Nutrient Distribution in Five Perennial Grain Species Exhibited by Light and Scanning Electron Microscopy

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ABSTRACT

The anatomy and histochemistry of mature caryopses of five perennial grains (Agropyron intermedium, A. trichophorum, Secale montanum, Hordeum bulbosum, and Tripsacum dactyloides) were studied by fluorescence and scanning electron microscopy. All five grains contain starch granules and protein in the starchy endosperm. Protein bodies, which often contain phytin inclusions, are abundant in the aleurone and embryo. Lipid deposits are primarily located in the embryo and aleurone. Caryopses of A. intermedium, A. trichophorum, S. montanum, and H. bulbosum are similar morphologically, having two populations of starch granules as well as continuous protein matrix in starchy endosperm tissue. The aleurone of Agropyron spp., T. dactyloides, and S. montanum are single celled in thickness. H. bulbosum has a multiple-layered aleurone (usually three cells thick). The caryopsis of T. dactyloides is nearly spherical, and the starchy endosperm contains spherical starch granules embedded in a matrix of small protein bodies. The embryo of T. dactyloides contains starch granules.

This study addresses comparative anatomy and nutrient distribution in caryopses of Agropyron intermedium (Host) P. Beauv., A. trichophorum (Link) K. Richt., H. bulbosum L., S. montanum Guss., and T. dactyloides L. L. and has provided basic information on possible uses of the grains of these plants.

MATERIALS AND METHODS

Samples

The samples consisted of caryopses of plants from four genera, five species, and included A. intermedium, A. trichophorum, H. bulbosum, S. montanum, and T. dactyloides. Agropyron spp., H. bulbosum, and S. montanum were supplied by Peggy Wagoner, Rodale Press, Emmaus, PA. T. dactyloides was provided by Chester Dewald, U.S. Department of Agriculture, Agricultural Research Service, Southern Plains Range Research Station, Woodward, OK.

Instrumentation

Two compound fluorescence microscopes were employed. Both microscopes contain similar exciter-barrier filter combinations. The Zeiss Universal Research microscope is equipped with a 100-W mercury illuminator (Osmar HBO 100W, Berlin) and two exciter-barrier filter combinations consisting of FC B (excitation filter, 450-490; beam splitter, FT 510; barrier filter, LP 520), and FC G (exciter filter, BP 546; beam splitter, FT 580; barrier filter, LP 590). The Nikon Fluophot microscope has a 200-W mercury illuminator (Osmar HBO 200W, Berlin) and the following two filter combinations: FC U (excitation filter, UV330-380; dichroic mirror, DM400; barrier filter, 420K) and FC B (excitation filter IF420-490, auxiliary filter, 460; dichroic mirror, DM505; barrier filter, 520W). In the above, FC = filter combination, U = UV excitation, B = blue excitation, and G = green excitation. Note that the same designation is given for FC G on both microscopes. Minimal visual differences were apparent between results obtained for the two microscopes. Therefore, for simplicity of presentation, only the excitation color is given in further descriptions.

Sample Preparation: Fluorescence Microscopy

Fixation and embedding: Caryopses were cut transversally into pieces approximately 1.5 mm thick and placed into fixative. Tissues were fixed in 2–3% glutaraldehyde with or without the addition of 2% formaldehyde (from paraformaldehyde) in phosphate buffer for preservation of general morphological structure and such tissue components as protein and starch. Calcium dichloride (CaCl₂) was added to some preparations in an effort to retain lipids in tissues, as calcium reduces extraction of cellular components (Hayat 1981). Where calcium was added to fixatives, cacodylate buffer was used instead of phosphate buffer to avoid the formation of insoluble Ca₃PO₄.

