

NOTE

Fluorescence Microscopy of the Pericarp, Aleurone Layer, and Endosperm Cell Walls of Three Sorghum Cultivars

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Pericarp color and the presence of a pigmented testa affect the quality of food products made from ground whole sorghum grain. The R and Y genes determine the genetic color of the sorghum pericarp, which can be red (R₋Y₋), yellow (rrY₋), or white (rryy or R₋yy). The presence of a pigmented testa is controlled by the B₁ and B₂ genes in the dominant state (B₁B₂) (Rooney et al 1980). Sorghum has been classed into three types by Butler and Price (1977). From work in this laboratory, we have found that this classification corresponds with the genetic traits of sorghum. Type I sorghums have genetically red, yellow, or white pericarps but do not have pigmented testae. Type II sorghums have genetically red, yellow, or white pericarps with pigmented testae. Type III sorghums have genetically red, yellow, or white pericarps with pigmented testae plus a brown pericarp (B₁B₂S₋). The dominant spreader gene (S₋), along with the pigmented testa, causes brown pigmentation in the epicarp, regardless of the pericarp color genetics (Rooney and Miller 1982).

The pigmented testa contains a class of polyphenolic compounds often called tannins. These compounds cause dark color in food products prepared from whole sorghums with a pigmented testa. White sorghums without pigmented testae can also produce off-color food products ranging from light tan to dark greenish-brown. The color changes have been attributed to the presence of nontannin, low molecular weight phenolic compounds in the grain that react with alkali. When the sorghum kernel is pearled, or abrasively milled, to remove the outer layers of the kernel, the products have considerably less color. A number of recent articles (Rooney and Murty 1982) deal with color in sorghum food products.

Hahn et al (1983) developed a high performance liquid chromatography (HPLC) method to determine levels of a number of phenolic compounds present in sorghum. Ferulic acid was the most abundant bound phenolic acid in six of the seven varieties analyzed. In four of the seven varieties, the highest level of free phenolic acid was also ferulic acid. Ferulic acid, like many phenolic compounds, autofluoresces in the blue region of the spectrum. Fluorescence microscopy, combined with microchemical techniques, has been used to determine the location of ferulic acid in wheat (Fulcher et al 1972, Smart and O'Brien 1979), barley (Fulcher and Wong 1980, Smart and O'Brien 1979) and oats (Fulcher 1982). The reported work was undertaken to demonstrate autofluorescence in sorghum and to investigate possible sources of the autofluorescence.

MATERIALS AND METHODS

Sorghum Samples

Three sorghum cultivars that illustrate major genetic differences among sorghums were selected: BTx3197 (RRyyb₁b₁B₂B₂SS), sorghum with a thick, white pericarp without a pigmented testa; Early Hegari (RRyyB₁B₁B₂B₂ss), sorghum with a thick, white pericarp with a pigmented testa and recessive spreader gene; and ATx623 × SCO103-12 (RRYyB₁b₁B₂B₂SS), sorghum with a thick, brown pericarp (genetically red) with a pigmented testa and a dominant spreader gene. All varieties were grown at Halfway, TX, in 1980.

Fixation and Embedding

Mature sorghum kernels were cut in half or quarters with a sharp, xylene-cleaned razor blade. The half kernels were fixed in 3% glutaraldehyde in a 0.025 M phosphate buffer (pH 6.8) for 48 hr at 4° C. Fixed specimens were dehydrated and embedded in a glycol methacrylate resin according to the procedure of Feder and O'Brien (1968).

Fluorescence Microscopy

Embedded kernels were sectioned with a rotary microtome (1 μm thick) and examined with a Zeiss Universal microscope equipped with a IIIRS epi-illuminating system and a 100-W mercury arc lamp. The excitation filter (trans λ max 365 nm) and the barrier filter (trans λ max >418 nm) were used to examine all sections.

