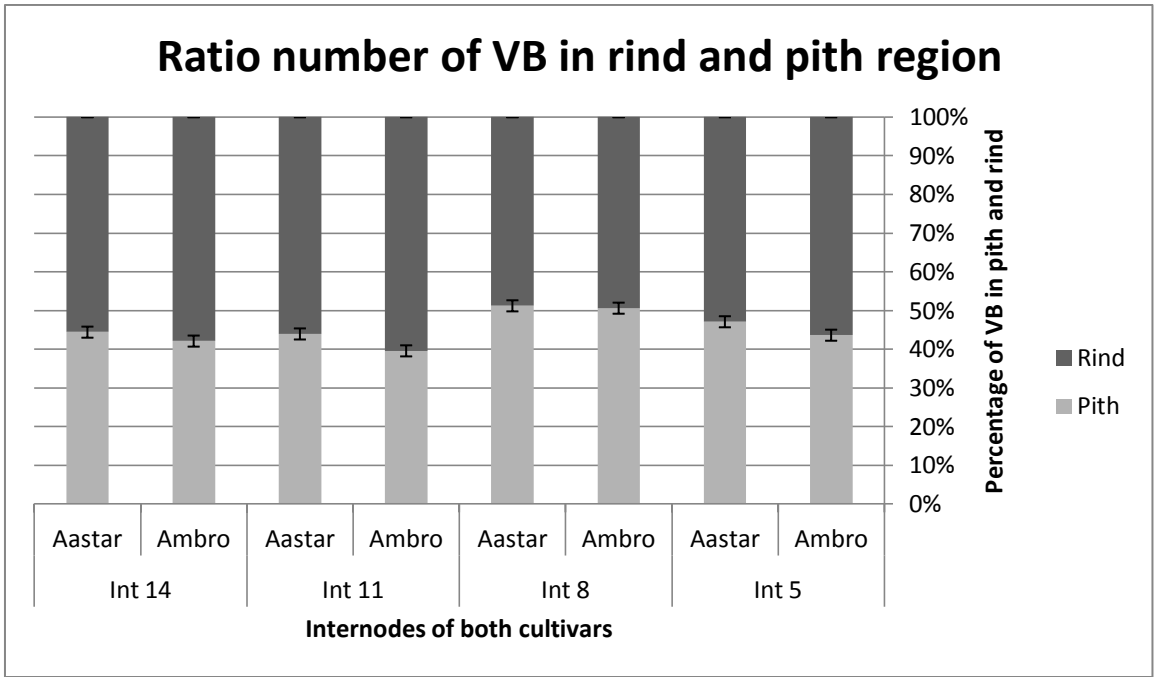


Figure 29: Percentage of the vascular bundles located in the rind and pith region of four different internodes in a maize plant harvested at time of anthesis.

In Figure 29 is shown how much of the total amount of vascular bundles is located in the rind and the pith region. As seen is more than half of the total amount situated in the rind region eventhough in surface it is smaller than the pith region. The graph shows that Ambrosini has always a higher percentage vascular bundles in the rind than Aastar.

