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Autofluorescence and HPLC analyses of phenolics in *Zea mays* L. stem cell walls

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SUMMARY

Cell walls from the internodes of various maize cultivars were analysed by ultraviolet fluorescence microscopy and histochemical staining before and after treatment with a number of chemicals known to extract wall substances. The material extracted with potassium hydroxide was quantitatively analysed with HPLC. This analysis showed an increase over time of the total cell-wall phenolic-acids in the ninth internode of different maize cultivars. During this time period however, the autofluorescence signal increased slightly in the parenchyma but decreased in the sclerenchyma. Chemical treatments of the cell walls of the various tissues effected different changes in the intensity of autofluorescence in these tissues. These differences help to explain the influence of cell-wall properties on the autofluorescence signal. The primary factor seems to be the amount of light penetration into the cell wall. This penetration depends on the packing of constituents which is a result of the structural and chemical composition of the cell wall.

Key-words: autofluorescence, cell walls, HPLC analyses, lignin, phenolic-acids, *Zea mays* L.

INTRODUCTION

In plant cell-walls, phenolics occur as polymerized phenolic-acids linked by ether-bonds, which represent the lignin content of the cell wall, and as phenolic-acids that are esterified at their carboxyl groups to hemicelluloses (Fry 1988). Walls containing ferulic acid bound to polysaccharides gives a negative phloroglucinol-HCl test for lignin (Harris & Hartley 1976).

In *Zea mays*, as in other Poaceae, autofluorescence occurs in nearly all the cell-walls. In the parenchyma of the stem cell-walls, the phenolic-acids, ferulic acid and *p*-coumaric acid esterified to hemicellulose are said to be the fluorescing substances (Harris & Hartley 1976; Hartley & Haverkamp 1984). The autofluorescence of phenolics in the parenchyma cell-walls of the stems of *Zea mays* was quantitatively analysed by Willemse & Den Outer (1988). This quantification showed that there was an increase in the content of phenolics in the stem-parenchyma cell-wall of the ninth internode over 70 days of culture, while in the fourth internode (nearest ground level) the content of phenolics increased over 160 days of

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culture. The difference in autofluorescence between internode 4 and internode 9 appeared to be caused more by the increase of phenolic content than by the increase of cell-wall thickness.

Chemical analysis of the phenolic content of maize plant-stems (Emons & Engels 1987; Boon 1989) however, shows an increase in phenolics in the ninth internode after 70 days of culture.

These contradicting data show the need for research into the cause of the difference in the quantity of phenolics measured by autofluorescence and by chemical analyses in stem cell-walls of maize.

In the present study the content of phenolic-acids linked to the hemicellulose moiety of the wall of different maize cultivars during growth, was quantitatively determined by HPLC. These figures are compared with the quantitative data from the autofluorescence of total phenolics. With the help of a number of chemical treatments, an attempt is made to explain the differences in the two quantifications. The consequences for the evaluation of autofluorescence, as used for instance in studies on lignin degradation (Akin *et al.* 1985), is discussed.

MATERIALS AND METHODS

HPLC analysis

For the HPLC analysis of phenolic-acids esterified to hemicelluloses the following maize cultivars were used: Brown midrib (BM), LG 11, and Eta Ipho. Plants sown in the field in April were harvested in July (81-day-old) and November (19-day-old). The ninth internode was freeze-dried and ground in a mill equipped with a 1 mm screen. A suspension of dried-milled internodes (5 g) was refluxed for 3 × 30 min in 80% ethanol (3 × 100 ml). After each extraction the residue was filtered and washed with 80% ethanol (50 ml). This ethanol fraction contains the phenolic-acids present in the cytoplasm and vacuoles, and the free phenolic-acids of the cell wall. The residue was dried and hydrolysed in 1 N NaOH, for 16 h at room temperature and centrifuged. The residue was washed with 2 × 50 ml of 1 N aqueous NaOH in centrifuge tubes. The combined supernatants were neutralized with 2 N HCl and concentrated to circa 250 ml. After acidification with 2·0 N HCl to pH 1, the solution was extracted with 3 × 300 ml distilled ethylacetate. The combined ethylacetate phases were dried with anhydrous Na_2SO_4, filtered and evaporated in a rotary evaporator. The residue was taken up in 0·02 M phosphoric acid at 60°C. Phenolic-acids were determined by HPLC (LiChrosphere column 10 ods 250 × 4·6 mm) in the alkali-hydrolyzed residue that remained after ethanol extraction and which was taken up in ethylacetate and dissolvable in phosphoric acid. The method was as described by Theander *et al.* (1981).