Microspectrofluorimetry

A Zeiss Zonax photometer was used to measure the relative intensity of emission spectra from 400 to 500 nm of the aleurone cell walls in all three sorghum cultivars after excitation at 365 nm. The photometer aperture was 0.5 mm in diameter and was placed directly on fluorescing aleurone cell walls. These emission spectra were compared to those of ferulic acid crystals excited under the same conditions.

Isolation of Cell Wall Material

Whole sorghum was ground through a 1-mm screen in a Udy Laboratory mill (Udy Corp., Fort Collins, CO). Cell walls were isolated by treating 150 g of ground whole sorghum with 0.15 ml of a bacterial α-amylase (Taka-therm, Miles Laboratory, Elkhart, IN) in 1.5 L of H₂O. The mixture was heated to 90° C and maintained at that temperature for 1 hr for starch liquification. After cooling to 37° C, 0.100 g of gasolase (saccharifying enzyme, Biochon, Lexington, KY) was added to the mixture. The system was sealed and stored at 38° C for two days. During this time, natural flora in the grain fermented the sugars released by the starch breakdown (Doherty et al 1983). The mixtures were centrifuged and washed. The residue was subjected to the method of Selvendran and Du Pont (1980) for further removal of intracellular material. This method consisted of wet-ball-milling the residue and then removing intracellular proteins, nonstarch polysaccharides, and starch by sequential extraction with aqueous sodium deoxycholate, phenol-acetic acid-water, and 90% (v/v) aqueous dimethyl sulfoxide. Cell wall material was monitored during the isolation procedure to ensure that there was no major loss in fluorescence.

Cell Wall Hydrolysis

The method of Krygier et al (1982) was used for base hydrolysis of cell wall material. One gram of cell wall material was hydrolyzed in 18 ml of 4 N NaOH for 5 hr at room temperature under vacuum. The sample was acidified to pH 2 with 6 N HCl. The mixture was partitioned four times with 10 ml of ethyl acetate. The ethyl acetate extracts were pooled and evaporated under vacuum at 32° C. The residue was dissolved in 2-3 ml of methanol. Total phenols were determined, using a high performance liquid chromatograph with a C18 column (Alltech Assoc., Deerfield, IL) and an ultraviolet (UV) detector (254 nm), according to the procedure of Hahn et al (1983).

RESULTS AND DISCUSSION

Fluorescence Microscopy

The three sorghum cultivars examined with fluorescence microscopy exhibited an intense blue autofluorescence in the aleurone cell walls (Figs. 1-3). The aleurone cell walls were thicker on the pericarp side of the cell (Figs. 1-4). The cross and tube cell walls showed blue autofluorescence of a lesser intensity than the aleurone cell walls. In Fig. 4, a cross-section through the aleurone layer showed the intense fluorescence in the cell walls. The center

aleurone cell was not cut and, with the cell wall still intact, the intense fluorescence can be clearly seen. A similar blue autofluorescence was also observed in the epicarp and mesocarp cell walls (Fig. 5). The pigmented testae in Early Hegari (Fig. 2) and ATx623 × SCO103-12 (Fig. 3) did not autofluoresce. The testa in both cultivars appeared to consist of two layers (Figs. 2, 3), which confirmed the report by Blakely et al (1979).

The aleurone cell walls were much thicker than those of the endosperm cell walls (Fig. 6). In Fig. 6, a peripheral endosperm cell is visible below the aleurone layer. The dark, spherical areas inside

