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C. F. Earp

L. W. Rooney

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FLUORESCENCE CHARACTERIZATION OF THE MATURE CARYOPSIS OF SORGHUM BICOLOR (L.) MOENCH

C.F. Earp and L.W. Rooney

Cereal Quality Lab, Dept. of Soil & Crop Sciences,
Texas A&M University, College Station, Texas 77843-2474

Abstract

Fluorescence microscopy was used to characterize the mature caryopsis of Sorghum bicolor (L.) Moench. Acid Fuchsin, a protein specific dye used in bright field microscopy, caused protein bodies and matrix in the sorghum endosperm to fluoresce. ANS (8-anilino-1-naphthalene sulfonic acid) also caused the protein bodies and matrix in the endosperm to fluoresce. Varietal differences in endosperm protein distribution were evident when viewed after staining with Acid Fuchsin. Nile Blue A caused fluorescence in neutral lipids such as those in the lipid bodies in the aleurone and scutellum of sorghum. Nile Blue A also caused fluorescence in two cuticular layers, one on the outside of the sorghum kernel and the other next to the aleurone layer. The bright field Sudan III and IV stains were used to confirm the presence of these cuticular layers. After staining with both Ehrlich's reagent and dimethylaminocinnamaldehyde, fluorescence due to aromatic amines was not observed in the aleurone of sorghum. After staining with diphenylborinic acid, a marker for flavonoids, fluorescence in the aleurone cell walls was observed. Periodic acid/Schiff's reagent was used to view starch in the sorghum endosperm. Acriflavine-HCl produced fluorescence in phytin granules in the scutellum of sorghum; no fluorescence was observed in the aleurone. When treated with cyanogen bromide and either barbituric acid or p-aminobenzoic acid, nicotinic acid deposits were detected in inclusions in both the aleurone and scutellum of sorghum.

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Direct inquiries to L.W. Rooney
Telephone number: 409 845 2925

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Introduction

Fluorescence microscopy of methacrylate-embedded samples provides a more rapid means for viewing samples than paraffin-embedded samples viewed with bright field microscopy. Paraffin-embedded samples are water impermeable and a lengthy staining series (often one hour or more) must be used to remove the paraffin to permit staining of the sections. When samples for fluorescence microscopy are embedded in glycol methacrylate, a resin which is water soluble, most fluorochromes can be added directly to the sections. Staining is usually complete in one to five minutes followed by a brief rinse. Fluorescence microscopy has the advantage over bright field microscopy in that chemical and structural information can be gathered. Chemical data results from the specificity of fluorochromes for particular compounds. When attached to a microspectrofluorometer, quantitative values can be determined for the fluorescent compounds of interest (Fulcher and Wong, 1980).

Fluorescence microscopy has been used to study the ultrastructure of wheat, oats and barley (Fulcher and Wong, 1980). Yiu et al., (1982, 1983) have also used fluorescence microscopy in the study of rapeseed structure and changes occurring during processing. In previous work from this laboratory, fluorescence microscopy of the pericarp, aleurone and endosperm cell walls of three sorghum varieties has been reported (Earp et al., 1983a). Congo Red and Calcofluor, both specific for mixed linkage β-glucans (Wood and Fulcher, 1978; Wood et al., 1983), were used to study β-glucans in sorghum (Earp et al., 1983b).

There are a number of reagents which can be used to stain proteins in fluorescence microscopy. Acid Fuchsin, a protein specific dye, has also been used in light microscopy (Gurr, 1960). Orange G binds basic amino acids and can be used to label proteins (Udy, 1956). Other fluorochromes such as ANS (8-anilino-1-naphthaline sulfonic acid) and Fluorescamine can also be used to view proteins. ANS fluoresces in aqueous media while Fluorescamine

must be used in acetone or similar solvents (Fulcher and Wong, 1980).

Nile Blue A, a water soluble dye, becomes an intense fluorescent yellow when in contact with neutral lipids. It is also a useful marker for cuticular layers. Since Nile Blue A is an aqueous dye, the problem of lipid extraction (such as occurs with Sudan dyes) is eliminated (Fulcher and Wong, 1980; Fulcher, 1982). Fulcher and Wong (1980) state that a non-extractive GMA-glutaraldehyde-urea resin must be used for high resolution analyses of lipids.

