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DISTRIBUTION OF AROMATIC COMPOUNDS IN COASTAL BERMUDAGRASS CELL WALLS USING ULTRAVIOLET ABSORPTION SCANNING MICROSPECTROPHOTOMETRY

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Abstract

The distribution of aromatic constituents, including lignin, in the leaf cell walls of "Coastal" bermudagrass (*Cynodon dactylon* (L.) Pers.) was investigated using scanning ultraviolet (UV) microspectrophotometry. Leaf blade sections and individual tissue types were scanned at three wavelengths representing the absorbance maxima (318, 287 and 250 nm) of aromatic constituents present in bermudagrass leaves. The measured absorbance data were printed in a geometric arrangement to produce an image of the distribution and amount of aromatic constituents among and within cell wall types which vary in digestibility. Differences in absorbance were observed among cell wall types, among walls of the same cell type, and at different sites in individual cell walls. Scans of the midvein at 10X magnification showed that various tissues and cells could be distinguished on the basis of UV absorbance. The abaxial sclerenchyma and mestome sheath gave the highest absorbance followed by those of the epidermis and parenchyma bundle sheath. The lowest levels of absorbance were observed in the mesophyll, parenchyma tissue and xylem tissue. Images produced from scanning individual cell walls at 100x magnification showed the heterogeneous nature of aromatic constituents within a cell wall. Varying the wavelength resulted in similar but not identical images, indicating that variations in the chemical structures of aromatic constituents in the cell wall can be detected using this technique.

Key Words: Microspectrophotometry, forage, digestibility, cell-wall components, aromatic compounds, Coastal bermudagrass.

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Introduction

Aromatic constituents in the walls of some cell types decrease the availability of wall polysaccharides to rumen microbes leading to low forage digestibility which, in turn, leads to low rates of animal production (for reviews see Hartley and Ford, 1989; Akin and Chesson, 1989). The distribution of aromatics, including lignin, in the walls of different cell types of forages is of importance in relation to their nutritional quality for ruminants.

Recently, ultraviolet (UV) absorption microspectrophotometry has been used to quantify the aromatics of individual cell walls of "Coastal" bermudagrass (*Cynodon dactylon* (L.) Pers) (Akin et al., 1990a; Hartley et al., 1990a). Measurements were taken by focusing manually on various sites of the different cell types, and, in general, it was found that walls with high absorbance in the UV region were those that were indigestible to rumen microbes. During this work it was noted that there were often large variations in UV absorbance of some cell types. These variations were not due to machine instability but to inherent differences among cells of similar type.

In the present work, the variations have been examined in detail by an automated imaging technique of UV absorption scanning microspectrophotometry using sections of the midvein of Coastal bermudagrass leaf. The imaging technique measured absorbance at the wavelengths in the UV region where absorption maxima were known to occur (Akin *et al.*, 1990a; Hartley *et al.*, 1990a). Parenchyma bundle sheath and mestome sheath cell walls were examined in more detail as these cell types are of particular importance in relation to the digestibility of forages. Cell walls of parenchyma bundle sheath show large variations in UV absorption and are slowly or partially degraded. In contrast, mestome sheath walls are representative of tissues with high UV absorption which are indigestible by rumen microbes.

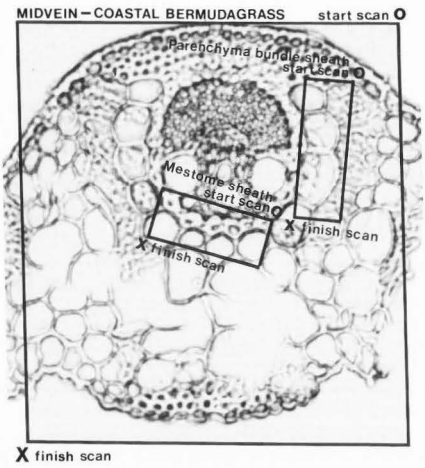

Figure 1. Midvein of Coastal bermudagrass leaf blade. Low power micrograph showing the locations of UV absorption imaging depicted in Figure 2 (shown on pages 30-31). Bar = 34 μm. Actual areas scanned for the midvein area was 220 μm (length) X 205 μm (width); for the parenchyma bundle sheath was 97 μm (length) X 22 μm (width); and for the mestome sheath was 334 μm (length) X 61 μm (width). Circle and cross denote the start and finish of scan respectively.