5. Conclusion

At first, it is proven that the leaf length is a good criterion to determine the 7th internode. Negatively the difference between the two cultivars was not found. So the statement that the average leaf length of Ambrosini is 65 cm and 60 cm for Aastar is not established in this report. The average leaf length of both cultivars is around 65 cm. In Figure 9 the leaf length variation within a plant is shown and a difference between cultivars is seen. It seems that Aastar develops faster than Ambrosini in the beginning. When maturing the difference becomes smaller.
Then is experienced that according to the executed experiments no visual difference in staining intensity, after different staining times, is found. From the literature is found that more important than the staining time, is the time between staining and imaging. Phloroglucinol has the characteristic to fade in time.
About the development of the vascular bundles, from the pith to the epidermis, no numbers were obtained during the experiments. The development is shown using pictures ranging from the epidermis to the pith of the maize stem. The pictures of the vascular bundles support the literature in chapter 2.4.2. There is stated that more thick-walled sclerenchyma tissue surrounds the vascular bundles laying in the rind region. On the other hand, the vascular bundles laying in the pith region are surrounded by thin-walled parenchyma. It is also observed that more layers of sclerenchyma surround the outer vascular bundles. When comparing the different internodes it is clear that the pictures of internode 5 are brighter red than the others. This proves that more lignification occurred. It's known from the literature in chapter 2.5.3 that thick-walled and more lignified cells are harder to digest. So in general all these parameters show that the older internodes are harder to digest than younger internodes.
When the thickness of the internodes was investigated it seemed that an increasing trend was seen from young to old. This supports the literature in chapter 2.2 that the maize stem decreases in diameter because of the formation of leaves. The transition from thick to thin is slowly and logically, as the bottom part of the maize stem has to support the whole maize plant and make sure it doesn't fall or break.
The total number of vascular bundles is increasing from top to bottom of the maize plant. This is mainly a result of the increasing stem diameter. In Figure 15 and Figure 17 a slight decrease is seen in the surface and the amount of vascular bundles in harvest 1067,5. This might be related to a drought period. As shown in chapter 2.6 the need of rainfall for maize is the highest around the time of anthesis. If then there is a shortage, the maize will redirect the moisture in the stem to other parts (tassel and kernel).
Furthermore the surface of the pith and rind region was visualized in chapter 4.1.7. These trends within the plant are clearly increasing from top to bottom. This is mainly because the stem diameter is also increasing. In Internode 5 the rind is the biggest and the pith the smallest. This is not only related to the stem diameter, it proves that internode 5 has more strength and rigidity. As one of the bottom internodes it carries a big part of the weight of the plant. In Figure 20 and Figure 22 there is again a slight decrease around harvest time 1067,5 due to drought.
Thereafter the vascular bundle density was described in the pith and rind region. A clear increasing trend is observed from top to bottom of the maize stem. This is due to bending off of vascular bundles in the nodes for the formation of leaves. Another reason is that vascular bundles start growing from the bottom of the plant. A significant difference is noted in both the vascular bundle density in the rind and pith

region. Ambrosini has clearly during the whole growth period a higher vascular bundle density. This might be a prove for the statement that Ambrosini is less digestible than Aastar (Struik, 2012). In the different internodes is seen that Ambrosini has a higher density of vascular bundles in the younger internodes, but is caught up by Aastar as older internodes are investigated.
The ratio rind and pith is in the young internodes constant around 80% pith and 20% rind. In the 5^{th} internode an increase is seen in rind region. This has to do with strength reasons. It also shows that the increase is steeper in Aastar than in Ambrosini what means that Aastar dries out faster than Ambrosini.
Finally the situation of the vascular bundles in the rind and pith region are compared. It seems that on average more than 50% of the vascular bundles lies in the rind region, which is only 20% of the diameter. The graph also shows that Ambrosini always has a higher percentage vascular bundles in the rind region than Aastar.

As final conclusion can be stated that vascular bundle density is the only big difference between the two cultivars. This seems to be the clearest reason for the better digestibility of Aastar compared to Ambrosini.

6. Discussion

The first bottleneck experienced during these experiments was the quantification of the staining. Phloroglucinol stains only lignin but, it results in a picture with lots of contrasts, ranging from light brown-red, to bright red. Because of the contrast a threshold should be used to get a black-white image. That way the white pixels can be measured in image J and a surface can be extracted.
Next, the measuring of the leaves should be done more precise. No difference is found between the cultivars in this experiment and this is partly due to inaccuracy. Rounding the numbers to integers increases the standard error, and so can results in none significant differences.
Furthermore the number of sclerenchyma layers was measured, this was done on the phloem side of the vascular bundle. Later seemed from literature that in young vascular bundles the sclerenchyma is equally divided around the vascular bundles, but when they develop further, the sclerenchyma becomes more located on the opposite side of the phloem (Boon, 2008).
Then the cell wall thickness was measured on fresh sections, stained with phloroglucinol. The magnification had to be so big, that the image became blurry. That way the measurements were not precise or relevant. The same bottleneck was experienced by Boon et al. (2005). Fresh sections cannot be cut thinner than 100 µm. When sectioning, the cell walls can be cut under an angle, therefore the cell wall might be overestimated.
For measuring the cell wall concentration again was tried to make a black-white image. This way the cell wall (=black) and cell lumen (= white) could've been distinguished. The downside was the thickness of the sections. Because of the thickness, the lumen was not white and not even when using a threshold, a black-white image was obtained. The sections were also cut under a certain angle. This also resulted in a bigger contrast, and the cell lumen was gray instead of white.
The incubation in rumen fluid was not successful. Before the experiment started a little pilot study was done with a few sections, attached with double sided tape to a microscopic slide. After 48h of incubation at 37°C the sections were loosened from the microscopic slide and not useful. As a result the experiment was not further executed.