Specifically, tissues were fixed overnight at 0–4°C in one of
the following fixatives: 1) a mixture of 2% glutaraldehyde and 2% formaldehyde in 0.05 M Na₂HPO₄/Na₃H₂PO₄ buffer, pH 7.0; 2) 3% glutaraldehyde in 0.05 M Na₂HPO₄/Na₃H₂PO₄ buffer, pH 7.0; 3) 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer (Glaevert 1974) with 2.5 mM CaCl₂, pH 7.2; or 4) 1% glutaraldehyde in 0.05 M cacodylate buffer with 2.5 mM CaCl₂. Additionally (fixative 5), samples were fixed in fixative 4 at 23°C for 3.5 hr. Tissues were rinsed in the same buffer, then dehydrated in an ethanol-butanol graded series (Jensen 1962) with 10–20 min per exchange. Samples prepared using fixatives 1–3 were infiltrated for three to five days and embedded in JB-4 (Polysciences, Warrington, PA) glycol methacrylate (Feder and O'Brien 1968) using heat polymerization (40–50°C for two days). Samples prepared using fixation schedules 4 and 5 were infiltrated for three to five days and embedded at 23°C using Historesin glycol methacrylate (LRB, Bromma Sweden).

Embedded samples were sectioned at 2–4 μm on a Sorvall Porter-Blum MT-2 ultramicrotome, mounted onto glass slides, and stained.

Staining procedures. Several stains were employed to demonstrate storage components and morphology in the grains.

1. Calcofluor. Sections on slides were briefly flooded with 0.01% aqueous calcofluor, rinsed, air dried, and mounted in immersion oil. Sections were then viewed using FC U and photographed (Fulcher and Wong 1980). Cell walls fluoresce blue with calcofluor. Note that autofluorescence of proteins also occurs at this wavelength and appears as a lesser intense blue than the cell walls.

2. Safranin O. Sections were placed in a Coplin staining jar containing 0.5% aqueous Safranin O and stained for 15 min (Revilla et al 1986). Slides were removed, rinsed under running tap water, air dried, and mounted in immersion oil or Eukitt mounting medium. Sections were viewed and photographed using FC B. Under these conditions, starch fluoresces yellow-green, proteins yellow to pink, and nuclei red.

3. Acid fuchsin. Slides containing sections were flooded with 0.01% acid fuchsin in 1.0% acetic acid, allowed to stain for approximately 2 min, rinsed in running water, air dried, and mounted in immersion oil. Sections were viewed and photographed using FC G (Fulcher and Wong 1980); storage proteins fluoresce red under these conditions.

4. Nile blue A. Sections prepared specifically to preserve lipids were mounted in 0.01% aqueous Nile blue A. As a check, hand sections were also observed following staining with Nile blue A. Lipid droplets appeared bright yellow when viewed using FC B (Fulcher and Wong 1980).

5. Acriflavine HCl (Yiu et al 1982). Slides were placed in a Coplin staining jar containing 0.01% acriflavine HCl in Walpole sodium acetate buffer (Pearse, 1960), pH 4.3, and stained for 15 min. Sections were rinsed briefly in 95% ethanol, air dried, and mounted in immersion oil. Sections were viewed and photographed using FC G. Phytic acid inclusions appear red under these conditions.

6. Periodic acid/Schiff’s (PAS). Sections were treated in a saturated solution of dinitrophenylhydrazine in 15% acetic acid to block preexisting aldehyde groups (Feder and O'Brien, 1968). The sections were then treated in 1.0% periodic acid, rinsed and stained in Schiff’s reagent (PAS kit, Sigma Diagnostics, St. Louis, MO). Sections were air dried, mounted in immersion oil, and observed using conventional brightfield optics. Carbohydrate groups containing vicinal hydroxyl groups, such as starch and cell walls, appeared pink to red (Fulcher and Wong 1980).

7. Iodine potassium iodide (IKI, Jensen 1962). Sections were stained briefly with a drop of IKI solution, rinsed, and mounted in water. The sections were viewed and photographed using either conventional brightfield conditions or Hoffman modulation contrast (similar to Nomarski DIC), in which starch granules appear black.