Materials and Methods

Preparation of Plant Material for Microspectrophotometry

Coastal bermudagrass (*Cynodon dactylon* (L.) Pers.) was grown as previously described (Hanna *et al.*, 1976) in Tifton, GA, and harvested at 6-week regrowth on 6 July 1988. The shoots (i.e., leaf and stem) were cut approximately 100 mm from the soil surface, frozen in carbon dioxide and stored at -10°C. The fourth leaf blade (fully expanded) from the apex was selected and horizontal sections (8 μm in thickness) were cut in a cryostat and stored in water.

UV Absorption Imaging Microspectrophotometry of Coastal Bermudagrass Midvein Sections

Leaf sections were mounted in glycerol on quartz slides with quartz cover glass and visualized using 10X and 100X ultrafluar quartz objectives and a Osram high pressure xenon (XB075W) lamp for transmitted light illumination. The numerical apertures of the 10X and 100X (glycerol immersion) objectives were 0.2 and 0.125 respectively. A 10X ultrafluar quartz objective was used as a condenser. Specimen scanning was accomplished using a computer-controlled Zeiss UMSP 80 microspectrophotometer equipped with a scanning stage and a UV grating monochromator positioned on the illumination side. Both the scanning stage and photomultiplier were controlled by APAMOS (Automatic Photometric Analysis of Microscopic Objects by Scanning) software which allowed photometric measurement of a rectangular field consisting of 10000 data points. Absorbance thresholds for measurement were 0 to 2.0. Prior to scanning, adjustment was carried out on an area devoid of plant material at 250, 287 or 318 nm depending on measuring wavelength. The reference value was taken as 100 percent transmittance.

The areas chosen for scanning included: entire midvein, parenchyma bundle sheath cells, abaxial and adaxial sclerenchyma, mestome sheath cells, xylem, mesophyll and phloem. Each scan was repeated three times for each wavelength and three separate leaves were evaluated to determine repeatability. For midvein area scans, a 220 X 205 μm area was defined for absorbance measurement at 250, 287 and 318 nm wavelengths using a bandwidth of 10 nm. A total of 8277 pixels were scanned using measuring increments of 2.5 X 2.25 μm. A 10X objective was used to accommodate the large area scanned and the measuring diaphragm was 10 μm.

Measurement areas of parenchyma bundle sheath and mestome sheath cell walls were defined (2 per midvein) and scanned at the three wavelengths. Measurement of these tissues was carried but using a 100X objective, a band width of 10 nm and a measuring diaphragm of 1 μm. For parenchyma bundle sheath cells, a total of 8712 pixels were measured at increments of 1.0 X 0.25 μm. For mestome sheath cells, a total of 5727 pixels were measured at increments of 0.5 X 0.75 μm.

The absorbance values (log scale) measured was printed in a geometric arrangement so that each pixel was represented by a number corresponding to UV absorbance. The software program allowed the operator to choose absorbance thresholds of interest and varied the numerical representations accordingly. The thresholds chosen in this case were 0.2 to 1.8. The resultant absorbance images were colored by hand so that each color represented a range of absorbance values.

Results

A light micrograph (10X) of the midvein shows the tissue areas scanned (Figure 1). Application of the UV absorbance scanning technique to the whole midvein using three wavelengths (318, 287 and 250 nm) resulted in absorbance images resembling the anatomical structure of the section (Figures 2 a-c). **(Note: Figure 2, color illustrations, are shown on pages 30-31).**