The eluting solvents of the HPLC analysis were 4% aqueous HAc and 4% HAc in methanol. Phenolic-acids were detected with an UV detector at 280 nm. Phenolic standards used include catecheic acid, chlorogenic acid, caffeic acid, epicatecheic acid, *p*-coumaric acid, ferulic acid, phloridzinic acid and quercitinic acid.

Autofluorescence analysis

For autofluorescence (AF) analysis, only the LG 11 cultivar was used. It was grown in a greenhouse with a temperature range of 18–23°C and a light regime of 8 h dark and 16 h light, starting 3 February. The AF analysis was done on 80- and 180-day-old plants from 1 mm thick stem sections taken from the ninth internode. The autofluorescence of the

parenchyma of the inner and outer part of the stem, of the cell walls of the subepidermal sclerenchyma and of the cuticle including the epidermis was measured.

The 1 mm thick transverse sections of the stem were placed on a slide in a solution of glycerine–0·1 M phosphate buffer (pH 7·2, 1:1). An area of $10 \times 10\ \mu m^2$ from each type of cell wall was measured using a 6 × ocular and 63 × objective lense.

The cytophotometer was composed of a Zeiss-junior microscope equipped with a Phloem incident UV light with an excitation wave length of 365 nm from a high pressure 100 Watt mercury lamp. A motor-drive Scott interference S20 filter for the emission spectrum was used and a RCA c 31034 photomultiplier with a fluke 412B high-voltage power-supply detected the signal. A Goerts RE 541 recorder registered the signals in mV.

Measurements were taken from at least 10 different areas of $10 \times 10\ \mu m^2$ each covering a part of the cell wall. The values were corrected for background effects by subtracting the value of a $10 \times 10\ \mu m^2$ area without cell walls. No correction was made for the increase in wall thickness from day 80 to day 180.

Using 1200 V power, the maximum of the emission spectrum (E_{max}) was determined in nm wavelength along with the intensity in mV of this wavelength (I), and the change in the intensity in the first 30 seconds at the E_{max}. The fading percentage (F%) was expressed as the percentage of decrease in intensity, if the E_{max} at 0 seconds = 100%. All AF intensity measurements were performed at 465 nm for parenchyma, at 470 nm for sclerenchyma, and at 500 nm for cuticle.

Chemical treatments

For chemical treatments of the cell walls the sections were extracted with:

(a) dioxane-water 9:1 with 0·5 ml HCl per 100 ml at 100°C, during 30 min, according to Higuchi (1978) extracting the less condensed 'lignin';
(b) 30% hydrogen peroxide/97% glacial acetic acid, 1:1 v/v (H_2O_2/HAC) at 100°C, extracting carbohydrates but leaving the cellulose (Desphande 1976);
(c) 1 N NaOH for 30 min at 60°C, with subsequent washing with 1 N HCl to neutralize the solution, extracting phenolic-acids that are ester-linked to hemicelluloses (Harris & Hartley 1976), as was also used for the HPLC analyses;
(d) 0·1 N ammonia according to Harris & Hartley (1976).

Treatment with glycerin–0·1 M phosphate buffer (pH 7,2, 1:1) was used as a control. After treatment the sections were transferred to glycerine–phosphate buffer which was the surrounding medium used during measurement.

The measurements started (time = 0) at the moment that the heating point was reached as given in treatment 1, 2 and 3. For treatment 4, the start of the extraction is taken as time = 0.