Fulcher and Wong (1980) demonstrated yellow fluorescence in the subaleurone tissues of oats after treating the section with diphenylborinic acid in 80% methanol. This compound has been used as a marker for flavonoid compounds on thin layer chromatograms. Use as a fluorochrome suggests that flavonoid compounds may be detected using this technique.

Using Ehrlich's reagent (2,4-dimethylaminobenzaldehyde), Fulcher and Wong (1980) determined the location of Ehrlich-positive structures in the aleurone cells of wheat, barley and oats. Fulcher and Wong believed that these structures contained ortho-aminophenol and ortho-aminophenyl glucose, two aromatic amines reported in wheat bran by Mason et al (1973) and Mason and Kodicek (1973a,b).

The periodic acid/Schiff's (PAS) reagent stains starch in fluorescence as well as light microscopy (Fulcher and Wong, 1980; Fulcher and Wood, 1983). An aldehyde blocking agent (such as 2,4-dinitrophenylhydrazine) must be used prior to the PAS reaction to minimize the effects of tissue aldehydes and those produced during aldehyde fixing (glutaraldehyde used in this study). The PAS reaction is a two-step procedure in which the sections are oxidized with periodic acid and then stained with Schiff's reagent or with Acriflavine-HCl (Fulcher and Wood, 1983). Acriflavine-HCl has also been reported to cause fluorescence in phytin granules (Fulcher, 1982).

To locate nicotinic acid deposits, sections are treated first with cyanogen bromide and then either with para-aminobenzoic acid to produce a yellow product (Feigl, 1966) or with barbituric acid to produce an orange-red product (Fulcher et al., 1981). Wheat, barley, oats and sorghum have been reported to contain significant amounts of this reaction product in the Type II aleurone inclusions which are high in nicotinic acid (Morrison et al., 1975).

The objectives of this paper were to adapt existing fluorescence microscopy techniques to studying sorghum structure. After optimizing procedures, the techniques were used to study the developing sorghum caryopsis (Earp, 1984).

Materials and Methods

Samples

The sorghum variety SC0103-12 was used. It is phenotypically brown (a genetically red pericarp) with a testa, dominant spreader gene and a thick pericarp.

The genetic description for SC0103-12 sorghum kernel characteristics is $RRYYB_1B_1B_2B_2$ SSzzII. The R and Y genes determine pericarp color. When both are dominant, the pericarp is red. The B_1 and B_2 genes control the presence or absence of the pigmented testa layer. Both genes must be dominant for a pigmented testa to develop. When the S gene (spreader gene) is dominant concurrently with the dominant B_1 and B_2 genes, pericarp color becomes phenotypically brown. The I or intensifier gene also affects pericarp color. When the I gene is dominant, pericarp color, usually red, will be much brighter than if the gene is recessive. A dominant Z gene produces a thin pericarp and the recessive condition is a thick mesocarp filled with starch granules. The sorghum variety SC0103-12 was selected for use in this study because the R, Y, B_1, B_2, S and I genes are all dominant causing each of the genetic characteristics to be expressed. Only the Z gene was recessive, which produced a thick pericarp.

Samples were grown at College Station, Texas, in 1983. Samples were collected at 34 days after anthesis (physiological maturity), placed on ice and then frozen at -4°C until fixed. Mature kernels of other varieties were also viewed, but SC0103-12 effectively demonstrates each of the kernel constituents. Double Dwarf Feterita is the other variety shown in the protein discussion.

Fixation and Embedding:

Mature sorghum kernels were halved or quartered and fixed in 3% glutaraldehyde in a 0.025 M phosphate buffer (pH, 6.8) for 48 hrs. at 4°C. Fixed specimens were dehydrated through an alcohol series and embedded in glycol methacrylate (Feder and O'Brien, 1968).