All the experiments with imaging on lower magnification (8x) were easier to execute. Nevertheless there are some things that should be taken into account when looking at the results. For measuring the surface of the pith and rind region, first a separation line had to be defined. This was done by drawing a line on the transition from thick-walled sclerenchyma tissue to thin-walled parenchyma. The outcome of this experiment is biased as the separation line is defined manually. Following, all the experiments where the surface is taken into account have also biased results.
Measuring the amount of vascular bundles in rind and pith region was also biased as the separation line was again defined manually. Consequently if the experiment is executed by somebody else the separation line between rind and pith can be different, resulting in a different outcome.
The correlation of a vascular bundle between the distance from the epidermis and the surface of the sclerenchyma sheath was not found. This might be due to a to small range of distances from the epidermis. The experiment was executed at the end with existing pictures, so the range was 400-600 µm from the epidermis.

Finally the first harvest time might have been too late to see big differences in stem composition.

7. Further research

To see more clear trends the first harvest date might have been too late in the growing period. The maize was almost fully grown and not much differences in composition occurred in the further growing season.

Some more experiments using fresh sections are:

- the correlation of a vascular bundle between the distance from the epidermis and the surface of the sclerenchyma sheath. Using a broad range of distances from the epidermis
- vascular bundles in the pith region should be bigger than in the rind region. This is possible to do using fresh sections and Image J.

A huge point of improvement is using Technovit instead of fresh sections. Much thinner sections can be cut using a rotary microtome (1-3µm), so much more detailed pictures can be made. In Figure 30 a vascular bundle is seen stained with toluidine blue. On this pictures much more detail can be seen and cell wall thickness can be measured more precise. Also the other experiments can be done with much more precision on Technovit sections.