The threshold absorbance levels chosen (0.2-1.8) allowed seven color groupings for comparison of absorbance among and within tissue types. At all three wavelengths, tissues were readily distinguished with sclerenchyma and mestome sheath showing the highest absorbance (> 1.6) and xylem and mesophyll the lowest (< 0.6); phloem, parenchyma bundle sheath and epidermal cell walls were intermediate and more heterogenous with respect to UV absorbance. Comparison of the midvein scans at the three wavelengths indicates similar but not identical absorbance images suggesting that some cell walls differ qualitatively in their aromatic constituents. Refractive differences may account for some of the variation among the three wavelengths and this is currently under investigation. Aromatic compounds absorbing at 318 nm were present at high levels (> 1.25) in the sclerenchyma and mestome sheath, at intermediate levels (0.95-1.55) in the inner tangential and radial walls of parenchyma bundle sheath cells and at low levels (< 0.95) in the outer tangential walls of parenchyma bundle sheath cells. Compounds which absorb at 287 nm were also present in high quantities in sclerenchyma and mestome sheath (> 1.85) and were present at intermediate levels (0.65-0.95) in mesophyll tissue. Differences between sclerenchyma and mestome sheath walls were visible at 250 nm, the latter having lower values. The outer and inner tangential walls of parenchyma bundle sheath cells showed differences at both 287 and 250 nm. It should be noted that this technique involves measuring at predefined incremental areas. Hence, walls of smaller cells (e.g., sclerenchyma) will be less well defined that those of large cells (e.g., parenchyma bundle sheath).

More detailed scanning of parenchyma bundle sheath and mestome sheath cell walls showed variation in the distribution and quantity of aromatic compounds within and among these cells (Figures 2 d-i; see page 31). Variation in concentration and distribution of phenolic compounds in parenchyma bundle sheath cell walls is shown for the three wavelengths in Figures 2 d-f. The mean absorbance was 0.666, 0.683 and 0.660 for 318, 287 and 250 nm, respectively. At all wavelengths the greatest absorbance was observed in the radial walls. As expected, absorption progressively decreased towards the lumen of each cell and was the highest in the middle lamella.

The high levels of absorption in the mestome sheath walls make it difficult to distinguish individual cells and differences among wavelengths at the threshold levels used here (Figures 2 g-i, see page 31). However, there were some areas of this tissue with lower absorbance values (0.95 to 1.25) which may represent a potential site of attack for rumen microbes. Unlike the parenchyma bundle sheath cell walls, the mestome sheath cell walls did not show a gradient decrease in absorbance towards the inside of the cell.

Discussion

This is the first report of the application of imaging UV absorption microspectrophotometry for the location of aromatic compounds in walls of different cell types. Absorbance was measured at 318 nm, 287 nm and 250 nm because the various cell wall types in Coastal bermudagrass leaf had been previously shown to give absorption maxima in these regions (Akin *et al.*, 1990a). It has been suggested (Hartley *et al.*, 1990a) that the absorption maximum at approximately 318 nm is due to the propanoid side chains of p-coumaric and ferulic acids that are ester-linked to the arabinoxylans of graminaceous cell walls (Kato *et al.*, 1983, 1987; Gubler *et al.*, 1985; Kato and Nevins, 1985; Mueller-Harvey *et al.*, 1986; Ishii *et al.*, 1990). The wavelength maximum at about 287 nm arises from the *p*-coumaroyl and feruloyl groups but can also arise from substituted truxillic and truxinic acids that are also apparently ester-linked to graminaceous cell walls (Ford and Hartley, 1990; Hartley *et al.*, 1990a, b) and from polymeric lignin (Goldschmid, 1971). The wavelength maximum at about 250 nm is probably due to general aromatic absorption (Dyer, 1965).

The present work shows that inherent biological variation in aromatic constituents within a particular cell wall, e.g., within the walls of parenchyma bundle sheath and mestome sheath (Figure 2), can be examined by the imagining technique. Such variations are likely to be of considerable importance in studies aimed at elucidating the mechanisms of degradation of cell walls by microbes. Differences in absorbance between the inner and outer tangential parenchyma bundle sheath cell walls, however, are not consistent with bacterial degradation of the outer tangential wall reported in an earlier study (Akin *et al.*, 1990a).