Sectioning and Microscopic Examination

Embedded kernels were sectioned with a rotary microtome with a glass knife. Sections (approximately 1µm thick) were examined with a Zeiss Universal Microscope equipped with an IIIRS epi-illuminating system and a 100-W mercury arc lamp. Objectives were Zeiss Neofluors. Fluorescence exciter/barrier filter combinations used and colors observed were:

Filter Combination:	FC I	FC II	FC III
Exciter:	365 nm	450-490 nm	546 nm
Barrier:	>418 nm	>520 nm	> 590 nm
Color Observed:	blue	yellow	red

Photographic Procedures

Fluorescence photomicrographs were taken with Ektachrome 400 film, with exposure times ranging from 30 sec to 3 min.

Fluorochromes Used

Acid Fuchsin: 0.01% aqueous solution for 1 min and washed with water 1 min. Viewed with FC III.

ANS (8-anilino-1-naphthalene sulfonic acid): 0.01% aqueous solution under cover glass. Viewed with FC I.

Nile Blue A: 0.01% aqueous solution under cover glass or dried and viewed. Viewed with FC II.

Autofluorescence: No fluorochrome was

added. Viewed with FC I.

Diphenylborinic acid: 0.05% w/v diphenylborinic acid (ethanolamine complex) in 80% methanol. Viewed with FC II.

Ehrlich's Reagent (2,4-dimethylaminobenzaldehyde): 0.5% in ethanol containing 1% HCl. A few drops were added to the slide and the slide was dried at 50-60°C. Viewed with FC II.

p-Dimethylaminocinnamaldehyde: Slides were prepared as shown for Ehrlich's reagent. Viewed with FC II.

Periodic acid/Schiff's Reagent: Sections were treated with 2,4- dinitrophenylhydrazine (saturated in 15% acetic acid) for 30 min and then washed for 30 min with water. Sections were then oxidized in 1% aqueous periodic acid for 10 min and washed 10 min. Sections were stained with Schiff's reagent for 10 min and washed with water to remove excess dye. Viewed with FC III.

Acriflavine HCl: 0.01% (w/v) acriflavine in H_2O adjusted to pH 3.1 with HCl. Slides were stained 5-15 min, rinsed in ethanol to remove excess dye and air-dried.

Cyanogen Bromide: Sections were suspended over a freshly prepared solution of cyanogen bromide (slowly add 10% potassium cyanide dropwise to 5-10 ml of saturated bromine water on ice until the solution just decolorizes with one drop KCN). Reaction complete in 5-10 min. then immerse section in p-aminobenzoic acid (2 g in 75 ml 0.75 N HCl and 25 ml ethanol) for 5-10 min. Viewed with FC II. Alternately, sections were treated with saturated barbituric acid (in 3% KH_2PO_4) as a substitute for p-aminobenzoic acid. Viewed with FC III.

Dyes used: Sudan III and IV

Excess Sudan IV was added to saturated solution of Sudan III in 70% ethanol. The solution is usable for several days. 70% ethanol was used for rinsing. Methacrylate embedded sections were stained for 6 or more hours as described by O'Brien and McCully (1981).

Results and Discussion

The sorghum endosperm is composed of cells filled with starch granules, protein matrix and protein bodies. The endosperm can be divided into three areas which vary in the proportion of starch to protein (Rooney and Miller, 1982). Directly below the aleurone is the peripheral endosperm which has some starch granules, many protein bodies and a large amount of protein matrix. The corneous endosperm has more starch and less protein than the peripheral endosperm. The floury endosperm has predominantly large starch granules with some protein bodies and matrix. Several protein-specific dyes were used to view the location of proteins in the mature sorghum caryopsis with fluorescence microscopy. Acid Fuchsin produced fluorescence in protein matrix and protein bodies (Figs. 1 and 2). ANS-induced blue fluorescence could also be seen in the peripheral and corneous endosperms (Fig. 3) and in the floury endosperm (Fig. 4). Using either of these protein dyes,

Captions of Figures 1-16 which are presented on two color plates in the following pages.