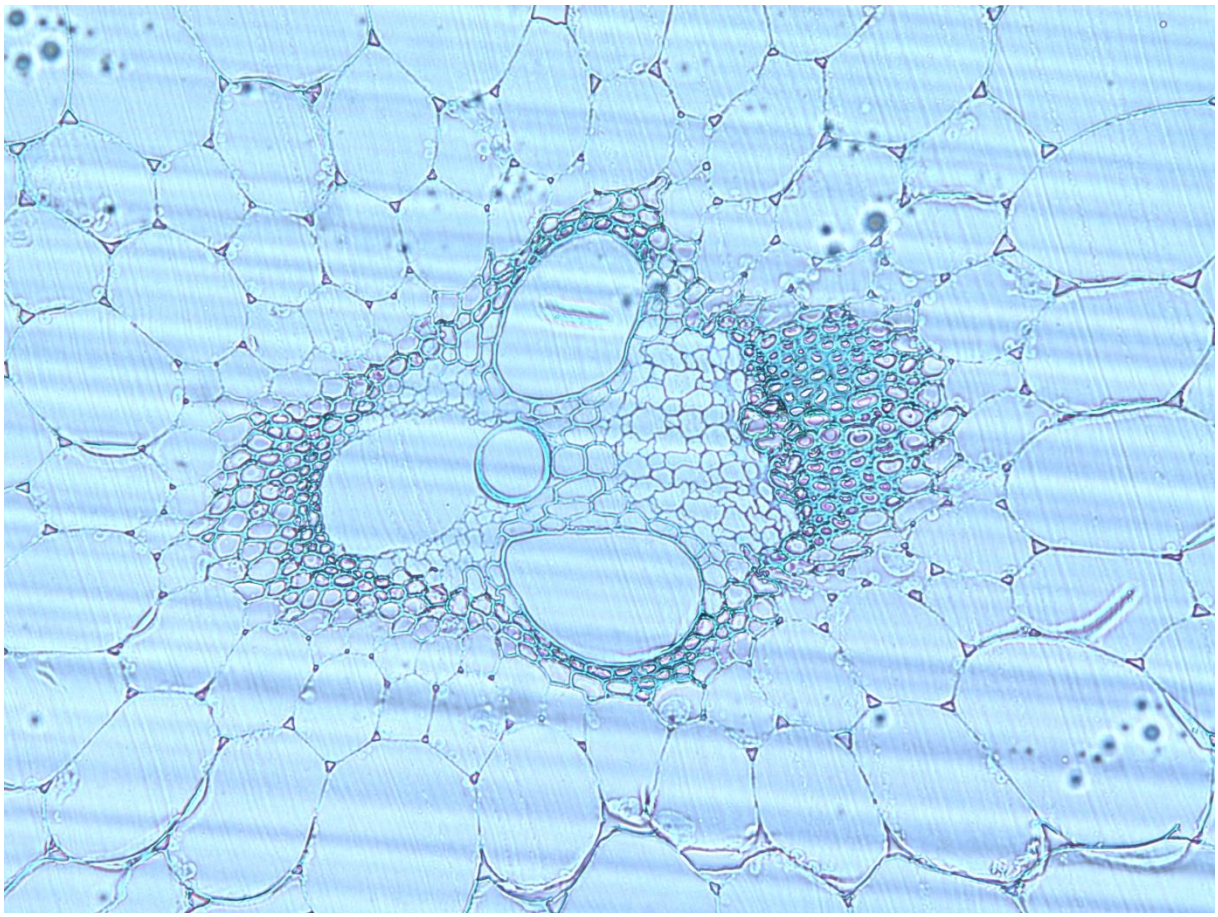


Figure 30: A vascular bundle in a Technovit section (2µm) stained with Toluidine Blue

In Figure 31 and Figure 32 Longitudinal sections can be seen from a vascular bundle. This way further research can be done about the vascular elements