In general, results from the imaging technique agree

with the manual focussing technique used previously for similar plant material (Akin et al., 1990a). This earlier work showed that at approximately 318, 287 and 250 nm, sclerenchyma and mestome sheath tissue, which were indigestible to rumen microbes, gave high absorbance. It also showed that mesophyll walls, which were totally digestible, gave low absorbance at these wavelengths while parenchyma bundle sheath and epidermal cell walls gave intermediate values. Phloem walls, which were totally digestible, gave in the present work higher values for absorbance than expected based on the earlier studies (Akin *et al.*, 1990b). This earlier work also showed that phloem walls gave a positive test with diazotised sulfanilic acid indicating the presence of phenolics. Hence for this cell type, the wall phenolics were not a barrier to microbial attack. The higher absorbance values of this tissue found in the present work may be due to aromatics in the cytoplasm. Absorbance of cytoplasm is not differentiated from the phloem walls by the imaging technique but it could be differentiated by the manual technique which focussed only on the cell walls.

The walls of the metaxylem cells, which are indigestible to rumen microbes, have not been examined previously by UV absorption microspectrophotometry. The imaging technique gave low absorbance values for these walls although the values might have been expected to be higher, i.e., similar to those for sclerenchyma and mestome sheath. Although their absorbance was low, metaxylem cell walls in Coastal bermudagrass leaf give a strongly positive test for lignin with acid phloroglucinol (Akin *et al.*, 1990b). It seems possible, therefore, that these cell walls do contain a high proportion of lignin but contain a low or negligible proportion of *p*-coumaroyl or feruloyl groups. These groups have high extinction coefficients (ϵ) in the 280-320 nm region due to their highly conjugated electronic systems. For example, the free acids have ϵ values of about 20000 in this region (Hartley *et al.*, 1990a). On the other hand, lignin has less conjugation leading to lower ϵ values (Goldschmid, 1971). Cyclobutane dimers of *p*-coumaric and ferulic acids that also occur in cell walls are also less conjugated. For example, 4, 4'-dihydroxytruxillic acid, a known constituent of graminaceous walls (Hartley *et al.*, 1988; Ford and Hartley, 1990) has an ϵ value at its wavelength maximum of 283 nm of about 3000.

The above results suggest that imaging UV absorption spectrophotometry will be a powerful tool for the investigation of the "lignification" of the plant cell wall in relation to biodegradation. The technique could also be employed for studies of the attack of microbes on plant tissues in relation to disease resistance.

Acknowledgments

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Discussion with Reviewers

J.R. Wilson: Does storing thin sections in water have any consequences for the subsequent determination of phenolics?
Authors: Previous studies (see Akin *et al.*, 1990a), aimed at quantifying and qualifying phenolics in different cultivars of bermudagrass, resulted in detection of variability using sections stored in water as described. This suggests that the aromatic compounds of interest, in terms of digestibility, are not lost from the cell walls examined following storage in water.

J.R. Wilson: I would prefer to see these photographs in the correct orientation rather than upside down.
Authors: The orientation of the micrograph was chosen to clarify the start, finish and angle of scanning without considering the the orientation of the abaxial or adaxial epidermis. Since the micrograph and image scans are already labelled, we have chosen to leave them as they were.

J.R. Wilson: The PBS walls of this species have pronounced light and dark striations in EM, it is interesting that these did not show up as differences in absorption. Please comment.
Authors: Variability in absorbance within the walls of the PBS cells were observed and can be seen in Figures 2d-f. Perhaps more detailed differences could be detected if a smaller area was defined and smaller step sizes could be used for measurements. This paper is intended to document the technique but we do intend to utilize the technique to answer such questions.

J.R. Wilson: Relating to the last sentence of paragraph 2 of **Discussion**, and the last sentence of the **Results** does "towards the inside of the cell", from outer tangential wall to inner tangential wall? This is not evident. Boundaries of walls between cells are not easy to distinguish (which seems a bit of a limitation to me).
Authors: In the case the **Discussion** paragraph we do mean from outer tangential to inner tangential. You are correct in that it is somewhat difficult to distinguish the cell wall boundaries. Familiarity with the material scanned and the scanning process helps but improvement is necessary. We expect to overcome some of this by varying scanning parameters.