P = pericarp; A = aleurone; PE = peripheral endosperm ; CE = corneous endosperm; M = mesocarp; PB = protein bodies; SG = starch granules; T = testa; LB = lipid bodies; SP = scutellar parenchyma; Ep = epicarp; CL = cuticular layer; En = endocarp; CW = cell wall; E = endosperm. The number on the bar in each figure indicates μm.

Figure 1: Acid Fuchsin staining of sorghum endosperm protein. SC0103-12 has a thick peripheral endosperm containing large amounts of protein matrix and protein bodies which fluoresce intensely red when stained with Acid Fuchsin.

Figure 2: Acid Fuchsin staining of sorghum endosperm protein. Double Dwarf Feterita has less protein in the peripheral endosperm than SC0103-12 (Fig. 1).

Figure 3: ANS staining of sorghum endosperm protein matrix and bodies. The peripheral and corneous endosperm regions are quite evident after staining with ANS.

Figure 4: ANS staining of sorghum endosperm protein bodies in the floury endosperm.

Figure 5: Nile Blue staining of lipid bodies in the aleurone layer.

Figure 6: Nile Blue staining of lipid bodies in the scutellum. Most of the lipids of sorghum are located in the germ.

Figure 7: Nile Blue staining of cuticular layers of sorghum above aleurone.

Figure 8: Sudan dye staining of cuticular layers on pericarp of sorghum and above aleurone.

Figure 9: No staining was used on this section. Autofluorescence (blue color) appears in the cell walls of the mesocarp. Pigmentation can be seen in the epicarp of SC0103-12.

Figure 10: Diphenylborinic acid treatment caused the aleurone cell walls to fluoresce (arrow) indicating possible presence of flavonoid compounds.

Figure 11: Periodic acid/Schiff reagent stains starch granules in the endosperm red.

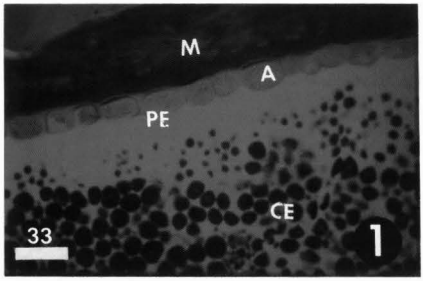
Figure 12: Phytin granules in the scutellum fluoresce red after treatment with Acriflavine-HCl.

Figure 13: No fluorescence was observed in the aleurone cells after Acriflavine-HCl treatment.

Figure 14: Treatment with cyanogen bromide and p-aminobenzoic acid produced a yellow-orange color in aleurone inclusions containing nicotinic acid.

Figure 15: Treatment with cyanogen bromide and barbituric acid produced an orange-red color in aleurone inclusions containing nicotinic acid.