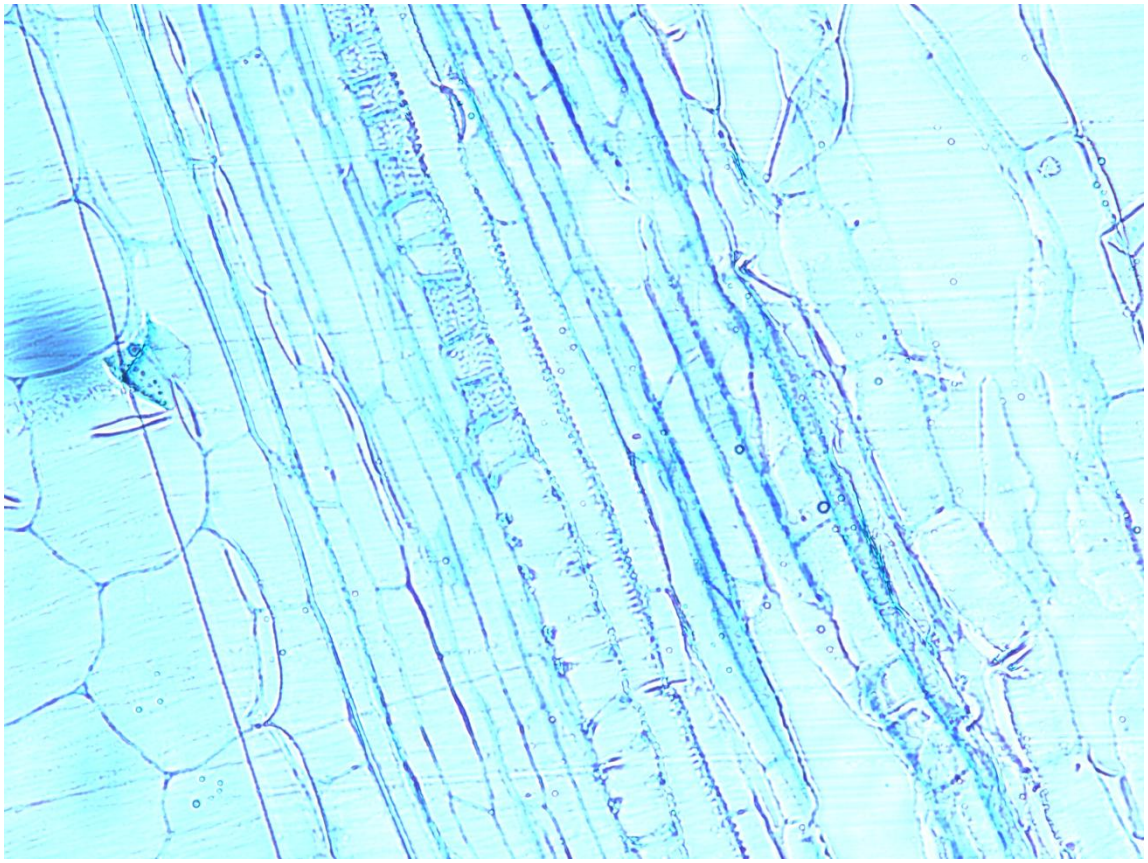


Figure 31: A vascular bundle in a Technovit section (2µm) stained with Toluidine Blue (longitudinal section) (1)

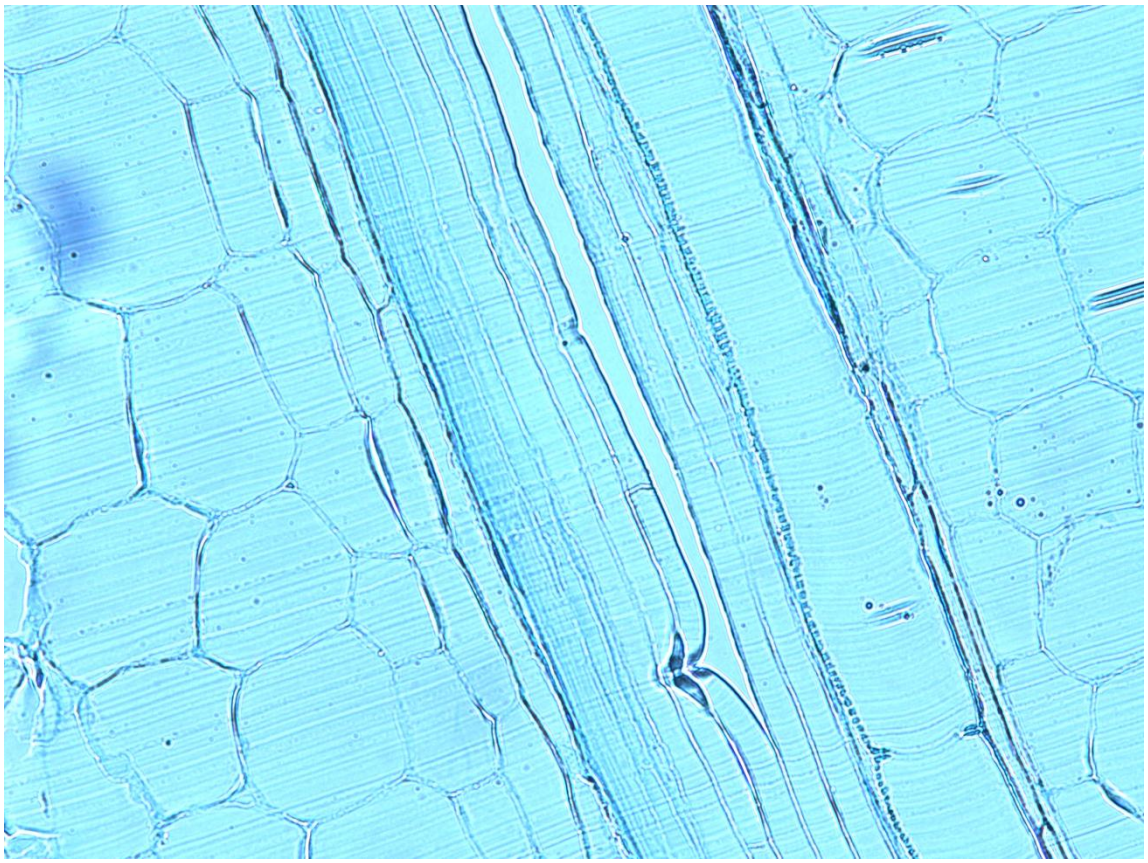


Figure 32: A vascular bundle in a Technovit section (2µm) stained with Toluidine Blue (longitudinal section) (2)

Using Technovit also has the advantage that much more different stainings can be used. This way proteins, fat and other substances can become visible.

To determine the lignification maybe in the future a better staining is invented to color thin sections.