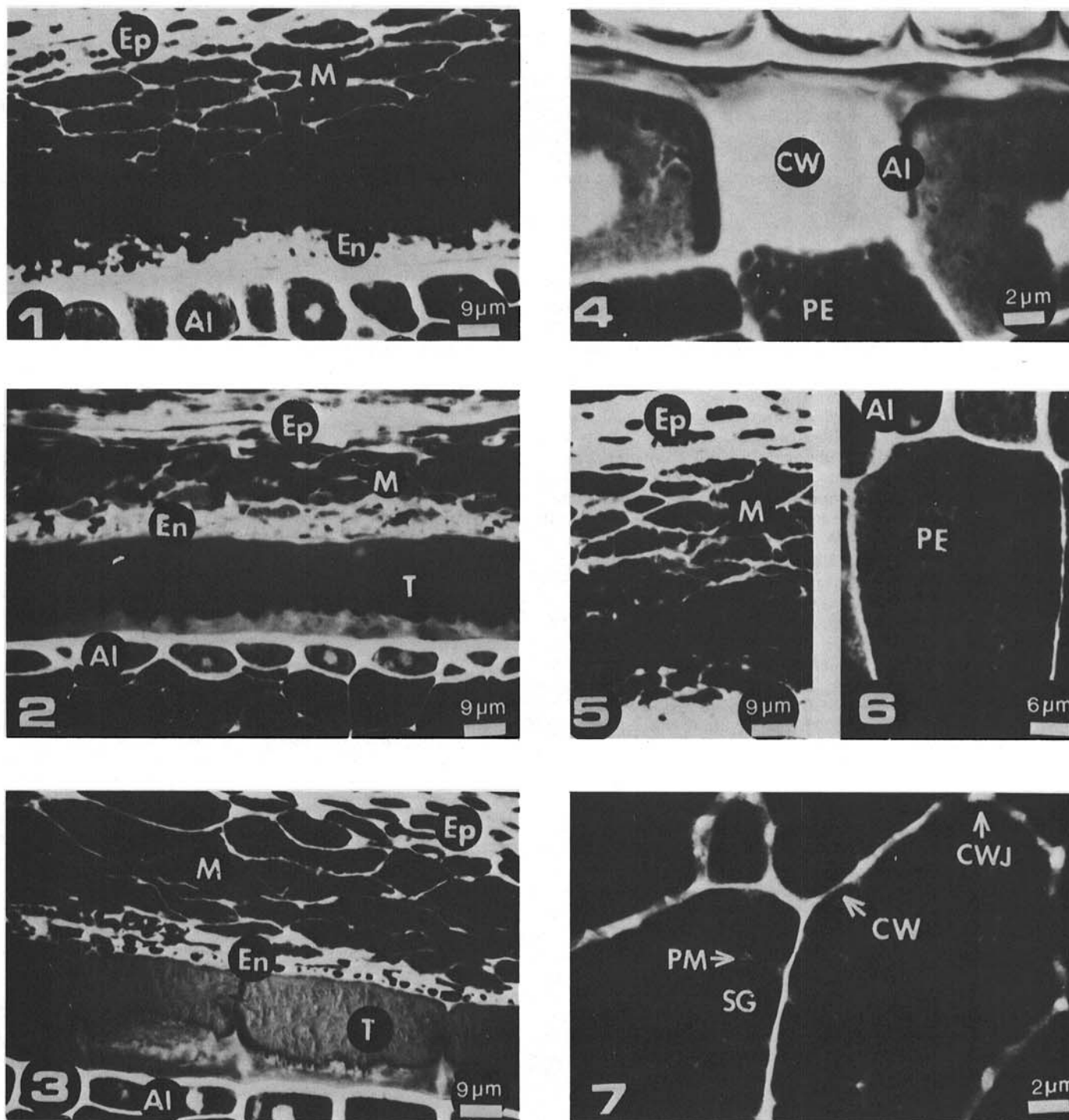


Fig. 1. Autofluorescence in pericarp, aleurone layer, and endosperm of three sorghum varieties. Ep = Epicarp; M = mesocarp; En = endocarp (cross and tube cells); Al = aleurone; T = testa; PE = peripheral endosperm cell; CW = cell wall; SG = starch granule; CWJ = cell wall junction; PM = protein matrix. **1,** BTx3197 (Type I sorghum). The pericarp is white. A pigmented testa is not present. **2,** Early Hegari (Type II sorghum). The pericarp is white. A pigmented testa is present. **3,** ATx623 × SCO103-12 (Type III sorghum). The pericarp is brown. A pigmented testa with dominant spreader gene is present. **4,** Cross section through aleurone layer showing autofluorescence in aleurone cell walls. **5,** Autofluorescence in mesocarp cell walls. **6,** Aleurone cell walls are thicker than endosperm cell walls. **7,** Floury endosperm cell walls showing bright spot at junction of adjacent cell walls.