Figure 16: Orange-red inclusions were also found in the scutellum after treatment with cyanogen bromide and barbituric acid (indicating presence of nicotinic acid).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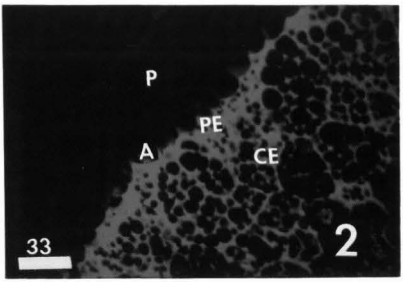
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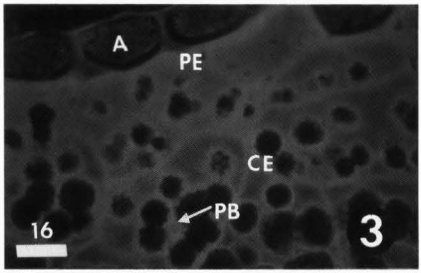
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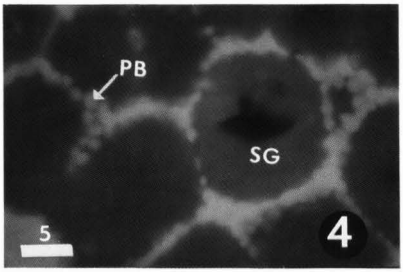
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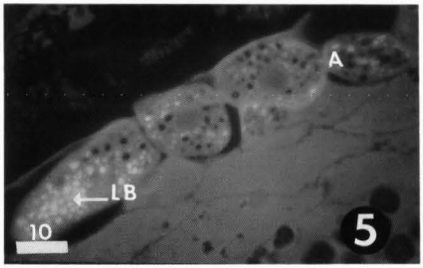
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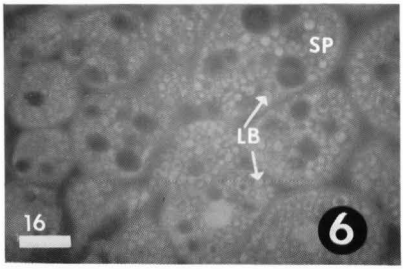
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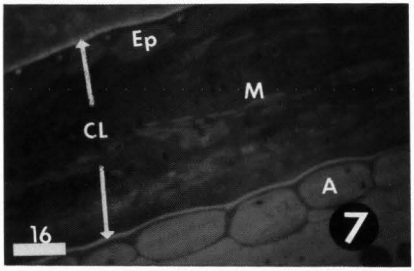
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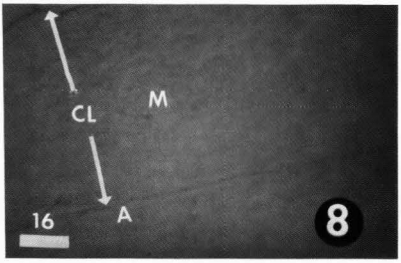
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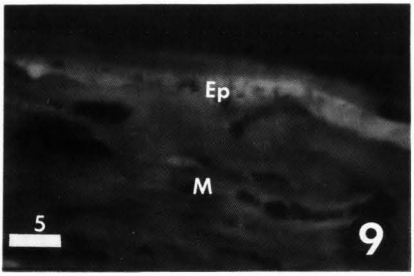
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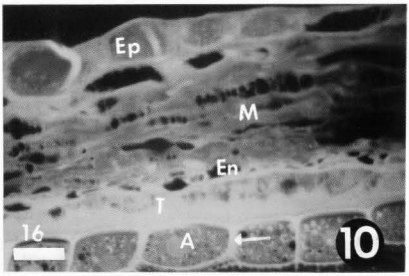
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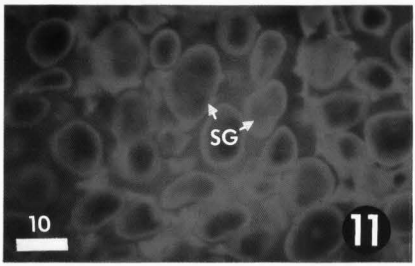
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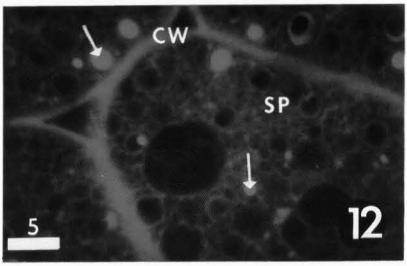
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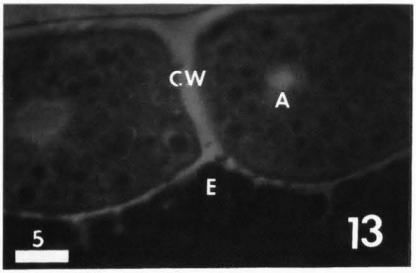
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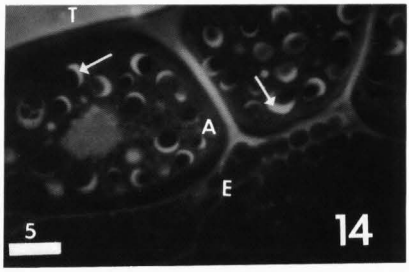
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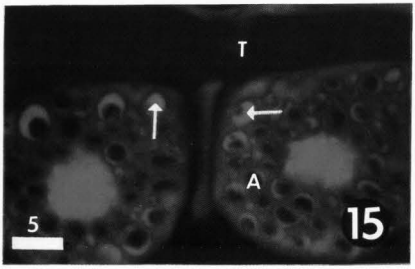
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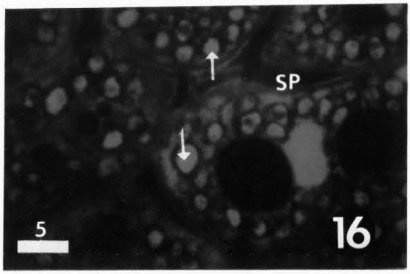
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the distribution of protein in the sorghum endosperm could be easily observed. The sorghum variety in Fig. 1 had a much thicker peripheral endosperm than the variety in Fig. 2. A number of other sorghum varieties have been observed. These two varieties show the extremes of varietal differences in protein distribution.