Finally to be sure that one cultivar is better digestible than the other it might be usefull to do mass spectrometry.

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Appendix A: Leaf length data internode 7

SAMPLING DATE 1

PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1: Ambrosini	65	64	63	67	65	64	65	68	63	65	65	67,5	67	69	
2: Aastar	66	66	65	64	64	63	66	62	64	65	68,5	66,5	60	65	
3: Aastar	63	64	64	66	66	62	63	64	66	63	70	65,5	70	63,5	
4: Ambrosini	64	67	68	66	67	61	60	67	67	62	67	68	67	65	
5: Aastar	65	66	63	67	62	64	68	63	64	60	61	62	64	65	
6: Ambrosini	64	63	65	63	67	64	63	62	63	65	67,5	68	67,5	64,5	
7: Ambrosini	66	64	66	64	66	65	66	61	61	66	67	68	65	65	
8: Aastar	61	65	63	62	63	62	66	64	65	62	62	61	65,5	63	

SAMPLING DATE 2

PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	62	66	64	64	64	66	68	67	63	66	68	69	63,5	70	
2	65	62	67	62	65	68	66	65	64	67	66	73	66,5	66,5	65
3	63	65	65	68	68	67	67	64	67	64	62	71	67	70,5	70,5
4	64	66	67	65	66	64	63	65	64	65	65	63	70	70	65,5
5	67	63	63	66	63	68	64	69	66	67	63,5	61,5	71	59	64,5
6	66	67	62	66	64	67	65	67	68	63	64,5	63	61	66	67
7	63	68	64	64	61	66	66	65	61	64	60	65	61	63	61
8	64	66	64	63	68	64	68	66	67	68	65	64	66,5	61	64

SAMPLING DATE 3

PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	67	63	64	67	65	64	65	65	67	66	63	63	69,5	67,5	70
2	64	65	67	68	67	66	66	65	64	63	65	64	67	68,5	69
3	68	67	68	66	69	63	67	65	67	68	61,5	63	70	72	68
4	68	67	64	67	70	63	63	65	67	67	72	65,5	63	68	
5	66	65	65	66	63	67	64	66	65	64	65	63,5	65	65,5	69
6	65	63	68	65	66	65	69	65	63	64	69	61,5	63	65	63,5
7	67	68	67	68	66	67	65	68	65	64	66,5	64	64,5	65	
8	63	66	68	64	68	67	67	65	66	65	67	66,5	60,5	67,5	65

SAMPLING DATE 4

PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	62	67	65	66	67	66	67	65	63	64	64	58	60	59	62
2	67	69	64	64	66	63	62	66	64	61	66	66,5	61,5	65,5	
3	65	65	67	65	61	63	67	62	66	63	62,5	57	65	67,5	66,5
4	64	63	68	67	66	65	68	65	63	65	67	61,5	68	70	65
5	68	65	64	65	65	64	66	70	67	66	64	67	67,5	64,5	60
6	69	66	60	62	69	64	63	65	70	66	67	69	66	64,5	67,5
7	65	67	62	63	67	65	70	68	63	67	61	66	72	73	
8	64	65	63	64	67	63	67	66	67	66	67	69,5	63,5	69,5	65

SAMPLING DATE 5

PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	66	63	67	66	64	64	66	64	68	63	62,5	69	71,5	63	64
2	65	64	66	67	65	65	64	63	64	68	67	65	66,5	66	67
3	67	66	67	67	64	65	63	66	65	63	68	64,5	67,5	60	67
4	66	65	66	65	68	68	63	64	63	64	62	65	67	60	69
5	67	66	65	66	66	63	62	67	65	68	60	68	62	67,5	61,5
6	63	67	63	64	63	68	64	63	68	63	60	63,5	60	67	67
7	65	65	68	68	63	64	66	64	64	65	60	62	62	63	67,5
8	63	67	64	63	64	64	67	65	64	67	71	63,5	67	66,5	67