L.H. Harbers: Please give the magnifications for Figure 2a, d, and g.
Authors: Magnification is indicated by a scale marker in Figure 1 which indicates the various locations and actual areas scanned. Figure 2 (a-i) are computer printouts of scans, not micrographs, and do not represent actual dimensions of area scanned.

L.H. Harbers: We have observed similar variations in cell walls of forage sorghum stem using fluorescence microscopy. Is it possible that there may be a wide variation in concentration and/or polymerization of aromatic constituents within individual cells?
Authors: It appears that variation within cells does occur in some cells such as the parenchyma bundle sheath cells at least in terms of concentration. It will be interesting to see the degree of inter- and intra-cellular variation as we examine other species.

Reviewer III: While there is obviously some merit in applying the Apomos measurement program to sections of the tissues described in this paper, there is also a requirement to ensure that the procedure is indeed measuring what the authors suggest it is measuring. In view of the **very** minor differences between the three chosen examples in each set, one wonders if these difference are really significant or whether, for example, they might represent refraction differences which would also be expected to be wavelength dependent. I would expect that use of settings at a variety of wavelengths (beyond those reported here) might also give some slight differences not unlike seen in Figure 2. It should not be terribly difficult to include an appropriate parallel set of measurements to ensure that one is indeed "seeing" differences in distribution of aromatics.
Authors: While recognizing the readers' concern, it is in fact impossible to repeat the measurements since these sections are no longer available. This paper is intended as an introduction to a technique and as stated in the text, we are aware of the possibility you have suggested.

Figure 2. UV absorbance imaging of the cell walls of the midvein of Coastal bermudagrass leaf.

(a) Imaging of whole midvein section at 319 nm using low magnification.

(b) As (a) but at 287 nm.

(c) As (b) but at 250 nm.

(d) Imaging of parenchyma bundle sheath at 319 nm using high magnification.

(e) As (d) but at 287 nm.

(f) As (d) but at 250 nm.

(g) Imaging of mestome sheath at 319 nm using high magnification.

(h) As (g) but at 287 nm.

(i) As (g) but at 250 nm.

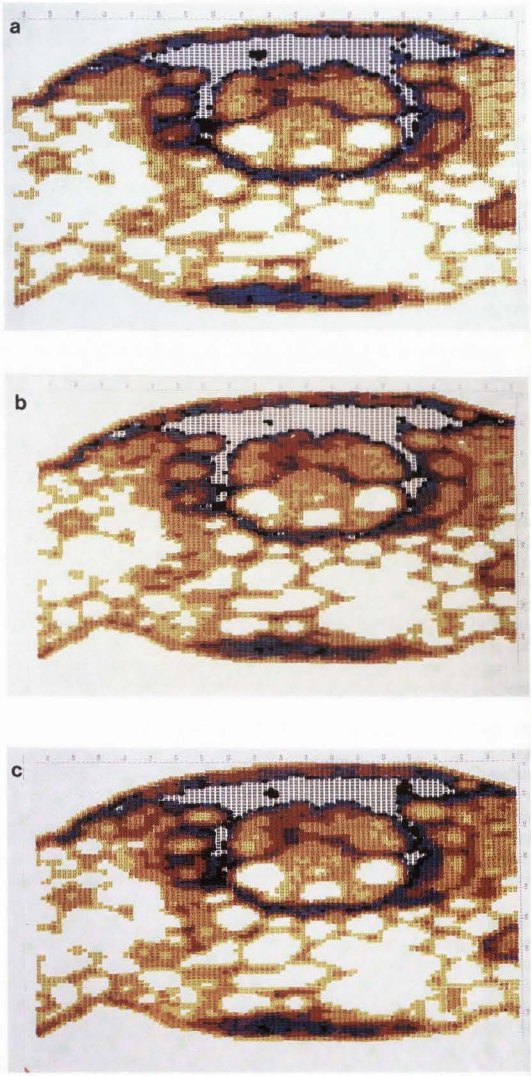

Figure 2.