The histochemical stain phloroglucinol–HCl was used to stain 'lignin' and chloro–zinc–iodine solution to stain cellulose; both are according to Jensen (1962). Samples were stained after 5 min of chemical treatment.

RESULTS

HPLC analysis

In the ethylacetate fraction *p*-coumaric acid and ferulic acid were present as the main phenolic compounds. A number of yet unidentified phenolic-acids were present in lesser amounts.

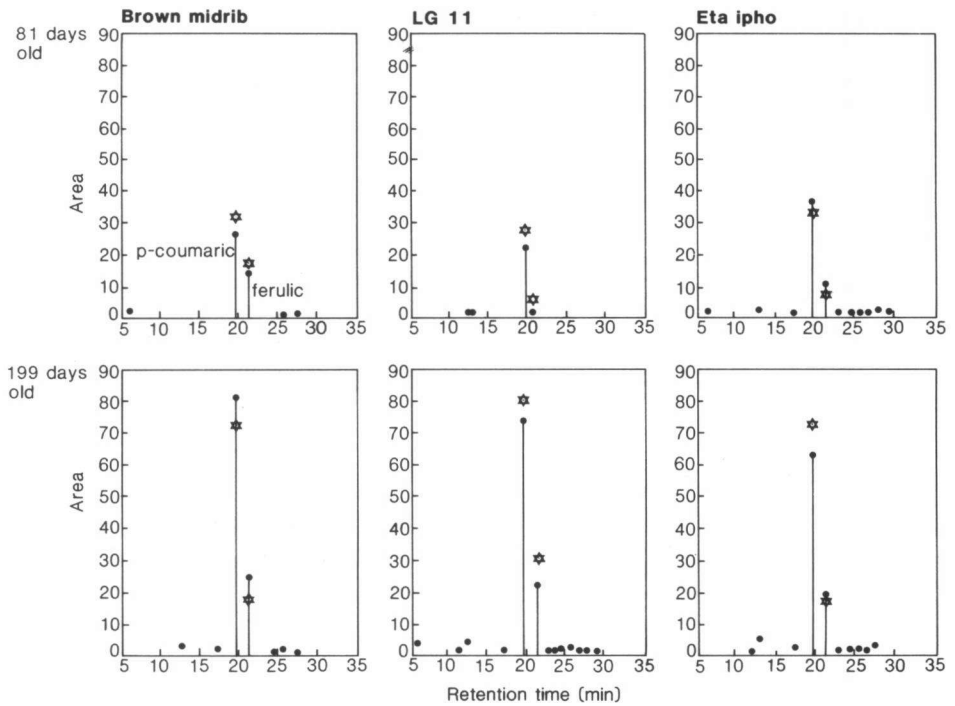

Fig. 1. Areas computed from HPLC analyses; (●) shows the amounts of phenolic acids in 81-day-old and 199-day-old ninth internodes of the stem of the maize cultivars Brown midrib, LG11 and Eta ipho of one experiment. (☆) shows mean amount of *p*-coumaric acid and ferulic acid measured in two other experiments.

Figure 1 shows the relative amounts of the phenolic-acids *p*-coumaric acid and ferulic acid present in 1 g of 81-day-old and 199-day-old ninth internodes, after determining the area under the curve from HPLC analyses of the ethylacetate fractions. In all three cultivars the amount of the phenolic-acids, *p*-coumaric acid and ferulic acid increased during maturation of the internode. In all cultivars, 81-day-old as well as 199-day-old, *p*-coumaric acid content was higher than ferulic acid content.

Autofluorescence intensity of untreated tissues

The results of the measured AF intensity of the inner and outer parenchyma, subepidermal sclerenchyma and cuticle of untreated sections in glycerine–phosphate buffer are given in the first column of Figure 2. The parenchyma showed a significant increase in AF intensity only in the outer parenchyma. AF of the primary cell-wall of the sclerenchyma was much higher than that of the secondary wall (Willemse & Den Outer 1988). The sclerenchyma showed a small decrease in intensity during growth. The cuticle showed no difference in intensity in the 180-day-old samples.