SAMPLING DATE 6

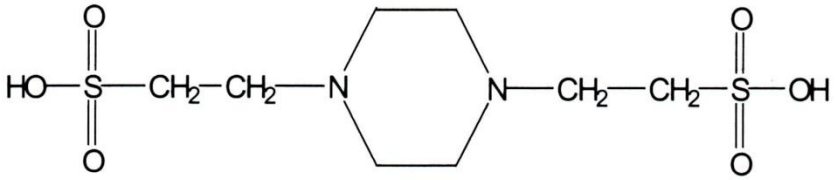
PLOT	LEAF LENGTH OF INTERNODE 7														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	61	66	66	64	66	65	63	64	67	65	72	60	66	61	61,5
2	66	67	65	65	54	64	64	61	62	66	66	71	67,5	63,5	63,5
3	64	66	64	66	65	65	66	63	67	62	65	67	69	62	66,5
4	65	64	69	65	66	63	63	65	68	65	65,5	63,5	69,5	61,5	62
5	67	66	66	67	64	67	67	66	66	70	60,5	63	64,5	62,5	
6	67	65	63	63	65	66	63	66	63	65	60	62	60	61	65,5
7	66	68	64	67	65	64	63	67	66	68	63,5	62	61	62,5	60
8	63	66	65	64	63	68	67	66	67	66	72	62	65	62,5	66,5

Appendix B: Pipes buffer

SIGMA

PIPES BUFFER
Sigma Prod. No. P6757

ProductInformation



CAS NUMBER: 5625-37-6
SYNONYMS: piperazine-N,N'-bis(ethanesulfonic acid); 1,4-piperazinediethanesulfonic acid

PHYSICAL DESCRIPTION:

Appearance: white powder
Molecular formula: $C_8H_{18}N_2O_6S_2$
Molecular weight: 302.4
Melting point: decomposes >300°C[1]
(pK_{a1} <3, but not usually reported)[1]; pK_{a2} = 6.8 at 25°C[1,2]
Effective buffering range: 6.1 - 7.5 (at 25°C)
$\Delta pK/\Delta T$ = - 0.0085[3]
No reported metal binding[1]

STORAGE / STABILITY AS SUPPLIED:

PIPES is expected to be stable for years at room temperature.

SOLUBILITY / STABILITY OF SOLUTIONS:

PIPES free acid is not very soluble in water (only 1 g per L at 100°C)[1], but its salts are very soluble in water at the pH normally used as a buffer. PIPES does form a clear, colorless solution in 1 N NaOH, and is soluble at least to 20% (w/w) in 1 N NaOH. (The resulting solution has a pH ≈ 6.)[2] PIPES becomes soluble as the pH rises above pH 7 (it is converted to the salt form).

HCl phloroglucinol

Appendix C: Paraformaldehyde

Preparing 8% para-formaldehyde (stock)

-Wear gloves and keep the fixative at all times in the fume-hood.
Add 40 ml demi water (of a final 50 mls) to 4 gr of para-formaldehyde powder (no 154 room 0044 cupb. A).

-Dissolving to a clear transparent solution needs some heat and NaOH.
Heat to 60°C on a heater with stirrer in the fume-hood. Be sure that the temperature does not rise above 60°C (thermometer). Do not mix in too much air during stirring; this reduces the fixation capacity. The solution will turn transparent after warming.
If necessary add a few drops of 10 N NaOH, until the fixative becomes clear. Cool the fixative to RT. (If needed filtrate before use). Adjust pH back to roughly pH 7 with HCl and indicator paper. Adjust to a final volume of 50 ml per 4 gr.

-Freeze the stock in aliquots of 10 ml at -20°C.
Prior to use thaw the frozen fixative by placing the tube in a beaker with warm water from hot tap). When the solution does not become transparent at RT, heat it slightly (with running hot tap water) and keep clear solution at RT. Stock solution can be stored for 4 Do not refreeze melted stocks.

Before tissue fixation mix the stock (i.e. fix 2x) with stock solution of buffer (i.e. buffer 2x) in ratio 1:1 to dilute to 1x.
Check with indicator paper if the pH is in the right range. The fixative is not buffered so should easily adapt to a buffer. Do not use the pH meter; the electrode will be damaged!

For background info on pfa, formalin and formaldehyde see:

http://publish.uwo.ca/~jkiernan/formglut.htm

Appendix D: Technovit procedure

Application:

Technovit 7100 is a hydroxyethyl-methacrylate which is particular useful for the embedding of soft tissues and the cutting of semi-thin sections with a large surface. Because the sectioning is easy and many types of staining are possible on the sections, this plastic is useful for studies at the light microscopical level.