Choice of fluorochromes is often dependent on the desired final color. If protein were to be viewed simultaneously with autofluorescence (blue color observed), Acid Fuchsin would be chosen since the protein would appear red. ANS would produce a bluish-white fluorescence which would be indistinguishable from the blue autofluorescence.

Sorghum lipids were located primarily in the aleurone cells (Fig. 5) and in the scutellum (Fig. 6.) by staining with Nile Blue A. The yellow fluorescence induced by Nile Blue A quenched quickly and at high magnifications it was difficult to photograph before the fluorescence disappeared. Some cereals such as oats often have lipid deposits in the endosperm (Fulcher, 1982). These were not observed in sorghum. Nile Blue A caused fluorescence in two cuticular layers, as shown in Fig. 7. Staining with Sudan III and IV dyes and observation with bright field microscopy were used to confirm the presence of these layers (Fig. 8). The fluorescent layer next to the aleurone was consistent with the reports by Morrall et al. (1981) and Glennie et al. (1984) of the presence of a cuticle between the integument and nucellus. This layer also corresponded to the testa as described by Zeleznak and Varriano-Marston (1982). Zeleznak and Varriano-Marston (1982) stated that "Not all mature sorghum grains contain an inner integument, but seeds in all grain sorghum caryopses have testae." The problem encountered here is the definition of testa. Some botany sources define the testa as the seed coat with no explanation of the original tissue from which it was formed. Other sources state that the testa or seedcoat is derived from the integuments (Esau, 1977). Blakely et al. (1979) described the development of the pigmented inner integument into what was termed the pigmented testa. The term "pigmented testa" has been used extensively in the sorghum literature. In sorghum, a pigmented testa (derived from the inner integument) occurs when the B_1 and B_2 genes are dominant. Glennie et al. (1984) described this layer as the polyphenol-containing layer which can confer bird resistance to the grain and is formed from the inner integument. In varieties where the pigmented testa is absent, the layer may be difficult to see because it is often nothing more than a thin layer of crushed cells no thicker than a cell wall. From this work and that previously reported by Morrall et al. (1981) and Glennie et al. (1984), there does appear to be a cutinized layer between the testa and the aleurone in sorghum.

Sorghum contains many phenolic compounds which can cause undesirable color formation in food products, especially when treated with alkali as in tortillas. It would be very useful to determine where these compounds are located. If they are predominantly in the pericarp, then milling could remove them and improve the color of flour used in food products. Autofluorescence indicated the presence of many phenolic compounds in sorghum that are primarily associated with cell wall material (Earp et al., 1983a). When viewed with FC I, pigmentation in the epicarp cells of SC0103-12 was also observed (Fig. 9). The pigmentation did not fluoresce, but could be viewed due to its coloration. An intense yellow fluorescence was produced in the aleurone cell walls of sorghum after treatment with diphenylborinic acid (Fig. 10). Fulcher and Wong (1980) used diphenylborinic acid as a possible fluorochrome for locating flavonoid compounds in cereals. They observed an increase in fluorescence in the crease of oats after treatment with diphenylborinic acid. This fluorescence may be indicative of flavonoid compounds, but may be due to a number of other phenolic compounds as well. Ehrlich's reagents and dimethylaminocinnamaldehyde have been used to locate amino phenols in the aleurone of several cereals but no Ehrlich-positive structures have been observed in the sorghum aleurone.