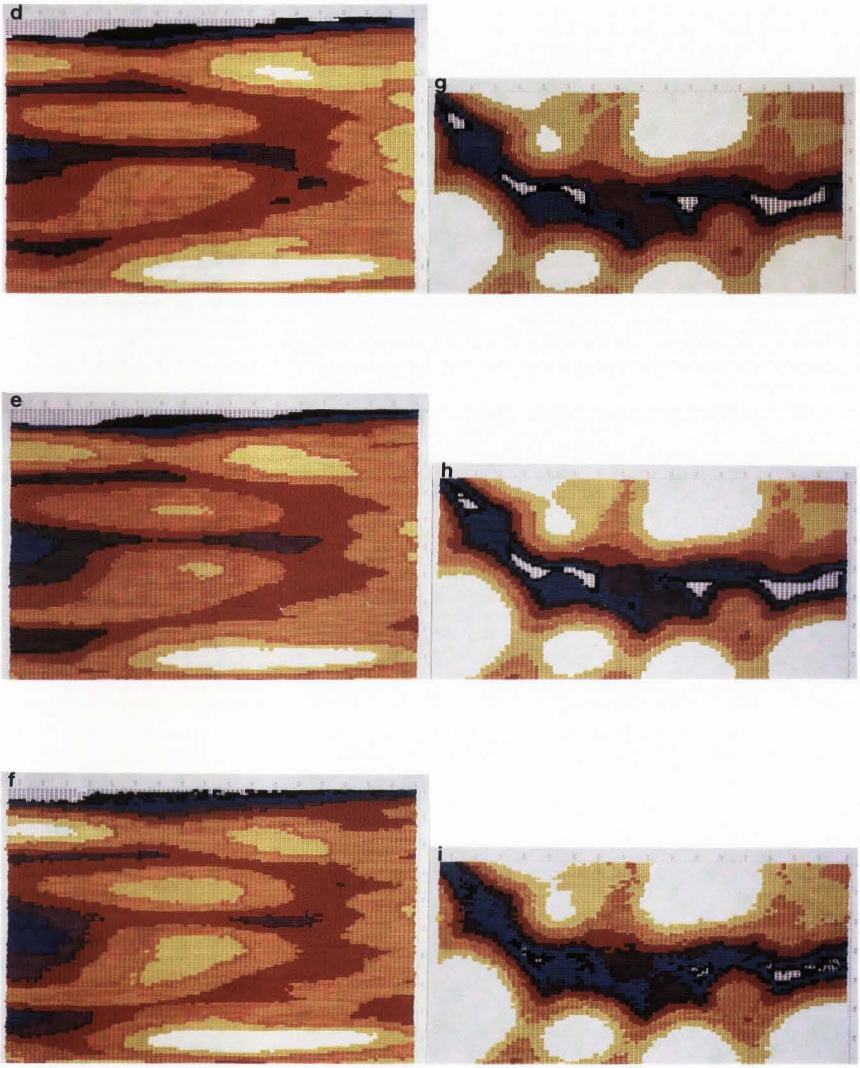

Figure 2. Cont'd.

J.A. Reffner: The author's use of 10X magnification and 100X magnification is incorrect since they are only referring to the objective magnification power and not the true system magnification. This error is most confusing when Figure 1 is referred to, in the text, as a 10X micrograph. The dimension marked on this micrograph for the midvein area is 220 X 205 μm and from this I calculate the magnification to be 370 to 395X!

Authors: The scale marker on Figure 1 gives its correct magnification. As stated in the **Materials and Methods**, 10X and 100X refer to the objective to give the reader an idea of the various ways the section was scanned.

J.A. Reffner: There is no independent verification that there are absorption maxima at the wavelengths monitored for aromatic constituents. The addition of absorption spectra of areas of various levels of aromatic constituents are necessary to support the authors' claims that they are recording changes in the aromatic content.

Authors: Esterified ferulic and p-coumaric acids are present in the cell walls of coastal bermudagrass and such esters have absorption maxima at approximately 285 nm and 320 nm [Smith MM, RD Hartley (1983) Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. **118**: 65-80]. Lignins have absorption maxima at about 280 nm (Goldschmid, 1971; text reference). As stated in the text, the wavelength maximum at about 250 nm is probably due to the general aromatic absorption.