Starch isolation

Starch was isolated from whole grains by washing ground material repeatedly with water using a modified technique of Knight and Olsen (1984). Caryopses (2–10 g) were ground using a mortar and pestle and sieved successively through 20-mesh and 60-mesh screens. The fine particles that passed through the 60-mesh screen were retrieved and mixed with water, stirred, and allowed to stand 20 min. More water was added to the suspension, and the mixture was stirred for 10 min. The suspension was then sieved through an 80-mesh screen, and the portion retained on the screen was washed and resieved. The resulting suspension was sieved successively through 150- and 270-mesh screens and allowed to settle for approximately 1 hr in a centrifuge tube. Floating debris and supernatant were removed and discarded. The precipitate was washed repeatedly by resuspending and centrifuging at 2,000 × g until it was completely white, which indicated that the isolate was relatively pure starch. The starch was allowed to dry at 23°C and was observed in a scanning electron microscope.

Scanning Electron Microscopy

Caryopses of T. dactyloides were cut into pieces (approximately 1 mm²), fixed overnight in buffered 2% glutaraldehyde, rinsed in buffer, and fixed 2 hr in buffered 1% OsO₄. Samples were then dehydrated in a graded series of ethanol, cryofractured (Humphreys et al 1974), and critical-point dried using CO₂ in a Polaron E3000 critical-point dryer. The dried samples were affixed to aluminum specimen stubs using carbon paint and were coated with gold in a Polaron E3100 sputter-coating unit. Samples were viewed and photographed in a Hitachi S530 scanning electron microscope (SEM) at 10 kV. Starch samples were air-dried, affixed to aluminum specimen stubs using double-stick tape, gold coated, viewed, and photographed at 4 kV in the SEM.

Measurements of Components

Starch granules and protein bodies (of T. dactyloides) were measured from scanning electron micrographs and in the light microscope. Starch granules from H. bulbosum, Agropyron spp., and S. montanum were found to vary considerably in size and shape. Thus, reported sizes and shapes are approximate. Protein bodies and starch granules of T. dactyloides were less variable, and reported sizes and shapes are more representative of that species. Values reported are from scanning electron micrographs taken from samples that were broken, mounted (as is) with epoxy onto SEM stubs. Samples were then photographed in the SEM at several magnifications, and measurements of components were taken with a ruler. Means and standard deviations were calculated and are presented here in parentheses after an estimated, rounded value, along with the number of measurements. In all cases, the numbers obtained from SEM micrographs agree with those taken directly from sections in the light microscope.

RESULTS

Figure 1 shows representative drawings of the four species.

Agropyron Species

A. trichophorum and A. intermedium were nearly identical

Fig. 1. Illustrations of medium longitudinal (a–d) and cross (a–c) sections of Agropyron sp. (a), Hordeum bulbosum (b), Secale montanum (c), and Tripsacum dactyloides (d).

Vol. 68, No. 4, 1991 377
morphologically and histochemically. *Agropyron* spp. exhibited a single layer of aleurone cells (Fig. 2a and b), which surrounds the starchy endosperm. Cell walls of the endosperm and aleurone exhibited blue fluorescence when stained with calcofluor and observed with FC U (Fig. 2a). Aleurone cells contained numerous protein bodies with phytin (a cationic salt of myoinositol hexaphosphoric acid) inclusions (globoïds), which were evident using FG following treatment with acriflavine HCl (Fig. 2b). Aleurone cells also contained lipid droplets (not shown, similar to those in T. dactyloides, Fig. 2h). Lipid droplets and protein bodies with phytin inclusions were also abundant in the embryo (germ). The embryo appeared similar to that of other grasses. The scutellum, the largest structure in the embryo, was adjacent to the starchy endosperm and contained protein bodies (not shown; scutellum similar to Fig. 2i). Phytic acid inclusions were evident within protein bodies after staining with acriflavine HCl. Protein bodies in the coleoptile were much smaller than in the scutellum and not as abundant. Lipid droplets were also located throughout the embryo (not shown, similar to S. montanum, Fig. 2f). Neither lipid droplets nor protein bodies were found in cells of the starchy endosperm. The material between starch granules appeared as a smooth matrix when viewed in the light microscope following staining with protein-specific dyes. Protein also autofluoresces in the near UV; thus, the proteinaceous material is visible in Fig. 2a between dark starch granules.