The AF intensity of the sclerenchyma cell-wall and of the cuticle was lower than that of the parenchyma cell-wall. This becomes clear when the AF data are reduced to a similar cell-wall surface and the differences between the AFs are expressed as percentages. If the measured AF intensity of a $10 \times 10\ \mu m^2$ area of the inner parenchyma cell-wall of an 80-day-old plant, which is $1{\cdot}3 \pm 0{\cdot}3\ \mu m$ thick, is considered to be 100%, then the intensity of the outer parenchyma cell-wall is 60%. The intensity of the sclerenchyma cell-wall, which

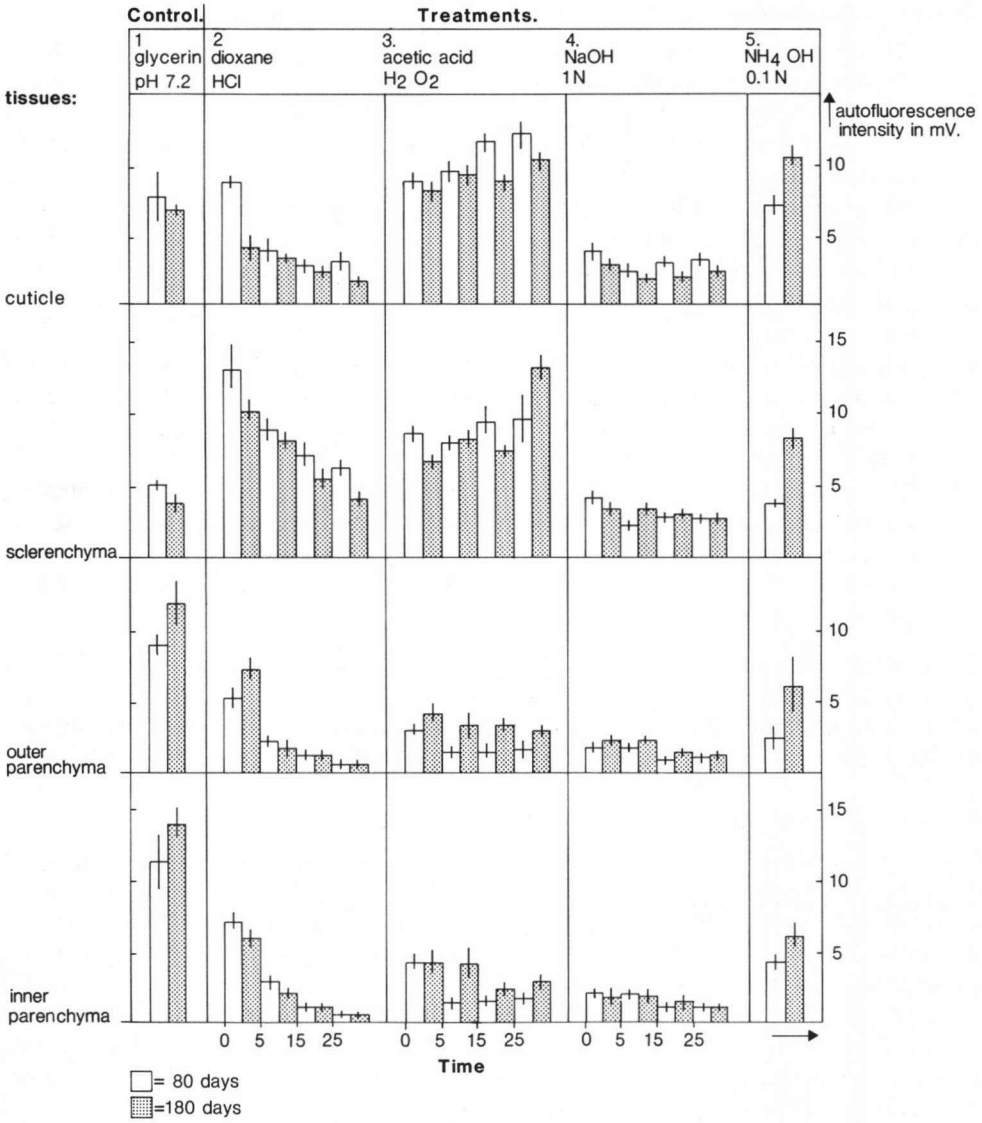

Fig. 2. Autofluorescence intensity of various untreated and chemically treated tissues of the 9th internode of an 80-day-old and a 180-day-old LG11 maize cultivar. In treatments 2, 3 and 4: 0, 5, 15, 25 means minutes after heating. The bars indicate the standard error.