Procedure:

The composition of the fixative can be changed if desired. The period of fixation, dehydration and infiltration can be varied depending on the type of tissue and the size of the specimen. Do not use heavy metals as fixatives and use the mixing mill in all steps.

- Fix in 2.5% glutaraldehyde and 2.5% formaldehyde in 0.1 M phosphate buffer pH 7.2 — 1-2 h
- Rinse in phosphate buffer — 4x15 min
- Rinse in double distilled water — 2x15 min
- Dehydrate in ethanol series 10, 30, 50, 70, 90, 96, 100, 100%, each step 30 min
- Stepwise plastic infiltration with pre-solution **A**:
  - **A** : 100% ETOH = 1:3 — 1 h
  - **A** : 100% ETOH = 1:1 — 1 h
  - **A** : 100% ETOH = 3:1 — 1 h
  - 100% pre-solution **A** — O/N

Pre-solution **A**

100 ml Technovit 7100
1 package hardener I
2.5 ml PEG 400

The addition of 1-5 % PEG 400 softens the Technovit and sectioning becomes easier. However, when using softer Technovit, sections should not cut too thin.
At room temperature, Pre-solution A can be used for about 4 weeks; cooling or freezing increases the period. (Cover well. It is important to let the Technovit come to room temperature prior to opening it, to prevent water getting into the embedding resin by condensation.)
Technovit which is too old turns pink. Polymerize the leftover in order to avoid leaving hazardous waste.

Polymerization solution **B**

15 ml pre-solution
1ml hardener II

Attention! Prepare this solution just before use and mix well. The polymerization starts immediately. After 10 min. threads are already formed and reorientation of the material is not possible anymore.

Be careful: Hardener I contains benzoylperoxide and hardener II contains dimethylsulfoxide. Prevent all contact with the skin.

Orientation during embedding:

- Cover the bottom of the mould with a thin layer of polymerization fluid (**B**) and position the sample properly with a pincet. The tissue has only been in the pre-solution **A**.
- Fill the mould with polymerization fluid. The bottom of the mould will be the cutting side.
- Cover fluid with plastic foil obtained from overhead sheet, otherwise the top layer will not polymerize because the oxygen in the air prevents polymerization.
- Polymerization time: 1 h at 37°C
- Store polymerized blocks in paper bags, because blocks will soften in plastic boxes.

Attachment of blocks with Technovit 3040 on specimen holder:

- Preparation of Technovit 3040: add three parts powder to one part fluid and mix well using a plastic pipette. The solution should be so fluid that one can still take it in a pipette. Work fast, within 3 min. the material is viscous and after 15 min. it is hard.
- Put the plastic block holders into the space of the embedding mould and take care that the contact surface between the holder and the Technovit is as large as possible.
- Glue the holder with freshly prepared Technovit 3040 and fill the holder.
- After 15 min. the glue is hard. Pull the blocks out of the mould with the aid of a vice. The blocks are now ready for sectioning with a rotation microtome.

The cutting and sticking of Technovit sections:

- If desired remodel the blocks using an iron saw. Smoothen the edges. The smaller the surface, the easier the sectioning.
- Sections of 2-5 µm are prepared on a rotary microtome. Do not cut too fast. For large sections (>0.5 x 0.5 cm2) often sectioning can be facilitated by guiding the section away from the knife holding it with tweezers. If too much curling occurs, remodel the block, smoothen the edges, or minimize its size. Take the section with dry tweezers or brush and bring it on the surface of water. Sections stretch the best in water of 25-35oC. Sections are transferred onto glass slides by putting a glass slide upright into the water and touching the section.
- Sections are fixed to the glass slide by evaporating the water at 80oC on a hot plate.

Enclosure of sections:

- Stain sections
- Add a drop of water, or aqueous embedding agent, or glycerol, or immersion oil.
- Enclose by covering the sections with a coverslip.

(De Ruijter, 2012)

Appendix E: Distance from epidermis in experiment: Development of vascular bundles from cortex to epidermis

Internode	Distance from epidermis
Int 5	130 µm
	341 µm
	790 µm
	1159 µm
Int 9	152 µm
	657 µm
	1050 µm
	1400 µm
Int 13	170 µm
	600 µm
	714 µm
	1350 µm
Int 17	250 µm
	880 µm
	1120 µm