The main storage components observed in the starchy endosperm were starch granules. Two distinct populations of starch granules were present in the starchy endosperm of both *Agropyron* spp. (Fig. 3a). Small starch granules were approximately 5 μm (5.24 ± 1.67, n = 236) in diameter and were roughly spherical in shape. Large starch granules were approximately 9 μm (9.14 ± 2.33, n = 56) X 20 μm (20.63 ± 4.42, n = 56) in the largest diameter and were disk-shaped. A continuous mass of proteinaceous material between starch granules in the starchy endosperm was evident in the light microscope. (Figures 2d and e, although taken of other genera, are representative of *Agropyron* spp.; protein appears as a pinkish background in Fig. 2d and red in Fig. 2e.)

**Hordeum bulbosum**

*H. bulbosum* had a multiple aleurone layer that was usually three cells thick over the starchy endosperm (Fig. 2c) and one cell thick adjacent to the embryo. Protein bodies, most of which contained phytin inclusions, were located in aleurone and embryo cells (especially abundant in cells of the scutellum, similar to T. dactyloides, Fig. 2i). Lipid droplets were located throughout the aleurone (not shown, similar to Fig. 2h) and embryo cells (not shown, similar to Fig. 2f). Starch granules, located only in the starchy endosperm (Fig. 2e), consisted of small spherical granules of about 4 μm (3.64 ± 1.40, n = 88) in diameter and larger ellipsoid-shaped granules of approximately 13 μm (12.64 ± 1.99, n = 22) X 18 μm (18.22 ± 3.51, n = 22) in diameter.

The protein matrix in the starchy endosperm was continuous, as in *Agropyron* spp. and *S. montanum*, but more of a protein gradient was evident (Fig. 2c, unstained portion in subaleurone represents protein). Protein was more abundant in the cells at the periphery, especially in the small cells adjacent to the aleurone layer, where starch granules were usually smaller and less abundant (Fig. 2e).

**Secale montanum**

*S. montanum* was similar in structure to *Agropyron* spp. A single layer of aleurone cells was generally found, although occasionally a double row was evident. Aleurone cells contained protein bodies with phytin inclusions (similar to Fig. 2b) and lipid droplets (similar to Fig. 2h). The starchy endosperm contained two populations of starch granules, smaller spherical granules approximately 5 μm (5.37 ± 1.87, n = 79) in diameter and larger disk-shaped granules approximately 16 μm (16.94 ± 6.37, n = 7) X 30 μm (30.23 ± 4.16, n = 7) in diameter.

The proteinaceous matrix between starch granules was evenly distributed throughout the starchy endosperm (Fig. 2e); no protein gradient was evident (i.e., more protein at the periphery). The embryo, especially the scutellum, contained protein bodies, most of which had phytin inclusions. Lipid deposits were abundant throughout the embryo and are shown in Fig. 2f for the scutellum.

**Tripsacum dactyloides**

The *T. dactyloides* carpyopsis was located inside hardened glumes; this arrangement was also noted in *Echinocloa turnerana* (channel millet, Irving 1983) and also occurs in rice (Juliano and Bechtel 1985). The seed, a relative of corn, is more spherical shaped than carpyopsis of other grains presented in this study (Fig. 1), but it had many similar features, including starch as the major storage component of the seed.

Starch granules in the starchy endosperm were roughly spherical, consisting of a single population of approximately 9 μm (8.94 ± 1.2, n = 66) in diameter (Figs. 2g and 3b) and exhibited birefringence through crossed polars (Fig. 3c). Small protein bodies, approximately 1 μm (1.15 ± 0.25, n = 68) in diameter (Figs. 2g and 3b), were located throughout the starchy endosperm and did not contain phytin inclusions. Protein bodies were numerous and tightly packed in the subaleurone. A gradient of proteinaceous material was evident, being greatest at the periphery (adjacent to the embryo and aleurone) and diminishing toward the central starchy endosperm.