is $3{\cdot}3 \pm 0{\cdot}4$ μm thick, is then 8%, and of the cuticle with a thickness of $4{\cdot}7 \pm 3$ μm is also 8%. For both the inner and outer parenchyma cell-wall of a 180-day-old plant with a thickness of $1{\cdot}8 \pm 3$ μm, the percentages remain the same, 100% and 60% respectively. In the sclerenchyma of a 180-day-old plant, which has a cell wall thickness of $5{\cdot}4 \pm 1$ μm, the percentage decreases from 8 to 4%. In the cuticle the percentage remains 8% at a thickness of $5{\cdot}5 \pm 3$ μm. In untreated tissue therefore, after correction for wall thickness, the similarity in intensity between 80-day-old and 180-day-old plants becomes even more clear for parenchyma, as does the decrease in intensity for sclerenchyma.

Changes in autofluorescence after chemical treatments

1. The dioxane–HCl treatment of inner and outer parenchyma-tissues (Fig. 2, column 2) caused a significant decrease of intensity to near zero after 25 min. In contrast, the sclerenchyma at first gave a significantly higher intensity, but this signal decreased gradually during the following 25 min. In the cuticle a slow decrease in intensity occurred.
2. After treatment with H_2O_2/HAc (Fig. 2, column 3) AF intensity of inner and outer parenchyma decreased and remained constant. The 180-day-old sample however, still showed a higher intensity than the 80-day-old sample. Both the sclerenchyma and the cuticle obtained and maintained a higher intensity but without a clear difference between the two tissues.
3. The NaOH treatment (Fig. 2, column 4) caused from time = 0, a rapid decrease in AF intensity in both inner and outer parenchyma-tissues. This also occurred in the sclerenchyma and cuticle. The AF intensity however, remained higher than that of the parenchyma.
4. The ammonia treatment (Fig. 2, column 5) resulted in a significantly lower intensity in inner and outer parenchyma-tissues. But here a difference in intensity of the two used samples can be stated. In the sclerenchyma and the cuticle the intensity of the 180-day-old sample increased. For all samples this treatment gave a higher AF in the 180-day-old plant.

For all samples a shift in the spectral maximum occurred after chemical treatment (Table 1). This shift is greatest in the parenchyma, and in this tissue the biggest shift (10%) occurs with the NH_4OH-treatment. The fading percentage increased drastically after all treatments, but differed for the various treatments (Table 1).

Histochemical staining

Cell-wall staining of parenchyma cell-walls with chloro–zinc–iodine, indicating the presence of cellulose, was always positive in untreated as well as treated samples. After treatment with dioxane/HCl, as well as with NaOH, the staining was stronger than before treatment. The sclerenchyma always reacted positively but after treatment with dioxane or NaOH the staining was even more intense.

Staining with phloroglucinol/HCl, a conventional stain for lignin was negative in inner and outer parenchyma. In the sclerenchyma it was positive both in the untreated and ammonia-treated sections. A weak reaction remained present after NaOH treatment. After dioxane/HCl and after H_2O_2/HAc treatment the reaction was weakly positive.