The periodic acid/Schiff's reagent caused fluorescence in starch granules. Starch granules in the floury endosperm are shown in Fig. 11. This reagent would be useful for viewing starch and other components of the endosperm simultaneously.

Phytin granules can be located with acriflavine-HCl as a fluorochrome. Phytin is inositol hexaphosphate bound usually with divalent cations such as Mg and Zn. Most cereals have phytin granules in both the aleurone cells and in the scutellum (Kent, 1975). Using this fluorescence technique, the phytin granules were observed in the scutellum (Fig. 12) but not in the aleurone cells of the sorghum varieties used in this study (Fig. 13). O'Dell et al (1972) cited that in corn, 88% of the phytin was in the germ. Kurien et al. (1960) reported that only 13% of the total phosphorus in sorghum was in the fibrous seed coat (pericarp). Wang et al. (1959) analyzed seven sorghum varieties and found that the germ contained the majority of phytin phosphorus (from 2-20 times more than in the bran). These previous studies tend to support the observation that phytin granules were not present in the sorghum aleurone. This would indicate that removal of the germ during milling would remove the majority of the phytin present in the sorghum kernel.

The cyanogen bromide technique cited by Fulcher and Wong (1980) was used to determine the location of nicotinic acid in sorghum. There were two variations used. In the first method, para-aminobenzoic acid was used to produce a yellow-orange color (FC II) in the nicotinic acid containing inclusions in the aleurone grains (Fig. 14). When barbituric acid was used instead of para-aminobenzoic acid, an orange-red color was produced (FC III) in the aleurone inclusions (Fig. 15) and in the

scutellum (Fig. 16). As mentioned earlier, choice of fluorochromes would be dependent upon the desired end-product color when viewed in combination with other fluorescent compounds.

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Discussion with Reviewers

P.J. Barnes: The authors claim that diphenylborinic acid produced intense yellow fluorescence in the aleurone cell walls of sorghum. Would they not agree that Fig. 10 shows intense yellow fluorescence not only in the aleurone cell walls but in the aleurone cell contents and all layers of the pericarp? Do they consider this yellow fluorescence in the other tissues to be due to flavonoids or to autofluorescence of other components? In the wheat grain, flavonoids are located primarily in the germ; did the authors detect flavonoid-type compounds in sorghum germ?
Authors: In Figure 10, the only real fluorescence is observed in the aleurone cell walls - possibly also in some of the aleurone inclusions. Other areas of the pericarp and the testa appear to have taken up the dye and are stained yellow but they aren't fluorescing. The one bright spot in the epicarp was a wrinkle in the section. We did not detect any flavonoid-type compounds in the germ of this sorghum variety.

P. J. Barnes: The authors state that the pigment in the epicarp cells did not fluoresce. Did they try all three filter combinations? Wheat pericarp is claimed to exhibit a yellow autofluorescence, although at relatively low intensity.
Authors: We did not see any fluorescence of the epicarp pigmentation under any of the filter combinations.

C. W. Glennie: Have the authors any information on the relationship between the peripheral endosperm thickness and the protein content of the various sorghum varieties?
Authors: We have seen no relationship between peripheral endosperm thickness and protein content. The only trend we have observed over the years is that brown sorghums (those with pigmented testa [inner integument]) have a thick peripheral endosperm. Since these sorghums have very floury endosperms, the thick, dense peripheral endosperm would function as nature's way to keep the seed intact.

P. J. Barnes: Differences are claimed between the two varieties in Figures 1 and 2. Could the differences in red fluorescence in the prints be a result of different exposure times? In Fig. 1, the aleurone fluoresces red and the peripheral endosperm shows the blurred effect found with over-exposing fluorescence: why does the aleurone in Fig. 2 not fluoresce red?
Editor
Apparently the picture P. J. Barnes had was over-exposed. The other photographs do not show this problem so we do not think this question is relevant.