Protein bodies, containing phytin inclusions, were abundant in aleurone cells (similar to Fig. 2h). The aleurone layer consisted of a single layer of cells (Fig. 2h), which were smaller and had thinner cell walls than *Agropyron* spp., *S. montanum*, or *H. bulbosum*.
Small lipid deposits, which appeared as brilliant yellow dots visible when viewed with fluorescence illumination after staining with Nile blue, were also abundant in the aleurone (Fig. 2h).

As in aleurone cells, embryo cells contained lipid deposits and protein bodies (Fig. 2f) with phytin inclusions (unstained, appearing to be dark holes, Fig. 2i). Starch granules ranging from 1 to 3 μm in diameter were located throughout the embryo (Fig. 3d).

DISCUSSION

The histochemical and morphological structure of the four genera (Agropyron, Hordeum, Secale, and Tripsacum) share similar features and exhibit typical Gramineae structure. Starchy endosperm, which consists of starch granules embedded in a proteinaceous matrix, is the major storage tissue in all grasses. Aleurone cells contain lipid and protein bodies with phytate inclusions. The embryo has numerous protein bodies of various sizes, some of which contain phytic acid. Small lipid droplets are located throughout the grass embryo and are especially abundant in the scutellum.

Agropyron spp., S. montanum, and H. bulbosum have continuous matrices of protein in starchy endosperm tissues—as does wheat (Parker 1980, Simmonds and O'Brien 1981). In the mature wheat caryopsis, a gradient in the staining pattern for protein is evident, with the staining heaviest at the periphery of the starchy endosperm (subaleurone and adjacent to the scutellum) and decreasing toward the center. This staining pattern was also evident in H. bulbosum, although the staining gradient was less than that of wheat. Agropyron spp. exhibited less of a gradient than H. bulbosum, and a protein staining gradient was not evident in S. montanum. A protein gradient was noted in T. dactyloides; however, the protein in the endosperm occurred as protein bodies.

Protein bodies of T. dactyloides endosperm were approximately 1 μm in diameter. Protein bodies have also been found in the endosperm of other Gramineae such as corn (personal obser-

Fig. 3. Scanning electron micrographs (a,b) and light micrographs (c,d) of two perennial grain species. a, Scanning electron micrograph of isolated starch of Agropyron intermedium, showing the disk-shaped large granules and spherical small granules. Isolated starch, such as in this micrograph, gives a clear indication of the shape of starch granules. Bar = 20 μm. b, Scanning electron micrograph of starchy endosperm cells of Tripsacum dactyloides, showing spherical starch granules (S) and protein bodies (PB). Identification of cellular components was made by staining sections for light microscopy before SEM observation. Bar = 20 μm. c, Light micrograph of an unstained section of the starchy endosperm and aleurone (AL) of T. dactyloides through crossed polarizers, showing birefringence of starch (S). Starch granules appear as the bright structures. Bar = 50 μm. d, Light micrograph of a section of the scutellum of T. dactyloides stained with iodine potassium iodide and photographed using Hoffman modulation contrast (brightfield) optics, showing darkly stained starch granules (S). Bar = 50 μm.
vation, Duvick 1961), rice (personal observation, Bechtel and Pomeranz 1978, Juliano and Bechtel 1985), oats (Fulcher 1986), and channel millet (Irvine 1983), where protein bodies are more abundant at the periphery. As in all grains, protein is a minor portion of the endosperm, whereas starch is abundant.

Starch granules were positively identified by their characteristic staining with IKI and PAS, as well as the birefringence they exhibited when viewed through crossed polarizers (Fig. 3c). Bates et al. (1981) did not see birefringence in isolated starch preparations of T. dactyloides and suggested that the lack of birefringence is perhaps due to the small size of the starch granules. However, in previous studies on Amaranthus, which has polyhedral starch granules approximately 1 μm in diameter (Irvine et al. 1981, Irvine and Becker 1985), starch was noted to exhibit birefringence (unpublished data). Bates et al. (1981) reported that, in T. dactyloides, starch was approximately 1 μm in diameter. However, in this study, starch granules of T. dactyloides were found to be about 10 μm in diameter, similar to those of corn (personal observation, Wolf et al. 1952). A few smaller granules (about 5 μm in diameter) were located in the subaleurone. Perhaps Bates et al. (1981) were measuring protein bodies, since they reported a lack of birefringence with polarized light and a diameter of 1 μm. Protein bodies, identified by staining reactions in this study, fit the description of the “starch granules” reported by Bates et al. (1981).