DISCUSSION

Chemical analyses of phenolics

In temperate grasses, concentrations of *p*-coumaric acid are generally lower than those of ferulic acid (Hartley 1972; Burritt *et al.* 1984; Theander *et al.* 1981). Exceptions have been found in wheat straw and barley straw (Jung & Fahey 1983). Tropical grasses generally contain much greater amounts of *p*-coumaric acid than ferulic acid (Ford 1986; Ford & Elliot 1987). The three maize cultivars reported here contain more esterified *p*-coumaric acid than ferulic acid in both 81-day-old and 199-day-old internodes (Fig. 1). In all cultivars *p*-coumaric acid and ferulic acid increase in the ninth internode. In LG 11 the ferulic acid content is low during the first 81 days. It should be noted here that complete

Table 1. Changes in autofluorescence of E_{max} and fading percentage (% F) in untreated and 5-min-treated 80-day (d)-old and 180-day (d)-old stem tissues

	E_{max} (nm)						% F after 30 min					
							At 465 nm					
	Inner- and outer-parenchyma		Sclerenchyma		Cuticle		Inner- and outer-parenchyma		At 470 nm Sclerenchyma		At 500 nm Cuticle	
	80 d	180 d	80 d	180 d	80 d	180 d	80 d	180 d	80 d	180 d	80 d	180 d
Control: glycerin, pH 7·2	465	465	475	480	500	505	28	25	16	30	25	13
Treatments: dioxane/HCl	495	495	480	480	490	490	76	76	42	40	21	13
H_2O_2/HAC	500	500	500	500	505	510	90	94	60	36	27	34
NaOH	485	485	475	480	510	510	60	50	13	22	3	7
NH_4OH	510	510	485	500	510	490	67	78	20	31	32	32

internodes were used for the HPLC analyses. Cells in the lower part are younger than those in the upper part. The relative abundance of ferulic and *p*-coumaric acid might be different in upper and lower part of one internode.

Boon has analysed our maize material by means of pyrolysis mass spectrometry (PYMS) (Boon 1989). The inner and outer parenchyma, subepidermal sclerenchyma and vascular bundles of the ninth internode, this is the fifth from the ground level, of a 180-day-old LG 11 plant were analysed separately.

This analysis showed that there are clear differences in the relative carbohydrate/lignin quantities, and in the phenolic components of the samples. The polysaccharides of the inner and outer parenchyma are also significantly different. The outer parenchyma is richer in pentosan relative to hexosan. The two phenolic acids *p*-coumaric acid and ferulic acid are present in abundance. It is probable that dimers of the phenolic-acids are also present. With pyrolysis mass spectrometry one cannot discriminate between phenolic-acids derived from esterified and from etherified material.

The PYMS analyses also showed that lignin in the maize internodes of LG 11 consists of an etherified lignin formed from the condensation of coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol.

Autofluorescence of phenolics in untreated cell walls

The amount of lignin in the complete ninth internode increases during 199 days of culture, but this increase is not correlated with the amount of AF intensity of the parenchyma and sclerenchyma. Also, the difference in concentration of ferulic acids of young and old LG 11 stem samples is not expressed in the AF measurements. Furthermore, although the cell walls of the sclerenchyma are thickening more than the cell walls of the parenchyma, the amount of AF intensity of the sclerenchyma remains even much lower than that of the parenchyma cell-wall. Thus, the measurements of the AF intensity are not at all representative of the increase in phenolic substances in these cell walls.

The AF intensity shows a difference between the inner and outer parenchyma, 100% versus 60%, probably due to the different composition of the carbohydrates. Qualitatively the AF, expressed as E_{max} in nm wavelength, reveals that the values for the parenchyma (465), sclerenchyma (475 nm), and cuticle (500 nm) are different. This probably reflects a difference in composition of the phenolics of these tissues.

Interpretation of autofluorescence in chemically treated walls

Dioxane/HCl acts as an organic solvent dissolving the less condensed 'lignin' (Higuchi 1978) and probably removes phenolic-acids and hemicelluloses, a procedure which seems to loosen the cell wall, permitting the UV light to penetrate and making lignin accessible. After this treatment the AF decreases in both types of parenchyma, and initially increases in the sclerenchyma up to approximately 2·5 times the original value. The increase of the AF signal in the sclerenchyma is then followed by a decrease. After removal of the esterified phenolic-acids, lignin substances also seem to be degraded by the treatment. During treatment, however, more lignin remains masked in the old sclerenchyma cell-walls than in the young walls. After prolonged treatment the AF signal is expected to decrease even further. The decrease of the AF intensity in the sclerenchyma is accompanied by an increase of cellulose staining and weak staining of the lignin 5 min after treatment indicating the loosening of the cell wall and degradation of the lignin.