the endosperm cell are starch granules, and the lighter areas surrounding the starch granules are protein bodies embedded in the protein matrix. Floury endosperm cell walls are shown in Fig. 7. Faint areas of fluorescence are present in the protein matrix, but the source of this fluorescence has not been investigated. Bright pockets of fluorescence also occur at the junction of adjacent cell walls (Fig. 7).

The scutellar parenchyma cell walls also showed the bright blue autofluorescence observed in the other cell walls. Ferulic acid has been identified as the major autofluorescent compound in the scutella of wheat and barley (Smart and O'Brien 1979). The scutellar epithelial cell walls were darker blue and were much less intensely fluorescent than the pericarp, aleurone, or endosperm cell walls.

When sections were exposed to ammonia vapor before being viewed, the bright blue fluorescence became a bluish-green color similar to that described by Fulcher (1982). Isolated cell wall particles also exhibited the bright blue autofluorescence of the intact sections. When exposed to ammonia vapor, the autofluorescent color in the isolated cell wall material also turned bluish-green.

Cell Wall Isolation and Analysis

Cell wall yields ranged from 3.0 to 4.0% of ground sorghum. The isolated cell wall material was negative to I₂/KI stain. Crude protein, (N × 6.25) was 10.1%, which was higher than that of barley and wheat cell wall preparations (Forrest and Wainright 1977, Selvendran and Du Pont 1980).

Base hydrolysis of the cell wall materials gave five major peaks by HPLC analysis, two of which were tentatively identified by retention time. When the cell wall extracts were chromatographed with a known amount of ferulic acid standard, the major peak for each variety coeluted with the standard, suggesting that ferulic acid is the primary phenolic compound in these samples. The highest level of ferulic acid was found in ATx623 × SCO103-12 (Type III) at a concentration of 5,790 µg/g cell wall sample, with lower concentrations of 4,993 and 2,213 µg/g sample in BTx3197 (Type I) and Early Hegari (Type II), respectively. *p*-Coumaric acid was also tentatively identified in the cell wall extracts of all three cultivars at concentrations of 1,040, 473, and 956 µg/g sample for BTx3197, Early Hegari, and ATx623 × SCO103-12, respectively. Two smaller unidentified peaks were eluted after ferulic acid. Another unidentified peak eluted early in the chromatogram. This peak was present in the cell wall extracts of Early Hegari and ATx623 × SCO103-12, but not in BTx3197, and was approximately one third the area of the ferulic acid peak.

Microspectrofluorimetry

Sections of all three sorghum cultivars were excited at 365 nm, and the relative intensity of the emission spectra was measured with a Zeiss Zonax photometer system. Maximum emission by ferulic acid was 440 nm. Maximum emission from the aleurone cell walls of BTx3197, Early Hegari, and ATx623 × SCO103-12 was 440, 480, and 450 nm, respectively. The emission spectra of BTx3197 was superimposable with that of ferulic acid. The shift in emission spectra of the other two cultivars may suggest that many phenolic compounds other than ferulic acid are present in the cell walls of Early Hegari and ATx623 × SCO103-12. It is also possible that the phenolic compounds in these two cultivars are bound to other cell wall components in the grain, thus shifting the maximum wavelength of the emission spectra.

CONCLUSIONS

Among nonbrown sorghums, there is a large variation in resistance to molding and weathering. Fungal invasion before grain maturity is termed molding, whereas invasion after grain maturity is termed weathering. Small, low molecular weight phenolic compounds may contribute to mold resistance. Because many of these phenolic compounds autofluoresce in the blue region of the spectrum, fluorescence microscopy is a useful tool in identifying their location, and microspectrofluorimetry can be used to determine levels of the fluorescing compounds.

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