H. bulbosum has a multiple aleurone layer, usually three cells thick, similar to cultivated barley (Jones 1969, Jacobsen et al. 1971). Rice also has a multiple aleurone layer (personal observation, Juliano and Bechtel 1985). Agropyron spp., S. montanum, and T. dactyloides exhibited a single aleurone layer around much of the caryopsis similar to those in wheat and oat (Fulcher 1986) caryopses.

Protein bodies were found in aleuernce and embryo cells of all five species (similar to Fig. 2i), and these protein bodies served as a storage location for phytin inclusions (Fig. 2b and 2i). Phytin inclusions are common in other cereal grains (Fulcher 1986) and other seeds (Lott and Buttsrose 1977). They were identified both by birefringence when viewed through crossed polarizers (Fulcher et al. 1981) and by staining with acriflavine HCl (Yiu et al. 1982), as shown in aleuernce cells of A. trichophorum (Fig. 2b). Figure 2i, although stained for protein, clearly shows where phytin inclusions are located inside protein bodies; they were previously identified by positive staining with acriflavine HCl and show in Figure 2i as lack of staining (dark areas).

In addition to an abundance of protein bodies, the embryo of T. dactyloides contained a few starch granules (Fig. 2d), especially in the scutellum. This was also noted for corn (personal observation). Starch granules were not found in the embryos of the four other species, which is similar to wheat (Swift and O’Brien 1972).

Another similarity to wheat was noted in the amino acid profile of the protein of Agropyron spp. (see the amino acid table in Lockhart and Hurt 1986). However, the protein content was reported to be higher than that of wheat, about 17% for A. trichophorum and 15% for A. intermediate versus 14% for wheat (Becker et al. 1986). Perhaps the higher protein content of the caryopses of Agropyron spp. is due to the proportionately larger embryo and aleuernce tissues compared to those of wheat, since protein is more highly concentrated in these tissues. The wheat kernel has been bred to have a large caryopsis, much of which is endosperm storage tissue, and thus a relatively small embryo. Embryo and aleuernce tissues in the gramineae typically have a greater percentage of protein than the endosperm.

The protein content of T. dactyloides was almost triple that of normal corn, 29.3% for T. dactyloides versus 10.9% for normal corn, and approximately the same protein content as that in high-protein corn, 26.2% (Paulis and Wall 1977). Amino acid profiles show that T. dactyloides is low in lysine (containing about half that of conventional grains), high in methionine (containing about twice that of conventional grains) (Lockhart and Hurt 1986; Bates et al. 1981), and high in leucine, similar to corn (Paulis and Wall 1977).

Compositional data on S. montanum and H. bulbosum was not available, however. Becker et al. (1986) have reported compositional data on a S. cereale × S. montanum hybrid (Michael’s rye), as 7.7% protein (N × 5.85) versus 13.4% protein for S. cereale. It is likely that the lower protein content of S. cereale × S. montanum is a consequence of the genetic complement of S. montanum, since S. cereale has twice as much protein as the hybrid.

CONCLUSION

Agropyron spp. and T. dactyloides show potential for development as perennial grain crops. Their favorable nutritional and morphological aspects, similarity to conventional grain crops, and perennial habit seem favorable to increase genetic diversity either as a crop or as germ plasm for hybridization with conventional crops. S. montanum and H. bulbosum also exhibit perennial habit and have morphological and histochemical features in common with conventional grain crops. Further studies are necessary to ascertain the value of using S. montanum or H. bulbosum as a crop species. However, the increase of genetic diversity through hybridization with conventional crops would be of value.

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LITERATURE CITED


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