H_2O_2/HAc removes hemicellulose and pectin and thus probably also phenolic-acids. However, it leaves lignin and cellulose intact (Desphande 1976). The procedure is used to

visualize cellulose microfibrils in non-lignified cell-walls (Emons 1988). With this procedure lignin becomes accessible to light. Therefore, the AF signal decreases in the parenchyma, but increases in the sclerenchyma and cuticle. Furthermore, the chemically measured differences between young and old stems become visible in the AF measurements.

NaOH removes phenolic-acids and leaves most of the lignin intact (Harris & Hartley 1976). This explains why AF decreases in parenchyma to a lower level than in sclerenchyma after this treatment. An alkali-soluble lignin component could be detected in combined pyrolysis mass spectrometry and HPLC studies of the same maize cultivars (A.M.C. Emons, pers. commun.) which explains why the treatment does not show the expected difference in lignin quantities in the sclerenchyma of young and old stems.

NH_4OH seems to discriminate between phenolic-acids esterified to hemicelluloses and etherified phenolics within the lignin molecule. It causes phenolic-acids to fluoresce green, while lignin fluoresces blue as before treatment (Harris & Hartley 1976). If NH_4OH affects the carbohydrates (Van der Valk *et al.* 1986) the phenolics probably become free to UV light. After treatment with NH_4OH the AF is higher in the older stems than in young stems, which correlates with the chemical analyses.

The relation between wall composition and autofluorescence

The fluorescence colour of carbohydrate esters of ferulic acid obtained from ryegrass cell-walls and separated by thin-layer chromatography changed from blue to green after treatment with 0·1 N NH_4OH (Harris & Hartley 1976). Ultraviolet fluorescence microscopy of transverse sections of maize internodes show a similar change in colour after NH_4OH treatment (Table 1) suggesting that these walls contain phenolic acids esterified to polysaccharides. In maize the presence of *p*-coumaric acid and ferulic acid is confirmed by the HPLC analyses (Fig. 1). Further evidence that the fluorescence of the parenchyma cell-wall is associated with the presence of phenolic-acids is given by the observation that treatment of internodes with 1·0 N NaOH greatly diminished the fluorescence. This treatment is known to remove esterified phenolic-acids from cell walls (Hartley 1972). Thus, the AF measurement of untreated material cannot give a reliable picture of the amount of lignin in cell walls, because phenolic-acids esterified to hemicellulose also fluoresce.

Chemical treatment strongly changes the AF of all cell types and each treatment produces its own characteristic AF. The AF signal is related to the chemical and structural composition of the cell wall. By loosening the sclerenchyma cell-walls with dioxane/HCl or H_2O_2/HAc, the AF intensity increases strongly. This reveals that the structural composition of the cell wall such as the way of coupling and packing of constituents, causing a tightening of the cell wall (Fry 1988) influences the AF.

We have to conclude that autofluorescence of untreated material is not a reliable method of determining quantitatively the amount of the phenolic-acids as well as the phenolics present in the lignin in cell walls. In the cell wall, a whole scale of phenolic substances is present, linked to each other and to other wall substances. Type and amount of these substances and the bonds between them determine whether UV light can penetrate the wall to produce a distinct AF signal.

Our experiments indicate that the hemicellulose moiety of the cell wall inhibits light penetration in untreated cell walls, acting as a physical barrier (Katrib *et al.* 1988). This phenomenon is comparable to the finding that stains penetrate more slowly in thicker cell-walls (Frey-Wyssling 1957). To determine the presence of lignin by means of auto-fluorescence, wall extraction has to be performed first, and the extracted material should

be chemically analysed. Such a use of chemical treatments can give a surplus value to AF measurements.

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REFERENCES

Akin, D.E., Willemse, M.T.M. & Barton, F.E. (1985): Histochemical reactions, autofluorescence, and rumen microbial degradation of tissues in untreated and delignified bermudagrass stems. *Crop Sci.* **25:** 901–905.

Boon, J.J. (1989): An introduction to pyrolysis mass spectrometry of lignocellulosic material: case studies on barley straw, corn stem and *Agropyron*. In: Chesson, A. & Orskov, E.R. (eds): *Physico-chemical characterisation of plant residues for industrial and feed use*. 25–57. Elsevier, London, New York.

Burritt, E.A., Bittner, A.S., Street, J.C. & Anderson, M.J. (1984): Correlation of phenolic acids and xylose content of cell wall with in-vitro dry matter digestibility of three maturing grasses. *J. Dairy Sci.* **67:** 1209–1213.

Desphande, B.P. (1976): Observations on the fine structure of plant cell walls. I. Use of permanganate staining. *Ann. Bot.* **40:** 433–437.

Emons, A.M.C. (1988): Methods for visualizing cell wall texture. *Acta Bot. Neerl.* **37:** 31–38.

— & Engels, F.M. (1987): Phenolic acids of maturing stems of maize cultivars differing in digestibility. *Acta Bot. Neerl.* **36:** 23.

Ford, C.W. (1986): Comparative structural studies of lignin–carbohydrate complexes from *Digitaria decumbens* (pangola grass) before and after chlorite delignification. *Carbohydrate Res.* **147:** 101–117.

— & Elliot, R. (1987): Biodegradability of mature grass cell walls in relation to chemical composition and rumen microbial activity. *J. Agric. Sci. Camb.* **108:** 201–209.

Frey-Wyssling, A. (1957): *Die pflanzliche Zellwand*. Springer-Verlag, Berlin, Göttingen, Heidelberg.

Fry, S.C. (1988): *The growing plant cell wall: Chemical and metabolic analyses*. Longman Scientific, Technical. Harlow, Essex, U.K.

Harris, P.J. & Hartley, R.D. (1976): Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. *Nature* **259:** 508–510.

Hartley, R.D. (1972): *p*-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility. *J. Sci. Food Agric.* **23:** 1347–1354.

— & Haverkamp, J. (1984): Pyrolysis mass spectrometry of the phenolic constituents of plant cell walls. *J. Sci. Food Agric.* **35:** 14–20.

Higuchi, T. (1978): Lignin structure and morphological distribution in plant cell walls. In: Kirk, J., Higuchi, T. & Chang, H.M. (eds): *Lignin biodegradation: microbiology chemistry and potential applications*. **1:** 1–20, CRC Press Inc., Boca Raton, Florida.

Jensen, W.A. (1962): *Botanical histochemistry*. Freeman & Company, San Francisco and London.

Jung, H.G. & Fahey, Jr, G.C. (1983): Nutritional implications of phenolic monomers and lignin: a review. *J. Animal Sci.* **57:** 206–219.

Katrib, A.L., Chambat, F.G. & Joseleau, J.D. (1988): Étude de quelques prétraitements favorisant la solubilisation de la Paille de Blé dans le solvant organique N-methyl-morpholine N-oxyde. *Holzforschung* **42:** 21–27.

Theander, O., Uden, P. & Aman, P. (1981): Acetyl and phenolic acid substituents in timothy of different maturity and after digestion with rumen micro-organisms or a commercial cellulose. *Agric. Environ.* **6:** 127–133.

Van der Valk, F., Boon, J.J. & Hartley, R.D. (1986): Curie-point pyrolysis-ms, -gc/ms, and -ms/ms of native, steam and ammonia treated OECD barley straw. In: Todd, J.F.J. (ed.): *Proc. 10th Int. mass spectrometry conference, Advances in Mass Spectrometry*, **Vol B:** 655–656. Wiley and Sons, New York.

Willemse, M.T.M. & Den Outer, R.W. (1988): Stem anatomy and cell wall autofluorescence during growth of three maize (*Zea mays* L.) cultivars. *Acta Bot. Neerl.* **37:** 39–47.